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Surface chemical analysis — Use of Total Reflection X-ray Fluorescence spectroscopy in biological and environmental analysis

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Surface chemical analysis — Use of Total Reflection X-ray Fluorescence spectroscopy in biological and environmental analysis

Analyse chimique des surfaces — Utilisation de réflexion spectroscopie des rayons X de fluorescence totale dans l'analyse biologique et de l'environnement

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Foreword

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The committee responsible for this document is ISO/TC 201, *Surface chemical analysis*.

Introduction

Total Reflection X-Ray Fluorescence (TXRF) spectroscopy is a reliable technique for chemical analysis. TXRF today is employed in electronic industry quality control. TXRF is also a powerful multi-elemental method for trace and ultra-trace analysis of different kind of samples that can be grouped as follows: environmental samples (as water, soil, aerosols, deposits, plants), geological and mineralogical samples (as ore, crystals, mineral raw materials), technological samples (as petroleum and petroleum products, thin films, wastes, metals, polymers), biological samples (as blood, serum, urine, human tissue), food samples (as fish, fruit, meat, nuts, mushroom), pharma and biomedical samples (as pharmaceuticals, cell culture media), archaeological, art, and forensic samples. Sample preparation is critical for the quantitative analysis and depends on the sample and its aggregate state.

Because of its capability to analyse different kinds of samples, TXRF is suitable for chemical metrology at the nanoscale, both for heavy metals and light elements in environmental and biological analysis.

The key advantages of TXRF are the following:

a) simultaneous multi-element trace analysis including halogenides;

b) analysis of very small sample amounts (lower than nanograms to microgram range depending on sample preparation and condition);

c) simple quantification using an internal standard and possibility of reference-free quantification;

d) suitable for various sample types and applications;

e) theoretically low matrix or memory effects;

f) relatively short time is required for measurement collection;

g) high-sensitivity, low-detection limits depending on sample (elements) matrix, preparation method, and instrumentation.

TECHNICAL SPECIFICATION ISO/TS 18507:2015(E)

Surface chemical analysis — Use of Total Reflection X-ray Fluorescence spectroscopy in biological and environmental analysis

1 Scope

This Technical Specification provides a framework on the uses of Total Reflection X-Ray Fluorescence (TXRF) spectroscopy for elemental qualitative and quantitative analysis of biological and environmental samples. It is meant to help technicians, biologist, doctors, environmental scientists, and environmental engineers to understand the possible uses of TXRF for elemental analysis by providing the guidelines for the characterization of biological and environmental samples with TXRF spectroscopy.

Measurements can be made on equipment of various configurations, from laboratory instruments to synchrotron radiation beamlines or automated systems used in industry.

This Technical Specification provides guidelines for the characterization of biological and environmental samples with TXRF spectroscopy. It includes the following: (a) description of the relevant terms; (b) sample preparation; (c) experimental procedure; (d) discussions on data analysis and result interpretation; (e) uncertainty; (f) case studies; and (g) references.

2 Normative references

No normative references cited in this document.

3 Terms, definitions, symbols, and abbreviated terms

3.1 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1.1
sample carrier
flat substrate where the specimen is deposited

Note 1 to entry: The reference surface corresponds to the flat surface of the sample carrier, where the residue lays. The most important feature of the sample carrier is to be a reflector/mirror for X-rays. Surface roughness, matrix, and contamination of the sample carrier have an impact on TXRF measurements.

3.1.2
residue
specimen that lays on the sample carrier to be measured

3.2 Symbols and abbreviated terms

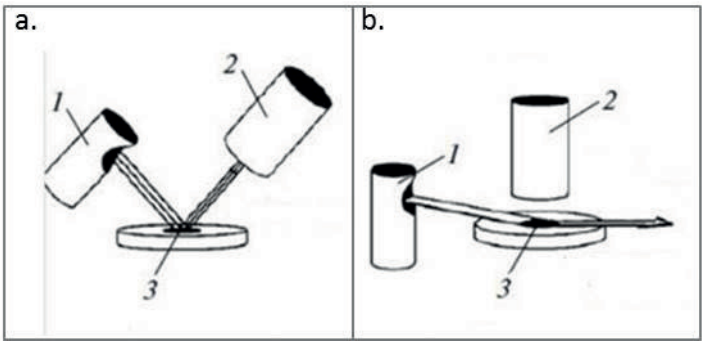
ppm	concentration in part per million range
ppb	concentration in part per billion range
MW	Microwave [used to describe the method of digestion (acidic MW digestion)]
APDC	Ammonium pyrrolidine dithiocarbamate
MIBK	Methyl isobutyl ketone
AAS	Atomic Absorption Spectroscopy
GF-AAS	Graphite Furnace Atomic Absorption Spectroscopy
ICP-MS	inductively coupled plasma-mass spectroscopy
IS	internal standard
INAA	Instrumental Neutron Activation Analyses
LPME	liquid phase microextraction procedure
FWHM	full width at half maximum
IR	infrared
QC	quality control
SR	Synchrotron radiation
XRT	X-ray tube
XSW	X-ray standing wave

4 Background

4.1 Preliminary remarks

TXRF is a surface elemental analysis technique often used for ultra-trace analysis of particles, residues, and impurities on smooth surfaces. TXRF is currently a key tool for wafer surface contamination control in semiconductor chip manufacturing.

In the TXRF experiment, the monochromatic X-ray beam impinges on the sample holder carrying the sample at very small angle, causing total reflection of the beam. The glancing beam angle shall be below the critical angle of X-ray total reflection, differently from XRF method, where both the glancing beam and the detection angle are at 45°.[1] Figure 1 shows XRF and TXRF geometries.



Key

1 X-ray generator

2 energy dispersive detector

3 irradiated sample area

Figure 1 — a. XRF conventional instrumental geometry: X-ray incident beam angle and fluorescence detector angle are 45° with respect to the sample surface; b. TXRF instrumental geometry: X-ray incident beam angle is near to 0° and detector position is 90° with respect to the sample surface.[2]

Due to the low glancing beam angle, in TXRF the detector can be arranged close to the sample leading to a higher fluorescence yield with respect to the conventional XRF geometry. The monochromatic X-ray beam illuminates the sample and it is totally reflected. The great inherent advantage of TXRF is the double excitation of the sample by both the primary and the reflected beams, leading to a doubling in the fluorescence intensity.

TXRF detection limits are comparable or better of those that can be obtained by FAAS, while the former technique is more sensitive in terms of total sample amount.[1] Table A.1 and Table A.2 show the comparison of the detection limits of TXRF, AAS, and ICP analysis of environmental and biological samples, respectively. An advantage of TXRF over AAS and ICP-OES and ICP-MS is the possibility to detect halogenides.

TXRF can be used to perform qualitative and quantitative multi-element analysis. For quantitative analysis, an internal standard, i.e. an element absent in the sample (for example, V, Sc, Ga, Ge, Se, Y, or Co), is added to the sample aliquot. Reference free TXRF analysis for quantification and qualification is technically possible and has been proposed several times and is employed at some facilities.

Resulting benefits of TXRF are significantly reduced background noise and matrix effects. In addition, a sample preparation procedure without digestion allows more accurate analysis of some volatile elements, as Hg, As, or Se, which can be reduced by the sample preparation. Table 1 shows a comparison of some characteristics of TXRF, AAS, and ICP-MS.

Light elements ($Z < 11$) are not detected efficiently with commercial spectrometers because their fluorescence signals are absorbed before detection. However, using the vacuum chamber spectrometer, the proper excitation energy (e.g. Cr-Ka, Rh-L...), and the suitable detector with Ultra Thin window, the detection down to Carbon is possible.

The detection limits for specific element depend on the X-ray tube. For example, by using Mo X-ray tube, only the L-lines of some elements (such as Cd, Sn or Sb) can be detected. Because of the low energy of L-lines and their possible overlapping with K-lines of Cl, K, and Ca, the quantitative analysis for these elements is not reliable.

Table 1 — Comparison between TXRF, AAS, and ICP-MS

Property	TXRF	AAS	ICP
Technique used	Non-destructive technique	Destructive technique (digestion required)	Destructive technique (digestion required)
Calibration	Single-standard calibration (internal)	Multi-standard calibration (external)	Multi standard calibration (external)
Sample presentation	solutions, suspensions, particles, thin films	solutions[a]	solutions[a]
Multi-element analysis	Yes	Sequential only	Yes
Digestion procedure	Not necessary	Yes	Yes

[a] GF-AAS and LA-ICPMS allow the analysis of solid samples for special applications.

5 Instrumentation

5.1 Instrumental requirements

The TXRF spectrometer consists of the following:

— sources of X-ray radiation like high-voltage generator and X-ray tubes or synchrotron radiation;

— a spectral modification element like a monochromator or a cut-off reflector, if necessary;

— sample station for handling the sample carrier;

— energy-dispersive detector;

— data acquisition unit.

Wavelength, voltage, and current of the X-ray tube, the presence of a spectral modification element (not always necessary), detector type, and glancing angle are the most relevant parameters. Today the performances of TXRF instruments are improving due to innovative X-ray optics.

5.1.1 X-ray sources of radiation

5.1.1.1 X-ray tubes

The X-ray tube is a device for the production of X-radiation via electrons striking an anode material. The electrons required are emitted from a heated cathode and are accelerated by a high voltage. Today, ceramic X-ray tubes cooled by air (low-power X-ray tubes) are also available.

