Analytical Methods

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: E. Margui, J. Dumic, I. Queralt, L. Bakovic and J. Jablan, *Anal. Methods*, 2020, DOI: 10.1039/D0AY01185A.

This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

PAPER

Simple and reliable determination of Zn and some additional elements in seminal plasma samples by using total reflection X-ray fluorescence spectroscopy

Eva Marguí^a, Jerka Dumic^b, Ignasi Queralt^c, Lada Bakovic^d and Jasna Jablan^{e,*}

Trace elements are essential for the normal spermatogenesis of mammals and play a critical role in sperm quality, but pathological processes *e.g.* inflammation, as well. Consequently, multi-elemental analysis of seminal plasma (SP) may provide significant information on physiological and pathophysiological processes occurring in the male reproductive tract.

Therefore, the development of a simple, fast and reliable method for seminal plasma (SP) analysis by total reflection X-ray fluorescence spectrometry (TXRF) could be useful for both, scientific and clinical studies. In this study, a detailed assessment of the sample preparation parameters and measurement conditions, including analysis of the shape and element distribution of the deposited residue on the reflector by micro X-ray fluorescence spectrometry, was carried out.

Using the best analytical conditions, limits of detection for trace elements were in the range of 0.04-0.3 mg/kg. Trueness and precision of the results, evaluated by spiked SP sample analysis, were in most cases acceptable with recoveries values in the range of 87-109 % and relative standard deviations 3-12% (n=5).

The developed TXRF method was applied for the analysis of several SP samples from patients with different diagnoses and results were compared with those obtained by ICP-OES. Among the studied trace elements with a role in the antioxidant defence system only Zn could be quantified and some differences in Zn concentrations among studied groups were observed. However, further studies on a large number of samples are required to define the exact relationship between element composition and semen quality.

^a University of Girona, Department of Chemistry, Girona, Spain

^b University of Zagreb, Faculty of Pharmacy and Biochemistry, Department of Biochemistry and Molecular Biology, Zagreb, Croatia

^c Institute of Environmental Assessment and Water Research, IDAEA-CSIC, Barcelona, Spain

^d General Hospital Zadar, Department of Laboratory Diagnosis, Zadar, Croatia

^e University of Zagreb, Faculty of Pharmacy and Biochemistry, Department of Analytical chemistry, Zagreb, Croatia E-mail:jjablan@pharma.hr

1. Introduction

Male infertility is a multifactorial disorder involving a wide range of factors including genetic, epigenetic, environmental, and lifestyle-related factors that affect sperm quality¹. The initial standard of care to evaluate male infertility is a basic semen analysis mostly based on spermatozoa number, morphology, and motility^{2,3}. However, it does not provide any information about sperm dysfunctions at the molecular level.

Seminal plasma (SP) has important roles in the regulation of semen coagulation and liquefaction, sperm motility and fertilization. It is composed of secretory products of glands in the male urogenital tract, such as peptides, proteins, lipids, saccharides, inorganic ions, nucleic acids, and small molecule metabolites *etc.* Consequently, changes in these components might reflect an ongoing pathological process in a specific organ. Therefore, SP represents a “gold mine” of potential biomarkers, which could significantly improve diagnostic/prognostic/treatment protocols, but also contribute to a better understanding of molecular mechanisms underlying pathological processes in the male urogenital system⁴. Human SP contains a variety of macro-, trace- and ultra-trace elements. Trace elements such as Zn, Cu, and Se are essential for normal spermatogenesis of mammals and play a critical role in antioxidant defense system thus being associated with sperm quality and inflammation^{5–8}. Furthermore, more recent studies have revealed adverse effects of certain elements *e.g.* Cu, Zn, Ca, Mg on human male reproduction^{9–11}. Some other elements, such as Pb, Cd, Cr, and Ni even in very low-level concentrations could adversely affect men reproductive health^{12–14}. Therefore, the measurement of various elements in SP could provide significant information on (patho)physiological processes occurring in male reproductive tract as well as in exposure prevention plans^{11,15}, thus playing an important role in the assessment of male reproductive health.

