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BIOTECHNOLOGY PROGRESS

NOTE

Cell Culture and Tissue Engineering

Rapid metal speciation of cell culture media using reversed-phase separations and inductively coupled plasma optical emission spectrometry

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Abstract

Cell culture media metal content is critical in mammalian cell growth and monoclonal antibody productivity. The variability in metal concentrations has multiple sources of origin. As such, there is a need to analyze media before, during, and after production. Furthermore, it is not the simple presence of a given metal that can impact processes, but also their chemical form that is, speciation. To a first approximation, it is instructive to simply and quickly ascertain if the metals exist as inorganic (free metal) ions or are part of an organometallic complex (ligated). Here we present a simple workflow involving the capture of ligated metals on a fiber stationary phase with passage of the free ions to an inductively coupled plasma optical emission spectrometry for quantification; the captured species are subsequently eluted for quantification. This first level of speciation (free vs. ligated) can be informative towards sources of contaminant metal species and means to assess bioreactor processes.

KEYWORDS
capillary-channeled polymer fibers, cell culture media, inductively coupled plasma-optical emission spectrometry, liquid chromatography, metal speciation

1 | INTRODUCTION

Far and away, the use of Chinese hamster ovary (CHO) cell lines is the predominant means of therapeutic, monoclonal antibody (mAb) production.[1,2] Cell culture media (CCM) provides nutrients to cell lines to support cell growth and mAb productivity.[3–6] There are two general classes of cell culture media: basal and feed. Basal media typically contains amino acids, carbohydrates, inorganic salts, and other constituents, and is introduced to a cell line at the beginning of a growth process. Growth media (basal or feedstocks) can be comprised of the same types of ingredients but the feed has higher concentrations than basal media, with the feed introduced during growth processes as supplemental nutrition.[7] For different types of feed, the balance between these nutrients is incredibly important, as small variations can affect various cell growth processes.[8] Transition metals (e.g., Cu, Fe, Mn, and Zn) are added as inorganic salts and serve as the active centers of enzymes, where they drive protein quality, and the ability to support growth.[9] While these metals are needed to promote cell growth, some may need to be limited due to harmful effects to the cells if they fall above certain thresholds.[10–12] Different suppliers often have formulations with varying trace metal contents, but additional variability in the expected concentrations can occur from trace metals introduced through raw (organic) material impurities and leaching from metallic preparatory equipment.[13–15] Even simple stir bars have been demonstrated to leach Fe, Cr, Ni, and Mn, which may impact the protein concentration, aggregation, and other critical quality attributes (CQAs).[16] While variation in suppliers is to be expected, metal concentration differences between lots from the same supplier have also been observed,[17] adding to the variability of trace metal ion contamination.

The metals of interest in this study include Fe, Zn, Co, Cu, and Mn, which are added to the specific test media as ferric ammonium

citrate, zinc chloride, cyanocobalamin, cupric sulfate pentahydrate, and manganous chloride tetrahydrate, thus some are added as inorganic salts, and some as ligated complexes. Each trace metal plays a certain role in the CCM to promote cell growth and productivity. For example, Mn serves as a cofactor for some enzymes in the glycosylation pathways.[18] Cu is critical to lactate metabolism in CHO cells. Increased Cu concentrations increase lactate consumption, which favors cell growth and productivity, while excess Cu can lead to toxicity through oxidative stress.[19] Zn influences the cell membrane structure and function, and deficiency can lead to cell death.[20,21] Co plays a role in protein glycosylation, but excess amounts can create damaging reactive oxygen species.[22] Iron is important for cell metabolism, replacing transferrin in chemically defined media.[23,24] Trace elements also play roles in oxidative stress, where lack of metal ion homeostasis can lead to increased concentrations of reactive oxygen and nitrogen species which can damage DNA, proteins, and lipids.[10–12] Each metal plays an important role in CCM, emphasizing the importance of monitoring and validating these concentrations throughout the mAb production process. Even slight metal variations in CCM have shown significant changes in monoclonal antibody production and the viable cell density.[8] Additional approaches have been investigated to add excess amounts of trace metals, specifically Mn and Zn. Excess Mn inversely effected viable cell density and specific productivity, while excess Zn increased monoclonal antibody production in CHO cells, but too much led to cytotoxic affects.[25–27] The different effects of each metals emphasizes the balance of metals needed for the optimal productivity.

