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DOI:

[10.1021/acs.analchem.9b03319](https://doi.org/10.1021/acs.analchem.9b03319)

Document Version

Accepted author manuscript

[Link to publication record in Manchester Research Explorer](#)

Citation for published version (APA):

Fruncillo, S., Trande, M., Blanford, C. F., Astegno, A., & Wong, L. S. (2019). A Method for Metal/Protein Stoichiometry Determination Using Thin-Film Energy Dispersive X-ray Fluorescence Spectroscopy. *Analytical Chemistry*. <https://doi.org/10.1021/acs.analchem.9b03319>

Published in:

Analytical Chemistry

Citing this paper

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A method for Metal:protein Stoichiometry Determination Using Thin-Film Energy Dispersive X-ray Fluorescence Spectroscopy

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ABSTRACT: A convenient approach is proposed for the quantitation of elemental cofactors in proteins and the determination of metal:protein stoichiometry, based on energy dispersive X-ray fluorescence spectroscopy (EDXRF). The analysis of proteins containing the metals Cu, Fe, Zn, Ca and also the non-metallic element P are shown as a demonstration of the generality of the method. In general, the reported method gives a limit of detection (LOD) and limit of quantification (LOQ) values in the low ppm range and requires only a few microliters of protein sample at micromolar concentrations. Moreover, sample preparation does not require any digestion steps before the analysis. The expected metal:protein stoichiometry was observed for each protein analyzed, highlighting the precision and accuracy of the method in all the tested cases. Furthermore, it is shown that the method is compatible with multi-meric proteins and those with post-translational modifications such as glycosylation.

Many proteins contain metal cofactors that are crucial for their structure or biochemical function. It is estimated that approximately one-quarter to one-third of all proteins require metals for their function.¹ Throughout nature, there is a large variation in the type of metal present, from the relatively small alkali earth metals (e.g., Ca and Mg) to heavier transition metals (e.g., Fe and Cu), as well as their stoichiometry within the protein. There is therefore a demand for convenient methods to identify and quantify the inorganic species that are present in proteins.

The most commonly used techniques for metal cofactor identification and quantification are derived from inorganic chemical analysis. They include inductively coupled plasma mass spectrometry (ICP-MS), which typically gives a limit of detection (LOD) at ppb levels; inductively coupled plasma optical emission spectrometry (ICP-OES), with a LOD at the ppm level; and atomic absorption spectroscopy (AAS), with a LOD between ICP-MS and ICP-OES.^{2,3} The concentration of the element of interest can then be correlated to the protein concentration (typically measured by a separate method) to give the metal:protein stoichiometry. However, despite their suitability for the study of metallic elements and the excellent detection limits, these techniques require sample pre-digestion and a relatively large amount of analyte (typically mL volumes at the relevant concentration). Protein stoichiometry can also be determined by whole protein (“native”) mass spectrometry (MS), though this method does not typically give absolute quantification and requires access of MS instrumentation that is not always readily available.^{4,5}

In the area of materials engineering, X-ray fluorescence spectroscopy (XRF) is a commonly used technique for the elemental analysis of inorganic materials.⁶ The principle of XRF is based upon the detection of X-rays emitted from sample atoms upon excitation, typically by irradiation with X-rays of

higher energy. The emitted X-rays are of energies that are characteristic for each element, therefore enabling the identification and quantification of the atomic composition of the sample. XRF-based methods can be applied to a wide range of samples, including solids, powders, liquids and suspensions; and does not require any sample pretreatment prior to analysis.

Total-reflection XRF (TXRF) is an XRF-based technique that has been used for the qualitative and quantitative analysis of metal cofactors in proteins.⁷⁻⁹ It is able to provide simultaneous analysis of several elements with LOD values in the ppm-ppb range, short analysis times (typically 2-30 min per analysis), with only a small amount of sample (few μg or μL). A related technique, microfocused particle induced X-ray emission (microPIXE), is also able to give similar performance, though it requires highly specialized equipment that is not widely available.¹⁰

An XRF technique that is more widely available is energy dispersive X-ray fluorescence (EDXRF). EDXRF has relatively higher detection limits compared to TXRF and microPIXE, but employs a less complex equipment design that has much lower instrumentation and maintenance costs; while retaining the characteristics of X-ray spectroscopy in terms of the qualitative and quantitative analysis of nearly all elements, in any physical form, as well as simplicity of sample preparation and operation.^{11,12} More recently, advances in EDXRF equipment design (e.g., the introduction of filters to reduce background signals, the ability to tailor voltage and current settings to improve the sensitivity to specific elements, the use of silicon drift detectors) have given improved signal-to-noise ratios, spectral resolution, throughput and reliability.⁶

By adapting the general principles of protein thin film TXRF, we report herein a generally applicable method that employs EDXRF for the rapid, convenient and affordable quantitation of inorganic cofactors in proteins and for accurate

determination of element:protein stoichiometry within a protein complex. The method is demonstrated on a range of proteins containing Cu, Fe, Zn, Ca and the biologically relevant non-metal P. These proteins include examples of prokaryotic and eukaryotic origin, multimeric proteins, as well as proteins bearing post-translational modifications.