Multi-elemental chemical analysis of SP samples represents a great challenge due to the complexity of matrix, presence of organic and inorganic substances as well as the limited sample amount available. In addition, the reliable quantification could be a problem due to a wide concentration range of elements of interest. Up today, several analytical techniques have been used for measuring metal concentrations in human SP, mostly with respect to semen quality

and infertility problems *e.g.* flame atomic absorption spectrometry¹⁶, graphite furnace atomic absorption spectrometry^{16–19}, inductively coupled plasma-mass spectrometry²⁰ or inductively coupled plasma-optical emission spectrometry (ICP-OES)^{21–23}. However, the use of these techniques usually requires a high dilution factor of the sample or the use of additional time-consuming sample treatments for the total destruction of the organic matrix. Consequently, there is a great interest in green analytical methods, which offer multi-elemental capability with a minimum sample treatment. In that sense, it is important to highlight the great potential of total reflection X-ray fluorescence spectroscopy (TXRF). To perform analysis under total-reflection conditions, samples have to be present as thin films by depositing a low quantity of sample (2–50 µL) on a reflective carrier. This procedure excludes matrix effects, such as absorption and secondary excitation, resulting in better detection limits in comparison with conventional XRF spectroscopy. Taking into account the basic principles of this technique, TXRF offers some advantages with respect to other spectroscopic techniques such as the low amount of sample required, which is of special interest in SP analysis, simultaneous multi-elemental capability and an easier quantification approach in a wide dynamic range by internal standardization²⁴. Besides, low power benchtop TXRF systems are cost-effective since they do not require gas or cooling media. This fact has promoted the application of such systems for trace element determination in many different fields^{25–29}. However, their use in SP analysis is very scarce and only one article dealing with the analysis of seminal plasma of men with varicocele has been recently published³⁰. In this paper, an evaluation of the sample treatment procedure and measurement conditions is not provided and the analysis of certified reference materials or spiked SP samples is not considered. Therefore, it is difficult to evaluate the analytical capabilities of the TXRF method and the quality of the results obtained.

In respect of the aforementioned premises, the aim of this study was to evaluate the real capability of benchtop TXRF as a simple and cost-effective technique for the determination of elements in SP samples that can play a critical role in antioxidant defence system and are associated with sperm quality and inflammation (Zn, Cu and Se). For that purpose, a careful assessment of experimental parameters regarding sample preparation and measurement conditions was performed, including the analysis of the shape and element distribution of the deposited residue on the reflector by micro X-ray fluorescence spectrometry. Analytical figures of merit, such as limits

Analytical Methods

PAPER

of detection, trueness and precision of the results were also evaluated to identify the possibilities and limitations of the developed TXRF method for SP samples analysis. Finally, the results of TXRF analyses of SP samples obtained from the patients with different diagnoses (normozoospermia, asthenozoospermia, and oligoasthenozoospermia) were compared with those obtained by ICP-OES.

2. Experimental

2.1. Instrumentation

TXRF measurements were performed using a benchtop TXRF spectrometer "S2 PICOFOX" (BrukerNano, GmbH, Germany). - Evaluation of TXRF spectra and calculation of analyte net peak areas were performed using the commercial software (Spectra Plus 5.3, Bruker AXS Microanalysis GmbH, Berlin, Germany) linked to the equipment. For comparison purposes, SP samples were also analyzed by ICP-OES (Agilent 5100, Agilent Technologies, Spain). Matrix-matched calibration standards (using the same solution employed to dilute SP samples) were used for ICP-OES quantification purposes. Additional spectrometer specifications and measurement conditions used for TXRF and ICP-OES analysis of SP samples are displayed in Table 1.

2.2. Reagents and materials

Stock solutions of 1000 mg L⁻¹ (ROMIL PrimAg[®] Monocomponent reference solutions) were used to prepare V, Ga, Y and Rh internal standard solutions. Ultrapure deionized water used for dilution of stock solutions and SP samples was obtained by Milli-Q purifier system (Millipore Corp., Bedford, Massachusetts). Triton X-100[™] (laboratory grade, Sigma-Aldrich) was used as diluting agent for preparation of SP samples before TXRF analysis. Silicone solution in isopropanol (Serva GmbH & Co, Germany) was used to coat all the quartz glass disc reflectors in order to obtain a hydrophobic film so as to facilitate sample deposition. To the best of our knowledge, there were no SP reference materials available. For this reason, the reference material Seronorm "Trace Elements Serum L-1" (Sero, Billingstad, Norway; Reference 201405, Lot:1309438) was used, taking into account the similar matrix in terms of the protein content. This lyophilized human serum material was reconstituted

in 3 mL of water, according to the instructions provided in the manufacturer's certificate³¹.