X-ray tube voltage is the high voltage applied to the X-ray tube that determines the relative intensities of the characteristic X-ray lines. The maximum allowed high voltage is normally 60 kV. The optimum yield of characteristic X-ray lines is obtained when the tube voltage (in kV) is about 3 to 4 times the energy of the absorption edge of the anode material (in keV).

The anode material of the X-ray tube is the material that is struck by accelerated electrons. The anode material determines not only the characteristic X-ray lines emitted by the X-ray tube but also the intensity of the Bremsstrahlung. The latter is proportional to the atomic number of the anode material and to the square of the applied voltage.

Types of X-ray tubes that can be used:

— Side-window tube X-ray tube from which the X-radiation is emitted perpendicular to the axis of the tube.

— Fine-focus X-ray tube side-window tube with a fine, collimated, either point or line-shaped beam profile.

— Mixed anode X-ray tube: a fine-focus X-ray tube with an anode composed of two different metals, whose emitted spectrum consists of the spectra of the individual anode components.

The element of the XRT cannot be properly detected, and other elements (such as Cd, Sn, Sb, and Pb when Mo tube is used) can be identified only by means of their L lines. When the quantification of these elements is necessary, the XRT source shall be changed.[3]

5.1.1.2 Synchrotron radiation

The use of synchrotron radiation (SR) as primary excitation source can increase the overall sensitivity of TXRF. Compared to X-ray sources produced by electron bombarding on metal targets, SR is produced as a natural by-product of circulating or oscillating electrons in a storage ring. It contains all the wavelengths of the electromagnetic spectrum and is 100 or more times powerful than an X-ray generator of a commercial instrument.[4]

The advantages of SR are:

— High incidental flux combined with low divergence which results in higher fluorescence intensities and so lower detection limits.

— Reduced elastic scattering of the incoming beam due to its linear polarization in the orbital plane (Detector has to be placed in the orbital plane to make use of this).

— The spectral background, which is already reduced by total reflection, is further lowered.

— The tunability of SR allows improving the sensitivity for special elements (by increasing the photon absorption cross section when setting the excitation energy right above the absorption edge of the respective element).

5.1.2 Monochromator

A monochromator is a device for the selection of a narrow spectral region. A dispersive medium is the main constituent of the monochromator. It consists of an arrangement of two multilayers which select the X-ray radiation, according to the Bragg law. Low-pass filters can also be used as monochromator but they are less effective. Crystal monochromators are also used with synchrontron radiation, especially if TXRF is combined with XANES, as the high spectral resolution is required for XANES.

5.1.3 Detector

Detector is one of the key tool for TXRF analysis. It collects the characteristic X-ray radiation emitted by the sample, according to the drift chamber principle. The following materials are usually used in solid-state detectors: Li doped Si or Ge single crystals, high purity Ge single crystals. These detectors are described by the following acronyms respectively: Si (Li), Ge (Li), Si-PIN, Si drift, or HPGe detectors.

Detector parameters are: energy resolution, efficiency, and dead time.

Resolution is the capability of distinguishing between two nearly equal wavelengths. The energy resolution of a detector expressed as the full width at half maximum (FWHM) of a peak. For Silicon-based detectors, the FWHM of Mn-Kα line at 5,9 keV is taken as reference value. Typical values for the resolution lie in the range from 120 keV to 150 keV, depending on the size of the detector crystal.

Detector efficiency is the ratio of output signal (peak intensity) to the input signal (photon rate) at a given energy. The detector sensitivity only accounts for the photons that reach the detector. The overall sensitivity of system taking into consideration the system geometry is usually significantly lower. The sensitivity varies within the range 10 % to 100 %, depending on the energy of radiation and the type of detector. An important issue of the detector efficiency is also the absorption of the Be window. To improve the efficiency in the low-energy range, ultra-thin windows (UTW) are used; this allows the detection of low energy radiation of elements. Only in the high-energy regions the thickness of the crystal influences the efficiency.

The dead time is the time period following the arrival of a photon in the detector in which the pulse processing takes place and during which no new photons can be registered. The dead time (dead time loss) is given as the ratio of the dead time to total time.

Depending on the detector, artificial signals and background noise might be created. Escape peaks, pile–up peaks, shelf and tail will appear in the spectrum depending on the energy that is registered by the detector. Thus, when a photon with definite energy escape from the detector-active region, an escape line appears. For silicone-based detectors, the escape peak lies at an energy which is lower than the main peak by an amount corresponding to the Si-Kα energy (1,74 keV). Pile-up peak is caused by the simultaneous processing of two X-ray photons that the detector registers as a single event: it is a sum peak appearing to the sum of energies of two intense lines in the X-ray spectrum. The incomplete charge collection in the detector causes shelf and tail lines. All detector artefacts have to be considered during the analysis and compensated for electronically or via computer operations.

5.1.4 Sample station

A sample station is used for automatic and repeated measurements of multiple samples. It also separates the radiated measurement area from the sample interface and protects the operator.

The data acquisition unit is a stand-alone computer, which is connected to the apparatus for control of the data acquisition, data evaluation, and storage.

5.1.5 Critical and glancing angle

The choice of the glancing angle of the X-rays beam is critical for TXRF analysis. Figure 2 shows the reflectivity of X-rays for different glancing angles.

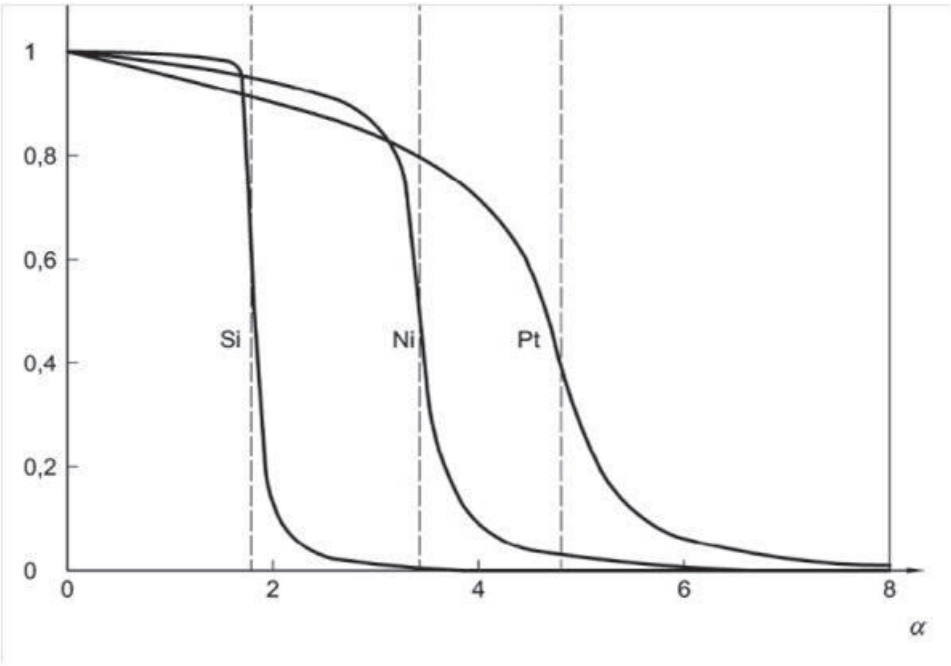


Figure 2 — Reflectivity curves of three different media calculated for E = 17,4 keV (Mo – Kα)

The critical angle of total reflection is given by Formula (1):

$$\alpha_{\text{crit}} = \frac{1{,}65}{E}\sqrt{\frac{Z_{\text{m}}}{A_{\text{m}}}\rho} \tag{1}$$

where

α_{crit} is the critical angle;

E is the photon energy in radiation;

Z_{m} is the mean atomic number of the medium;

A_{m} is the mean relative atomic weight of the medium;

ρ is the density of the medium.

To have total reflection, the glancing angle for TXRF experiment shall be smaller than the critical angle. Since the critical angle depends on the substrate and the beam energy, the glancing angle might require subsequent adjustments.

5.2 Quality control of TXRF spectrometer

5.2.1 Stability check of X-ray beam

Before starting the measurement, the stability of the X-ray beam shall be checked according to the procedure suggested by the spectrometer producer. To operate, the main switches of the TXRF spectrometer and the high voltage shall be on.

A generalized procedure for stability check with a specific example is reported as follows:

a). Preparation of a suitable test sample: Transfer 10 µl a standard solution (1 g/l, e.g. Ga) on TXRF sample carrier.

b) Data acquisition: Define Region of Interest (e.g. 8,9 keV to 9,5 keV in case of a Ga standard). Run repetitive measurements.

c) Data evaluation: Qualitative prove of the count rate in dependence of the measurement time until the count rate reaches a stable value.

d) Required failure action: Authorized service engineer needs to be consulted in case of a deviation of the count rate of more than 1 % after 30 min run.

5.2.2 Spectroscopic resolution

The spectroscopic resolution has a significant influence on the analytical performance of a TXRF spectrometer. The monitoring of the parameter is performed as described in the following procedure:

A generalized procedure for monitoring the spectroscopic resolution with a specific example is reported as follows:

a) Checking the instrument status: 60 min warm-up phase at maximum tube power.

b) Preparation of a Mn standard: Transfer 10 µl of a 1 g/l Mn solution on TXRF sample carrier and dry. Target value for count rate is >5,000 cycles per second.

c) Data acquisition: Set measurement time (live time) to 60 s to 120 s and store acquired spectrum.

d) Data evaluation: Calculate FWHM following the specific procedure of the TXRF device and save new value.

e) Required failure action: An authorized service engineer needs to be consulted in case of a FWHM value with a deviation of more than 10 % compared to the delivery status.

