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TITLE:

Quantification of Metal Leaching in Immobilized Metal Affinity Chromatography

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KEYWORDS:

immobilized metal affinity chromatography, hydroxynaphthol blue, HNB, Ni-NTA, metal contamination, His-tag

SUMMARY:

We present an assay for easy quantification of metals introduced to samples prepared using immobilized metal affinity chromatography. The method uses hydroxynaphthol blue as the colorimetric metal indicator and a UV-Vis spectrophotometer as the detector.

ABSTRACT:

Contamination of enzymes with metals leached from immobilized metal affinity chromatography (IMAC) columns poses a major concern for enzymologists, as many of the common di-and trivalent cations used in IMAC resins have an inhibitory effect on enzymes. However, the extent of metal leaching and the impact of various eluting and reducing reagents are poorly understood in large part due to the absence of simple and practical transition metal quantification protocols that use equipment typically available in biochemistry labs. To address this problem, we have developed a protocol to quickly quantify the amount of metal contamination in samples prepared using IMAC as a purification step. The method uses hydroxynaphthol blue (HNB) as a colorimetric indicator for metal cation content in a sample solution and UV-Vis spectroscopy as a means to quantify the amount of metal present, into the nanomolar range, based on the change in the HNB spectrum at 647 nm. While metal content in a solution has historically been determined using atomic absorption spectroscopy or inductively coupled plasma techniques, these methods require specialized equipment and training outside the scope of a typical biochemistry laboratory. The method proposed here provides a simple and fast way for biochemists to determine the metal content of samples using existing equipment and knowledge without sacrificing accuracy.

INTRODUCTION:

Since its inception by Porath and co-workers[1], immobilized metal affinity chromatography (IMAC) has become a method of choice to quickly separate proteins based on their ability to bond with transition metal ions such as Zn^{2+}, Ni^{2+}, Cu^{2+}, and Co^{2+}. This is most commonly done via engineered poly-histidine tags and is now one of the most common chromatographic purification techniques for the isolation of recombinant proteins[2]. IMAC has also found applications beyond recombinant protein purification as a way to isolate quinolones, tetracyclines, aminoglycosides, macrolides, and β-lactams for food sample analysis[3] and as a step in identifying blood-serum protein markers for liver and pancreatic cancers[4,5]. Not surprisingly, IMAC has also become a method of choice for the isolation of a number of native bioenergetics enzymes[6-10]. However, successful implementation of these purification methods for studies on enzymatically active bioenergetic proteins is dependent on the presence of negligible levels of metal cations leached from the column matrix into the eluate. The divalent metal cations commonly used in IMAC have known pathologic biological significance, even at low concentrations[11,12]. The physiological effect of these metals is most pronounced in bioenergetic systems, where they can prove lethal as inhibitors of cellular respiration or photosynthesis[13-15]. Similar issues are unavoidable for the majority of protein classes where residual contaminant metals can interfere with a protein's biological functions or characterization with biochemical and biophysical techniques.

While the levels of metal contamination under oxidizing conditions and using imidazole as an eluant are typically low[16], protein isolations performed in the presence of cysteine reducing agents (DTT, β-mercaptoethanol, etc.) or with stronger chelators like histidine[17,18] or ethylenediaminetetraacetic acid (EDTA) result in much higher levels of metal contamination[19,20]. Similarly, since metal ions in IMAC resins are frequently coordinated by carboxylic groups, protein elutions performed under acidic conditions are also likely to have much higher levels of metal contamination. Metal content in solutions can be assessed using atomic absorption spectroscopy (AAS) and inductively coupled plasma-mass spectrometry (ICP-MS) down to a limit of detection in the ppb-ppt range[21-24]. Unfortunately, AAS and ICP-MS are not realistic means for detection in a traditional biochemistry lab as those methods would require access to specialized equipment and training.