2.3. Studied seminal plasma samples

SP samples were obtained from leftovers of semen samples collected for diagnostic purposes, as a part of the standard procedure of infertility diagnostics and treatment. Semen samples were collected in the hospital by masturbation after 3-5 days of sexual abstinence in disposable urinal flasks and processed according to a standardized protocol³². Then, after liquefaction for 30 min at r.t., seminal plasma was separated by slow centrifugation (400g, 10 min, r.t.) to avoid cell lyses, transferred and re-centrifuged (12000g, 10 min, r.t.). Colloid silica was not used during the separation step in any case. Supernatants were stored at -80 °C until analyses were performed. According to the results of the semen analyses, performed by a licensed specialist of laboratory medicine (EuSpLM), the samples were described as normozoospermic (normal number of sperm and normal sperm motility), asthenozoospermic (normal number of sperm but reduced sperm motility), and oligoasthenozoospermic (abnormally low number of sperm and reduced sperm motility).

2.4. Spiked seminal plasma samples

In order to evaluate the limits of detection and quality of the obtained results for elements that could be present in SP samples but that were not detected in the SP samples studied, a spiked SP sample was prepared. For that purpose, 0.2 g of SP were spiked adding an amount of a multi-element standard solution containing Cr, Mn, Fe, Co, Ni, Cu, Se and Cd to obtain a final concentration of 0.9 mg kg⁻¹. The mixture was homogenized by vortex agitation.

Table 1 TXRF and ICP-OES instrumental parameters and measurement conditions.

S2 PICOFOX TXRF benchtop spectrometer	
X-ray tube anode	Tungsten (W)
Current intensity	1 mA
Voltage	50 kV
Monochromator	Multilayer
Detector	SDD, R<149 eV Mn-K α)
Measurement time	2000 s

Agilent 5100 ICP-OES spectrometer	
Nebuliser	Pneumatic concentric
RF power	1200 W
Plasma flow	12 L min ⁻¹
Torch configuration	Radial
Detector	Multichannel charge transfer
Wavelengths	K (766.491nm), Ca (317.933nm), Zn (202.548nm), Cu (327.395nm), Fe (238.204nm)

R: resolution
RF: radio frequency power

2.5. Sample preparation

2.5.1. Dilution for TXRF analysis

The best sample preparation conditions for SP samples analysis by TXRF were carefully assessed (see section 3.1 for additional details).

The selected conditions for SP samples analysis were as follows: sample solutions were prepared by weighing 0.2 g of each SP sample and adding 0.2 g 1% Triton X-100 water solution (final sample dilution was 1 : 1). Then, an adequate amount of the 200 mg kg⁻¹ Y solution (internal standard) was added to obtain a final concentration of 5 mg kg⁻¹ of Y. The mixture was homogenized by vortex agitation for 10 s and an aliquot of 5 µL was transferred onto a siliconized quartz glass reflector and dried subsequently using an IR lamp.

2.5.2. Dilution for ICP-OES analysis

For comparison purposes, concentration levels of some elements in SP samples were also determined by ICP-OES. For that, 0.2 g of SP sample were diluted 1:20 with a water solution containing 0.01 mmol L⁻¹ EDTA, 0.004 % NH₃ and 0.07 % Triton X-100³³.

3. Results and discussion

3.1. Sample preparation and TXRF analysis

The main goal of this study was to develop a simple, sustainable and reliable method for multi-elemental analysis of SP samples. For that, dilution was studied as sample treatment strategy and other more sophisticated sample treatments such as microwave digestion or cold plasma ashing, which have been used in biological sample analysis by TXRF^{34,35}, were not considered.

In order to define the best analytical conditions for TXRF analysis of SP samples, several experimental parameters regarding the sample preparation (internal standard, dilution agent and dilution factor) and TXRF measurement conditions (sample deposition volume, drying mode and measuring time) were studied.

