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Determination of nutrient and toxic elements in food reference materials by suspension preparation and TXRF analysis

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Abstract

There is a need to maintain nutrient element adequacy through food consumption. Due to the risk of toxic element exposure during food consumption, an accurate and precise characterisation approach is required. This makes the development of a simpler and faster procedure a great concern, especially in Indonesia, where time-consuming methods such as atomic absorption spectroscopy (AAS) and inductively coupled plasma-mass spectrometry (ICP-MS) are still dominant. Therefore, the present work aimed to evaluate a suspension technique for food matrices in combination with TXRF S4-TSTAR (total reflection X-ray fluorescence) in Indonesia. The examination focused on standard reference materials (SRM) such as 1570a spinach leaves (SL), 1548 typical diet (TD), 1566b oyster tissue (OT), and 8418 bovine muscle (BM). The concentration of elements was determined by mixing each SRM with internal standard gallium (Ga). This was followed by the comparison of the concentration and sensitivity of Ga to each element in SRM. The TXRF performance was evaluated by quantifying nutrients such as calcium (Ca), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), and toxic element namely arsenic (As). The trueness and precision were calculated through %bias, %recovery, coefficient of variance (%CV), and HorRat ratio (*r*). The recovery of all elements in SRMs was within 81.20 - 103.35%, except for SRM TD and BM which were 61.73 - 91.70 and 73.78 - 99.41%, respectively. The CV of all SRMs was within the range of 0.63 - 9.54%, except for SRM BM and HorRat ratios which were 2.10 - 25.22 and 0.12 - 3.21%. Based on the results, the concentration of Zn was in good agreement with the primary method of neutron activation analysis (NAA). This showed that TXRF S4-TSTAR had good trueness and precision on SRM food matrices, and could be a promising method to be applied for element characterisation in Indonesian nutritional research.

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Introduction

Macronutrients such as Ca, K, and Na, and micronutrients like Fe, Zn, Se, Cu, and Mn (de la Guardia and Garrigues, 2015) play an important role in human metabolism (Yong *et al*., 2015; Jin *et al*., 2017). Although the lack of food nutrients leads to malnutrition, stunting, and a weakened immune system (Saunders and Smith, 2010; Gashu *et al*., 2016), excessive micronutrient intake also causes a variety of adverse health effects (Pike and Zlotkin, 2019). Previous research stated that toxic elements including As originating from soil pollution, food storage, and processing (Nerín *et al*., 2016) can contaminate foods. Therefore, the determination of nutrients and toxic elements in the food is indispensable to fulfil nutrient adequacy, and avoid the accumulation of toxic elements.

In Indonesia, the characterisation of nutrient and toxic elements in food is mostly conducted using AAS as a conventional and time-consuming method (Diharmi *et al*., 2019; Köhler *et al*., 2020; Hadju *et al*., 2021), as well as ICP-MS, which is high-cost and requires complex sample preparation (Indriana *et al*., 2012; Koesmawati and Arifin, 2015). Therefore, total reflection X-ray fluorescence (TXRF) is used as a developing multi-element method to overcome several characteristics that are suitable for the aforementioned obstacles. TXRF has been widely applied to nutrition and food research across the world (Marcó Parra, 2011; De La Calle *et al*., 2013; Dalipi *et al*., 2017a; Gama *et al*., 2017; Allegretta *et*

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al., 2019; Marguí *et al*., 2022), but it has not been found in Indonesia. This makes the explanation of the use of TXRF in food and nutrition research difficult. The method is cost-effective because it is fast (100 - 1000 s of analysis time), has low sample consumption, and can be used in the analysis of micro samples (µL or mg) (Tölg and Klockenkämper, 1993; De La Calle *et al*., 2013). Due to these advantages, TXRF can become a forefront and promising method in nutrition research, especially for the characterisation of nutrient and toxic element composition in food samples in Indonesia.

