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Research Article

Biotherapeutic Formulation Factors Affecting Metal Leachables from Stainless Steel Studied by Design of Experiments

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Abstract. Trace amounts of metals are inevitably present in biotherapeutic products. They can arise from various sources. The impact of common formulation factors such as protein concentration, antioxidant, metal chelator concentration and type, surfactant, pH, and contact time with stainless steel on metal leachables was investigated by a design of experiments approach. Three major metal leachables, iron, chromium, and nickel were monitored by inductively coupled plasma-mass spectrometry. It was observed that among all the tested factors, contact time, metal chelator concentration, and protein concentration were statistically significant factors with higher temperature resulting in higher levels of leached metals. Within a pH range of 5.5–6.5, solution pH played a minor role for chromium leaching at 25°C. No statistically significant difference was observed due to type of chelator, presence of antioxidant, or surfactant. In order to optimize a biotherapeutic formulation to achieve a target drug product shelf life with acceptable quality, each formulation component must be evaluated for its impact.

KEY WORDS: biologics formulation factors; metal chelator; metal leachables; protein drug product/drug substance manufacturing; protein formulation stability.

INTRODUCTION

Biotherapeutic products are predominately formulated as liquids or freeze-dried products delivered by injection, and therefore fall into the category of parenteral formulations. In order to develop a stable formulation with an acceptable shelf life, excipients such as solvents/cosolvents, polymeric and surface-active compounds, chelating agents, anti-oxidants, preservatives, buffers, bulking agents, protectants, and tonicity adjusters, have been used in parenteral formulations.

The majority of biologics products consist of a buffer, a tonicity modifier, a cryo- or lyoprotectant, and a surfactant (1–4). In some cases, a metal chelator and an antioxidant are also added to the formulation to protect the protein from oxidation. Polysorbate 80 and 20 are the most commonly used surface-active compounds. The concentration of polysorbate 80 employed in most biologics formulations is ≤0.2 mg/mL. To complex the trace amount of metal ions inadvertently present in parenteral formulations, salts of EDTA (mainly sodium edentate; $Na_2EDTA.2H_2O$), have been used as chelating agents. The content of $Na_2EDTA.2H_2O$ in pharmaceutical preparations is generally between 0.005% and 0.1% (*w*/*v*, 0.05–1 mg/mL; 5). Diethylenetriaminepentaacetic acid (DTPA) is an alternative metal chelator approved for parenteral use and has demonstrated a comparable capacity to inhibit metal-induced mAb instability (6). L-methionine has been used as an antioxidant to protect labile methionine residues in proteins from oxidation. L-histidine buffer is commonly used to adjust the formulation pH for optimal stability and solubility.

Trace amounts of metal ions are invariably introduced into biotherapeutic bulk solution during drug substance and/or drug product manufacturing, shipping, and storage. The metal ions inadvertently introduced into products either arise from the excipients or leach into solution from contact with manufacturing equipment. Even though parenteral grade excipients are used, residual amounts of metal ions are present, mostly at part per billion (ppb) levels or below. Some plastic packages, glass vials, and/or rubber stoppers can also be the sources of metal leachates (7). Salts of tungsten oxide were reported to migrate from prefilled syringes into a biotherapeutic protein product (8). Barium was also observed leaching from glass vials (9). However, the major source of the metal contamination in biotherapeutic drug products is the contact with stainless steel during drug substance and/or drug product manufacturing, as well as during shipping and storage processes, such as transfer tanks, cryotanks, compounding tanks, stainless steel piping, header tanks, and filling needles.

Trace amount of metals can have a profound negative impact on the stability of the product. Site specific metal-protein binding can induce secondary and tertiary structural changes resulting in the formation of protein aggregates (10,11). Metals can induce product degradation reactions, such as oxidation

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(6,12–14), fragmentation (6,13), and aggregation (6,12,15). Such degradation reactions can potentially jeopardize the quality of the products by altering physicochemical properties as well as reducing stability and shelf life. In addition, excessive metal ions, especially heavy metal ions, introduced into a biological system may lead to safety concerns. Although metal ions are too small to induce an immune response, they can conjugate with proteins to form metal–protein complexes, potentially resulting in immunogenicity (16). Iron has been implicated in the aggregation of proteins responsible for the pathophysiology of several neurodegenerative diseases, such as Alzheimer and Parkinsons (15).

