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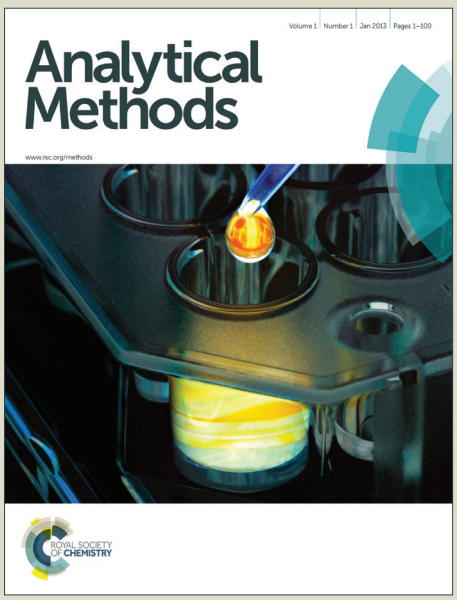

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Using X-ray Fluorescence to Measure Inorganics in Biopharmaceutical Raw Materials

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Small deviations in metal content found in biopharmaceutical raw materials can have detrimental effects on cell activity and growth. Here we report the use of a portable energy-dispersive x-ray fluorescence (EDXRF) spectrometer for elemental analysis of powdered raw materials (hydrolysates and chemically defined media) to help maintain consistent therapeutic protein quality and production. Unlike traditional metal analysis techniques such as inductive coupled plasma mass spectrometry (ICP-MS), EDXRF analysis requires no sample preparation and acquisition times range from 2 to 10 minutes for a sub-ppm limit of detection for elements such as Cu and Zn. However, issues with sensitivity, matrix interferences and calibration standards have prevented EDXRF from being adopted in the biopharmaceutical industry. This paper presents an alternative method to overcome these limitations, involving: measuring raw materials before dilution to ensure the largest metal concentration; the use of wavelet transforms to process EDXRF spectra, removing background and matrix variability; and utilizing the resultant spectral intensity to correlate to cell culture process parameters before developing calibration standards. Finally, a brief case study will outline the methodology and illustrate the high throughput of the EDXRF spectrometer for identifying the raw material and quantify the key trace metal associated with process attributes.

Introduction

Metals in cell culture media can affect cell growth and production in different ways. For example V is linked to cytotoxicity[1], Mn to glycosylation[2], and Cu and Zn to yield and performance[3, 4]. Minor metal variations can have a large impact on bio-therapeutic consistency and can lead to the termination of batch processing during drug manufacturing, costing time and money to resolve[5-7]. Although there is a large body of literature discussing the importance of nutrient-relevant inorganic elements for good cell growth, there is very little discussion correlating the variability of elements in raw materials with cell performance. One reason is that elemental analysis in the pharmaceutical industry is typically performed with atomic absorption spectrometry (AAS), inductively coupled plasma-atomic emission spectrometry (ICP-AES), and/or inductively coupled plasma-mass spectrometry (ICP-MS)[8]. These methods all tend to be time and labor intensive with regards to sample preparation and instrument maintenance. Moreover, AAS and ICP instruments are large, expensive and costly to operate, using large quantities of purified gas. A promising alternative for rapid multi-element analysis that is cheaper, smaller, and requires little to no sample preparation is energy-dispersive x-ray fluorescence (EDXRF).

X-ray fluorescence refers to the emission of "secondary" radiation (fluorescence) from a material after excitation by high energy x-rays[10]. The energy of the secondary radiation is unique to the element and its intensity can be directly related to the abundance of that element, i.e., its concentration. There are several different types of XRF spectrometers. Wave-length dispersion x-ray fluorescence (WDXRF) has been explored to measure heavy metals in active pharmaceutical ingredients[9]. Its adoption in the industry has been limited due to a balance between efficiency versus cost and size. Total x-ray fluorescence (TXRF) has also been explored for testing trace metals in active pharmaceutical ingredients, medical samples, and serum.[11-14] Although TXRF is ~100 times more sensitive than EDXRF, it is best suited for liquid samples and hence necessitates sample preparation for powder samples. In contrast, an EDXRF spectrometer can measure many different matrices including liquids, powders and solids. Advances in research have enabled the fabrication of EDXRF spectrometers that are safe, easy to use, have good spectral resolution, sub parts per million (ppm) sensitivity, fast acquisition times, and reproducible results. The use of a portable EDXRF spectrometer has been successfully demonstrated for concentrations on the order of 1-10 ppm (mg/kg) for many FDA-regulated products such as infant cereals, grains and