As compared to conventional EDXRF, TXRF is different in its geometry, as shown in Figure 1. The detector is located very close to the sample carrier (3 mm), thus leading to high sensitivity and low background noise. The incidence and reflected angle are 0°/90°, which allows the total reflection of the X-ray indecent beam when it impinges on a thin layer sample. The total reflection obtained in TXRF was due to a very small critical angle (< 0.1°). Since the method required a thin-layer sample, matrix effects are usually neglected (Klockenkämper and Von Bohlen, 1999). Generally, acid is used to destruct samples in TXRF food analysis applications (Espinoza-Quiñones *et al*., 2010; Marcó Parra, 2011; Antosz *et al*., 2012; Dalipi *et al*., 2017a; Planeta *et al*., 2021). This procedure is a time-consuming sample treatment, and also uses harmful reagents namely acid vapours that can harm the detector; hence, a simple suspension is usually considered. Allegretta *et al*. (2019) quantified six microgreen genotypes using the suspension method on P, S, K, Ca, Cl, Mn, Fe, Ni, Cu, Zn, Br, Rb, and Sr.

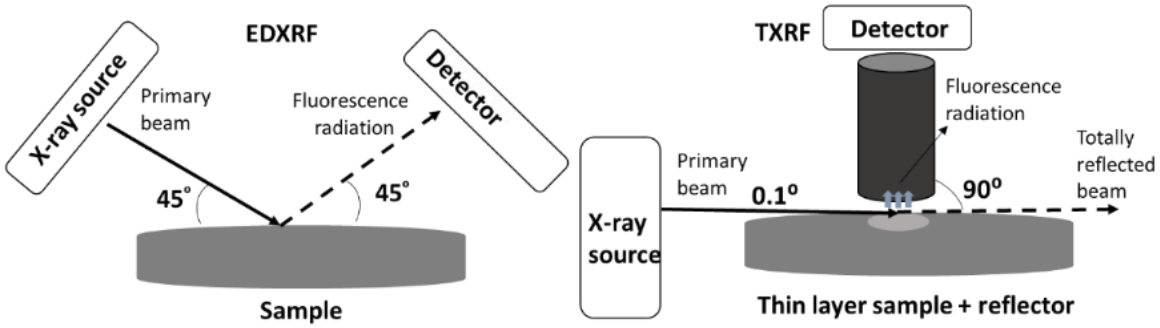

Figure 1. Conventional EDXRF (left) and TXRF (right) schemes.

Although there are several advantages of TXRF, its information on the nutrient application in Indonesia is limited. Therefore, the present work focused on the establishment of TXRF application in food matrices through the evaluation of TXRF S4-TSTAR performance by determining nutrient (Ca, Zn, Cu, and Mn) and toxic (As) elements in various food matrices - standard reference material (SRM). The SRMs applied were 1570a spinach leaves (SL), 1566b oyster tissue (OT), 8418 bovine muscles, and 1548 typical diet (TD) using the simple suspension method. The evaluation was carried out through an assessment of trueness and precision to validate the performance of TXRF S4-TSTAR on element characterisation in SRM food matrices. Subsequently, the TXRF data were compared to the results of the primary neutron activation analysis (NAA). The present work also served as a preliminary assessment of the TXRF S4-TSTAR application in Indonesia. When the TXRF method is validated, it could be implemented in the country's future food research.

Materials and methods

Sample carrier preparation

The sample cleaning procedure was based on the recommendation stated in Hagen Stosnach (Bruker, 2017b). The sample carrier (glass quartz) was mechanically cleaned using fluff-free alcohol tissues, and placed into the washing cassette that was put in the large beaker filled with Extran MA 02 liquid as a cleaning agent. The beaker was placed in an Elmasonic 100 H ultrasonic water bath at 45°C for 60 min. The washing cassette was taken out, and rinsed with deionised water. Subsequently, another 1,000 mL was placed in a beaker containing sufficient HNO_3 5N, simmered in the ultrasonic water bath at 45°C for 60 min, and rinsed with deionised water. The sample carriers were dried under an infrared lamp. The dried sample carrier was scanned individually by TXRF. The spectrum of a clean sample carrier is shown in Figure 2. To obtain a clean sample carrier, it is critical to ensure that only Si, Ar, and Mo peaks were recorded. The Si peak was generated by the

quartz glass substance of the sample carrier, the Ar peak was seen because the measurement chamber was not flushed with N_2, and the Mo peak was caused by Compton and Rayleigh scattering. Subsequently, the cleaned sample carriers were siliconised by depositing 10 µL of silicone solution into the centre of the clean one. This was followed by drying on the hotplate to create a hydrophobic surface of the quartz, and form a sample droplet during the preparation.