3.1.1. Internal standardization

In TXRF analysis, quantification is usually carried out directly by internal standardization. This method is based on the addition of an element named internal standard (IS) which should not be present in the sample, not interfere with the target elements and have an adequate XRF response. The concentration of the found element can be calculated using the equation:

$$C_i = \frac{C_{IS} \cdot N_i \cdot S_{IS}}{N_{IS} \cdot S_i}$$

View Article Online
DOI: 10.1039/D0AY01185A

where

C_i = concentration of the element i to be analysed

C_{IS} = concentration of the internal standard

N_i = analyte net peak area

N_{IS} = IS net peak area

S_i = instrumental sensitivity for the analyte

S_{IS} = instrumental sensitivity for the IS

After qualitative analysis, it was considered to use V, Ga, Y, Rh as internal standard candidates due to the absence of these elements in the original sample. In Figure 1, TXRF spectrum for SP sample using different internal standards at 5 mg kg⁻¹ concentration level was shown. It can be observed that V is not a good candidate to be used as internal standard due to the poor instrumental sensitivity of this element when using a W X-ray tube²⁷. The use of Ga as internal standard should be also avoided due to the overlapping of Ga-lines with Zn-lines observed in SP sample. From the results of elemental quantification obtained it was found that there were no statistically significant differences using Y and Rh as IS. In view of the obtained results, Y was finally selected as IS since it can be determined by both Mo and W TXRF systems.

Once the best IS was selected, the evaluation of its spatial distribution in the dried SP residue on the reflector was performed. 2D µ-XRF mapping of Y distribution in SP sample is shown in Figure 2. For comparison purposes, Zn distribution (analyte) in the same sample was also displayed. As it is shown, Y (IS) and Zn (analyte) distribution was not homogeneous but both elements followed the same pattern and were accumulated at the borders of the drop deposited on the reflector (ring effect). These findings highlighted the necessity of analysing the whole residue on the reflector to obtain representative element concentrations. This fact is of special relevance if micro-TXRF systems are used. However, in the present study, the diameter of the sample spot deposited on the reflector

Figure 1 TXRF spectrum for a seminal plasma sample using different internal standards at 5 mg kg⁻¹ concentration level.

($\varnothing$: 4.0 mm) was suitable to perform the analysis according to the TXRF instrument's user manual and thus sample homogeneities issues are not critical for obtaining representative results.

3.1.2. Sample treatment

A preliminary study was performed to test the effect of the dilution agent on the sample deposition on the reflector. The direct analysis of SP was discarded because the resulting residue on the reflector was detached from the surface of the quartz reflector. In regard to the previous findings on dilutions agents used for the analysis of biological samples³⁶, two dilutions agents were tested for preparing SP samples: deionized water and 1% Triton X-100 water solution. In both cases, the diameter of the sample spot deposited on the reflector ($\varnothing_{\text{Triton 1\%}}$: 4.0 mm, $\varnothing_{\text{water}}$: 3.0 mm) was suitable to perform the analysis according to the TXRF instrument's user manual. Nevertheless, the drop was better deposited and faster dried on the reflector surface when using Triton 1% as diluting agent. To select the best dilution agent element concentrations measured in SP samples were analyzed and compared with those obtained by ICP-OES analysis. Zn was used as element model in view of the presence of its element in the target SP samples (Figure 1) and its relevance in human male reproduction [9-10]. As it is shown in Figure 3A, no statistically significant differences in Zn content were found when SP samples were diluted with water or Triton 1%. Finally, a solution of 1% Triton X-100 in water was selected as a dilution agent for preparing SP samples since as it was stated above the drop was better deposited and easily dried on the reflector surface. Once established the dilution agent, several experimental tests were performed to study the effect of the dilution factor (1:1, 1:2, 1:5) on Zn determination in SP samples. Comparable results were obtained between TXRF and ICP-OES results regardless the dilution factor used for TXRF analysis of SP

Figure 2 2D mappings for distribution of Zn and Y in a seminal plasma sample deposited on a reflective carrier. Sample preparation conditions: seminal plasma diluted 1:1 with Triton 1% (v/v), sample deposition volume: 10 µL. Mapping conditions (m-XRF): 50 kV, Al1000 µm, collimator: 0.3 mm, grid: 15x15 points, time per point: 200s.

samples (Figure 3B). Nevertheless, when using higher dilution factors, a major dispersion of the results was found (RSD: 7% (dilution 1:1), 11% (dilution 1:2), 13% (dilution 1:5)) and thus, for further experiments, SP samples were diluted using a ratio 1:1 with Triton 1%.

3.1.3. Sample deposition volume

The influence of sample deposition volume on the reflector was estimated using sample aliquots of 5 and 10 µL. The use of lower deposition volumes (lower than 5 µL) was discarded due to a higher uncertainty of the results when such small deposition volumes are used. The diameter of the sample spot on the reflector obtained by both volumes (5 and 10 µL) was adequate, considering the volume spanned by excitation beam and the detector viewing (10 µL: $\varnothing$: 5.5 mm, 5 µL: $\varnothing$: 4.0 mm). Results showed that there were no differences regarding the element concentrations when using 5 or 10 µL to carry out TXRF measurements (Figure 3C). Thus, 5 µL sample volume was defined as a suitable deposition volume for TXRF analysis of SP samples, due to shorter drying-time on the reflector.