vegetables, liquid dietary supplements[15] and metal impurities in tablets.[16-19] Although previously explored for soy-hydrolysate[20], the potential for EDXRF in measuring nutrient-relevant metals in powdered raw materials for the biopharmaceutical industry has not been fully realized.

In this paper, the methodology and analytical figures of merit for using an EDXRF spectrometer to quantify trace metal levels in powdered biopharmaceutical raw materials, with minimal sample handling, are presented. The method includes wavelet transformation, similar to Arzhantsev *et. al*[19], to remove the background "Bremsstrahlung" radiation and filter the raw spectra for better selectivity and sensitivity. Since the accuracy, precision and detection limits are a function of the element's sensitivity and measurement duration, a simple formula to help determine an appropriate acquisition time for the elements of interest is given. Finally, the methodology is validated in a case study, which was able to identify the key ingredient and trace metals responsible for variability in a product attribute.

Materials and methods

Samples

Elemental analysis of different nutrient powders was explored. The samples were prepared for EDXRF analysis by weighing 5 g of powder into a double open-ended disposable XRF cup (SC-4231: Lab Premier Supply, Port St. Lucie, FL) capped with 4 μm thick ultralene windows (SPEX Certiprep, Metuchen, NJ). To minimize matrix effects and maximize packing density, XRF cups with powder samples were gently tapped on a hard surface 5-10 times. Due to the fine grain size (10 - 400 μm) of the nutrient powders no further packing was necessary.

Reference Standards

Both liquid and powder reference standards spanning a wider range of concentrations than the raw materials were used to test the linearity of the EDXRF spectrometer. For liquid standards, ultrapure water was mixed with varying volumes of either 1000 ppm V, Mn, Fe, Ni, Cu, Zn, As, Se, Br, Rb, Sr, or Mo solution purchased from SpexCertiPrep. Powder calibration standards used an L-glutamine (Sigma Aldrich) base matrix spiked with different concentrations of the metal solutions. The mixture was frozen using liquid nitrogen and lyophilized for 1 day. The remaining powder was ground with mortar and pestle and transferred to an XRF cup. A total of 20 powder reference standards and 28 liquid reference standards were made with concentrations ranging from 0 – 100 ppm.

Portable EDXRF Spectrometer

All XRF experiments use the Tracer III-SD energy dispersive hand-held XRF spectrometer (Bruker AXS Hand-held, Inc., Kennewick,WA) outfitted with a rhodium (Rh) x-ray tube and a 10 mm^2 XFlash silicon drift detector. The multichannel analyzer resolution is approximately 20 eV per channel and the instrument resolution is < 155 eV (Mn Kα, 1500 cps). The spectrometer operates at a voltage of 40 kV, and a current of 30 μA for integration times ranging from 60-600 sec. A filter composed of 0.001" Ti and 0.012" Al is used to optimize the excitation conditions for elements ranging from titanium to silver (Ti-Ag). A collimated elliptical x-ray beam, ~ 10 mm x 7 mm, is emitted at 53° onto the samples, which are positioned on an automated sample changer (DeWitt Systems, Inc., North Augusta, SC). Stable multi-elemental discs supplied by Brammer Standards are used once a month to monitor for EDXRF drift and intensity.