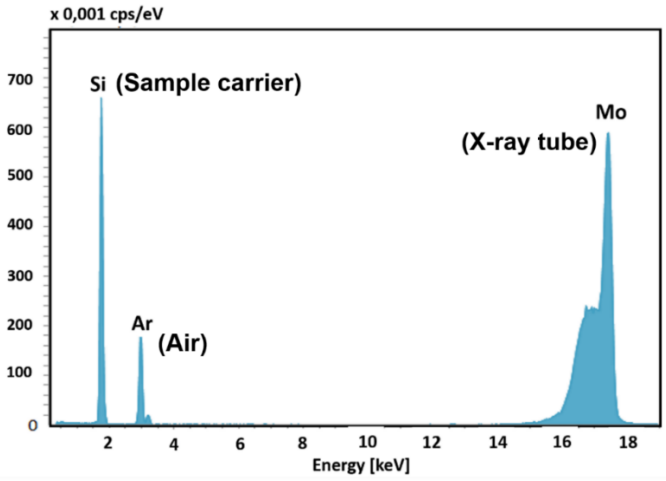

Figure 2. Spectrum of clean sample carrier. Si: sample carrier (quartz), Ar: air, and Mo: X-ray tube.

Standard reference material (SRM) sample preparation

The sample preparation scheme was based on the illustration stated in Figure 3. A total of 100 mg of SRM sample from the National Institute of Standards and Technology (NIST) (SRM NIST 1570a spinach leaves (SL), 1548 typical diet (TD), SRM NIST 1566b oyster tissue (OT), and 8418 bovine muscles (BM)) were weighed and placed in a polyethylene (PE) vial. Each sample was dissolved in 1% Triton X-100 as a dispersion agent into a 5 mL solution to enhance the sample homogeneity. Subsequently, 10 µL of 1,000 mg/L Ga was added as the internal standard solution, sonicated for 10 min, and homogenised using a vortex for ± 20 s. De La Calle *et al*. (2013) and Dalipi *et al*. (2017a) recommended 5 - 20 µL sample deposition volume to avoid matrix effects and detector damage, while Dalipi *et al*. (2017a) showed that better LODs were obtained using higher volumes (10 µL). Therefore, 10 µL of the suspension was pipetted and deposited onto a 30 mm hydrophobic quartz sample carrier, thus forming a droplet, which was dried on a hotplate. The diameter of the sample droplet must not exceed 10 mm (Bruker, 2017a; Marguí *et al*., 2022), and each

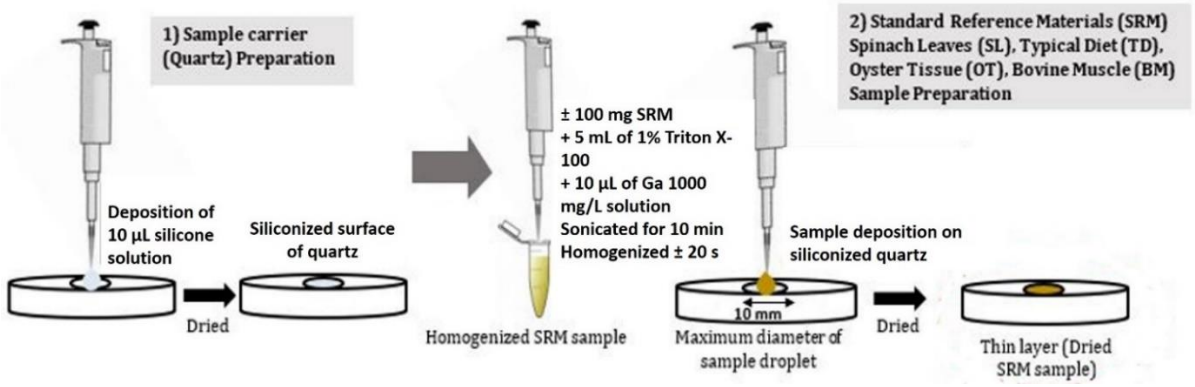

Figure 3. Preparation of thin-layer SRM (SL, TD, OT, and BM) sample by suspension method.

sample was analysed for 1,000 s using a 50 kV molybdenum (Mo-K).