Biotherapeutic products are predominately delivered by intravenous injection. Additionally, the majority of biological products are formulated as a liquid or as a lyophilized dry powder and packaged in vials. The likelihood of an interaction between packaging and a liquid solution dosage form is considered high and considered medium for a powder product. Therefore, biotherapeutic products fall into the category of highest concern from an extractables and leachables perspective (17–19). In the USA, the requirement for the proper evaluation of extractables and leachables is governed by the Federal Food, Drug, and Cosmetic Act as codified in 21 Code of Federal Regulations, parts 210 and 211. Furthermore, specific guidance for monitoring and control of leachables as part of requirements for the primary packaging of drug products has been issued by the regulatory authorities (17,19).

The stainless steel grade commonly used in biopharmaceutical applications is called 316 L, a low-carbon (<0.03%) alloy containing mainly iron, nickel, and chromium. This grade is called austenitic stainless steel and the alloy is designed to reduce its propensity for corroding by reducing the precipitation of chromium carbides (actually mixed iron/chromium carbides) along grain boundaries of stainless steel by keeping carbon below 0.03%.

Since the biologic drug product in solution interacts with the manufacturing equipment, it is useful to understand their role in the leaching process. The impact of individual biotherapeutic formulation factors, commonly utilized buffers, the relationship of the solution volume and contact surface area, metal chelating agent concentration and type, and solution pH, on metal leachables from 316 L stainless steel was studied, respectively (20). In this paper, six major formulation factors: protein concentration, solution pH controlled by L-histidine, metal chelator concentration and type ($Na_2EDTA.2H_2O$ *vs.* DTPA), antioxidant (methionine), and surfactant (polysorbate 80), were investigated to elucidate the effect of the biotherapeutic formulation component in the presence of other components on the quantity of metal leachables from 316 L stainless steel over time at storage temperatures of −40°C, 2–8°C, and 25°C. The presence of multiple factors makes it efficient to study their impact by a design of experiments (DOE) approach. Such DOE studies are in accordance with the principles of quality by design as suggested in ICH Q8 (21) since the impact of multiple factors and their potential interaction can be evaluated, thus providing an understanding of product during all phases of production and enhance the probability of developing a robust formulation.

MATERIALS AND METHODS

Materials

As in our previous work (20), rectangular 316 L stainless steel coupons were utilized for this study. Prior to use, the coupons were chemically passivated as described earlier (20). The dimensions of the coupons were 2 cm (length)×1 cm (width)×0.1651 cm (thickness) with a surface area of ~5 cm^2. The 10-mL type I Schott glass vials and serum stoppers (West 4432/50 Gray B2-40 and Flurotec coated, West Pharmaceutical Services, Lionville, PA, USA) were used after they were cleaned and autoclaved.

An IgG2 monoclonal antibody produced in-house was used in this study. The mAb was formulated in 20 mM histidine at pH 5.5. USP/EP grade L-histidine and L-histidine hydrochloride monohydrate, analytical grade $FeCl_3$, Na_2EDTA dihydrate, and DTPA were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). EP/USP grade polysorbate 80 was obtained from J. T. Baker (Meriden, CT, USA). USP/F.C.C. grade L-methionine was obtained from J. T. Baker (Phillipsburg, NJ, USA) and parenteral grade α,α-trehalose dihydrate (low endotoxin) was obtained from Ferro Pfanstiehl Laboratories, Inc (Cleveland, OH, USA). Polyvinylidene fluoride (PVDF), 0.22 μm, filters were obtained from Millipore Inc. (Billerica, MA, USA).

Methods

Inductively Coupled Plasma-Mass Spectrometry

Metal ion concentrations in the testing solutions were quantitated by an inductively coupled plasma-mass spectrometry (ICP-MS) method which utilized an Agilent 7500 CX ICP-MS system equipped with an auto-sampler according to our published method (20). The instrument was qualified, calibrated, and maintained regularly to ensure the system suitability. Prior to analyzing the samples at each time point, an external calibration curve for each element measured was revalidated. The same linear range as in previous work (20), 1–500 ppb for iron and 1–100 ppb for chromium and nickel, was maintained.