RESULTS AND DISCUSSION

Experimental design and instrument calibration. It was envisaged that by employing thin-film analysis it would be possible to minimize the amount of sample material required and avoid distortions of the spectra associated with thick samples that could lead to less accurate quantification.¹³ To further maximize the signal-to-noise ratio, the analyte solutions were made up in a TRIS-acetate buffer, which had previously been reported to give good results with TXRF.⁷ This buffer is particularly suitable as it contains no elements with an atomic number >11, thus avoiding the introduction of any peaks that may overlap with the signal of interest and minimizing any intra-element absorption or enhancement effects.

For calibration, a series of thin-film standards for each element were produced from salt solutions of known concentrations by drying an 8 μ L droplet of the solution. EDXRF spectra were then recorded and calibration curves were obtained by plotting the peak area versus the original concentration of the solution (Figure 1). These plots gave good linear correlations indicating that film thickness or small variations in the size of the film had a negligible effect on the quantification.¹³ Since very low concentrations of each element were analyzed, the spectra were recorded for 1600 s in order to maximize the LOD while maintaining a reasonable duration for the proposed analysis. The calculated LOD and LOQ values were found to be in the low ppm range (Table 1), equivalent to nmol-pmol quantities of the element.

Table 1. Best linear fit calibrations for each element.

Element	Linear best fit equation ^a	R ²	LOD (ppm)	LOQ (ppm)
Cu	$I_{Cu} = 1.31[Cu] + 0.71$	0.997	1.1	3.5
Fe	$I_{Fe} = 0.86[Fe] + 24.16$	0.991	0.9	3.2
Zn	$I_{Zn} = 0.13[Zn] + 0.50$	0.993	0.8	2.7
Ca	$I_{Ca} = 0.56[Ca] + 60.39$	0.989	4.4	14.6
P	$I_P = 0.25[P] + 56.21$	0.986	5.4	18.8

^a $I_{element}$ represents the signal intensity in the EDXRF spectrum for the element (in cps), [Element] the concentration of the element (in ppm) and R² the correlation coefficient for a linear regression.

The light elements Ca and P emit photons of relatively low energy that are poorly transmitted through air,² thus to mitigate this effect the sample chamber was purged with helium prior to recording the spectra. Even so, these elements showed the highest LOD and LOQ values. Moreover, P, Ca and Fe generally showed the highest levels of background signal compared to all the other tested elements (also evidenced by the high baseline in the calibration fit), likely due to their presence as common contaminants in Mylar films and other materials commonly used during EDXRF analysis such as polypropylene and polyester films. Nevertheless, these data indicate that the LOD and LOQ are sufficiently low to allow for the proposed protein analysis.

Protein analysis. Elemental analysis was undertaken on 10 proteins containing Cu, Fe, Zn, Ca and the non-metallic ele-

ment P (Table 2). Thin-films were prepared from protein solutions of known concentration, and their EDXRF spectra recorded (Figure 2). By applying the calibration line equation in Table 1, the concentration of the metal in the original analyte solution was determined. Subsequently, using the previously measured protein concentration (expressed as the concentration of the monomer), the element:protein stoichiometry could then be calculated (Equation S1 in Supporting Information).

Table 2. Element:protein stoichiometry.

Entry	Protein	Element	Range of protein concentrations tested (μ M)	Obtained E:P ^a (\pm SEM)	Expected E:P
1	CotA	Cu	35-120	3.97 ± 0.10	$4^{14,15}$
2	MvBOx			4.13 ± 0.12	
3	CytC	Fe	200-500	1.03 ± 0.05	1^{16-18}
4	AHb1			0.98 ± 0.04	
5	HRP			0.99 ± 0.05	
6	ADH1	Zn	35-140	2.18 ± 0.11	2^{19}
7	CaM1	Ca	140-400	4.01 ± 0.20	4^{20-22}
8	CML7			3.85 ± 0.08	
9	CML19			3.60 ± 0.12	
10	HRP		250-500	1.85 ± 0.12	2^{17}
11	UbiX	P	600-800	1.30 ± 0.20	1^{23}

^a E:P represents the element:protein stoichiometry. The mean values \pm SEM from at least six independent samples are presented.

For all the proteins analyzed the ratios of the element to protein were in good agreement with the values obtained through other techniques (see refs. in Table 2). For robust stoichiometry determination, it was found that the concentration of the metal should ideally lie between the LOQ and the highest calibrant concentration used in the construction of the calibration curve. Thus, heavier elements and proteins with multiple binding sites required lower amounts of protein for successful analysis.