Quantification of element concentration

TXRF analysis was performed on a Bruker S4 T-STAR, and the spectrometer was equipped with Mo X-ray tube anodes, metal-ceramic, air-cooled, a Ni/C multilayer monochromator, and a silicon drift detector (SDD) of 60 mm^2 with a < 149 at Mn-Kα energy resolution. The sample used was prepared as a thin layer. A grazing incidence angle of less than 0.1° was required to obtain total reflection. The detector was located a few millimetres from the sample carrier to detect the radiation effectively (Bruker, 2017b).

After being irradiated with a Mo-K beam source, the sample will excite the atom's electron, thus creating an unstable atomic state and releasing characteristic X-rays in fluorescence (De La Calle *et al*., 2013). The intensity of this characteristic fluorescence energy is proportional to the element concentration. The sample's elements were quantified using the calibration sensitivity curve with a specific sensitivity value. In this method, the sample was mixed with Ga as an internal standard, which was chosen as a reference element with the sensitivity of

Ga being 1. The sensitivity and concentration of Ga were used to quantify the elements' concentration in the sample using Eq. 1:

$$C_i = \frac{C_{IS} \cdot N_i \cdot S_{IS}}{N_{IS} \cdot S_i} \quad \text{(Eq. 1)}$$

where, C_i = element concentration, C_{IS} = internal standard (Ga) concentration, N_i = element net count rate, N_{IS} = internal standard (Ga) net count rate, S_i = element sensitivity factor, and S_{IS} = internal standard (Ga) sensitivity factor.

TXRF performance criteria in trace analysis were evaluated by calculating the limits of detection (LOD) and quantification (LOQ) of an element within the sample using Eq. 2:

$$\text{LOD} = \frac{3 \cdot C_i \cdot \sqrt{N_{BG}}}{N_i}$$

$$\text{LOQ} = \frac{10 \cdot C_i \cdot \sqrt{N_{BG}}}{N_i} \quad \text{(Eq. 2)}$$

where, C_i = element concentration, N_i = fluorescence peak area in counts, and N_{BG} = background area under the fluorescence peak (Dalipi *et al*., 2017a; Allegretta *et al*., 2019).

Validation of TXRF method

The validation of TXRF was conducted using SRM NIST SL, TD, OT, and BM by comparing the results to the certified values through trueness and precision calculations. The trueness represents the closeness of the analysis result to the actual value, and was evaluated by %recovery (%R) using Eq. 3. The %R represents the percentage of the analyte recovered when the test sample is carried out. According to the Association of Official Analytical Chemistry guidelines (AOAC, 2012), the acceptable %recovery for 1 ppm concentration is 75 - 120%, and 10 ppm is 80 - 115% in laboratory validation.

$$\%R = \frac{X\,test}{X\,ref}\ x\ 100\% \quad \text{(Eq. 3)}$$

where, *Xtest* = analytical result, *Xref* = certificate value, and %R = %recovery.

The precision was expressed as a coefficient of variance (%CV) in Eq. 4. Generally, a %CV of < 5% is deemed acceptable (Machin *et al*., 2008) and must not be exceeded by 15% (EMA, 2011).

$$\%CV = \frac{SD}{\bar{X}}\ x\ 100\% \quad \text{(Eq. 4)}$$

The Horwitz (HorRat) ratio (*r*) was calculated using Eq. 5 to obtain a more representative value for the repeatability precision. The acceptable range of HorRat values is 0.3 - 1.3. The corresponding Horwitz predicted relative reproducibility standard deviation (PRSDR) and relative standard deviation of reproducibility (RSDR) were calculated using Eqs. 6 and 7, respectively, with C expressed as a mass fraction (Horwitz and Albert, 2006).