3.1.4. Drying mode

The drop of liquid sample deposited on a reflector has to be completely dried before TXRF analysis. In this study, two different drying modes were tested for SP samples deposited on the quartz reflectors, an infrared (IR) lamp and a hot plate set at 80 °C. Similar results, in terms of element concentrations, were obtained using both drying modes highlighting that the drying step was not a critical parameter for preparing SP samples (Figure 3D). Finally, an IR lamp was used in further experiments due to shorter drying-time on the quartz reflector.

PAPER

Analytical Methods

3.1.5. Measurement time

The measurement time is a parameter that can influence significantly on the repeatability of the obtained results. For that a test was conducted to evaluate the relative standard deviation (RSD) obtained for element concentrations in five replicate measurements of a spiked SP sample (section 2.3) at different measurement times (500, 1000 and 2000s). It was found that for elements present at high concentrations ($> 10 \text{ mg kg}^{-1}$, i.e., K, Ca and Zn), the effect of measurement time on RSD values was not significant and in all cases RSD values lower than 4% were obtained. However, for the elements present at lower concentrations, the repeatability of the results could be significantly improved by increasing measurement time. For instance, for Br analysis, RSD values were lower when the measurement time was increased from 500s (RSD~9%) to 2000 s (RSD~5%). A decrease was even more pronounced for low Z elements present in concentrations lower than 1 mg kg^{-1} . Concerning these results and taking into account the necessity of trace elements detection in SP samples, a measurement time of 2000 s was selected (RSD~10 %).

3.2. Analytical figures of merit of the TXRF method

3.2.1. Evaluation of the limits of detection

Limits of detection (LODs) were estimated by analysing a real SP sample and using the 3σ approach²⁴. LODs for other elements that can be important for diagnosis^{11, 12-14} but were not detected in the studied SP samples were also evaluated by analysing a spiked SP sample with Cr, Mn, Fe, Co, Ni, Cu, Se, Pb and Cd at a concentration level of 0.9 mg kg^{-1} . As it can be seen in Table 2, the highest limits of detection were found for elements such as K and Ca, which presented a weak fluorescence yield in comparison to mid and high Z elements. However, this is not a major issue in SP samples since these elements are present at high concentrations ($> 50 \text{ mg/kg}$). Limits of detection for other target elements were in the range of

0.04-0.3 mg/kg. These values are appropriate for instance for the determination of interesting elements related with human male reproduction such as Zn. But they are not enough for a proper determination of other trace elements present at lower concentrations (i.e. Cu ($63\text{--}133 \mu\text{g/L}$)³⁰, Se ($38\text{--}56 \mu\text{g/L}$)³⁰, Cd ($0.1\text{--}50 \mu\text{g/L}$)¹⁴). Nevertheless, in a previous publication we demonstrated that limits of detection for light-medium Z elements with K-line detection (K-Sr) and high Z elements with L-line detection (i.e., Pb) were one order of magnitude lower when using the Mo-TXRF system³⁷. So, the possibility to detect elements in SP samples that can play a critical role in antioxidant defence system and sperm quality (i.e, Se and Cu) using the developed method can be also feasible using Mo TXRF systems.

3.2.2. Evaluation of results trueness

In order to study the quality of the results obtained using the TXRF method, the reference material was analyzed and concentration values determined are summarized in Table 3. For comparison purposes, results obtained by ICP-OES analysis are also included.

In general, good agreement between TXRF, ICP-OES and reference results was found for most elements. An interesting aspect of the proposed TXRF method is the determination of bromine that is difficult to be measured by other spectrometry techniques such as ICP-OES and it could be of interest in some clinic studies³⁶.

Nevertheless, it was not possible to verify the trueness of the obtained results for some relevant elements in the field of sperm quality such as Se due to the low concentration of this element present in the serum RM. For this reason, it was considered appropriate also to evaluate the trueness of the obtained results by

Table 2 Limits of detection (mg/kg) for the analysis of seminal plasma samples by the developed TXRF method (W X-ray tube, measurement time: 2000 s).