As is common to most other biological processes, the concept of metal speciation is relevant to mammalian cell processes productivity.[28] In truth, the roles of metal speciation have been far more studied in biota than animal systems.[29,30] Thus, one cannot simply assess the "total metal" content within a system (CCM here) to understand all aspects of efficacy or toxicity. The chemical form, and redox state of these metals drive cellular chemistry and biology.[31] In fact, researchers have used cellular uptake as a means of assessing metal speciation.[32] In the case of "free" metals, oxidation states dictate the production of problematic free radicals via basic processes such as the Fenton reaction.[12,23] In the case of CCM, one must be concerned with the speciation effects of metals, in principle, as various forms are added and potentially interact. Additionally, the speciation can reveal potential contaminants within the media based on the identity of the initial metal speciation states added during preparation. Taken a step further, CCM can contain 50–100 compounds, all of which have the potential to form ligands with the aqueous metal ions. Clearly, there are several unknowns in this arena, in part due to a lack of analytical methods. Described here is a rapid, straightforward method to perform what might be termed a first-level of metal speciation analysis; elucidating the fraction of free vs. ligated metals. In some fields, this difference would be described as extractable or labile vs. bound species.[31,33]

The most common techniques to quantify metals in CCM include inductively coupled plasma - mass spectrometry (ICP-MS) and inductively coupled plasma-optical emission spectroscopy (ICP-OES).[34] ICP-MS has lower detection limits in comparison to ICP-OES, but is limited by lower matrix tolerance and relatively high capital cost and operational requirements. ICP-OES provides a less expensive option with better matrix tolerance, albeit with higher overall detection limits. We describe here the use of ICP-OES to first determine total metal content in three priority, chemically defined CCM basal and feed stocks developed as part of the Advanced Mammalian Biomanufacturing Innovation Center (AMBIC) research consortium. To establish benchmark performance for ICP-OES as a method for trace metal quantification in these media, quantitative analysis of Fe, Co, Cu, Zn, and Mn in CCM was performed via ICP-MS as well. In addition to quantification, the speciation of the metals in CCM is an important parameter to access the bioavailability of the metals in the media. To differentiate the speciation states (free vs. ligated) of the metals in the various media, trilobal polypropylene (PPY) capillary-channeled polymer (C-CP) fibers were employed as the stationary phase during the reversed phase solid phase extraction process, with packing and separation conditions previously optimized.[35–37] C-CP fiber columns allow for rapid processing in a format the costs <$5 per column, which can be used over tens of cycles. This effort builds substantially on the initial demonstration of using the fiber phase to isolate free and ligate metals for a singular medium.[37] In this scenario, free ions or hydrophilic species in CCM pass unretained through the column, while hydrophobic bound (ligated) species are retained and subsequently eluted in modest-strength organic solvent. The free ions and ligated species were then analyzed with ICP-OES to determine the respective speciation states of the metals. The present method provides rapid determination of the first-level of speciation of the target metals in the media, potentially providing insights into the sources of metals in the media and chemical changes, which may occur through the course of CHO cell processing.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

HPLC-grade acetonitrile (ACN) (VWR Chemical, Radnor, PA), a milli-Q water (DI-H_2O) (deionized water; 18.2 MΩ-cm, purified with Millipore, Merck, Germany) purification system, and trifluoroacetic acid (TFA) (Sigma-Aldrich, Milwaukee, WI, USA) were used for sample and mobile phase preparation. The media evaluated here are part of a development project from the Advanced Mammalian Biomanufacturing Innovation Center (AMBIC), a National Science Foundation I/UCRC, designated as AMBIC Reference Basal Medium 1.1, AMBIC Reference Feed A 1.1, and AMBIC Reference Feed B 1.1. The media were formulated and packaged by MilliporeSigma (Burlington, MA, USA), and Lonza (Walkersville, MD, USA).[38] In general terms, the basal and Feed A media are composed of both metals and organic nutrients (amino acids, sugars, etc.), while Feed B is composed solely of a mixture of amino acids. The metal species and their concentrations according to the mixture formula are presented in Table 1. The metal complexes of interest in the media include ferric ammonium citrate,

TABLE 1 Metal species' composition in Advanced Mammalian Biomanufacturing Innovation Center 1.1 basal and Feed A media