Previous work by Brittain[25,26] investigated the use of hydroxynaphthol blue (HNB) as a way to identify the presence of transition metals in solution. However, there were several internal contradictions in the data[20] and those works failed to offer an adequate protocol. Studies by Temel et al.[27] and Ferreira et al.[28] expanded on Brittain's work with HNB as a potential metal indicator. However, Temel developed a protocol that makes use of AAS for sample analysis, using HNB only as a chelating agent. Ferreira's study used the change in the HNB absorbance spectra at 563 nm, a region of the free-dye HNB spectra that overlaps heavily with the spectra of HNB-metal complexes at pH 5.7, making the assay sensitivity fairly low as well as resulting in relatively weak metal binding affinity[20]. To address issues in our own lab with Ni^{2+} leaching from IMAC, we have expanded the work done by Brittain[25,26] and Ferreria[28] to develop an easy assay capable of detecting nanomolar levels of several transition metals. We showed that HNB binds nickel and other common for IMAC metals with sub-nanomolar binding affinities and form 1:1 complex over a wide range of pH values[20]. The assay reported here is based on these findings and utilizes absorbance changes in the HNB spectrum at 647 nm for metal quantification. The assay can be

performed in the physiological pH range using common buffers and instrumentation found in a typical biochemistry lab by using colorimetric detection and quantification of metal-dye complexes and the associated change in absorbance of the free-dye when it binds to metal.

PROTOCOL:

1. Assay component preparation

1.1. Determine the chromatography fractions to be assayed using optical absorbance at 280 nm or alternative methods of protein quantification to identify the protein enriched fractions.

NOTE: For this work, we used a diode array UV-Vis spectrophotometer. To increase throughput, a plate reader capable of measuring UV-Vis absorbance can be used.

1.2. Preparation of necessary assay components

1.2.1. Prepare or obtain 10-100 mM buffer ("Sample Buffer") with a pH between 7 and 12.

NOTE: Common biochemical buffers such as Tris, HEPES, MOPS, and phosphate at neutral or basic pH are all acceptable for the assay. Tricine and histidine can be used but will require calibration curves as they both substantially chelate metal ions. An example of calibration for histidine is shown in reference[20].

1.2.2. Prepare a 12% w/v (20-fold concentrated) solution of hydroxynaphthol blue (HNB) dispersion in the Sample Buffer using 120 mg of HNB reagent for each milliliter of stock solution prepared.

CAUTION: Exposure of HNB to the eye can cause serious damage and irritation. Eye protection should be used when handling HNB and hands should be washed thoroughly after handling.

NOTE: HNB is sold as a dispersion on KCl by major suppliers of scientific reagents. As such, actual concentration in solution will vary from different manufactures, batches, and where in the bottle the HNB dispersion is taken from. Ideally, an absorbance between 0.5-0.8 at 647 nm after 20-fold dilution of the stock should be achieved.

2. Sample preparation and measurement

2.1. Preparation of the spectrophotometer for data collection

2.1.1. Turn on and warm up the UV-Vis spectrophotometer. Set the spectrophotometer to collect data at 647 nm.

NOTE: If the spectrophotometer allows, additionally collect data at 850 nm, or some other wavelength without significant changes related to the dye-metal and dye spectra, to be used for

a baseline correction.

2.1.2. Blank the spectrophotometer using the Sample Buffer.

NOTE: Quartz or disposable plastic cuvettes may be used. Quartz cuvettes are preferred for quantitative analysis as they will allow higher accuracy and precision over disposable plastic cuvettes. However, plastic cuvettes block UV light, which may be present in the measuring beams of some diode-array spectrophotometers. Exposure of HNB to intense UV light causes notable dye degradation and an unwanted slow absorbance decrease that can be confused with slow metal binding (for example, see Figure 1 in Supporting Information of Reference 20).

2.2. Preparation and absorbance measurement of control

2.2.1. Prepare a control solution containing 50 μL of HNB stock per milliliter of total assay volume. To ensure good mixing of all samples, pipette the small volumes first then add the Sample Buffer followed by mixing by pipetting. Diluted HNB solution should be prepared fresh but HNB stocks can be stored at 4 °C and shielded from light for weeks without significant degradation.