$$\text{HorRat (r)} = \frac{RSD_r}{PRSD_R} \quad \text{(Eq. 5)}$$

$$\text{PRSDR} = 2{*}C^{-0.15} \quad \text{(Eq. 6)}$$

$$\text{RSDR} = \frac{SD}{\bar{x}} \times 100 \quad \text{(Eq. 7)}$$

Neutron activation analysis of Zn

Approximately 50 - 60 mg of each SRM (SL, TD, OT, and BM) were weighed and placed into a polyethylene vial. Subsequently, each SRM was sealed by heating the vial's cap, wrapped in aluminium foil, and ready to be irradiated along with mixed standards. The irradiation technique in the reactor followed the same procedure as stated in previous research (Damastuti *et al*., 2012). The NAA was employed as a comparison technique because it is a more sensitive, primary, less contaminated method, and the dilution of the sample is not needed (Tölg and Klockenkämper, 1993).

Results and discussion

Quantification of elemental concentration in SRMs

Figure 4 shows the performance of the TXRF suspension method in four different SRM spectra namely SL, TD, OT, and BM. The Ca's peak was obtained at the lowest energy of k-α1 at 3.692 keV, followed by Mn at 5.899 keV, Fe at 6.404 keV, Zn at 8.693 keV, Cu at 8.404 keV, and As at 10.543 keV. It was also observed that SRM SL had the highest Ca content, SRM BM had the lowest Ca content, whereas SRM OT had the highest Zn content, and SRM TD had the lowest Zn content. In Figure 4, other elements such as S, K, and Cl were also observed; however, they were not further discussed because the present work focused on nutrition such as stunting-related