Metal	Species	Basal media (μg ml^{-1})	Feed A (μg ml^{-1})
Fe	$(NH_4)_5$ [$Fe(C_6H_4O_7)_2$]	34.9	175
Mn	$MnCl_2$	0.001	0.058
Co	Cyanocobalamin	3.9	19.4
Cu	$CuSO_4$	0.067	0.34
Zn	$ZnCl_2$	1.9	9.8

manganous chloride tetrahydrate, cyanocobalamin (vitamin B12), cupric sulfate pentahydrate, zinc chloride. Of these primary species, the iron and cobalt complexes would be anticipated to be retained on the C-CP fiber phase, while the others would pass unretained. A solution of 2% nitric acid was diluted from concentrated trace metal grade nitric acid (VWR, Radnor, PA, USA) and used as the solvent for all samples analyzed by ICP-OES. Standards of 1000 μg ml^{-1} of Fe, Co, Cu, Zn, and Mn, (High Purity Standards, Charleston, SC, USA) were used to make a multi-element solution for the calibration curves required for the quantification of metals in the CCM samples. A 1 μg ml^{-1} Y (High Purity Standards, Charleston, SC, USA) solution in 2% nitric acid was prepared from a 100 μg ml^{-1} stock solution and used as the internal standard for all solutions. Each analytical determination was accomplished using the standard addition method, with spikes ranging from 0.01 to 1 μg ml^{-1} for each element. In this way, the sample species existed in aqueous (unretained) and mixed solvent (column-eluted) media, and did not require separate calibration curves. The standard addition curves for each metal in all three media types are included as Supporting information as demonstration of the efficacy of the method.

2.2 | Column preparation

PPY fibers were melt extruded by the Department of Material Science and Engineering at Clemson University as previously described.[39] PPY fibers sufficient to meet target interstitial fraction values (equating to ~630 fibers) were pulled through 30 cm of polyether ether ketone (PEEK) tubing (0.78 mm diameter) (Cole-Palmer, Vernon Hills, IL, USA) and washed with DI-H_2O, ACN, and DI-H_2O to remove any anti-static coatings on the fiber stationary phase. The column packing and separation conditions were optimized and described previously.[35–37]

2.3 | Instrumentation

Reversed phase (RP) separations were performed using a simple, HPLC pump from a Shimadzu Prominence LC-20AD liquid chromatograph (Shimadzu, Kyoto, Japan). Fiber columns were mounted in place of the standard stainless steel columns. Sample volumes of 20 μl were injected onto the column and two vials were placed at the column exit to collect the respective unretained and solvent-eluted fractions. Column loading studies on the PPY column were performed on a Dionex Ultimate 3000 HPLC system consisting of a quaternary pump with a variable wavelength detector, with absorbance measurements taken at 216 nm. A Thermo Scientific iCAP 7200 ICP-OES (ThermoScientific, Waltham, MA, USA) was used for all OES measurements. The instrumentation included a 3-channel, 12-roller peristaltic pump with an enhanced matric tolerance (EMT) torch, a duo (axial and radial) plasma and RF generator, and a charge injection device (CID) detector with continuous choice of wavelengths (166–847 nm) all controlled by Otegra ISDS software. ICP-OES measurements were taken in the axial position and the optical emission was monitored for each element. Co, Cu, Fe, Mn, Y, and Zn emission was monitored at 228.616, 224.700, 259.940, 257.610, 371.030, and 206.200 nm, respectively. The ICP-OES plasma had an auxiliary gas flow of 0.50 L min^{-1} and a coolant gas flow of 12 L min^{-1}. The RF power was set to 1150 W and the peristaltic pump speed was set to 45 rpm.

2.4 | Methods

The free ions (unretained) and hydrophobic species (retained) of CCM were separated using the reversed-phase PPY column platform. A diagrammatic representation of the analytical process is shown in Figure 1. The respective media samples were injected onto the PPY C-CP fiber column in DI-H_2O+ 0.1% TFA (MP A) at a flow rate of 0.5 ml min^{-1}. The retained hydrophobic species were subsequently eluted in ACN + 0.1% TFA (MP B). The TFA is not expected to complex with the metals due to the minimal amount of time (<6 min) in the flow and low concentration of TFA (0.1% v/v) the actual sample is exposed to, where extensive periods of time and high temperatures are needed to form metal-TFA complexes.[40,41] Additionally, if the metal-TFA complex was formed, that species would be expected to be unretained, and so elute with the initial free metal in any case. The metal species populations were collected in two fractions; the first fraction was composed of unretained free ions in MP A, while the other fraction was the hydrophobic species in the MP B wash. For both the free ion and hydrophobic collections, the solvents were removed by evaporation, reconstituted with 10 ml 2% HNO_3, where 2 ml was aliquoted and spiked to create a standard addition response curves with a multi-element solution and reconstituted in 2% HNO_3 to a total volume of 6 ml prior to ICP-OES analysis. The efficacy of the ICP-OES measurements was validated with ICP-MS measurements of the same material at the laboratories of AMBIC member-company, MilliporeSigma, at their St. Louis facilities. ICP-MS measurements were determined via a 10-element calibration curve and corrected with internal standards. As a general rule, the total elemental content values were