EDXRF Analysis

The EDXRF spectra are collected using Bruker's software (S1PXRF 3.8) and analyzed with an in-house program written in Matlab (MathWorks, Natick, MA ver. R2012a) utilizing the Matlab wavelet toolbox (MathWorks, ver. R2012a). The program loads the EDXRF spectra, calculates the normalized wavelet coefficients corresponding to the energy for the element of interest, and compares it with ICP-MS results to compute the metal concentration.

Inductive Coupled Plasma- Mass Spectrometry (ICP-MS)

ICP-MS measurements of nutrient powders were used to calibrate the EDXRF spectrometer. Both an in-house ICP-MS (ELAN DRCII, Perkin Elmer) and an ICP-MS from an external lab (Exova Inc., Santa Fe Springs,CA) were used. See supplemental information for ICP-MS parameters of operation.

Cell Lines and Culture Conditions

A mouse myeloma (NS0) cell line was used for monoclonal antibody production. A fed-batch process with this cell line was performed in 15 kL bioreactors containing 10 kL cell suspension. The feed for cell culture was prepared using nutrient powder. Small-scale studies were performed in 1 L shake flasks containing 200 ml cell suspension. To evaluate the effect of Cu on cell metabolism, a set of experiments was carried out with copper (II) sulfate pentahydrate (J.T Baker) added to cultures containing low-Cu nutrient powders.

EDXRF Wavelet Transform Analysis

Figure 1a shows the EDXRF spectrum for a nutrient powder used in cell culture media. The sharp spectral peaks below 18 keV are the fluorescent signatures of metals found in the powder; most noticeable are K at 3.3 keV, Fe at 6.4 keV, Zn at 8.6 keV and Br at 11.9 keV. The broad signal from 10-35 keV, often referred to as the Bremsstrahlung continuum, is associated with elastically and inelastically backscattered x-rays. Elastic scattering refers to x-rays that do not lose any energy when scattered from the medium and inelastic refers to x-rays that impart energy to the medium. A filter can be used to shift this background to optimize the excitation for particular elements of interest. In this case, a filter suited to detect elements ranging from titanium to silver is used. The peaks at 20.2 keV and 22.7 keV are the elastic backscattered peaks associated with the Rh target and the broader more intense

peaks at 19 keV and 21 keV result from inelastic (Compton) scattering associated with the Rh parent peaks. The intensity of the elastic and inelastic scattering is affected by the sample matrix, i.e., its state (solid, liquid, or powder), the material type, and/or its density. It is therefore challenging to measure elements with energies located on top of the background envelope. However, use of wavelet transformation with normalization to the Compton peak intensity reduces errors by removing variation in the EDXRF background as well as improving selectivity of the peak by removing high frequency noise from the spectrum. The first use of wavelet transformation for EDXRF analysis was by Arzhantsev *et al.*[19] to provide a pass/fail criterion for impurities in pharmaceutical tablets. Here, the method is developed further by incorporating spectral normalization for higher accuracy.

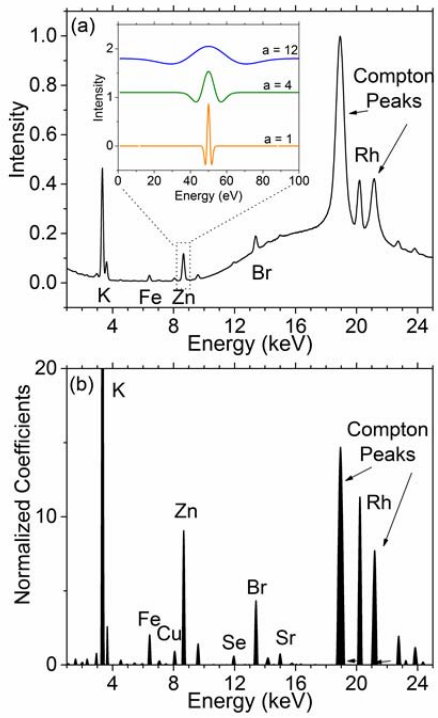

Fig. 1 Wavelet transforms of EDXRF spectra. (a) EDXRF spectrum of a nutrient powder. The broad signal from 10-35 keV, the Compton and Rh peaks, are due to emission from the EDXRF analyzer itself. The sharp peaks for energies < 18 keV correspond to x-rays emitted from metals in the raw materials. The inset plots show the Mexican hat wavelet for different scaling parameters. The wavelets are translated across the EDXRF spectrum and at each location multiplied and integrated. The resulting coefficients for a scaling parameter a = 4 and normalized by the peak Compton intensity at a = 12 are plotted in (b). The maximum value is used to calculate the concentration of metals in raw materials.