5.2.3 Energy calibration

Energy calibration is performed with respect to the position of measured X-ray lines of the elements of calibration samples. Energy calibration is performed by adjustment of channel/energy ratio or gain correction of the amplifier hardware directly by software. TXRF instrument has one of the above correction functions or both. Energy calibration should be carried out frequently enough to ensure that the uncertainties if the analysis are not significantly increased. A set of reusable samples could be used for the calibration of the energy. For this purpose, residue samples prepared from single or plural element solutions, or sample carriers coated with pure metals can be employed.

A generalized procedure for the gain correction with a specific example is reported below.

a) Initial condition: Gain correction shall follow after completion of the stability check.

b) Preparation of a test sample: Transfer 10 µl of a 1 g/l standard solution on TXRF sample carrier and dry.

c) Data acquisition: Set measurement time to 60 s to 120 s, start measurement, monitor count rate; it shall exceed 5,000 cycles per second.

d) Calculation of new gain value: Calculate the gain factor following the specific procedure of the TXRF device and save new value.

e) Repetition of the gain correction: During automatic measurement jobs, the gain correction should be repeated regularly, if possible once per two hours.

f) Required failure action: Strong changes of the gain values indicate abnormal deviations for the nominal value. In this case, a service engineer should be consulted.

g) Storage of the test sample: This test sample can be used for up to six months if it is stored safely in a containment and does not show significant contaminations.

5.2.4 Sensitivity test

The sensitivity is determined by the measurement of single element sample and it is defined as the detected fluorescence intensity normalized by mass, time, and tube current. The sensitivity is primarily affected by the quality of the beam adjustment and by the deterioration of the X-ray tube. When the sensitivity decreases below 60 % of the original value, the quality of the beam adjustment shall be checked. If the sensitivity is still low after the adjustment, XRT shall be substituted.

6 Specimen preparation

6.1 Preliminary remarks

TXRF is used for the analysis of liquids, with or without pre-concentration treatment, and solid samples, after decomposition, dissolution or suspension procedure, deposited on carrier with high reflectivity. Liquid samples shall be dried before measurements. In some cases, as for aerosols, sample can be directly collected on the sample carrier.

Detailed information on types of sample carriers and sample treatment procedures for the analysis of liquid and solid samples in chemical analysis by TXRF are presented in this section.

6.2 Sample carriers

6.2.1 Choice of sample carriers

The choice of the proper sample carrier shall consider the elements to be measured, chemical resistance of the reflector material and its spectral background. In Table 2, advantages and disadvantages of the most commonly used sample carriers materials are given.

Table 2 — Advantages and disadvantages of commonly used sample carrier materials

Material	Advantages	Disadvantages
Plexiglass	Inexpensive Disposable	High background Frequently presence of S
Quartz glass	Very hard Very pure Easy to clean	Not stable against HF Presence of Si peaks Expensive
Sapphire	Chemically stable Durable	Presence of Al peaks Very expensive Natural material, can contain impurities
Silicon wafer	Very pure	Presence of Si peaks Expensive It should be cut and sized
Soda lime glass	Inexpensive Disposable	Possible presence of impurity peaks

The mechanical performance of sample carriers is crucial: despite the fact that the reflectors are usually made of very hard material, they can be damaged by inappropriate handling, increasing the background.

When using non-disposable reflectors, a proper cleaning of the surface shall be ensured to avoid contamination. Contamination on the sample carriers will result in systematic errors of both qualitative and quantitative analysis.

6.2.2 Cleaning procedure for sample carriers

In the course of time, TXRF users have developed specific procedures for the cleaning of reflectors. The representation of the main cleaning procedure steps is shown in Figure 3.

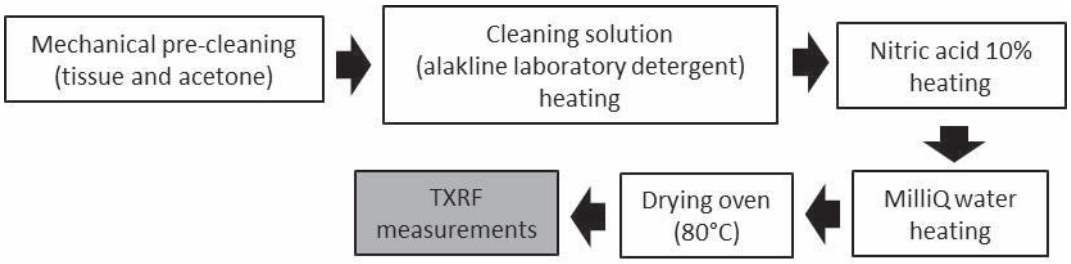


Figure 3 — Main steps followed for the cleaning of sample carriers

The time for each step has to be tested. Usually, to ensure the complete cleaning of sample carriers, the whole procedure takes about 2 to 3 h.

After the cleaning procedure, a blank spectrum has to be measured for each reflector in order to ensure the absence of contamination.

It shall be noted that the cleaning procedure and surface preparation of the carriers have impact on the quantification procedure. In particular, the surface of sample carrier might be modified during the cleaning and preparation procedure and this modification is not reproducible, leading to systematic errors in the analysis. Disposable carriers might show different backgrounds. Thus it is strongly suggested to record the background of the sample carrier before sample deposition and make the estimation of the background signal.

In the case of aqueous samples after the drying step, non-hydrophobic carriers (i.e. quartz reflectors) shall be coated with silicon solution and subsequently dried in order to favour the deposition of the target sample within a small spot on the cleaned reflector.

6.3 Sample treatment procedures for chemical analysis by TXRF

The sample preparation is not important in case the quantification is performed by XSW. In the other cases, several treatment procedures have been proposed for the analysis of liquid and solid samples by means of TXRF. Usually, the preparation processes allow to obtain thin layer sample (thickness less than 100µm) on the reflector carrier. The choice of proper sample preparation is crucial in order to ensure the total reflection conditions. A flow chart describing the sample preparation steps for liquid and solid materials is shown in Figure 4.

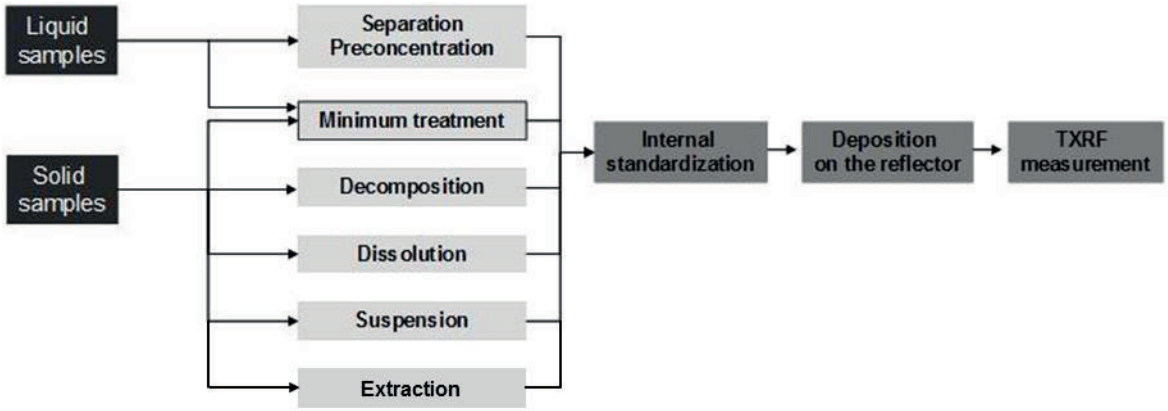


Figure 4 — Sample preparation procedures for the analysis of liquids and solids by TXRF

Internal standardization is one of the most used strategies for quantification purposes in TXRF analysis: it is based on the addition of a defined amount of liquid mono-element standard [internal standard (IS)] to the sample. To select the concentration of the internal standard, one shall verify the following conditions:

a) The IS is not present in the sample, usually Ga, Co, V, Y or some other Rare Earth Element are used;

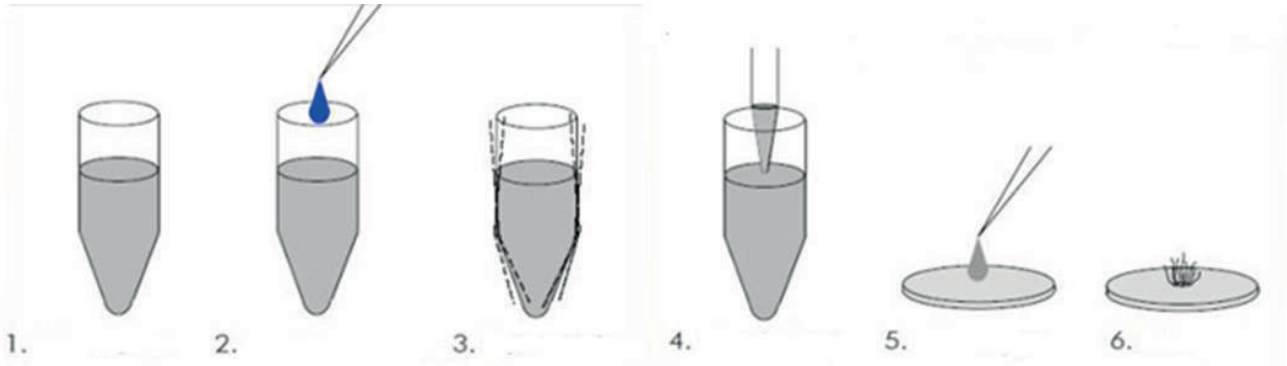
b) The fluorescence lines of the IS do not interfere with the elements to be identified and quantified;

c) The concentration of the IS shall be in the average concentration range of the elements to be quantified.