UV Spectrophotometer for mAb Concentration

The mAb concentrations in the formulations were determined using a Cary UV spectrophotometer coupled with a diode-array detector (Varian Inc., Lake Forest, CA, USA). The extinction coefficient of 1.43 (1 mg/mL at 280 nm) was determined by the Edelhoch method (22). The system was calibrated and maintained regularly. Quartz cuvettes with a 1 cm path length were utilized. The formulations were gravimetrically diluted to 0.5 mg/mL using the corresponding placebo and then equilibrated to room temperature before testing. The corresponding placebo was used as the reference solution to zero the instrument. The absorptions for the formulations were measured at the fixed wavelength of 280 nm.

RP-HPLC Analyses for Chelator Concentration

The concentrations of both Na_2EDTA and DTPA in the formulations were determined using an Agilent 1100 system coupled to a diode array detector (6,20). The system was calibrated and maintained regularly to ensure the system suitability. The method accuracy was evaluated by a spike recovery test. Prior to sample analysis, external calibration was reestablished with varying levels of Na_2EDTA and DPTA simultaneously. The separation was monitored by UV detection at 254 nm.

Experimental Design

The experiment was designed by StatEase Design Expert® (v7.1.6) (Stat-Ease Inc., Minneapolis, MN, USA). In this study, six formulation factors of protein concentration, pH, anti-oxidation agent, metal chelator, metal chelator concentration, and surfactant concentration were investigated. In order to reduce the substantial resources needed for the full design of 2^6 (64) formulations, a fractional factorial design, $2^{(6-2)}$, of 16 formulations was adopted. This design is shown in Table I. Five of the formulation factors (factors A–E) are continuous while factor F, chelator type, is a categorical factor. The design incorporated 16 boundary runs (F1–F16) along with seven additional formulations, five of which were center points (F17–F21), and the other two formulations (F22–F23) were controls not containing any protein while other components at center points. The drawback for this design is that there could be aliasing between the significant factors which would require additional knowledge or experiments to deconvolute as to which interaction is actually significant.

The formulations were prepared in 20 mM histidine buffer with 84 mg/mL α,α-trehalose dihydrate, except the formulations F20 and 21, which were included to explore the effect of sugar. The mAb concentration ranged from 5.0 to 20.0 mg/mL with 12.5 mg/mL as center point. These concentrations were confirmed by UV absorption at 280 nm. The formulation pH was controlled at 5.5–6.5 (due to consideration of protein stability) with the center point at pH 6.0, and these were confirmed by a pH meter at ambient room temperature. The concentrations of methionine ranged from 0.0 to 0.1 mg/mL with 0.05 mg/mL as the center point. The surfactant (polysorbate 80) concentration ranged from 0.0 to 0.2 mg/mL with the center point of 0.1 mg/mL. The metal chelator concentration ranged from 0.0 to 0.1 mg/mL with 0.05 mg/mL as the center point, as confirmed by RP-HPLC. The chelator was either Na_2EDTA or DTPA. At a pH of 5.5–6.5, DTPA (H_5A) exists in equilibrium between H_2A^{3-} and H_3A^{2-} (23). For simplicity, from here on, the term DTPA is used regardless of its ionization form.

The formulations were sterile filtered through a 0.22 μm PVDF filter. Aliquots of 5 mL were filled into 10 mL autoclaved glass vials containing stainless steel coupons as the samples accompanied by the vials containing no coupons as negative controls. The vials were sealed with the autoclaved stoppers and placed upright in stability chambers maintained at −40°C over 6 months, as well as 2–8°C and 25°C over 3 months. During storage, the testing solutions were not allowed to come into contact with the stoppers eliminating the potential leachables from the stoppers confounding the study.