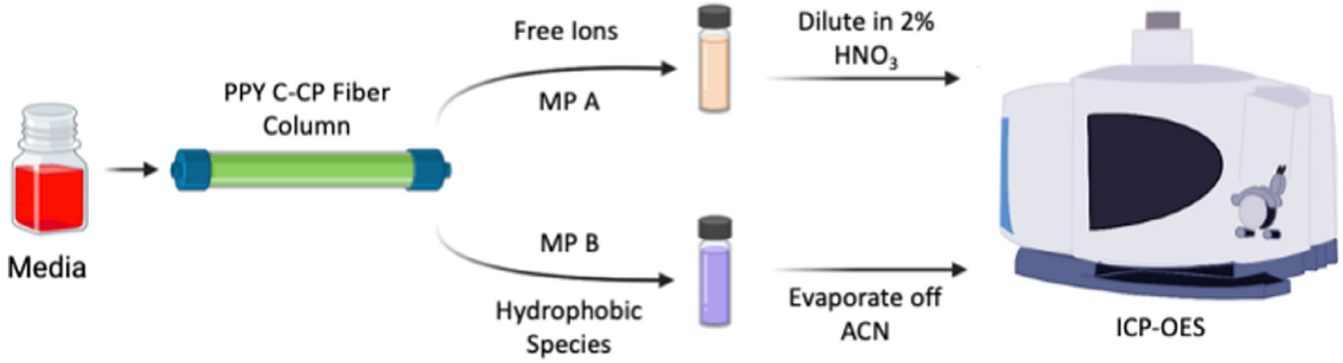

FIGURE 1 Diagrammatic representation of the isolation of free and ligated metals in cell culture media (CCM) on PPY C-CP fiber columns followed by quantification with inductively coupled plasma optical emission spectrometry (ICP-OES)

in agreement to within ±20%, relative, for the two techniques. With larger discrepancies existing for the cases where the concentration values were close to ICP-OES limits of detection versus the far more sensitive ICP-MS determinations. In terms of the respective recoveries of for the metals quantitation, the two methods yielded values generally within a factor of 2 versus the media formulae, with Fe being an outlier as it exhibited recoveries of <20% by both methods. While there was no evidence of precipitates in the media samples, further investigation as to the losses is certainly in order.

3 | RESULTS AND DISCUSSION

3.1 | Fiber column loading capacities

Chromatographic separations were performed to isolate the media into two fractions: the unretained free ions and the retained (hydrophobic) chelated species. These chelated metals could be the result of the elements being added to the formulation as complexes or formed in situ. Given the high composition of organic nutrients (amino acids sugars, etc.) for each of the media types, there was a concern that the columns could be overloaded by those organic species. Such overloading would result in non-retention of ligated metals. Prior to the quantification of each fraction, the binding capacity of the hydrophobic species. Feed A was selected as it contains the highest concentration of metals and organic species (~61 g L^{-1}). Various volumes of CCM were loaded onto the PP fiber column to determine the largest injection volume and providing proportional recoveries of the retained fraction as determined via the UV absorbance response. The chromatograms for the injection of increasing injection volumes of Feed A are depicted in Figure 2a. Clearly seen are the increased-breadth injection peaks of the unretained species as the injection volume is increase from 5–80 μl. At a time 4 min after the injection, the solvent is changed to mobile phase B, and so those retained, hydrophobic species are eluted from the column. Here, it is clear that the recovery increases steadily as a function of the injected sample volume. As the volume of media injected increases, the peak height and areas of the hydrophobic species peak increases. It is important to note that this response is a reflection of all of the potential forms of hydrophobic molecules absorbing at this wavelength.