This procedure is applied to liquid samples, digested samples, and suspensions. The use of internal standard reduces the inaccuracies due to sample deposition on the sample carrier.

6.3.1 Liquid samples

For liquid samples with light matrix, TXRF analysis should be performed by depositing a small volume on suitable reflector. For the quantitative analysis, the aliquot of 500 µL to 1 000 µL of the original liquid sample is transferred to the container with a pipette and the sample is prepared by adding the suitable amount of internal standard solution. The mixture shall thoroughly be homogenized (automatic sample shaker is strongly suggested) and the drop of the solution is deposited on the carrier and dried (see Figure 5).



Key

1 aliquotation of some mL
2 addition of some µL of internal standard
3 homogenization by shaking
4 aliquotation of some µL
5 pipetting on a clean carrier
6 drying by evaporation

Figure 5 — Sample preparation steps for the TXRF analysis of liquid[1]

If only a small quantity of liquid is available, the internal standard (IS) can be added to the sample directly on the carrier. However, by these procedures, homogeneous distribution of the internal standard is not certain.

For the analysis of organic liquid samples, the IS shall be diluted in an organic solvent to guarantee the homogeneous distribution of the IS in the sample.

Few microlitres (from 5 µL to 20 µL) of solution are needed for the sample preparation. The diameter of the sample spot on the carrier shall be within the beam size (usually less than 10 mm) in order to ensure the complete exposition of the drop to the X-ray beam. If a higher sensitivity for specific element is needed, multiple droplets (from two to a maximum to be estimated with respect to the critical mass) should be deposited sequentially on the same spot, allowing each droplet to dry before the following deposition. The drying step should be performed in vacuum or by a dryer, with IR-lamps, laboratory ovens, or heating plates. Contamination effects shall be excluded by testing a reference sample. Nanodroplets or pico-droplets are suggested to attain reproducible spot and decreasing the drying time (20 s to 170 s for 2 nL droplets).[5] A new technique for the deposition of standard solutions in Total Reflection X-ray Fluorescence spectrometry (TXRF) using pico-droplets generated by ink-jet printers and its applicability for aerosol analysis with SR-TXRF are discussed in literature.[6][7] This sample preparation methods can be used when small quantity of the sample is available.

If in the liquid sample the concentration of salts is high, the dry spot on the carrier appears inhomogenous. In order to comply with the thin layer condition, dilution of the liquid sample is then necessary. Dilutions by ultrapure water or diluted solutions of a commercial non-ionic detergent (i.e. Triton® X-114[1)]) are suggested. Alternatively, the addition of a film-forming lubricant, e.g. polyvinyl alcohol (PVA), can be used to facilitate homogenous sample layer formation.[8]

If the element concentration to be quantified is lower or close to the detection limits, preconcentration procedures can be applied. The determination of volatile elements and the attainment of element speciation information is also possible when using specific sample treatments before TXRF analysis. For instance, Hg, difficult to measure because of sample losses during the drying step, can be determined at trace levels by with the help of complexing agents.[9]

1) Triton® X-114 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

6.3.1.1 Sample preconcentration

Sample preconcentration treatments are commonly used for analysis of liquid samples by means of atomic spectroscopic techniques such as flame atomic absorption spectroscopy (FAAS) or inductively coupled plasma atomic emission atomic spectroscopy (ICP-AES).[10] These procedures can also be applied for the preparation of TXRF samples.

The preconcentration procedure suggested for TXRF analysis are based on complexation and extraction of the analytes. In Figure 6, the main steps of this procedure are shown.

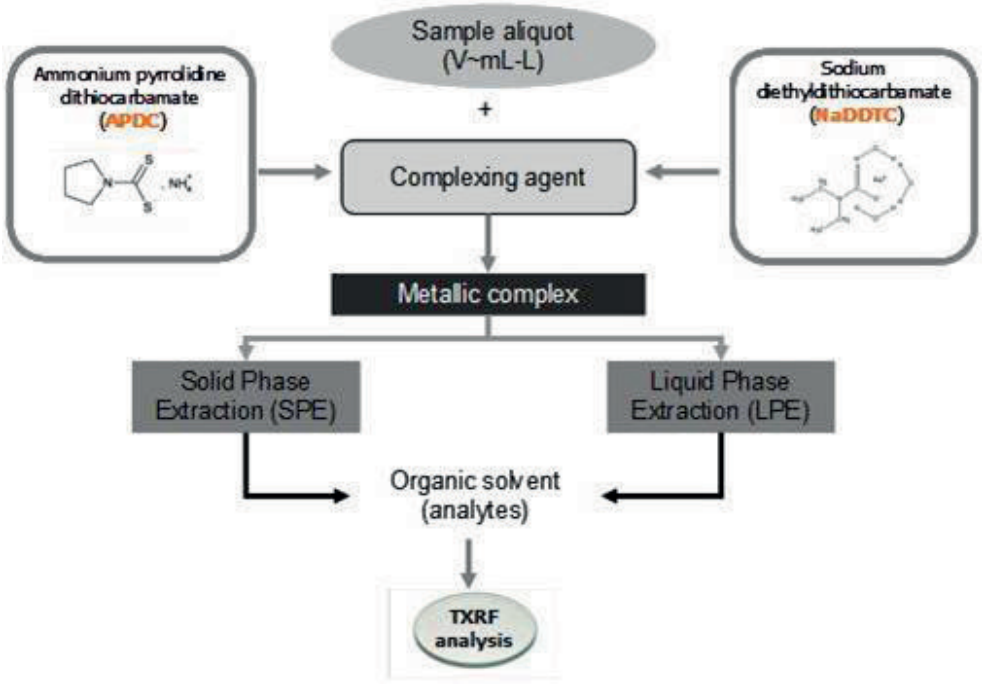


Figure 6 — Main steps of preconcentration procedures based on complexation and extraction of the analytes prior TXRF analysis

Solid-liquid extraction and liquid-liquid extraction are based on the complexation of elements under investigation using carbamates followed by solvent extraction with an organic reagent. Then, the water-immiscible liquid phase is collected and dried on a sample carrier before analysis.

A reduction of limits of detection can be achieved by the use of cation exchange columns to decrease cationic metal content in the target solution. Using this approach, cation species are retained in the cation exchange column whereas anionic species remain in the target solution and can be therefore determined. This procedure has been already applied for selenium determination in soil leachates containing high amounts of Zn and Ca.[11] [12]

Since the choice of the treatment can influence the results, it is strongly suggest to test at least two different methods for the analysis.[13]

6.3.2 Solid samples

TXRF technique can be applied to the analysis of solid samples with or without sample treatment procedures (i.e. dissolution, suspension, decomposition).

The direct analysis of fine powder sample entails the deposition of few grains on the sample carrier by means of an clean cotton wool bud (Q-tip) as sampling device. This procedure can be applied for the analysis of scarce or high valuable samples. With a dry cotton bud, a few grains are rubbed of carefully from the area of interest and deposited on a sample carrier coated with a thin layer of vacuum grease. Since the detector is placed close to the carrier, sample thickness shall be lower than detector distance

from carrier surface (i.e. 0,5 mm) to prevent damages. This preparation procedure is not suitable for quantitative analysis and only the element percentage can be obtained. In Figure 7, an example of sampling of small particles of an ink-written letter from a parchment is shown.

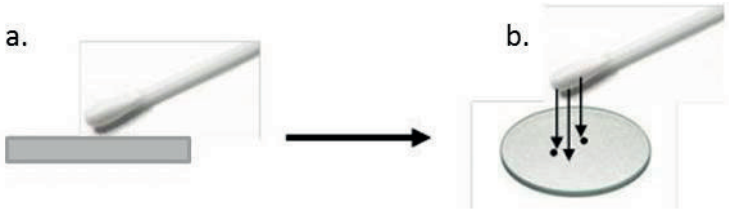


Figure 7 — Sampling of small particles of an ink written letter from a parchment for TXRF analysis, a. Sampling using a Q-tip; b. Deposition of small particles on the sample carrier

For quantitative analysis, solid samples have to be dissolved or transferred into a suspension.

Dissolution procedures similar to those employed for atomic spectroscopic analysis can be used to achieve limits of detection at the mg/kg to µg/kg range. The choice of the preparation procedure depends on the sample. Always, high-quality reagents (i.e. Suprapur quality) have to be used in order to avoid contaminations. All the reagents shall be checked before use. The use of a microwave oven is suggested to avoid analyte losses and contamination from other samples or from the surroundings. Often it is necessary to concentrate the solution to achieve the required sensitivity and preconcentration treatments shall be performed.