LOD	K	Ca	Cr	Mn	Fe	Co	Ni	Cu	Zn	Se	Br	Rb	Cd	Pb
mg kg ⁻¹	2.6	1.9	0.33	0.26	0.16	0.15	0.13	0.11	0.10	0.05	0.04	0.04	0.05	0.07

analysing a spiked SP samples with a set of trace elements of potential interest at a concentration level of 0.9 mg kg^{-1} . Results are displayed in Table 4. As it is shown, in general quantitative recoveries were obtained in most cases demonstrating the potential use of the developed TXRF method for the analysis of this type of samples. Additionally, Zn content determined in the SP sample ($70 \pm 7 \text{ mg/kg}$, $n=3$) was compared with the content obtained by ICP-OES (67.0 ± 0.5

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Analytical Methods

PAPER

mg/kg, n=3) and a good agreement between both values was found. This fact confirmed also the suitability of the TXRF method for the reliable quantification of Zn in SP samples.

3.2.3. Evaluation of results precision

Total repeatability of the TXRF method was estimated by analyzing five independent replicates of the aforementioned spiked SP samples. Further, one reflector containing 5 µL of sample solution was measured five times under the same conditions and the associated RSD was also calculated. This uncertainty was related to the sample positioning inside the TXRF system, instrument stability and counting statistics. Therefore, by means of error propagation, the uncertainty due to sample preparation (dilution and deposition of the SP sample on the quartz reflector) can also be estimated. In

Table 3 Results obtained in the analysis of the control material SeronormTM Trace Elements Serum L-1 (dilution 1:1, Triton 1%, n=3) by means of the developed TXRF method. For comparison purposes, element concentrations in seminal plasma samples determined by ICP-OES (dilution 1:20) are also included. Results are expressed as mean concentration values of three replicates with the associated standard deviation.

Element	Units	TXRF	ICP-OES	Reference ^a
K	mg/L	100 ± 20	121 ± 5	127 ± 26
Ca	mg/L	90 ± 10	74 ± 3	86 ± 17
Zn	mg/L	1.3 ± 0.1	1.06 ± 0.02	1.097 ± 0.213
Br	mg/L	0.67 ± 0.06	n.m.	0.724 ^b
Cu	µg/L	1300 ± 200	980 ± 58	1088 ± 171
Fe	mg/L	1.65 ± 0.3	1.25 ± 0.2	1.47 ± 0.3

^a Value ± analytical uncertainty (95% confidence interval), ^b Indicative value, n.m: not measured

Table 4 Results obtained for the analysis of a spiked SP samples with Cr, Mn, Fe, Co, Ni, Cu, Se, Cd and Pb at 0.9 mg/kg concentration level. Results are expressed as mean of four independent replicates ± standard deviation.

Element	TXRF method	Recovery (%)
Cr	1.08 ± 0.09	114
Mn	0.8 ± 0.1	87
Fe	1.05 ± 0.05	109
Co	0.97 ± 0.08	104
Ni	0.78 ± 0.07	83
Cu	0.94 ± 0.04	99
Se	0.95 ± 0.06	100
Cd	0.89 ± 0.05	95
Pb	0.78 ± 0.03	83

Figure 4, an example of the results obtained for Zn (>10 mg/kg), Br (1-10 mg/kg) and Se (<1 mg/kg) is displayed. As it can be seen the the global precision was acceptable with RSD values in the range of 2.7-12.3%. From Figure 4 it can also be deduced that most part of the uncertainty of element present at higher concentrations (i.e., Zn, Br) is related to the sample preparation procedure, whereas, the uncertainty for ultratrace elements (i.e, Se) is mainly a consequence

of the sample positioning inside the TXRF system, instrument stability and counting statistics.

3.3. Application of the method to SP samples

To further prove applicability of the TXRF method for real SP samples, a set of 21 SP samples of three different diagnostic groups (normozoospermia, asthenozoospermia, and oligoasthenozoospermia) were analyzed.

Unfortunately, only Zn could be quantified in all the SP samples and other interesting elements, which can affect human male reproduction (Cu, Se or other toxic elements such as Cd), were not detected. These results were also confirmed by ICP-OES analysis of the same SP samples. In Figure 5, a comparison of Zn concentrations obtained by the developed TXRF method and ICP-OES is displayed. As it is shown, no statistical significant differences (at 95% confidence range) were found between both datasets. This fact demonstrated the usefulness of the developed TXRF method for SP analysis but in a simpler (quantification by internal standardization) and cost-effective way. As can be seen in Figure 6, at a first glance, it seems that Zn content in SP samples from patients with diagnosis of oligoasthenozoospermia is lower than in SP with other diagnoses (normozoospermia and asthenozoospermia). However, further studies on a large number of samples are required to determine the relationship between the

Figure 4 Contribution of instrument and sample preparation uncertainties to the global precision of the TXRF results obtained in the analysis of SP samples.