2.2.2. Allow the control to incubate for a minimum of 3 min at room temperature.

NOTE: A longer incubation time may be necessary for samples at alkaline pH or in the presence of phosphate due to formation of poorly soluble metal complexes resulting in slower equilibration.

2.2.3. Measure and record the absorbance at 647 nm for the control sample.

2.3. Preparation and absorbance measurement of samples

2.3.1. Prepare the assay samples by mixing 50 μL of HNB stock with 950 μL of appropriately diluted protein fractions with the Sample Buffer.

NOTE: Since metal contamination levels reported in literature vary by a factor of more than 1000 depending on elution conditions[16,20], it may be necessary to try a few dilutions of the assayed protein fractions with the Sample Buffer (see step 1.2.1 above) to achieve absorbance changes within the dynamic range of the assay.

CAUTION: Nickel and other metals used in IMAC are known skin irritant, suspected of being carcinogenic, and are capable of damaging the kidneys and blood after prolonged exposure. Gloves and eye protection should be used when handling protein samples prepared with IMAC.

2.3.2. Allow the sample to incubate for a minimum of 3 min at room temperature.

NOTE: The limiting step of the assay in terms of time invested is the incubation step. The data for

this paper was collected using a single quartz cuvette that was carefully washed between each sample. Even with the added washing time and preparation of the HNB stock, data collection for 14 samples and the control took approximately an hour and a half and as such, the protocol can be easily completed without the need for interruption.

2.3.3. Repeat steps 2.2.4 and 2.2.5 for each fraction that will be measured.

NOTE: If several cuvettes will be used for multiple samples, samples should be prepared in a way that allows for comparable incubation time and exposure to ambient light.

3. Metal quantification

3.1. Determining the concentration of metal in each sample

3.1.1. Find the difference of each sample absorbance at 647 nm from the HNB control.

3.1.2. Determine the metal concentration (in µM) using the formula below:

$$[Ni^{2+}] = \frac{DF \ * \Delta Abs_{647}}{3.65 * 10^{-2} * l}$$

where *DF* is the dilution factor for the assay fraction, ΔAbs_{647} is the absorbance change at 647 nm, 3.65×10^{-2} represents the extinction coefficient of HNB (ε=36.5 $mM^{-1} \cdot cm^{-1}$ see Reference[20] for more details) and *l* is cuvette's optical path in cm.

REPRESENTATIVE RESULTS:

The spectrum of free HNB at neutral pH (black line) and representative spectra of fractions assayed for Ni^{2+} from the isolation of MSP13ED1[29] are shown in **Figure 2**. A successful assay series should demonstrate a decreased absorbance at 647 nm compared to the HNB control, which corresponds to the formation of HNB complexes in the presence of a transition metal. A failed assay would be indicated by an increase in absorbance at 647 nm. Alternatively, more than 90% decrease from initial absorbance at 647 nm would indicate too high metal content and a need to assay more diluted fractions. An assay with no absorbance changes from the free HNB control does not necessarily indicate a failure. It is possible that samples contain essentially no leached metals. However, this is unlikely and any sample showing no absorbance change should be prepared and measured again, preferably with less dilution, to confirm the result. In total, most failures to observe the expected absorbance change can be attributed to improper pipetting during sample preparation, an inadequate incubation time prior to measurement, or pH values outside the recommended 7-12 range.