The stoichiometry values obtained by this method are not dependent on the quaternary structure of the protein since the calculations rely only on the protein monomer concentration. For example, AHb1 forms a homodimer^{18,24} with one Fe atom per monomer, and this method gives a metal:monomer ratio of 1:1 (Entry 4, Table 2); while ADH1 forms a homotetramer¹⁹ with two Zn atoms per monomer, giving a metal:monomer ratio of 2:1 (Entry 6, Table 2) The analysis of individual elements was also not affected by the presence of other elements or post-translational modifications. For example, commercial batches of eukaryotic proteins CytC and HRP include varying degrees of acetylation and glycosylation, respectively,^{16,17,25} but analyses of both proteins gave the expected Fe stoichiometry of 1:1 (Entries 3 & 5, Table 2). Simultaneous quantification of multiple elements from a single protein is possible, as for HRP, which contains both a catalytic Fe and two structural Ca atoms (Entries 5 & 10, Table 2).

In principle, EDXRF can also be used for the quantification of non-metal elements, provided the photon energies are within the detectable range. As an example, the protein UbiX contains a single riboflavin-5'-phosphate (flavin mononucleotide, FMN) cofactor, which itself contains a single P atom. Here, the EDXRF-determined P:protein ratio was consistent with previous literature,²³ although the SEM was relatively large

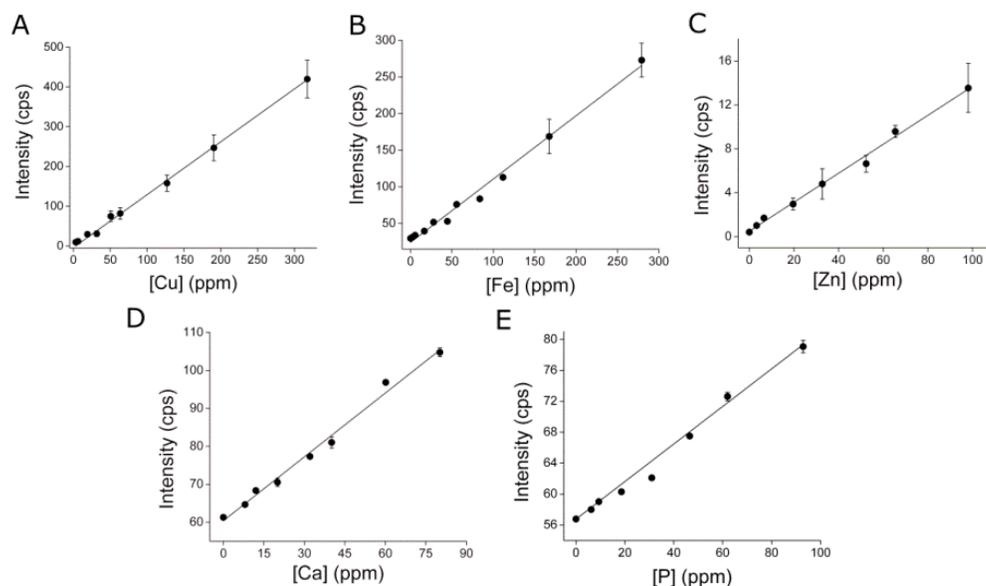


Figure 1. Calibration plots of EDXRF peak area against element concentration for (A) Cu at 0-317 ppm, (B) Fe at 0-279 ppm, (C) Zn at 0-98 ppm, (D) Ca at 0-80 ppm and (E) P at 0-93 ppm.