This journal is © The Royal Society of Chemistry 20xx

Analytical methods 2020 | 7

Please do not adjust margins

PAPER

Analytical Methods

Figure 5 Zn concentration in SP samples (n=21) determined by the proposed TXRF method and by ICP-OES. Results are expressed as mean concentration values of three independent replicates.

presence of trace elements in SP and semen quality (sperm number and motility).

4. Conclusions

A simple and reliable TXRF method has been developed for the determination of trace elements in SP samples. Although the

Figure 6 Comparison of Zn concentrations in SP samples with three different diagnoses (asthenozoospermia n=6, oligoasthenozoospermia n=5 and normozoospermia n=7) using the developed TXRF method.

minimum element concentrations that can be detected using the TXRF method are higher than those associated with ICP-based techniques, the method is suitable for the reliable quantification of elements such as Zn, which play a critical role in antioxidant defense system and it has been associated with sperm quality and inflammation. Moreover, the proposed method has several advantages over other spectroscopic techniques including simultaneous multi-elemental capability, low sample amount required for analysis (significant for SP samples), low consumption of reagents and consumables since no gas or cooling media are required and easy quantification by internal standardization (external calibration is not needed). All these advantages make the TXRF method very attractive for element composition analysis of SP and provide a good basis for further application of TXRF in clinic studies.

Acknowledgments

"This article/publication is based upon work from COST Action CA18130 ENFORCE TXRF, supported by COST (European Cooperation in Science and Technology)."

COST (European Cooperation in Science and Technology) is a funding agency for research and innovation networks. Our Actions help connect research initiatives across Europe and enable scientists to grow their ideas by sharing them with their peers. This boosts their research, career and innovation.

<http://www.cost.eu/>

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

Conflict of interest

The authors declare that they have no conflict of interest.