Wavelet transformation is a signal processing technique based on discretizing a signal, s, by multiplying it by a finite, oscillatory function, ψ (called the wavelet) and integrating the result[21]. In the case of EDXRF, the signal is the spectrum and so s and ψ are written as functions of energy E. Since the EDXRF spectral features are Gaussian in shape, the Mexican Hat wavelet (the negative normalized second derivative of a Gaussian function) is selected as the oscillatory function[19]. Mathematically it is given by,

$$\psi(E) = \frac{2}{\sqrt{3}\pi^{1/4}}(1 - E^2)\exp\left(-E^2/2\right). \quad (1)$$

This "mother" wavelet can be further decomposed using the scale parameter a and translation parameter b such that,

$$\psi_{a,b}(E) = \frac{1}{\sqrt{a}}\psi\left(\frac{E-b}{a}\right). \quad (2)$$

The wavelets for three different scaling parameters are depicted in the inset of Fig. 1a. As the wavelets are translated across the spectrum, they are multiplied with the EDXRF signal and integrated. The resulting wavelet coefficients are thus calculated by,

$$C_{a,b}(E) = \int_R s(E)\psi_{a,b}(E)dE. \quad (3)$$

Wavelet transformation is a type of bandpass filter. Wider wavelets pass wider spectral features; narrower wavelets pass narrower spectral features. The optimum value for the scale parameter a is that which passes the spectral features of interest but dampens other features[19]. The optimum a for the EDXRF spectral features is 4 while that for the Compton peak is 12. In contrast to Arzhantsev *et al.*, who only considered a = 1 and a = 4 for limit tests, we use the intensity of the Compton peak when a = 12 to normalize a = 4 coefficients to determine metal concentrations. Normalizing helps reduce noise associated with instrument intensity fluctuations, dead time, and sample presentation. The resulting normalized coefficients for a nutrient powder are plotted in Figure 1b.

Our wavelet transform algorithm to calculate metal concentrations is as follows:

1. Acquire spectral data,
2. Calculate the coefficients for a = 4 and a = 12,
3. Normalize all coefficients for a = 4 by the Compton peak when a = 12,
4. Select the normalized peak intensity for the elements of interest,
5. Determine the concentration by relating the peak intensity to reference standards or ICP-MS measurements used to calibrate the EDXRF.

Results and discussion

The performance of the EDXRF was evaluated for typical trace metals in powdered biopharmaceutical raw materials, namely elements from V to Mo with concentrations from 0.5 ppm to 100 ppm.

Selectivity

As seen in Fig. 1a, the Bruker portable EDXRF spectrometer can measure a large range of elements in one scan (i.e., Al-U) including both nutrient-relevant metals and impurities. Since the specificity is related to the inter-orbital transitions and the detector resolution, interferences may result from the overlap between multiple transitions or from sum peaks. Sum peaks refer to two x-rays arriving simultaneously at the detector and hence registering at a higher energy. This is common for highly concentrated materials typically composed of heavier metals such as $FeCl_3$ salt, but has not been observed for nutrient powders where the heavy metal content is typically less than 100 ppm. Within the 160 eV resolution of the EDXRF spectrometer, the only potentially problematic overlap in nutrient powders was the secondary $K\beta_1$ and dominant $K\alpha_1$ transitions between Fe-Co and Br-Rb pairs. In nutrient powders measured to date, these overlaps have been negligible because the concentrations of Fe and Br are less than 10 ppm. For more details on the effects of transitional overlap on the accuracy for higher concentrations, see supplemental information. Linearity Since most nutrient-relevant elements in raw materials are present in mixing ratios less than 100 ppm, the linearity of the EDXRF method was determined with liquid reference standards spanning 0-100 ppm. In all cases a fit with $R^2 > 0.99$ was observed. At higher concentrations, typically greater than 500 ppm, emission/reabsorption between elements could lead to a misrepresentation of the number of secondary x-rays produced. The linear relationship between concentration and normalized peak intensity means that critical nutrient-relevant elements in raw materials can be assessed even before calibration by correlating the peak intensity with cell growth performance parameters.