For suspensions, diluted solutions of commercial non-ionic detergents (i.e. Triton® X-114[1)]) are commonly used. The sample shall be powdered, with grain sizes smaller than 75µm by using suitable devices (mortar, ball mill etc.). Typically, 20 mg to 100 mg (depending on the available sample amount and homogeneity) of the sample are weighed in a lockable container with a volume of about 1 ml to 5 ml of the detergent solution. A blank sample is also needed. The choice of adequate suspension concentration (sample/dispersant ratio) is crucial: if the condition of thin layer is not reached, the analysis is not performed under total-reflection conditions and important errors on the analytical results are expected. [14]

After the dissolution or suspension of the sample, to perform quantitative analysis, the proper amount of IS shall be added. After thorough homogenization, few microlitres of the mixture are dropped to the sample carrier. Otherwise, the sample can be weighted and the given mass of IS added. The concentrations are usually calculated in µg/kg.

If solid samples contain high amount of organic matter, as in the case of some biological samples, the separation of the matrix by cold plasma or microwave ashing is strongly suggested to improve the analytical results. In fact, these procedures allow the gentle oxidation of organic matter, which also reduces the thickness of samples optimizing the conditions for TXRF experiment.[15]

6.3.3 Preparation of the Internal Standard solution

For the preparation of the Internal Standard, the use of balance instead of volume is strongly suggested, since the weight value is generally more accurate and precise. An example is reported below.

EXAMPLE In this example, a generalized procedure for the preparation of an IS solution of Ga with concentration 1 mg/l from a standard solution with concentration 1 000 mg/l is reported. Perform two-step dilution from the original standard (Ga concentration [Ga] = 1000 mg/l): a) First dilution 1:10 from [Ga] = 1 000 mg/l to [Ga] = 100 mg/l; b) Second dilution 1:100 (in the specimen) from [Ga] = 100 mg/l to [Ga] = 1 mg/.

7 Data Collection and Storage

7.1 Preliminary remarks

TXRF spectra recorded with energy dispersive detectors can be analysed qualitatively and quantitatively. Position and shape of liquid residues can be different for each sample changing the X-ray intensities. Therefore quantitative analysis of liquid residues is usually performed by means of internal standard addition. Quantification of each element with respect to the internal standard element is performed using relative sensitivity factors.

7.2 Data collection

At least three independent specimens shall be prepared and analysed. If the required confidence level is not reached for the considered elements, more independent samples shall be tested. Measure and analysis conditions shall be registered for all the tests.

It is a common practice to display graphically the collected data in real time on the monitor. At the end of the scan, spectrum data are manually or automatically saved and stored in the computer. Corrections for live time, if not automatic, shall be considered after spectrum collection.

Whenever possible, it is recommended to save the data according to ASCII code, in two columns, where one column reports the energy of the collected point and the other column reports the number of counts detected at the given energy.

8 Data Analysis

8.1 Qualitative analysis

Peaks in the spectrum shall be present. Peaks are distinguished to be excitation X-rays, Compton line of the excitation X-rays, excited X-rays from substrate material, excited X-rays from sample materials and artefact caused in the detector. All the peaks of the identified elements shall be detected in the spectrum. Escape, shelf and pile-up peaks shall be recognized. In the analysis, attention should be paid to select the elements present in the sample carrier (i.e. Si) and in the environment (i.e. Ar).

8.2 Quantitative analysis

8.2.1 Preliminary remarks

Based on the analysis conditions that have been set and stored beforehand, concentration values for the selected elements are calculated from the data by means of the internal standard method or by other methods that shall be specified. It shall be noted that the conditions specified above shall be fulfilled for a reliable quantification. The main effects that can compromise the analysis reliability are the lateral spreading of the dried droplet and the vertical thickness of the residues. The lateral spreading affects the effective solid angle of detection, thus can lead to underestimation of the deposited mass, while the vertical thickness affects the fluorescence intensity by changing the irradiance of the X-ray Standing Wave (XSW) field.[3][16] These effects depend mainly on two experimental parameters: specimen thickness and area-related mass of carrier covering. These parameters should be in a range of values experimentally determined for the specific matrix and/or theoretically estimated for the particular conditions.[1] The maximum values should be defined by taking into account, at least, the geometry of excitation, the limited counting capability of the detector, and X-ray absorption of the matrix. The minimum values should be determined by considering the detection power for the area-related mass and the wave field across the carrier for the thickness.

8.2.2 Background correction

Computer-based removal of the background contribution from the measured X-ray spectrum is usually performed. The corrected spectrum consists mainly in the contribution of the X-ray fluorescence radiation

coming from the sample. There are several methods of background correction. One is the application of digital filtering to the measured spectrum. An example of this is the Fourier transformation with which the low and high Fourier frequencies are filtered out and the medium Fourier frequencies are taken as the background-corrected spectrum. Alternative methods consist in subtracting from the spectrum the approximated background curve calculated by selecting few points in the spectrum. Smoothing algorithms can be applied to the background-corrected spectrum.

8.2.3 X-ray intensities of each element

The analytical signal is the count rate of the characteristic emission of the element. To determine the count rate, the net area of the characteristic emission peaks shall be calculated from the spectrum by fitting procedures using proper software. The area of the element peaks is obtained. Net intensity is calculated by subtracting the background and considering the line overlapping, possibly due to the presence of peaks belonging to other elements, escape peaks, or pileup peaks. The proper sample preparation as a thin layer is crucial to obtain reliable quantitative results.

8.2.4 Experimental derivation of relative sensitivities

Standard samples are required for calibration procedure and to assess the quality of quantitative results. The correlation between the intensities experimentally obtained by the standard samples and the nominal concentration is defined. If the conditions described above are fulfilled, the correlation is linear.

The relative sensitivity on the element named x with respect to the reference element named b is given by the ratio between the absolute sensitivities of x and b, whereby the sensitivities are determined under identical measurement conditions. Relative sensitivities are calculated according to Formula (2):

$$S_x = \left[\frac{\left(\frac{I_x}{C_x} \right)}{\left(\frac{I_b}{C_b} \right)} \right] \tag{2}$$

where

C_x is the nominal concentration of the analyte x;

I_x and I_b are the net intensities of the peaks of x and b respectively;

S_x is the relative sensitivity of x with respect to b;

C_b is the nominal concentration of b.

8.2.5 Quantification by means of internal standard

Absolute sensitivities are seldom used in TXRF, while for calibration relative sensitivities with respect to selected element, referred as internal standard, are preferred. For the determination of relative sensitivities, different standard solutions of multi-elemental or single element are used. They are spiked with some known amount of the internal standard and measured. Elements not present in the sample shall be selected, such as Ga, Y, or Rare Earths. Relative sensitivities are used for quantification by

means of internal standard addition. The concentration of the selected element (named x) calculated with respect to the known internal standard element (named s) are calculated according to Formula (3):

$$C_x = \frac{I_x}{I_s}\frac{S_s}{S_x}C_s \quad (3)$$

where

C_x is the concentration of x;

I_x and I_s are the net intensities of the peaks of x and s respectively;

S_x and S_s are the relative sensitivities of x and s respectively;

C_s is the nominal concentration of *s*.

Other quantification methods such as standard addition methods[17] and external calibrations[18] can be used. External calibration is also usually employed when pre-concentration procedures are performed before the TXRF analysis.

8.2.6 Statistical treatment

To have statistical significance of data, at least three independent trials (residues deposited on sample carriers) shall be considered for each sample specimen (sample solution or suspension to be measured already added with standards) and the level of confidence set for the measurements shall be checked. Eventually, more trials shall be performed to achieve the desired level of confidence.

9 Information required when reporting TXRF analysis

9.1 Preliminary remarks

ISO 17025 suggests in a general way the reporting. As a general rule, the scientific report should contain all the information necessary to reproduce the experiments. Thus the complete report of TXRF analysis should contain the accurate description of the instrument and the experimental details. This section reports the general guidelines to report TXRF analysis.

9.2 Experimental details

When reporting TXRF experiments, it is important to include all the following experimental details:

a) the kind(s) of X-ray source used (i.e. anode W-tube);

b) the excitation X-rays used (i.e. W-LII-MIV);

c) the voltage applied to the X-ray source (i.e. 30 kV);

d) the current applied to the X-ray source (i.e. 750 µA);

e) the glancing angle used, (i.e. 0,60°, optional information if available);

f) the collection time (i.e. 600 s);

g) the detector area (i.e. 1 cm^2);

h) the required level of confidence (i.e. 5 %);

i) the specimen identification:

— phase (i.e. liquid, solution, or solid, powder);

— number of specimens (i.e. three independent specimens were prepared from the sample solution);

— others: photo of the residues, etc.;

j) the type and surface treatment of sample carriers:

— material (e.g. quartz glass not disposable);

— optical flatness grade (polished, 4 Lambda);

— siliconization (i.e. added with 10 µl of silicon solution);

k) the information about internal standard:

— compound, solution description, and concentration (e.g. Ga in nitric acid standard for AAS);

— method used to prepare it (by volume, by weight and detailed description of steps);

l) sample preparation:

— number of deposited sample carriers (i.e. three sample carriers were prepared by one specimen solution);

— volume or mass of specimen deposited (a total volume/mass of 30 µl/30 ng deposited);

— drop/drops deposited (3 drops of 10 µl are deposited one over the other after drying);

— drying procedure (e.g. in air at temperature less than 60°C);

m) sample pretreatment (i.e. addition of fixation agents);

n) sample dilution ratio applied (i.e. 1,01).

9.3 Analysis procedures

When reporting on TXRF analysis procedures, it is important to include the following information:

a) software corrections used (shelf, escape, pileup, if any other);

b) list of all the detected elements;

c) list of the elements inserted in the fitting procedure;

d) list of all the quantified elements.