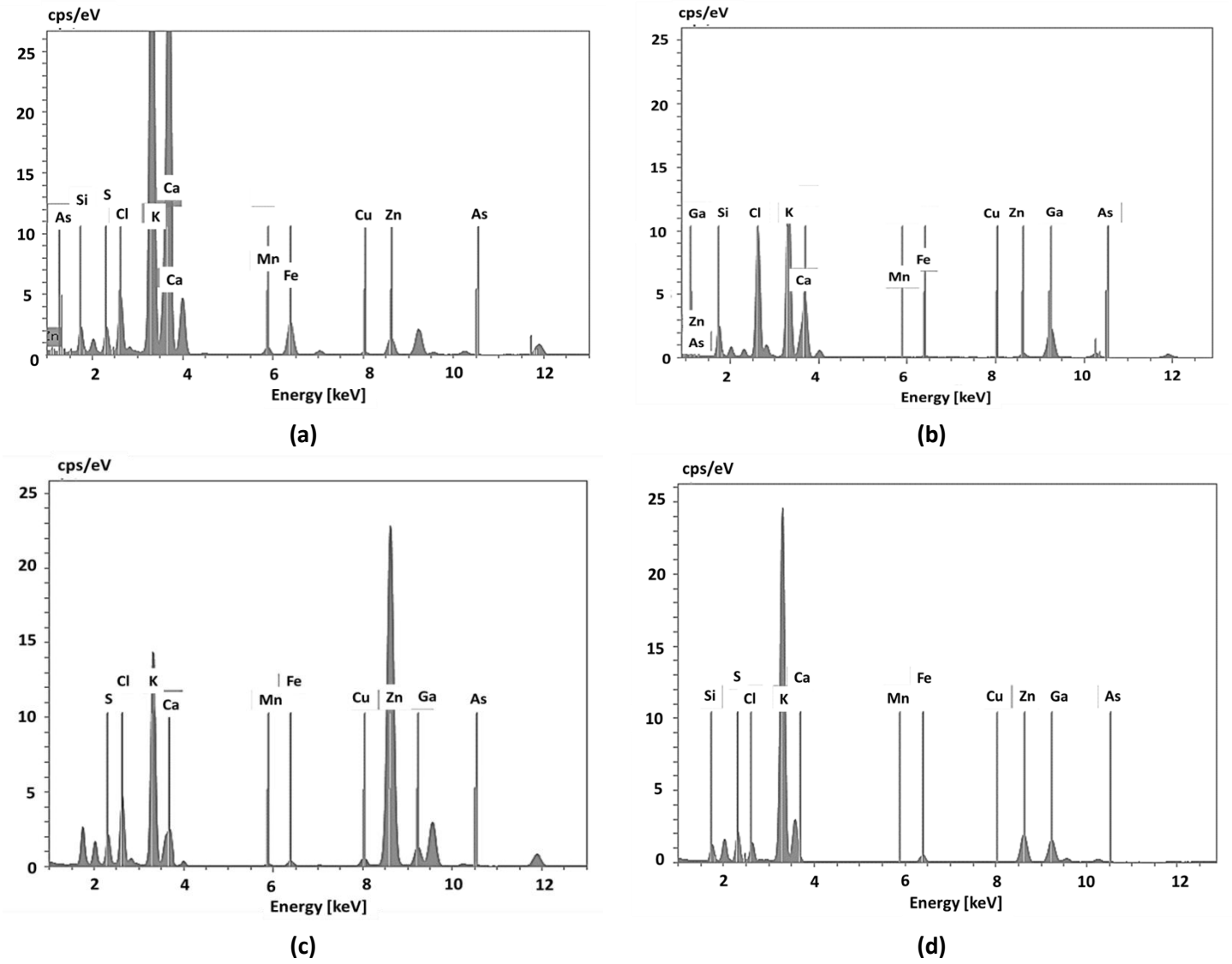

Figure 4. TXRF spectrum of **(a)** SRM NIST 1570a SL, **(b)** SRM NIST SRM 1548 TD, **(c)** SRM NIST 1566b OT, and **(d)** SRM NIST 8418 BM. SL: spinach leaves, TD: typical diet, OT: oyster tissue, and BM: bovine muscle. Each peak of elements represents the peak from k-α1 lines.

elements in Indonesia, which include Ca, Fe, and Zn (Beal *et al*., 2018; Sanin *et al*., 2018; University of Indonesia, 2019; Ramadhani *et al*., 2019).

There is a need to know the LODs and LOQs estimated from spectra parameters for each element in SRMs. The LOD represents the minimum detectable concentration of elements in the sample during concentration determination. The LOQ is the lowest concentration at which the analyte is reliably detected, and fulfils some predefined goals for bias and imprecision. The amount of sample deposited on the carrier was 10 µL to obtain better LOD (Dalipi *et al*., 2017a). Apart from the measurement period, the sample matrices were also critical for obtaining the lowest detection limits. Table 1 shows that both the LOD and LOQ generally decreased with increasing element atomic number. The LOD range of Ca, Fe, Zn, Cu, Mn, and As were 1.07 - 1.57, 0.16 - 0.40, 0.08 - 0.23, 0.07 - 0.13, 0.09 - 0.29, and 0.05 - 0.07, respectively. Meanwhile, the LOQ range of Ca, Fe, Zn, Cu, Mn, and As were 3.55 - 5.23, 0.49 - 0.85, 0.22 - 0.65, 0.16 - 0.42, 0.71 - 0.97, and 0.16 - 0.25, respectively. These results indicated that the higher the atomic number, the lower the limit of detection.

The results in Table 1 show that the lowest LOD of all SRM was 0.05 ppm (50 ppb). This confirmed that TXRF had good sensitivity, and could detect the elements down to the ppb level. This is

because the TXRF experimental set-up geometry reflects the entire beam on the reflector with an angle of less than 0.1°, which improved detection limits (Streli, 2006; Dalipi *et al*., 2017b). However, in some SRMs specifically SL, TD, and BM, the arsenic element could not be quantified since the concentration value was below the LOD.

Table 1. Statistical data of TXRF accuracy and precision in all SRMs.