Accuracy, Precision, Limit of Detection and Limit of Quantification

For a specific EDXRF configuration (i.e., operating voltage and current) the accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) are most strongly influenced by the sample matrix and acquisition time. Figure 2a plots Cu concentrations measured with ICP-MS against the corresponding EDXRF intensity as determined with wavelet transformation for a nutrient powder. Included are the residuals of the linear fit. There is a good linear relationship with $R^2 > 0.90$ and uniform variance as noted from the residuals, for trace levels of Cu ranging from 0.6 ppm - 2.5 ppm. Note that the fit values, relating ICP-MS to EDXRF peak intensities, are used to calibrate the EDXRF for new lots of nutrient powders. Figure 2b shows how the standard deviation (σ) decreases with the square root of the acquisition time for Cu in nutrient powders. Each point represents 36 separate measurements. At approximately 600 s, the variation in an individual measurement is approximately equal to the sample to sample variability. To further improve the accuracy, it is best to increase the number of scans. In general, the acquisition time and number of scans should be set by the precision needed for the cell culture process[22] where the standard error (σ_e) is defined by the intercept of the square root fit (σ_1), the number of scans (n) and the time (t) such that $\sigma_e = \sigma_1/\sqrt{n \times t}$. For the Cu example given, a precision of 0.2 ppm was achieved for triplicate measurements with a 600 s integration time.

The parameter, σ_1 is related to the x-ray absorption probability and is therefore specific to the element, excitation conditions and phase of the matrix. In order to assess the sensitivity of the handheld EDXRF for a wide range of elements, the liquid and powder reference samples were used (see Fig. 2c). The line fit should only be used as a guide to the eye and is not necessarily representative of elements not measured. Nonetheless, for the experimental conditions reported here, the sensitivities for both liquid and powder samples appear to have the same parabolic trend with elements from Cu to Rb (Z numbers 29-37) with σ_1~5 ppm. Since the distribution of residuals from 36 measurements points is Gaussian, the LOD and LOQ can be defined as 3×SE and 10×SE, respectively. Therefore, to achieve 1 ppm LOD for Se a measurement time of 225 s is required. On the other hand, a similar LOD detection for V would require 12 hours. The current EDXRF configuration is not ideal for measuring trace levels of Mn and V.

The decreased σ_1 for powders is a consequence of the density and composition of elements, which influences the x-ray absorption depth. In liquids, the depth of penetration is typically less than 100 µm whereas in powdered raw material is closer to 5 mm. Therefore, the signal is larger in powders than liquids for the same concentration.