Annex A
(informative)

Comparison of detection limits of TXRF, AAS, and ICP-MS

Table A.1 — Detection limits found in environmental analysis

Sample	Element	Method				
		TXRF	SR-TXRF	FAAS	GFAAS	ICP–MS
		Mo-Kα (µg/L)	white beam (µg/L)	(µg/L)	(µg/L)	(µg/L)
Tap water	Cr	1,7[21]	0,10[19]	3[18]	0,004[22]	0,003[23]
	Mn	1,3[21]	0,07[19]	—	—	0,007[23]
	Fe	0,8[21]	0,06[19]	5[18]	0,1[22]	—
	Ni	0,7[21]	0,05[19]	6[18]	0,07[22]	0,005[23]
	Cu	0,6[21]	0,05[19]	1,5[18]	0,014[22]	0,04[23]
	Zn	0,4[21]	0,06[19]	1,5[18]	0,02[22]	0,15[23]
	Br	0,4[21]	—	—	—	—
	Pb	0,4[21]	0,24[19]	15[18]	0,05[22]	—
	Hg	0,58[20]	—			
	Cl		2,48[19]			
Waste water		W-tube (mg/L)	(µg/L)	(mg/L)	(mg/L)	(µg/L)
	Cr	0,24[24]	0,14[19]		0,02[25]	0,08[26]
	Mn	0,12[24]	0,13[19]			0,001[27]
	Fe	0,07[24]	0,12[19]			0,03[26]
	Ni	0,07[24]	0,1[19]			0,10[26]
	Cu	0,06[24]	0,14[19]		0,08[25]	0,02[27]
	Zn	0,05[24]	0,25[19]		0,01[25]	0,02[27]
	Cd	0,003[24]			0,01[25]	0,000 1[27]
	Pb	0,01[24]	0,58[19]		0,009[25]	0,000 5[27]
Lichens		Mo- Kα (mg/L)		(mg/L)		(mg/L)
	Fe	0,008[28]		20[29]		—
	Zn	0,005[28]		3[29]		—
	Mn	0,011[28]		—		—
	Cu	0,005[28]		0,2[29]		0,1[30]
	Ni	0,005[28]		1[29]		0,2[30]
	Pb	0,003[28]		0,1[25]		0,05[30]

Table A.1 *(continued)*

Sample	Element	Method				
		TXRF	SR-TXRF	FAAS	GFAAS	ICP–MS
		Mo-Kα (µg/L)	white beam (µg/L)	(µg/L)	(µg/L)	(µg/L)
Soil		Mo- Kα (µg/g)		(µg/g)		(mg/kg)
	Cr	17[31]		1,5[29]		—
	Mn	16[31]		—		0,002 1[32]
	Fe	13[31]		800[29]		—
	Ni	8[31]		0,4[29]		0,002[32]
	Cu	6[31]		0,5[29]		—
	Zn	5[31]		6[29]		0,006[32]
	Pb	6[31]		0,2[29]		0,000 6[32]
Sediment		(µg/g)	(mg/kg)	(mg/kg)		(µg/g)
	Cr	<1[34]	0,03[19]	0,06[33]		
	Mn	<0,9[34]	0,03[19]	0,04[33]		
	Fe	5[34]	0,02[19]	0,32[33]		<10[34]
	Ni	<0,5[34]	0,03[19]	0,32[33]		
	Cu	<0,5[34]	0,03[19]	0,04[33]		
	Zn	1,2[34]	0,03[19]	0,15[33]		
	Pb	<0,6[34]	0,11[19]	0,1[33]		
Filters		Mo- Kα (ng)				
	Cr	15[35]				
	Mn	14[35]				
	Fe	11[35]				
	Ni	7[35]				
	Cu	6[35]				
	Zn	4[35]				
	Pb	4[35]				

Table A.2 — Detection limits found in biological analysis

Sample	Element	Method		
		TXRF	AAS	ICP–MS
		W Mo,Kα (µg/mL)	(µg/L)	(ng/mL)
Blood	K	5[36]; 0,97[37]*		—
	Ca	1,5[36]; 0,54[37]		—
	Fe	0,6[31]; 0,16[37]		—
	Cu	0,3[31]; 0,11[37]	0,02[38] (mg/L)	—
	Zn	0,3[31]; 0,09[37]	0,04[38] (mg/L)	—
	Ni	0,4[31]; 0,12[37]		0,188[39]
	Se	0,04[36]; 0,07[37]		0,49[39]
	Rb	0,04[36]		0,019[39]
	Pb	0,4 (µg/g)[31]		

Table A.2 *(continued)*

Sample	Element	Method		
		TXRF	**AAS**	**ICP–MS**
		W Mo,Kα (µg/mL)	**(µg/L)**	**(ng/mL)**
Urine		**Mo,Kα (µg/mL)**	**(µg/L)**	**(ng/mL)**
	Cr	0,18[37]	0,05[40]	0,26 µg/L[40]
	Mn	0,12[37]	<1[41]	0,01[39]
	Fe	0,08[37]		
	Ni	0,06[37]	0,1[42]	0,063[39]
	Cu	0,05[37]		0,047[39]
	Zn	0,05[37]		0,506[39]
	Pb	-	0,206[42]	0,017[39]
Hair		**Mo – Kα (mg/L)**		
	Ni	0,25[43]	0,11 µg/g[44]	
	Cu	0,47[43]	0,52 µg/g[44]	
	Pb	0,88[43]	-	
Pharmaceutical		**Mo – Kα (mg/L)**	**(ng/g)**	
	Cr	0,17[45]	4,8[46]	0,13 (µg/L)[47]
	Fe	0,13[45]	-	0,2 (µg/g)[47]
	Ni	0,09[45]	7,2[46]	0,05 (µg/L)[47]
	Cu	0,08[45]		47,1 (ng/L)[47]
	Zn	0,07[45]		109 (ng/L)[47]
	Se	0,07[45]		24 (ng/L)[47]
	Pb		5,8[46]	0,08 (µg/L) (15 % HNO_3)[47]

Annex B
(informative)

Case studies of TXRF analysis for environmental applications

B.1 General

Today environmental monitoring is one of the most important and most advanced analytical tasks: monitoring of contaminated land, determination of hazardous elements in air and water, classification of waste materials, ashes, sludge, and the specification of products for later recycling and disposal – new legislation forces the analysis of elements at lowest concentration levels in a huge variety of different material types.

TXRF is used to analyse total elements in soil (samples are suspended in detergent, pipetted onto carriers, and dried) and in soil water extracts after centrifuging the same sample. Lower detection limits are in the parts per million concentration range for suspended soil and parts per billion levels in soil water extracts. The total element concentration profiles (essentially spectra) are used for fingerprint analysis of soils, to capture key mineralogical differences.

Trace element analysis in water is one of the most common applications of TXRF method. With reference to water matrix, TXRF technique can be used for similar sample types such as: snow or rain water, tap water, drinking, deionized and mineral water.[59][60] Air particulate and aerosols can be modelled as thin film samples and it is possible to perform their direct chemical quantitative analysis.[3] Moreover, the use of plants and other living organisms as biomonitors to detect the spread of different trace element in air particulate is widely used.[28]

Lichens are used as biomonitors to examine the environmental pollution because of their capability of enriching their tissues with airborne trace element. TXRF quantitative analysis of the lichen samples is performed by the internal standard addition procedure.[28]

Filters are suitable for TXRF analysis. TXRF allows nondestructive multi-element analysis of trace and ultratrace amounts of elements. Using filters as the sample, systematic errors associated with the extraction, dilution or digestion are avoided.[3]

Sewage can be analysed by TXRF method after a fast sample treatment without digestion.[24]

The TXRF method allows a simple sample preparation procedure for environmental monitoring compared with AAS and quantification does not require external calibration.

The following table shows environmental matrices that can be analysed using TXRF, a brief description of the sample preparation procedure and the elements typically analysed.

Table B.1 — Environmental matrices analysed by TXRF

Matrix	Typical amount	Sample preparation	Typical elements
Soil and sediment	>25 mg	Dried, ground powder +1 % aqueous solution Triton X-100[1)], IS* Ga[48]	V, Cr, Ni, Cu, Zn, As, Se, Hg, Pb
Natural water	5 µL to 100 µL (multiple pipetting and drying steps)	Direct analysis IS Ga[21]	Mg, Cl, K,Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ba, Se, Br, As, Pb
Groundwater	as before	Direct analysis IS Ga[31]	Fe, Ni, Cu, Zn, Sr, Pb
a PEM – personal environmental monitors.			

Table B.1 *(continued)*

Matrix	Typical amount	Sample preparation	Typical elements
Bioindicator (lichens, algae, bio-films)	150 mg (or less)	Dried + acid digestion + diluted with water IS Ga[28,53]	K, S, Ca, Ti, Mn, Fe, Ni, Cu, Zn, Cr, Cl, Br, Sr, As, Pb, Rb
Particulate matter–Filter	10 µm PEM[a]	Acid digestion[49] Sample is collected directly on sample carrier[50] Filter is a sandwiched between two thin sheets of polypropylene[3]	Ti, Cr, Mn, Fe, Ni, Cu, Zn, Pb, Ba, Rb.
Sewage (waste)	5 µL to 20 µL	Digestion with aqua regia + filtration IS Ga[51] alternatively direct analysis	As, Pb, V, Cr (complete), Co, Cu, Mo, Ni, Zn.
Sludge	>20 mg	Dilution with distilled water IS Ga[52]	As, Pb, V, Cr (complete), Co, Cu, Mo, Ni, Zn.
Sea water		Direct analysis[54] [55] [56]	V, Mn, Fe, Co, Ni, Cu, Zn, Pb and U
Fly ash		[57]	
Leaching solution		[58]	

[a] PEM – personal environmental monitors.