To demonstrate the application of this assay, we analyzed 2 His-tag membrane scaffold proteins MSP1E3D1 (isolated as in Denisov, I. G. et al.[29]), MSP2N2 (isolated as in Grinkova, Y. V.[30]), and a novel 3-heme c-type cytochrome GSU0105 from *Geobacter sulfurreducens,* which was recombinantly expressed in *E.coli* and eluted with 500 mM imidazole. The elution profiles of Ni^{2+} from a Ni-NTA resin column (see **Table of Materials**) and the associated protein elution profiles

for these 3 proteins are shown in **Figure 3**. Any protein will have a unique nickel elution that may or may not align with the protein elution profile as measured at 280 nm. For example, **Figure 3C** shows that the protein and Ni^{2+} content of each fraction for GSU0105 are significantly shifted from one another while the fractions for MSP1E3D1 and MSP2N2 (**Figure 3A,B**) that contain the most protein also have the highest Ni^{2+} content. **Figures 3A,B** also illustrate that metal content may not be evenly distributed among fractions collected using IMAC. Depending on column packing, the composition of the elution buffer, pumping equipment and conditions, it is possible to have metal elute in consecutive fractions at very different concentrations independent of the protein content of those fractions.

FIGURE AND TABLE LEGENDS:

Figure 1. Structure of hydroxynaphthol blue (HNB). In the functional pH range of the assay, all sulfonate groups and one of the hydroxyl groups are ionized.

Figure 2. Absorbance spectra for selected MSPE31D fractions and HNB. The relative absorbance of three fractions of MSPE31D (colors) compared to an HNB control (black thick line) are shown. Samples were prepared in 20 mM Tris, pH 7.5.

Figure 3. Ni^{2+} quantification for 3 representative His-tag proteins. Protein and Ni^{2+} elution profiles for (**A**) MSPE31D, (**B**) MSP2N2, and (**C**) GSU0105. Protein elution was performed using 300 mM, 300 mM, and 500 mM imidazole, respectively. Ni^{2+} quantification was performed in 20 mM Tris, pH 7.5.

DISCUSSION:

Colorimetric detection of metals using HNB provides a simple way to quantify the degree of protein contamination by transition metal ions from IMAC resins. As we established in Ref. 20, Ni^{2+} binds to HNB with 1:1 stoichiometry and the dissociation constant for the Ni-HNB complex changes with pH. However, the complex K_d is in the sub-nM range for all recommended (7-12) pH values. In practical terms, it means that all Ni^{2+} in any tested fractions will bind to HNB as long as no other strong chelating reagents, like EDTA, are present. All these properties together result in linear Ni^{2+} titration curves, which we experimentally observed. In that report[20], we also established that the spectral changes due to metal-dye complex formation will be the same over the entire 7-12 pH range. The detection is limited by the minimal reliable absorbance change measurements (10^{-4} – 10^{-3} OD depending on the spectrophotometer used) corresponding to 2.7-27 nM Ni^{2+}. The upper range is limited by the amount of HNB present. In our work, we use ~15 µM, corresponding to ~0.6 OD at 647 nm. However, this can be increased up to 50-80 µM HNB, if needed. Practically, we observed Ni^{2+} contamination levels in chromatographic fractions comparable or higher than the upper limit forcing us to make 10- to 50-fold dilutions of assayed fractions. However, this additional dilution step can increase relative errors while determining the nickel concentration in a fraction.

Though we have not investigated the details, it appeared that binding of other metals used in IMAC resins (Co, Zn, Fe) also has sub-µM dissociation constants and virtually no overlap between

dye and dye-metal absorbance spectra at 647 nm, the peak wavelength of free HNB. Therefore, complete metal binding to the dye and the associated spectral changes of the dye can be used for absolute metal determination over the entire recommended pH range.

Execution of the protocol is straightforward and depends most heavily on proper laboratory technique. Modern spectrophotometers have highly linear responses and dynamic ranges of 3-4 orders of magnitude. Consequently, the most likely place for the introduction of error in the method is through the pipetting steps for sample preparation. As described in this text, the method is based on the quantification of metal content based on the difference in the HNB absorbance peak at 647 nm from a free HNB control and samples with HNB complexed with a metal. If care is not taken to accurately pipette the HNB aliquots or the buffer volumes, comparison of the control and sample absorbances at 647 nm becomes a point of error. Similarly, poor pipetting of protein fractions for sample preparation can skew the perceived concentration of metal in a fraction. It is recommended that, due to the sensitivity of the assay, any pipettes being used for analyses where precise quantification is required be calibrated prior to use.