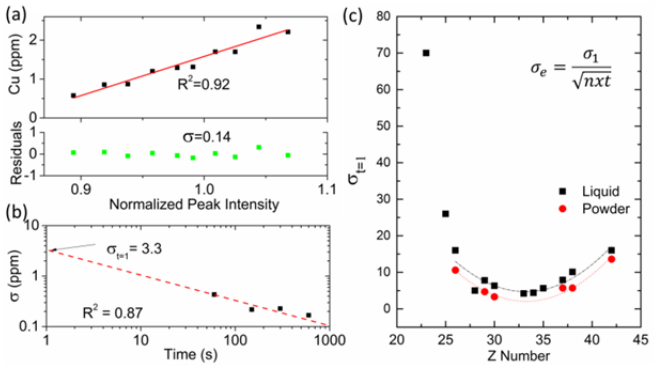

Fig. 2 (a) Cu concentrations as measured by ICP-MS and compared to the EDXRF normalized peak intensity show the linearity of EDXRF for concentrations from 0.5-2.5 ppm. The red line is a linear fit and the green points are the corresponding residuals in ppm. The standard deviation (σ_r) is calculated from the sum of squares of the residuals. (b) The EDXRF precision is shown as a function of the acquisition time for Cu in nutrient powders. Each point represents the standard deviation of 36 measurements. The precision decreases with the square-root of time (t) and is represented by the fit $\sigma(t) = \sigma_{t=1}\sqrt{t}$. (c) A generalized plot showing the precision at time t = 1s for different elements found in liquid and powder reference standards. The fits are guide lines only. The error (σ_e) is calculated as a function of time (t) and number of scans (n).

Table 1 summarizes the analytical figures of merit for Cu and Zn for two different acquisition times. The repeatability was calculated for 6 back-to-back measurements on the same day and the intermediate precision was determined as the error over several days. The accuracy of the method, determined by

comparing EDXRF and ICP-MS concentrations for new lots of nutrient powders, is within 20% for Cu and Zn . In contrast to ICP-MS, the EDXRF measurements of powdered nutrient materials were measured as is without acid digestion or filtration thus reducing errors associated with sample handling.

Table 1 Analytical figures of merit for Cu and Zn in nutrient powders.

Characteristic	Cu	Zn
Acquisition Time	3×600sec	600 sec
Accuracy	15%	20%
Repeatability (ppm)	0.1	0.2
Intermediate Precision (ppm)	0.1	0.2
Specificity	Pass	Pass
Limit of Detection (ppm)	0.3	0.5
Limit of Quantitation (ppm)	0.9	1.7
Linearity (ppm)	0.6 to 2.4	0.5 to 8
Range (ppm)	0.6 to 2.4	0.5 to 3.5

Challenges Related to Matrix Effects

There are three main challenges related to the sample matrix when using the EDXRF to measure trace metals. One is associated with the density and crystal structure. A non-uniform sample presentation between different lots of the same material makes comparison difficult because the path the x-rays follow changes. Fortunately, most raw materials of interest are finely grained powders ranging from 10 - 400 µm, which pack densely thus alleviating the need for milling or dilution.

The second potential challenge that might affect accuracy is associated with the phase state of the sample. Since x-rays absorb differently between solids and liquids, this can produce erroneous intensities if a nutrient powder is hygroscopic. The nutrient powders measured to date were able to be stored in ambient conditions for up to one month with no observed difference in the metal concentration. Only when ultrapure water was added to the sample to raise the water content above 2% did metal concentrations vary (work ongoing). Therefore, deliquescent samples would require a concentrated solution or material sealed under a dry environment to minimize errors associated with increased water content.

Finally, slight differences in the reflectivity, i.e., ability to scatter x-rays, between materials can interact with instrumental line leakage to cause relatively large errors when measuring trace metals. Instrumental line leakage is the emission of x-rays by metals-in this case Ti, Cr, Fe, Cu, and Zn-within the spectrometer; these x-rays backscatter from the sample and offset the signals of interest. For example, L-glutamine makes a fairly good reference standard for many chemically defined media composed of a similar base, but the slight changes in composition, change the reflectivity and hence the accuracy by as much as 2 ppm for metals matching the backscattered instrumental lines. This makes it difficult to develop universal reference standards to calibrate the EDXRF and developing calibrations standards for each matrix is impractical. However, only the EDXRF intensity, which is linear with concentration, needs to be acquired to compare lot-to-lot consistency of raw materials. When an element is found to vary and correlate to process parameters then a one-time external calibration against ICP-MS is used to relate the peak intensity to the concentration of the element of interest. This strategy manages cost and complexity, while ensuring vigilance against adulteration of the raw material.