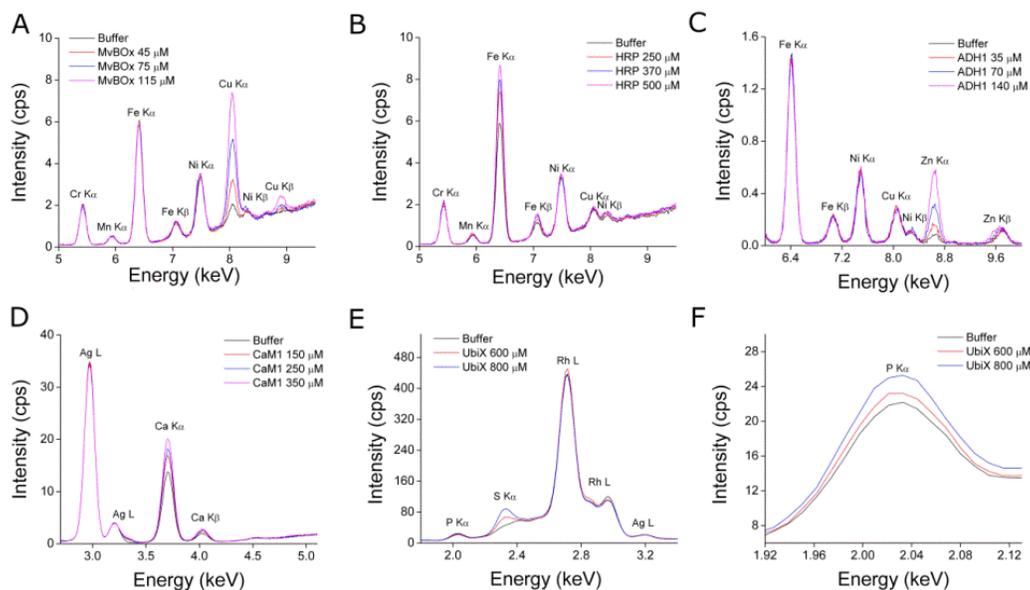


Figure 2. Illustrative EDXRF spectra of proteins samples at different concentrations. Overlaid spectra of (A) Cu-dependent MvBOx, (B) Fe-dependent HRP, (C) Zn-dependent ADH1, (D) Ca-dependent CaM1, (E) P-containing UbiX, and (F) showing a magnification of the P peak from the spectra shown in (E). The Rh signal observed in (E) is given by the Rh anode of the instrument. The spectra acquisition settings used for each element are indicated in Table S2.

due to the low sensitivity of the instrument towards light elements (Figure 2E). In comparison, elements such as Fe and Cu gave appreciable differences in spectral intensities even with differences in the concentration of the analyte solutions as low as 5 μM . Nevertheless, this method can be used to confirm or exclude the presence of cofactor binding in proteins of interest. In the case of CytC, this protein has two possible phosphorylation sites,²⁵ but no signal for P was detectable above the background even at the highest protein concentration that was analyzed, thus indicating that the CytC used in this study was in fact not phosphorylated.

Protein quantification represents a crucial component of the stoichiometry calculations. In theory, S can be detected and quantified by EDXRF, so it could be used for simultaneous protein quantification by measuring the S present in Cys and

Met residues of the protein. However, because S is a relatively light element giving only weak signals, and overlapping signals are present in the spectral region between 2.2 and 2.6 KeV caused by the Rh anode, quantification would only be possible when large amounts of S were present. For example, in the case of UbiX that has 14 S atoms, at a protein concentration of 600 μM (equating to an S concentration of 269 ppm) it was clearly possible to detect the presence of S (Figure 2E). However, the LOD was extremely high (estimated >180 ppm) making simultaneous analysis with other cofactors inconvenient at best, and impossible in cases where this value was higher than the solubility of the protein of interest.

CONCLUSIONS

In the present work a simple and accurate thin-film EDXRF method has been developed and validated for the determination of metal stoichiometry within protein complexes. This approach was demonstrated with a range of proteins containing Cu, Fe, Zn and Ca, as well as with one protein containing the non-metal P. The method gives LOD values between 1 and 6 ppm and LOQ between 3 and 20 ppm with a minimal consumption of sample (pmol), and does not require pre-digestion prior to analysis. It is generalizable across proteins of different molecular weights, oligomerization states, the type(s) and number of bound metal(s), as well as post-translational modifications (e.g. glycosylation, and acetylation). The quantitation of multiple elements within a single protein is also possible.

Though only a selected range of biologically relevant elements was investigated using existing EDXRF equipment, in principle this type of spectroscopy allows the quantification of any element between Na and U, provided the appropriate instrument setup is available. By combining the fact that only minimal sample preparation is required and EDXRF instruments are relatively widely available, this method represents a competitive alternative to other methods for the quantitation of elements in proteins. It is intended that the method reported here can be employed for the analysis of novel synthetic proteins containing transition metals such as Rh or Ru;²⁶ the validation of currently uncharacterized metalloproteins where the metal binding capability is only predicted through sequence-based bioinformatic analysis; or in the quality assurance of recombinantly produced metalloproteins.

ASSOCIATED CONTENT

Supporting information

Experimental Methodology; Table S1: Characteristics of proteins analysed with EDXRF; Table S2: EDXRF instrument settings.

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Author Contributions

SF and LSW designed the research. SF and MT performed the experiments. SF, MT, AA and LSW analyzed the data. All authors wrote and edited the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

SF acknowledges from the University of Manchester (UK) and the Agency for Science and Technology Research (Singapore) for an A*STAR Research Attachment Programme PhD studentship. CFB and LSW acknowledge funding from and the N8 Agrifood Programme (UK). MT and AA acknowledge funding from the University of Verona (Italy) (FUR 2018). The authors acknowledge the use of the School of Materials X-ray Diffraction Suite at the University of Manchester and the technical support, advice and assistance provided by Dr. John E. Warren.

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