SRM (*n* = 6)	Element	Measured value (mg/kg)	Certified value (mg/kg)	Bias (%)	R (%)	CV (%)	HorRat (*r*)	LOD	LOQ
SRM NIST 1570a spinach leaves (SL)	Ca	15297 ± 33	15260 ± 660	-0.24	100.24	3.80	1.02	1.24	4.13
	Fe	nd	na	nd	nd	nd	nd	0.16	0.49
	Zn	70.7 ± 0.3	82.3 ± 3.9	14.12	85.88	9.04	1.08	0.08	0.27
	Cu	10.03 ± 0.08	12.22 ± 0.86	17.95	82.05	9.16	0.82	0.09	0.30
	Mn	63.4 ± 0.3	76.0 ± 1.2	16.14	83.86	7.31	0.86	0.21	0.71
	As	nd	0.068 ± 0.012	nd	nd	nd	nd	0.05	0.16
SRM NIST 1548a typical diet (TD)	Ca	1804 ± 6	1967 ± 113	8.30	91.70	0.63	0.12	1.07	3.55
	Fe	21.79 ± 0.17	35.30 ± 3.77	38.27	61.73	9.54	0.95	0.40	0.58
	Zn	19.6 ± 0.12	24.6 ± 1.79	20.42	79.58	6.65	0.65	0.15	0.22
	Cu	1.74 ± 0.04	2.32 ± 0.16	25.17	74.83	5.33	0.36	0.07	0.16
	Mn	5.00 ± 0.10	5.75 ± 0.17	13.07	86.93	5.6	0.45	0.09	0.69
	As	nd	0.20 ± 0.01	nd	nd	nd	nd	0.05	0.16
SRM NIST 1566b oyster tissue (OT)	Ca	868 ± 4	838 ± 20	-3.5	103.35	4.51	0.78	1.40	4.66
	Fe	167.12 ± 0.68	205.80 ± 6.80	18.80	81.20	8.09	0.78	0.21	0.70
	Zn	1419 ± 4	1424 ± 46	0.35	99.65	4.01	0.75	0.23	0.65
	Cu	59.2 ± 0.3	71.6 ± 1.6	17.32	82.68	8.36	0.97	0.10	0.35
	Mn	18.0 ± 0.2	18.5 ± 0.2	2.75	97.25	2.51	0.24	0.29	0.97
	As	7.18 ± 0.25	7.65 ± 0.65	6.10	93.90	6.41	0.54	0.07	0.25
SRM NIST 8418 bovine muscle (BM)	Ca	110± 2	145 ± 20	25.03	74.97	25.2	3.21	1.57	5.23
	Fe	58.74 ± 0.37	71.20 ± 9.20	17.88	82.12	2.10	0.36	0.25	0.85
	Zn	128 ± 1	142 ± 14	9.62	90.38	7.44	0.97	0.12	0.40
	Cu	2.11 ± 0.07	2.86 ± 0.45	26.22	73.78	7.41	0.52	0.13	0.42
	Mn	0.37 ± 0.11	0.37 ± 0.09	0.59	99.41	11.05	0.59	0.30	1.00
	As	nd	0.009 ± 0.003	nd	nd	nd	nd	0.06	0.18

n: number of data repetition, na: not available, nd: not detected, R: recovery, CV: coefficient of variance, LOD: limit of detection, and LOQ: limit of quantification. Values are represented as mean.

Trueness assessment of TXRF

The results of the trueness evaluation of SRM SL, TD, OT, and BM are displayed in Table 1, which was assessed through %bias and %recovery calculation. For all elements, SRM SL was recovered at 82.05 - 100.24%, SRM TD at 61.73 - 91.70%, SRM OT at 81.20 - 103.35%, and SRM BM at 73.78 - 99.41%. For As quantification, most of As was not quantified because its content was below the LOD of As (0.05 mg/kg). This element was only detected in SRM OT as it contained higher As (7.18 mg/kg) with excellent trueness; %recovery of 93.9% and %bias of 6.1%. These trueness values of all elements were acceptable based on AOAC guidelines (AOAC, 2012). All SRM samples were well suspended with the present method resulting in good trueness, except for elements in SRM TD and BM as shown in Ca and Cu, as well as Fe and Cu, respectively. This occurred because SRMs with complex protein matrices such as SRM BM and TD were not perfectly soluble at room temperature. Therefore, further modification using an ultrasonic and acid digestion technique can be used as

an alternative method to remove the matrices' influence from the sample, and achieve higher recovery (Wagner and Boman, 2003). Since the lower value of Ca in SRM BM is due to the absorption effects of the matrix, external calibration might be necessary (Dalipi *et al*., 2017a). Based on the results, the TXRF suspension method showed excellent performance in determining the nutrient elements of Ca, Fe, Zn, Cu, and Mn, as well as a toxic element of As in all SRMs, except for Ca and Cu in SRM BM, as well as Fe and Cu in SRM TD.