The next section discusses EDXRF measurements of nutrient powders used for cell culture and demonstrates how useful correlations were observed between EDXRF peak intensities and cell-culture variables.

Biopharmaceutical Investigations Using EDXRF Spectrometer

Discrepancies in the cell culture performance of NS0 cell lines pointed to variability in raw materials. The portable EDXRF spectrometer was used to screen raw materials, both powders and liquids used in the cell culture process to determine the most likely source. Cu level for one of the nutrient powders correlated to cell metabolism in the 15 L bioreactor (Fig. 3a). To obtain quantitative concentrations to help validate the hypothesis, 10 lots of nutrient powders spanning the range of EDXRF intensities were used to calibrate the EDXRF against ICP-MS. Using the EDXRF calibrated results, several small-scale shake flask experiments were performed, supplementing low-Cu lots with ~0.5 ppm Cu to match the level of high-Cu lots. The results consistently showed that the supplemented and high-Cu lots behaved the same, confirming that Cu variability in the nutrient powder impacted the cell metabolism (Fig. 3b).

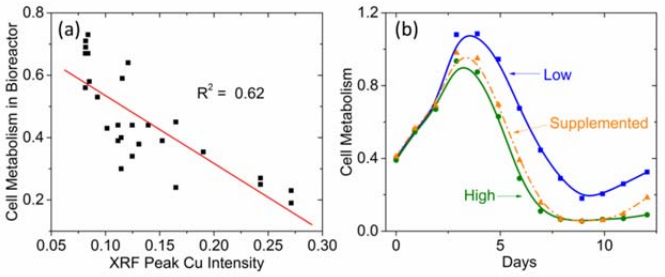

Fig. 3 Plots illustrating EDXRF analysis of raw materials for prediction of cell performance in biopharmaceutical investigations. (a) Each point in the plot corresponds to a cell metabolism-Cu concentration pair associated with a particular lot of nutrient powder. The trend was significant enough to warrant further investigation. (b) Time series measurements of cell metabolism from a shake flask experiment for two different nutrient powders where the Cu concentrations were measured to be low and high. The yellow line refers to media and feed preparation where a stock of copper sulfate was used to supplement the low-Cu lot to reach the high-Cu lot concentration.

Conclusions

The use of an EDXRF spectrometer for detecting variations in nutrient-relevant elements in biopharmaceutical raw materials which impact product consistency was demonstrated. Here, we showed one example of Cu variability, as measured by the EDXRF, on product attributes from NS0 cell lines and have since applied the EDXRF successfully to a number of other manufacturing investigations. The advantages of the EDXRF in comparison to ICP-MS include limited sample handling and maintenance, and click-of-a-button acquisition. Like ICP-MS, analyzing the EDXRF spectral data can be challenging and a good understanding of potential interferences between multiple transition lines and indirect excitations is beneficial. In the case

of biopharmaceutical powdered raw materials, many of the nutrient-relevant metals are less than 100 ppm and hence the interferences are minimized or nonexistent. One potential disadvantage with EDXRF is the lack of quantitative results for an uncalibrated matrix. However, if a correlation between EDXRF intensity of a trace metal and product attribute is found, then only a one-time calibration is required. Also a fairly good approximation (± 2 ppm) is still possible by calibrating the EDXRF with L-glutamine reference standards. Finally, the signal-to-noise ratio was improved by using wavelet transform analysis to reduce noise associated with the broad background "Bremsstrahlung" radiation. Due to the success of the portable EDXRF spectrometer for fast, no-sample-preparation, high-throughput measurements, more robust bench-top EDXRF systems are being explored.

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Notes and references

[a] Biogen Idec Inc., 14 Cambridge Center, Cambridge, MA 02142, United States.

Electronic Supplementary Information (ESI) available:

1-ICP-MS parameters of operation.

2-Effects of wavelet transformation on the overlap between the secondary Fe $K\beta_1$ and dominant Co $K\alpha_1$ transition. See DOI: 10.1039/b000000x/

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