B.2 Multi-element determination in waste water effluents

The monitoring of heavy metals in industrial waste water effluents is an important activity for many laboratories. Of special interest is the screening of elemental composition (i.e. As, Ba, Cd, Cu, Cr, Sn, Fe, Mn, Ni, Pb, Se, and Zn concentration) of inlet effluents and quantitative analysis of outlet effluents. This information are mandatory to study the efficiency of chemical treatment process aimed to eliminate metals and to comply with current established concentration limits. For this application, fast analytical methodologies which entail simple sample preparation are desired.

Considering the high amounts of suspended solids and the viscosity of industrial waste water samples, the preliminary evaluation of the effects of the sample on the TXRF analysis shall be checked in order to verify the sample preparation. For routine and screening analysis of industrial inlet and outlet effluents, TXRF analysis can be performed by depositing 20 µL of the internal standardized sample on a quartz glass reflector and using a measuring time of 1 000 s.[24] Using such conditions, the accuracy of the concentration values obtained by using Y as internal standard is typically ±10 % of the nominal concentration value. Relative standard deviation for the analysis of three independent replicates is also satisfactory, with values for the detected elements less than 10 %. A further enhancement of analytical quality of TXRF results can be achieved using more sophisticated sample treatments as microwave acid digestion.

Since TXRF analysis is performed directly on the raw waste waters, less sample manipulation and lower amounts of reagents are needed and, therefore, cost and time are reduced for the analysis. Additional advantages of the TXRF method are the multi-elemental information of the sample, the easy quantification through internal standard, and low operating costs when benchtop systems are used (they do not require cooling media and gas).

In conclusion, TXRF is suitable for the analysis of waste waters, having sufficient analytical performance and short-time for the experiment, thus increasing the productivity of industrial laboratories.

B.3 Determination of trace amounts of selenium in soil samples

Selenium soils concentrations are an important issue because of the small difference between the nutritious requirements and toxic effects. Thus the accurate determination of its value is very important

and challenging due to low concentrations within complex matrices that hamper the analysis in most spectroscopic techniques.

The use of direct analysis of soil suspensions by TXRF is interesting as a fast and relatively simple methodology for a qualitative determination of the soil multi-elemental composition. Moreover, TXRF can be successfully applied to determine the Se content when extremely small amount of sample is available (20 mg of soil are required) provided that the Se content is sufficiently high. A further enhancement of Se detection limit can be achieved using more sophisticated sample treatment on the digested soil sample. However, when using additional sample treatments focused on Se determination, information on other elements is not reliable. Selenium detection limit for direct analysis of the soil digest by TXRF is 18,4µg/L for 0,76 mg/kg of soil.[17] However, the detection limit achieved for Se is not suitable for most of environmental applications since the worldwide average concentration of Se in soils is ~0,4 mg/kg. Sensitivity can be improved when decreasing the Fe concentration in the soil digests (~700 mg/L) by the application of a liquid-liquid extraction procedure using ethyl ether or simply the introduction of Cr absorber between the sample and the detector. Using both approaches, the detection limit for Se is significantly higher (about 35 %) compared to the direct analysis of the soil digest. In any case, to get accurate results, soil concentrations of Se in the mg/kg range have to be present.

On the other hand, when Se content in soil samples is below the mg/kg level, additional sample preparation step is mandatory. Indeed, to extract Se from soil matrices, the liquid phase microextraction procedure (LPME) improves the detection limit of the TXRF analysis. The main advantages of this method are the low consumption of solvents, low cost, speed, and simplicity of operation. With the LPME sample preparation, Se detection limit of 1,1 µg/L in the soil digest corresponding to 0.05 mg/kg in the soil was achieved with only 2 mL of soil digestion solutions. This result makes TXRF suitable for the analysis of such a sample. Indeed, by using this simple and low cost sample preparation strategy, accurate results can be obtained at the low mg/kg range and the Se detection limit is almost 10 times lower than the worldwide mean concentration of Se in soils. Thus, the proposed method combining LPME and TXRF analysis is suitable for analysis. Moreover, the Se detection limit achieved when the LPME procedure was applied before the TXRF experiments is in most cases much better than those obtained by using XRF spectrometry or INAA and TXRF is also competitive with other popular spectrometric techniques such as GFAAS, ICP-MS, and AFS.[17]

Annex C
(informative)

Case studies of TXRF analysis for biological applications

Metallomics is a new frontier in the investigation of trace elements in biology and is expected to develop as an interdisciplinary science complementary to genomics and proteomics. The term metallomics was introduced by a Japanese scientist, Haraguchi, to distinguish functions of an organism in which metals are involved. In analogy with proteomics, the subject of metallomics as of a branch of study appears to be metallome. So, metallomics refers to "the study of all possible species of a metal or metalloid in a specific cell or a tissue by means of their qualitative and quantitative analysis".[61]

Indeed, trace elements are increasingly used as dietary supplements in the prevention of diseases and as clinically effective therapeutics. Trace elements has became a critical research area in applied sciences due to the unique reactivity to their respective enzyme. Essential trace elements or micronutrients are those with concentrations below 50 mg/L in humans.[62][63][64]

In this frame, TXRF is a useful tool for various clinical applications due to the small amount of required sample, achievable detection limits, and ease-of-sampling. Because trace metals such as zinc, iron, copper, and selenium play important roles in cellular and molecular processes in biology, the major goal of metallomics in biology and medicine is to facilitate the dissection of the specific biological functions associated with these trace elements.[62][65] The field of metallomics is comprised by a group of powerful quantitative technologies that can be used to determine the availability of metals in the complex biological environment.

Many kinds of samples can be analysed directly by TXRF, however some of them need a pre-treatment procedure. Some examples are following described.

A "**whole blood sample**" contains the plasma together with all types of blood cells. Several digestion methods using nitric acid have been compared and one of the main problems is that whole blood contains a large amount of Fe. Removal of excessive amount of Fe could be accomplished by extraction with HCl and methyl isobutyl ketone (MIBK).[63][66]

Blood plasma is a straw-coloured liquid component of whole blood in which the blood cells are suspended. It makes up about 55 % of the total blood volume. It is mostly water (93 % of the volume) and contains dissolved proteins, glucose, mineral ions, hormones. It can be generally stated that the layer thickness from blood plasma samples enables the direct determination of elements with an atomic number greater than 56 (Fe) without any sample pretreatment.[63][66][77]

Serum is blood plasma without blood–clotting factors. For serum analysis, there are two techniques widely used. In the first method, the peak intensity of the studied element is standardized to the Compton scatter peak of X-ray tube with Mo anode. The advantage of this method is that the sample is transferred directly to the quartz carrier after centrifugation without any pretreatment method or additive, thus reducing error sources. The second and more classic method comprises the use of an internal standard for quantification.[63][66]

Urine is a typically sterile liquid by-product of the body secreted by kidneys. The determination of transition metal ions from human urine is usually carried out after removal of the high Cl content by direct addiction of HNO_3 on the carrier, to remove a lot of background and pile-up peaks (detection limits are in the range of 1 to 2 µg/L). In the case of urine sample, Co is used as internal standard.[66]

Celebrospinal fluid is a colourless bodily fluid found in the subarachnoid space and in the ventricular system around and inside the brain and spinal cord, so the brain "floats" in it. This fluid can be analysed by direct TXRF. Background disturbance from the NaCl content can be eliminated by adding nitric acid to the sample located on the carrier plate. Low Z elements such as Na, Mg, Cl, K, Ca can also be quantified using Sc as internal standard.[63]

Teeth, bones, and **hair** are some of the animal or human tissues examples that can be analysed by TXRF in two ways: after a complete digestion or after cutted (into thin layers) preparation. Digestion preparation is time-consuming and can cause cross-contamination. For this reason, direct approach, based on tissue slices cut with a microtom, is preferred.[63]

Liver (biopsy sample) is an example of cells tissue analysed by TXRF. Cells–based experiments mostly involve cancer cells cultures. Cancer cells were fractionated by centrifugation and digested with nitric acid for 24 h without heating.[63]

Wheat flour is one of the food component analysed by TXRF for the quantification of Se. Se is an essential trace element involved in several major metabolic pathways and immune functions. The wheat sample has grain 50µm and for that reason grinding is not required. 50 mg of sample is weighted and suspended in aqueous Triton X-100 solutions. Y can be used as internal standard.[62]

The following table shows biological matrices analysed using TXRF, a brief description of the sample preparation procedure and elements typically analysed.