The primary limitations of the method come with the functional pH range of the assay and the presence of strong chelating agents. The assay is best utilized in a pH range from 7-12. Below pH 7, the spectrum of the free HNB dye changes, losing the peak at 647 nm used for quantification[20]. Above pH 12, many metal hydroxides begin to precipitate, including those of metals commonly found in IMAC resins, making quantification slower and less reproducible. While the alkaline maximum does not pose a significant problem as purification procedures rarely call for such a high pH, the acidic minimum is more likely to be a limiting factor. Since the detection limits for Ni^{2+} and other transition metals are approximately 1000-fold lower than metal contamination levels demonstrated above (**Figure 3**), the low pH limit for the assay can be circumvented by dilution of the assayed acidic protein fractions in buffers with neutral pH values and sufficiently high buffering capacity. Alternatively, the pH of analyzed fractions can be adjusted or the HNB stock solution can be more strongly buffered to maintain the desired pH after mixing.

If the isolation procedure for the protein being purified requires the use of reagents with known or suspected transition metal chelating properties, a modification of the method would be necessary to allow for proper quantification of leached metals. A standard curve would need to be prepared using the chelating agent used for protein elution and known concentrations of metal standards to accurately quantify the concentration of leached metal in the presence of the chelator. An example of metal quantification in the presence of histidine is available in Kokhan & Marzolf[20].

Accurate quantification of metals in biological samples is still largely dependent on the use of analytical techniques and instrumentation, such as AAS and ICP-MS, that remain outside the realm of the typical biochemist[31,32]. Bonta et al. have described the simple preparation of biologic samples on common filter paper for analysis by ICP-MS, however, their method still relies on non-standard instrumentation for a biochemist[31]. The method we describe allows for the measurement of metal content in a sample to be taken without additional training on new instrumentation or outsourcing to others. Similar colorimetric protocols have been developed

for metal analysis in biological samples[33]. However, the method described by Shaymal et al.[33] relies on a fluorescence assay using a commercially unavailable fluorescent probe that gives a higher limit of detection than that in this paper. Considering the relative ease by which the described method can be performed and the recent interest in the development of portable metal detecting protocols for aqueous samples[34,35], it could be easily adapted for field testing of water samples. As a portable test, our method could be modified for use with a portable spectrophotometer for quantification or as a qualitative measure to identify samples for further analysis at a fixed testing location.

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DISCLOSURES:

The authors have nothing to disclose.