The relative recovery of the eluted hydrophobic species is plotted in Figure 2b as the integrated peak areas (mAU × min) versus injection volume for triplicate injections. The repeatability of the responses/recoveries provides confidence in the overall isolation process. The peak area begins around 10 mAu × min at 5 μl of media and increases with volume injection, then plateaus to ~30 mAu × min for the 60 μl injection of media. The plateau indicates that the column has reached saturation, wherein the column is no longer quantitatively retaining additional hydrophobic species from the CCM samples. Since the ligated metal species are no longer fully retained, these species would contribute to the unretained fraction, biasing the results at higher injection volumes. Based on these results, separations were performed using 20 μl injections to ensure the column was not overloaded, while yielding good standard deviations in peak area/recoveries.

3.2 | Relative metal speciation

The eluate of each fraction was collected and analyzed with ICP-OES to determine the extent to which metals were ligated or not. The percentage of each metal in the respective fractions are presented for the three CCM formulations tested in this work in Figures 3a–c. The orange-colored region represents the fraction of free ions, and the purple region represents the hydrophobic species. As previously mentioned, the metals of interest in this study were added to the media as ferric ammonium citrate, zinc chloride, cyanocobalamin (vitamin B12), cupric sulfate pentahydrate, and manganous chloride tetrahydrate. As such, each of the metals except Co (and perhaps Fe) are expected to exist as free (aqueous) ions. The respective fractions for the basal media are shown in Figure 3a, in which all metals are found to primarily exist as free ions or hydrophilic species. The presence of Co as a free ion could indicate the breakdown of vitamin B12 into free Co ions in basal media as reversed phase chromatographic separations have shown the retention of that species to hydrophobic stationary