View Article Online
DOI: 10.1039/D0AY01185A

Analytical Methods Accepted Manuscript

References

- 1 A. Agarwal, A. Mulgund, A. Hamada and M. R. Chyatte, *Reprod Biol Endocrinol*, DOI:10.1186/s12958-015-0032-1.
- 2 S. C. Esteves, *Int Braz J Urol*, 2014, **40**, 443–453.
- 3 S. Gunes, G. N. T. Hekim, M. A. Arslan and R. Asci, *J Assist Reprod Genet*, 2016, **33**, 441–454.
- 4 M. J. Roberts, R. S. Richards, R. A. Gardiner and L. A. Selth, *Biomarkers in Medicine*, 2015, **9**, 77–80.
- 5 A. T. Alahmar, *J Hum Reprod Sci*, 2019, **12**, 4–18.
- 6 R. Dada and S. Bisht, in *Male Infertility: Understanding, Causes and Treatment*, eds. R. SINGH and K. Singh, Springer Singapore, Singapore, 2017, pp. 151–165.
- 7 M. M. Hashemi, N. Behnampour, M. Nejabat, A. Tabandeh, B. Ghazi-Moghaddam and H. R. Joshaghani, *Romanian Journal of Internal Medicine*, 2018, **56**, 15–20.
- 8 S. Bisht, M. Faiq, M. Tolahunase and R. Dada, *Nat Rev Urol*, 2017, **14**, 470–485.
- 9 S. E. Chia, C. N. Ong, L. H. Chua, L. M. Ho and S. K. Tay, *J. Androl.*, 2000, **21**, 53–57.
- 10 W. Y. Wong, G. Flik, P. M. Groenen, D. W. Swinkels, C. M. Thomas, J. H. Copius-Peereboom, H. M. Merkus and R. P. Steegers-Theunissen, *Reprod. Toxicol.*, 2001, **15**, 131–136.
- 11 A. Kasperczyk, M. Dobrakowski, S. Horak, J. Zalejska-Fiolk and E. Birkner, *J Trace Elem Med Biol*, 2015, **30**, 153–159.
- 12 S. Telišman, *Arhiv za higijenu rada i toksikologiju*, 1995, **46**, 459–475.
- 13 A. Pizent, J. Jurasović and S. Telišman, *Journal of Trace Elements in Medicine and Biology*, 2003, **17**, 199–205.
- 14 J. Sun, G. Yu, Y. Zhang, X. Liu, C. Du, L. Wang, Z. Li and C. Wang, *Biol Trace Elem Res*, 2017, **176**, 27–36.
- 15 A. P. Drabovich, P. Saraon, K. Jarvi and E. P. Diamandis, *Nat Rev Urol*, 2014, **11**, 278–288.
- 16 P. J. Parsons and F. Barbosa, *Spectrochimica Acta Part B: Atomic Spectroscopy*, 2007, **62**, 992–1003.
- 17 B. J. Bolann, R. Rahil-Khazen, H. Henriksen, R. Isrenn and R. J. Ulvik, *Scand. J. Clin. Lab. Invest.*, 2007, **67**, 353–366.
- 18 F. Atig, M. Raffa, B.-A. Habib, A. Kerkeni, A. Saad and M. Ajina, *BMC Urol*, 2012, **12**, 6.
- 19 A. H. Colagar, E. T. Marzony and M. J. Chaichi, *Nutrition Research*, 2009, **29**, 82–88.
- 20 U. Marzec-Wróblewska, P. Kamiński, P. Łakota, M. Szymański, K. Wasilow, G. Ludwikowski, M. Kuligowska-Prusińska, G. Odrowąż-Sypniewska, T. Stuczyński and J. Michałkiewicz, *Biol Trace Elem Res*, 2011, **143**, 167–177.
- 21 H. Vanhoe, *J Trace Elem Electrolytes Health Dis*, 1993, **7**, 131–139.
- 22 B. Kollander, M. Andersson and J. Pettersson, *Talanta*, 2010, **80**, 2068–2075.
- 23 J. Yoshinaga, *Nihon rinsho. Japanese journal of clinical medicine*, 1996, **54**, 202–206.
- 24 E. Margui, *X-ray fluorescence spectrometry and related techniques: an introduction*, Momentum Press, New York, NY, 2013.
- 25 H. Schwenke, P. A. Beaven and J. Knoth, *Fresenius J Anal Chem*, 1999, **365**, 19–27.
- 26 M. Schmeling, *Physical Sciences Reviews*, DOI:10.1515/psr-2017-0161.
- 27 P. Wobrauschek, *X-Ray Spectrometry*, 2007, **36**, 289–300.
- 28 V. N. Salomone, M. Riera and C. Muniain, *Int J Exp Spectroscopic Tech*, 2017, **2**, 1–6.
- 29 R. Fernández Ruiz, *Developments in Analytical Chemistry*, 2014, **1**, 1–14. DOI: 10.1039/D0AY01185A
- 30 M. I. Camejo, L. Abdala, G. Vivas-Acevedo, R. Lozano-Hernández, M. Angeli-Greaves and E. D. Greaves, *Biol Trace Elem Res*, 2011, **143**, 1247–1254.
- 31 Sero - Seronorm Trace Elements Serum, <https://www.sero.no/products/seronorm-trace-elements-serum/>, (accessed November 5, 2019).
- 32 WHO | WHO laboratory manual for the examination and processing of human semen, <http://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/>, (accessed November 5, 2019).
- 33 T. Živković, B. Tariba and A. Pizent, *Journal of Analytical Atomic Spectrometry*, 2014, **29**, 2114–2126.
- 34 S. Woelfl, M. Mages and F. Encina, *Spectrochimica Acta Part B: Atomic Spectroscopy*, 2003, **58**, 2157–2168.
- 35 E. Marguí, P. Ricketts, H. Fletcher, A. G. Karydas, A. Migliori, J. J. Leani, M. Hidalgo, I. Queralt and M. Voutchkov, *Spectrochimica Acta Part B: Atomic Spectroscopy*, 2017, **130**, 53–59.
- 36 E. Marguí, J. Jablan, M. Gerić, S. Inić, A.-M. Domijan, R. Janušić, B. Šarčević, I. Queralt and V. Garaj-Vrhovac, *Anal Bioanal Chem*, 2019, **411**, 1659–1670.
- 37 R. Dalipi, E. Marguí, L. Borgese, F. Bilo and L. E. Depero, *Spectrochimica Acta Part B: Atomic Spectroscopy*, 2016, **120**, 37–43.