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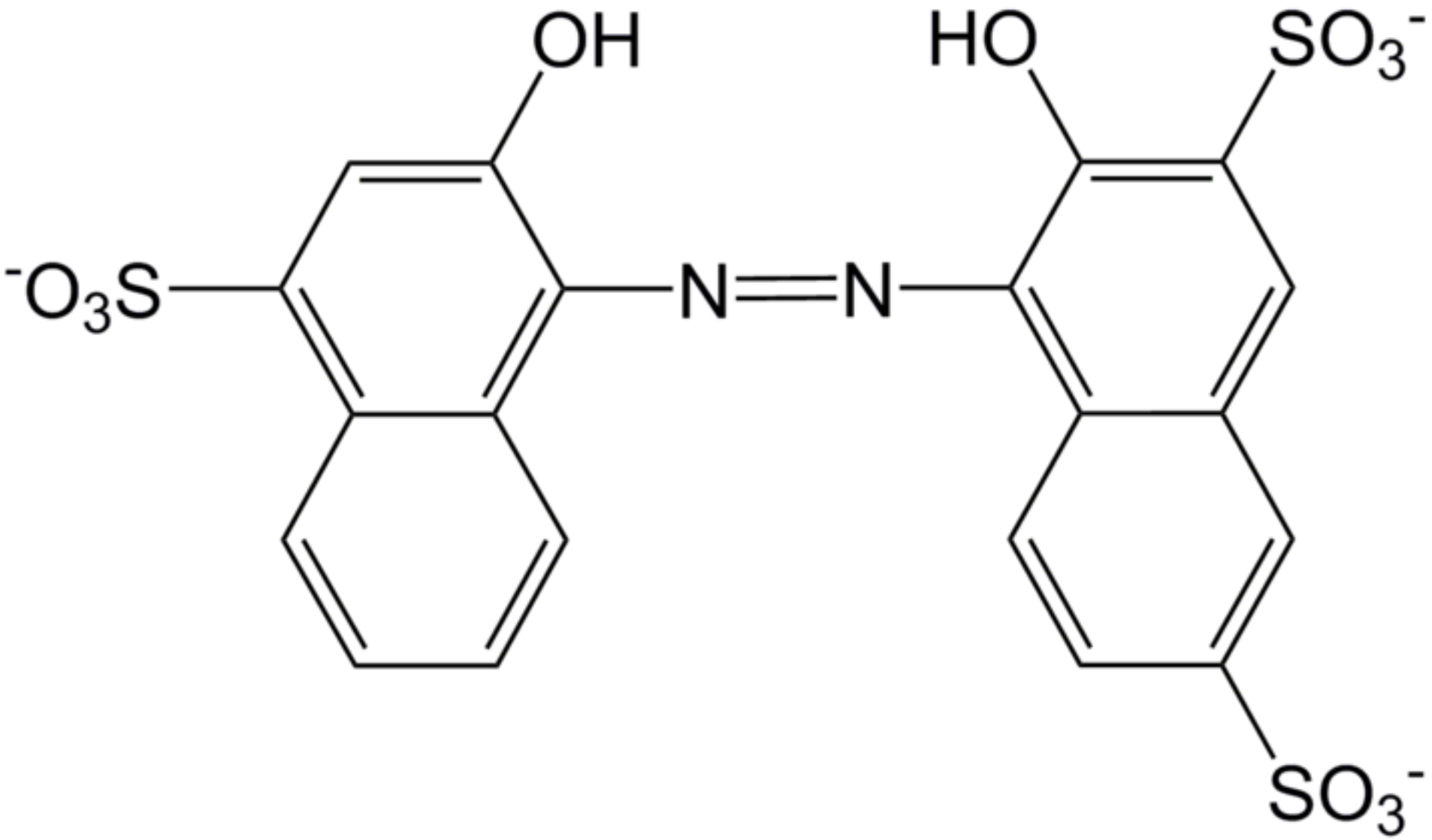