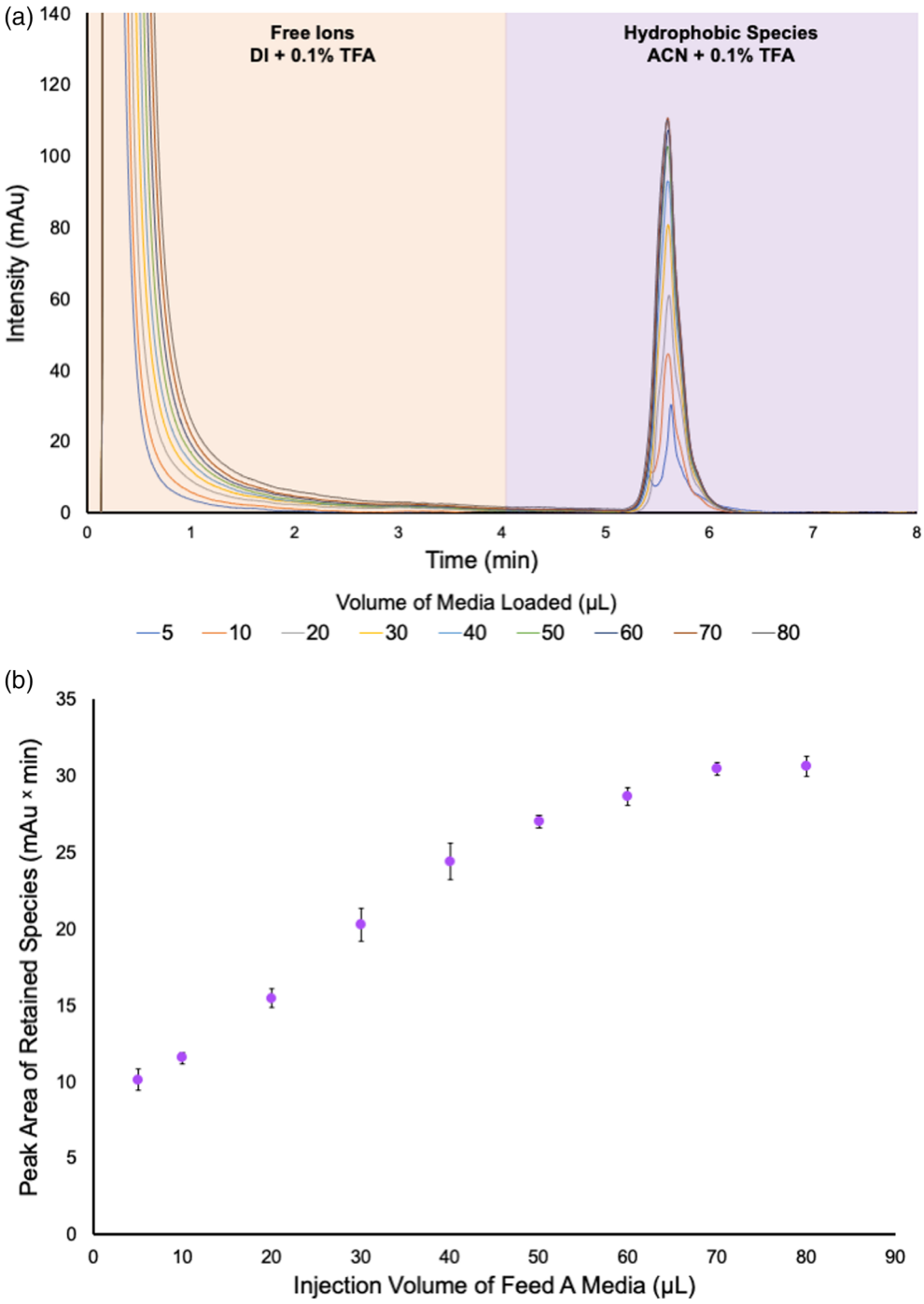

FIGURE 2 Feed A media loading on a PPY C-CP fiber column. (a) Representative chromatograms obtained for injections ranging from 5 to 80 µl. Free ions are eluted (unretained) in the loading conditions where the mobile phase is DI + 0.1% trifluoroacetic acid (TFA), while the hydrophobic species are eluted with +0.1% TFA. (b) Integrated peak areas for the elution of retained species at each loading

phases.[42,43] The presence of Cu and Mn at ligated levels of >5% suggests some amount of in situ coordination chemistry. The percentile distributions of the metal species in Feed A CCM are presented in Figure 3b. These distributions are significantly different from the basal media values, with the Feed A fractions existing in the ligated forms ranging from 9.7% for Fe, to 78.8% for Co, meaning there are less free metals available for uptake by cells. According to the formulations, the metal species introduced in Feed A medium are the same as that for the basal media. The principle difference should be the relative concentrations, where the metal concentrations in the feed are approximately 5× greater than those of the basal media in the case of Fe, Co, Cu, and Zn, while that of Mn is ~60× higher. By the same token, organic constituents' content are also increased by ~5× in Feed A. Macroscopically, the total solute concentration of the basal medium is ~17 g L^{-1}, while that of Feed A is ~61 g L^{-1}. Thus, it would not be expected that the disparity would come from differences in the stoichiometries between the two media. In fact, Feed A does not contain three of the amino acids that are present in the basal medium, so there are fewer potential ligand species. It is interesting to note that the total elemental recoveries for the metals (determined by direct ICP-OES and MS) do not differ appreciably (0.58%–10.7% differences, excluding Mn, a common leaching contaminant) between