Table C.1 — Biological Matrices analysed with TXRF

Matrix	Typical volume	Sample preparation	Typical element
Blood-Whole	<500 µL	a) Acidic digestion, precipitation with APDC alternatively 1:1 dilution with distilled water, IS Ga[63][66]	a) Mn, Ni, Cu, Zn, Se, Rb, Pb
		b) Acidic W-digestion, Fe separation with MIBK, Ni as internal standard[63]	b) S, K, Ca, Cu, Zn, Se, Br, Rb, Pb
Plasma	<500 µL	a) Direct analysis or dilution with water, IS Ga[63]	a) Cu, Zn, Se, Br, Rb b) Gd[69]
Serum	<500 µL	Direct analysis or dilution with water IS Ga, V or Y[63]	S, Cl, K, Ca, Fe, Cu, Zn, Se, Br, Rb
Urine	<1 mL	a) Direct analysis IS Ga[63] treatment with nitric acid to evaporate Cl b) Direct analysis IS Co[63]	Cr, V, Mn, Fe, Cu, Zn, Se, As, Br, Rb, Pb b) Pt c) Gd[69]
Cerebrospinal fluid	µL	Direct analysis IS Ga[63]	Cl, K, Ca, Cr, Mn, Fe, Ni, Cu, Zn, Br, Rb
Teeth	100 mg to 300 mg	Acidic digestion IS Y[63]	Ca, Cr, Mn, Fe, Ni, Cu, Zn, Sr, Pb
Bones	100 mg to 300 mg	Acidic digestion (170 °C, 12 h) and adding Y as IS[63]	Mn, Fe, Cu, Zn, Sr, Pb
Hair	100 mg to 300 mg	Acidic digestion, precipitation with APDC and Ga as IS[63]	Ni, Cu, Zn, Sr, Pb
Liver (biopsy sample)	mL	Acidic MW- digestion and adding Y as IS[63]	Cr, Mn, Fe, Ni, Cu, Zn, Rb, Pb
Proteins	µg	direct preparation of protein suspension, standardless quantification	S, Fe, Cu, Zn
Cell culture media	<1 mL	direct analysis[67]	Cr, Mn, Fe, Zn, Cu, Se
Fermentation	<1 mL	direct analysis or dilution of suspension IS Co	P, S, Cl, K, Ca, Fe, Ni, Cu, Zn, Br, Rb, Sr, W, Mo, Pb
Wheat flour	50 mg	Suspended in aqua Titron 100 solution and adding Y as internal standard[62]	Se, P, S, Cl, K, Ca, Mn, Fe, Ni, Cu, Zn, Br, Sr, Rb.
Honey	1 g	Acidic(HNO_3+H_2O_2) MW digestion, using Ga as internal standard[68]	Ti, Cr, Mn, Cu, Zn, Pb, Br, Rb, Sr, As, Se

Table C.1 *(continued)*

Matrix	Typical volume	Sample preparation	Typical element
Plant material, leaves	10 mg	samples were ground and sieved through a mesh size of 50 lm. Powdered samples were stored in polyethylene tubes at 4 °C. One mL of extractant solution containing 1 mg/L of Ga was added.[53]	P, K, Ca, Mn, Fe, Cu, Zn
Roots		extraction[70]	K, Ca, Mg, Mn, Fe, Zn
Fish and oysters		[71]	Cr, Mn, Fe, Ni, Cu, Zn, Se
Plankton	500 µg to 100 mg	Nitric acid and hydrogen peroxide are used at 100 °C[72]	Ti, Mn, Fe, Ni, Cu, Zn, Se, Rb, Sr, Pb, P, S, K, Ca
Algae	20 to 200 µg	Slurry samples were prepared from freeze-dried algae by sonication. Solution samples were prepared by vapour-phase microwave digestion.[73] [74]	P, K, Ca, Mn, Fe, Cu, Zn, Pb Cu, Fe, Mn, Zn
Beverages: wine, juices, liquid nutritional products		Direct analysis[75] [76]	K, Ca, Mn, Fe, Rb, Sr

Four different methods of direct analysis of biological samples are used and described below: 1. Direct analysis with internal standard; 2. The method of Compton peak standardization; 3. *In situ* microwave digestion; 4. *In situ* chemical modification.

Direct analysis with internal standardization can determine concentration at low levels (µg/L) adding the analyte in the sample solution. The self-absorption and the scattering shall be minimized by an adequate sample preparation.[37] Tissues, whole blood, serum blood, urine, amniotic fluid samples can be measured with this method.

The Compton peak of TXRF spectrum depends to the sample mass. Therefore the area of this peak can be considered as a reference internal standard. This method takes the matrix effects into consideration. It is applied for the determination of Fe, Cu, Zn and Se in serum samples.[37]

In situ microwave digestion is a useful method for analysis of samples available in small amounts as biopsy tissues, blood samples for children. The micro-digestion increases the signal-to-background ratio, particularly of low energy elements, improving detection limits and providing higher recovery rates for most of the analytes.[37] [65]

In situ chemical modification methods are used for samples that need a specific preparation. Samples such as urine, amniotic fluid that contain high levels of chlorine shall be analysed with chemical modification.

For a given compound to be properly used as chemical modifier in TXRF, it shall meet two general requirements: (i) alter a physical-chemical property of system (analyte or matrix) according to the case, (ii) leave a thin film.[37]

In the growing field of **biomedical** and **biopharmaceutical** applications, TXRF will play a crucial role for a rapid determination of metals. Examples are: the rapid monitoring of Gd contrast agents in blood and urine,[69] analysis of chemotherapeutics[8] in cell suspensions, and analysis of trace metal catalyzers in active pharmaceutical ingredients.[78][79]

Annex D
(informative)

Theoretical derivation of relative sensitivity factors

The X-ray intensity ΔI_{L} of a measured spectrum L of element A, generated from the very thin layer Δt is given by:

$$\Delta I_{\mathrm{L}} = I_{0,\lambda_p} \exp\left\{\left[\left(\mu/\rho\right)_{M,\lambda_P} \rho t \cos ec\phi\right]\right\}\left(\mu/\rho\right)_{M,\lambda_P} \rho \Delta t \cos ec\phi \frac{w_A\left(\mu/\rho\right)_{A,\lambda_P}}{\left(\mu/\rho\right)_{M,\lambda_P}}$$
$$\times \frac{\gamma_A 1}{\lambda_A} \omega_A g_L \frac{\delta\Omega}{4\pi} \exp\left\{\left[\left(\mu/\rho\right)_{M,\lambda_L} \rho t \cos ec\psi\right]\right\} \quad \text{(D.1)}$$

where

I_{0,λ_p} is the glancing X-ray intensity at wavelength _P;

$\left(\mu/\rho\right)_{M,\lambda_P}$ is the mass absorption coefficient for the X-rays glancing on specimen M;

ρ is the density of specimen M;

w_A is the mass fraction of element A in the specimen;

$\left(\mu/\rho\right)_{A,\lambda_P}$ is the mass absorption coefficient for the X-rays glancing on element A;

γ_A is the jump ratio of the series shell of element A at the absorption edge;

ω_A is the fluorescence yield of the series shell of element A;

g_L is the relative transition probability of the measured spectrum L;

$\left(\mu/\rho\right)_{M,\lambda_L}$ is the mass absorption coefficient of measured spectrum L at wavelength _L for specimen M.

Assuming that the value of the depth t is very small, the two exponential factors can be approximated to 1.

$$\exp\left\{-\left[\left(\mu/\rho\right)_{M,\lambda_P}\rho t\cos ec\,\phi\right]\right\}=1$$
$$\exp\left\{-\left[\left(\mu/\rho\right)_{M,\lambda_L}\rho t\cos ec\,\psi\right]\right\}=1 \quad \text{(D.2)}$$

Adding the double excitation of total reflection, Formula (D.2) can be expressed as following:

$$\Delta I_L = 2I_{0,\lambda_p} w_A \left(\mu/\rho\right)_{A,\lambda_P} \frac{\gamma_A - 1}{\gamma_A} \omega_A g_L \frac{\delta\Omega}{4\pi} \rho\Delta t \cos ec\phi \quad \text{(D.3)}$$

As the value of the depth t is very small, the following relation can be applied:

$$w_A \rho t = C_A \left(A_{\gamma,A}/N_A\right) \quad \text{(D.4)}$$

where

C_A is the atomic surface density of element A;

$A_{\gamma,A}$ is the atomic mass of element A;

N_A is Avogadro's number.

The absolute sensitivity can be then expressed as Formula (D.5):

$$S_A = \frac{I_L}{C_A} = 2I_{0,\lambda_p} \frac{\delta\Omega}{4\pi}\left(A_{\gamma,A}/N_A\right)\cos ec\phi\left(\mu/\rho\right)\frac{\gamma_A - 1}{\gamma_A}\omega_A g_L \quad \text{(D.5)}$$

The relative sensitivity factor S_R is thus given by Formula (D.6):

$$S_R = \frac{S_A}{S_{RM}} = \frac{\left(\mu/\rho\right)_{A,\lambda_P}\frac{\gamma_A - 1}{\gamma_A}\omega_A g_L \cdot A_{r,A} \cdot E_A}{\left(\mu/\rho\right)_{RM,\lambda_P}\frac{\gamma_{RM} - 1}{\gamma_{RM}}\omega_{RM} g_{\lambda_{RM}} \cdot A_{r,RM} \cdot E_{RM}}_L \quad \text{(D.6)}$$

where

$A_{r,RM}$ is the relative atomic mass of the RM element;

E_A and E_{RM} are the attenuation factors in the solid-state detector for wavelengths λ_L and λ_{RM} respectively.

The above formula is based on the assumption that the specimen has a uniform density and a smooth surface, that monochromatized X-rays with no divergence are used, and that no multiple scattering or excitation by other elements present occurs.

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