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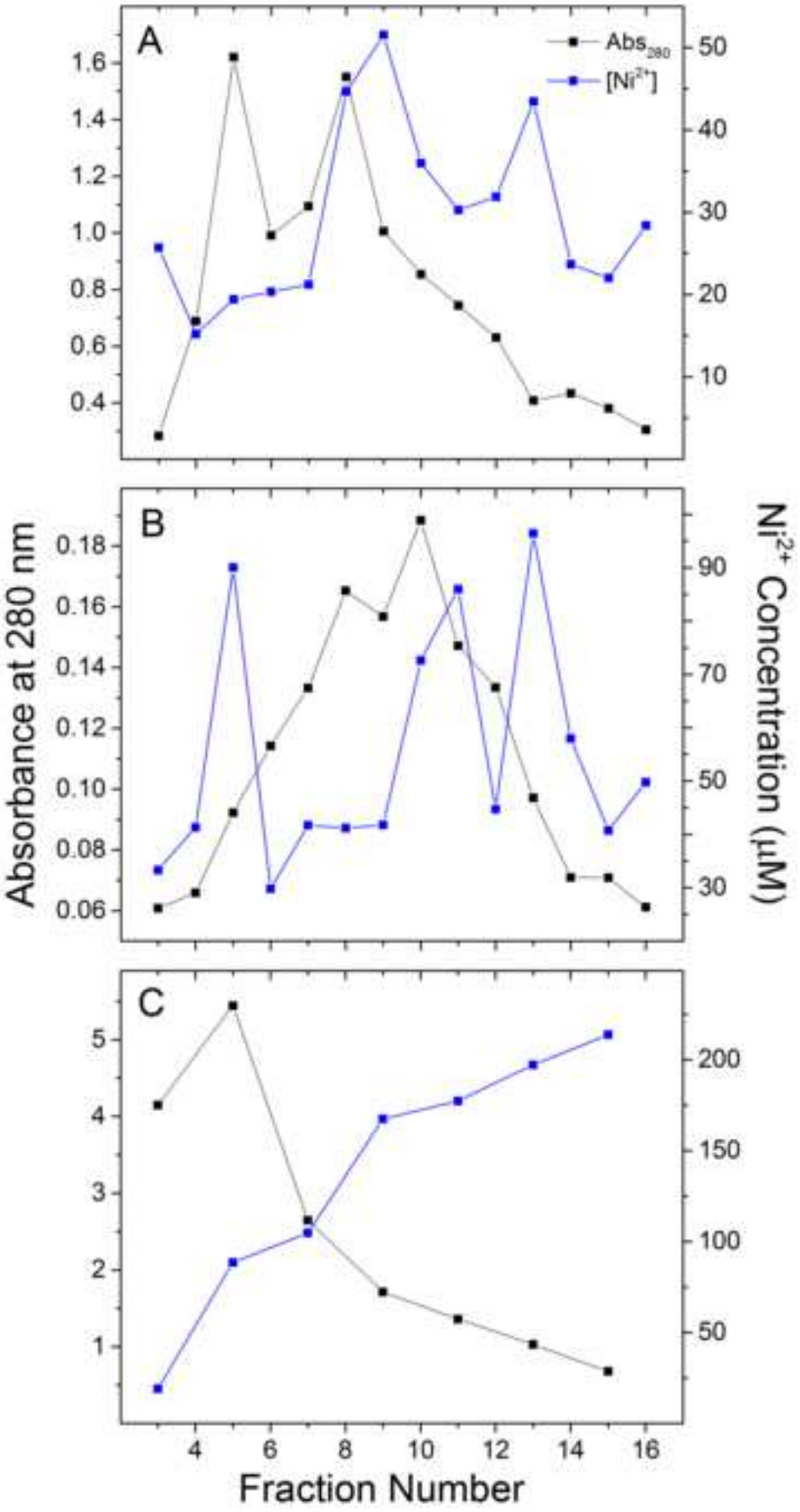