Precision assessment of TXRF

Precision is the absence of random error, which is used to measure the statistical variance of the procedure, and is expressed as %CV and HorRat ratio (*r*). The results in Table 1 show that the CV for all elements was 3.80 - 9.16% for SRM SL, 0.63 - 9.54% for SRM TD, 2.51 - 8.36% for SRM OT, and 2.10 - 25.2% for SRM BM. The HorRat ratio for SRM SL, TD, OT, and BM were 0.82 - 1.08, 0.12 - 0.95, 0.24 - 1.97, and 0.36 - 3.21, respectively. TXRF had a precise result on nutrient elements, namely Ca, Fe, Zn, Cu, and Mn, as well as toxic element (As) determination. The %CV and HorRat ratio were in the acceptable repeatability range according to AOAC and other guidelines (EMA, 2011; AOAC, 2012; Couto *et al*., 2013). This suggested that TXRF had high precision when determining trace elements in various SRM (SL, TD, OT, and BM) sample matrices. For real vegetation, food, fish, and meat samples, several parameters such as particle size, fat, or protein content need to be taken into account to obtain homogeneous suspension, and gain a precise and accurate result (Dalipi *et al*., 2017a; Marguí *et al*., 2021).

Comparison of TXRF and NAA

The trueness and precision of Zn in all SRMs by TXRF were also assessed using NAA as a primary method. Figure 5 shows that the comparison between NAA and TXRF results gave a slope of 0.9793 with a correlation coefficient (R^2) of 0.9977. This correlation result was obtained by comparing six replicates data of Zn concentration in each SRM with NAA. The curve showed that both TXRF and NAA had a good correlation, and provided a similar result for Zn concentration in each SRM. TXRF was shown to be in good agreement with the NAA as the most reliable and low-error method. Therefore, the TXRF method can be used as an alternative to NAA for Zn element determination in food matrices samples because it implies a similar value.

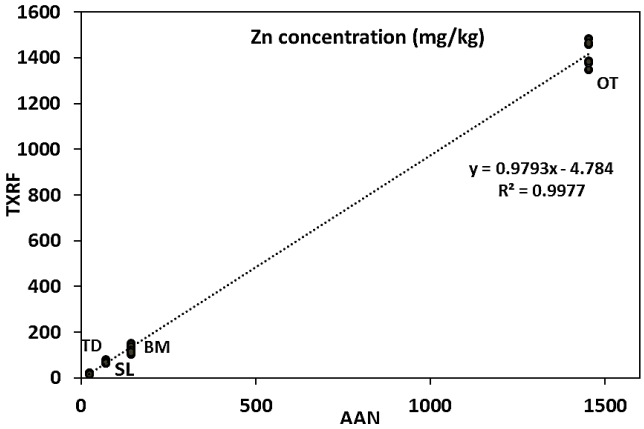

Figure 5. Comparison of Zn concentration resulting from TXRF and NAA (n = 6 for each SRM TD, SL, BM, and OT). SL: spinach leaves, TD: typical diet, OT: oyster tissue, and BM: bovine muscle.

Conclusion

The present work showed that the TXRF S4-TSTAR set up with the suspension method could offer a simple way to perform screening and reliable quantitative analysis of food sample matrices in SRMs in Indonesia. The detected limits verified that TXRF S4-TSTAR could analyse down to ppb level. These results represented good trueness and acceptable precision in examining nutrient elements namely Ca, Fe, Zn, Cu, and Mn, and toxic element (As) in the SRM SL, TD, OT, and BM. The comparison with NAA also confirmed that the method was in good agreement with NAA trace element (Zn) determination. This indicated that the TXRF S4-TSTAR could be applied in food analysis in the future elemental characterisation for nutrition research, particularly in Indonesia, where TXRF S4-TSTAR has not been applied. However, further study is needed to use the method for the analysis of real samples with different matrices, and those which involve the preparation procedures such as acid digestion for complex sample matrices to obtain complete solubilisation.

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