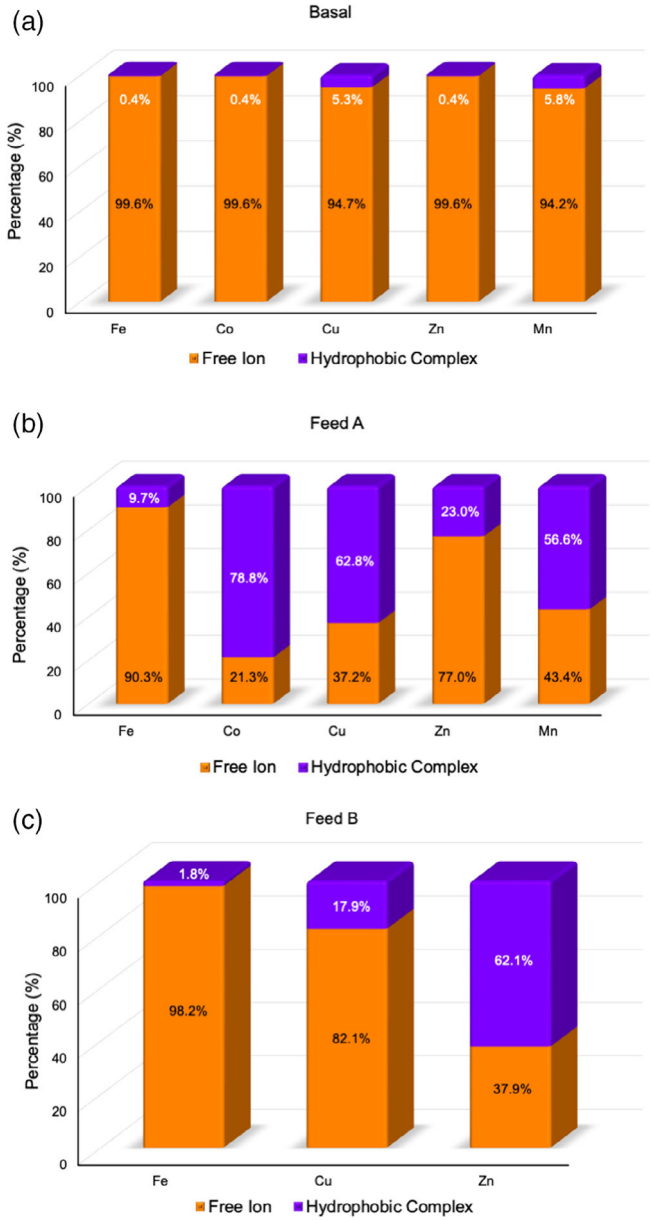

FIGURE 3 Percentages of free ions (orange) and hydrophobic species (purple) in the various CHO cell growth media as determined by fiber column isolation and inductively coupled plasma optical emission spectrometry quantification. (a) Basal, (b) Feed A, and (c) Feed B media

the two formulations. That is, the differences in the metals' determined concentrations based on the two formulations are observed quantitatively. Therefore, the question remains as to why the fraction of free versus bound metals changes so dramatically. One possibility lies in the fact that the media are sourced from different producers, who likely get their raw materials from different manufacturers. These sorts of variations, may or may not have impact on CHO cell productivity, but only after such differences can be readily identified can those sorts of studies be undertaken.

As stated above, the formulation of the Feed B media is solely composed of amino acids. As such, there should be none of these key metals present. By the less-sensitive ICP-OES method, Fe, Cu, and Zn were detected at levels much lower that the basal and Feed A media; μg ml^{-1} for Fe and ng ml^{-1} for Cu and Zn (all were seen via ICP-MS). The percentage for each state for Feed B media are shown in Figure 3c. As no free metals were added to Feed B media, all metals present would be resultant of impurities in raw materials, sample preparation solvents, or contamination from hardware component leaching. Interestingly, Fe and Cu are determined to exist predominately as free ions, while Zn has an appreciable hydrophobic (ligated) fraction. This might suggest that the primary starting materials for the various metals in the media may be the cause of the contamination, or that simply ionic Zn may react more extensively with the amino acids in Feed B.

4 | CONCLUSION

There is intense interest in the biotherapeutics manufacturing community in understanding the role of metals in productivity and quality attributes of CHO cell bioreactors. As detailed studies evolve, there is a key need in the development methods, which can assess not only the quantity of metals in different feedstocks, but also the metal speciation state. The chemical form of the introduced metals likely will have an effect on the free metal to ligate metal ratios, and this ratio (and the chemical specifics) will effect intracellular processes. Likewise, the ability to monitor changes in speciation could also provide insights into cellular processes or serve as means of process monitoring. The use of polypropylene C-CP fiber isolation columns provides a rapid, low cost means to affect what could be termed first-level metal speciation. The two-step process, where aqueous metal ions pass unretained from the microcolumns, followed by a simple organic solvent elution of ligated species, is readily implemented with subsequent ICP-OES or MS detection. Indeed, one could easily envision a direct flow through process with on-line introduction to the plasma.

In this particular demonstration, three types of CHO cell growth media were evaluated regarding the fraction of metals existing as free or ligated states. While the formations were specific to deliver the metals in a specific form, appreciable differences were determined by media type (basal and feeds). While the total metal recoveries were as expected by direct ICP-OES/MS, the differences in the fractionation amongst the media types was unexpected. This work does not attempt to assess which form of the specific metals is most effective, but highlights that these differences in speciation could have implications into the observed variability, and that those differences need to be considered by bioreactor operators. As documented previously, variability across media lots is a major source of concern in general. In addition, while the present method does not provide absolute speciation, it may serve as a useful screening tool and potential means of monitoring bioreactor processes.

AUTHOR CONTRIBUTIONS

Sarah K. Wysor: Data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); writing – original draft (lead); writing

– review and editing (supporting). **Katja J Hall:** Conceptualization (lead); data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting). **R. Kenneth Marcus:** Conceptualization (lead); funding acquisition (lead); project administration (lead); resources (lead); supervision (lead); writing – original draft (supporting); writing – review and editing (lead).

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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