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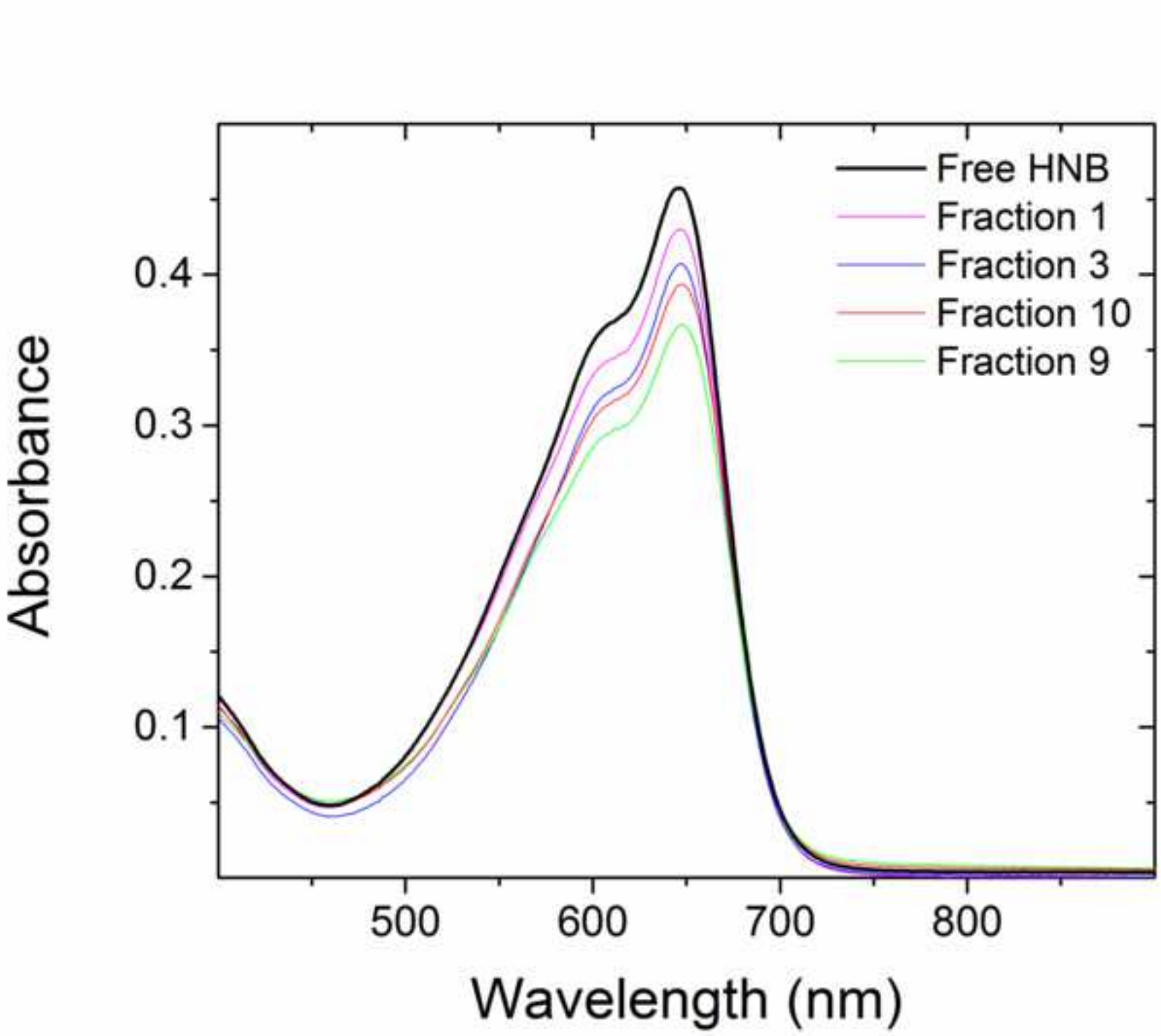

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Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2xYT broth	Fisher Scientific	BP9743-500	media for E.coli growth
HEPES, free acid	BioBasic	HB0264	alternative buffer
HisPur Ni-NTA resin	Thermo Scientific	88222	
Hydroxynaphthol blue disoidum salt	Sigma-Aldrich	219916-5g	
Imidazole	Fisher Scientific	O3196-500	
Imidazole	BioBasic	IB0277	
MOPS, free acid	BioBasic	MB0360	alternative buffer
Sodium chloride	Fisher Scientific	S271-500	
Sodium phosphate	Fisher Scientific	S369-500	alternative buffer
Tricine	Gold Bio	T870-100	
Tris base	Fisher Scientific	BP152-500	
Triton X-100	Sigma-Aldrich	T9284-500	

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9/20/19

Dear Dr. Wu,

We would like to sincerely thank you for your constructive feedback. We have addressed your comments and suggestions in the manuscript. Our changes should be visible via "Track changes". We believe that the revisions will make our manuscript much stronger and you will find them adequate and satisfactory to accept our manuscript for publications.

Specifically, to address **editorial comments** we made the following changes:
1. All co-authors and I have thoroughly proofread the manuscript and corrected a few spelling, grammar, and punctuation issues.
2. We removed mentioning of commercial brands on p. 3
3. We specified in 2.2.2 and 2.3.2 that incubations should be performed at room temperature.

Sincerely,

Oleksandr Kokhan