ICP-MS was performed on each formulation sample at the initial time point and 3 months for the storage temperature of 2–8°C and 25°C, and at both 3 and 6 months for storage

Table I. Biotherapeutic Formulation Components Studied to Explore their Capacity in Leaching Metal Ions from 316 L Stainless Steel

	A	B	C	D	E	F	
Formulation ID	mAb Conc. (mg/mL)	pH	Chelate Conc. (mg/mL)	Methionine Conc. (mg/mL)	Polysorbate 80 (mg/mL)	Chelator type	Comment: sugar
F1	5.0	5.5	0.0	0.0	0.0	DTPA	Trehalose
F2	5.0	5.5	0.0	0.1	0.0	Na_2EDTA	Trehalose
F3	20.0	5.5	0.0	0.1	0.2	Na_2EDTA	Trehalose
F4	20.0	5.5	0.0	0.0	0.2	DTPA	Trehalose
F5	20.0	5.5	0.1	0.0	0.0	Na_2EDTA	Trehalose
F6	20.0	5.5	0.1	0.1	0.0	DTPA	Trehalose
F7	5.0	5.5	0.1	0.1	0.2	DTPA	Trehalose
F8	5.0	5.5	0.1	0.0	0.2	Na_2EDTA	Trehalose
F9	20.0	6.5	0.0	0.1	0.0	DTPA	Trehalose
F10	20.0	6.5	0.0	0.0	0.0	Na_2EDTA	Trehalose
F11	5.0	6.5	0.0	0.1	0.2	DTPA	Trehalose
F12	5.0	6.5	0.0	0.0	0.2	Na_2EDTA	Trehalose
F13	5.0	6.5	0.1	0.1	0.0	Na_2EDTA	Trehalose
F14	5.0	6.5	0.1	0.0	0.0	DTPA	Trehalose
F15	20.0	6.5	0.1	0.0	0.2	DTPA	Trehalose
F16	20.0	6.5	0.1	0.1	0.2	Na_2EDTA	Trehalose
F17	12.5	6.0	0.0	0.05	0.1	Na_2EDTA	Trehalose
F18	12.5	6.0	0.05	0.05	0.1	DTPA	Trehalose
F19	12.5	6.0	0.05	0.05	0.1	Na_2EDTA	Trehalose
F20	12.5	6.0	0.05	0.05	0.1	DTPA	None
F21	12.5	6.0	0.05	0.05	0.1	Na_2EDTA	None
F22	0.0	6.0	0.05	0.05	0.1	DTPA	Trehalose
F23	0.0	6.0	0.05	0.05	0.1	Na_2EDTA	Trehalose

temperature of −40°C to explore the impact of the formulation components on metal leachables from 316 L stainless steel.

RESULTS AND DISCUSSIONS

The amount of metal ions leaching into biological products is dependent on many variables, such as formulation components, solution pH, contact materials, drug substance/drug product contact surface area with stainless steel, surface area processing procedure, contact duration and temperature, *etc*. The leaching capacity of individual biotherapeutic formulation factor, commonly utilized histidine/HCl, histidine/acetate, succinate, acetate and citrate buffers, the relationship of the solution volume and contact surface area of 1–5 mL/cm^2, metal chelator Na_2EDTA *vs.* DTPA at the concentration of 0.0–0.1 mg/mL, solution pH 5.0–9.0, on metal leachables from 316 L stainless steel was studied, respectively (20). In this paper, the focus was to investigate the effect of each formulation component in the presence of others, as described in Table I, on metal leachables from 316 L stainless steel. All stainless steel coupons used in this study were made from the same sheet of stainless steel to ensure consistency of the material. The 316 L stainless steel was made in the USA (US: S31603), composed of 62–72% iron, 16–18.5% chromium, and 10–14% nickel, along with a trace amount of carbon, magnesium, manganese, silicon, nitrogen, phosphate, and sulfur (24). Three major components, iron, chromium, and nickel in the testing solutions were quantified by ICP-MS to evaluate the amounts of metal ions introduced into the product. The other elements such as magnesium, manganese, silicon, phosphate, and sulfur were not monitored in this study because our feasibility study suggested that the amount of these leached into solution was several order of magnitudes lower than that of three major elements (iron, chromium, and nickel). Silicon can also arise from packaging such as stoppers in greater amounts than via leaching from stainless steel containers and was thus not monitored.

At each time point, both the sample vial (containing the stainless steel coupon) and its negative control (containing no stainless steel coupon) were pulled for metal ion determination. Prior to ICP-MS analysis, the samples were equilibrated to ambient room temperature by storing them in the laminar airflow hood overnight at ambient room temperature. In order to eliminate the interference of any metals possibly introduced from the glass and raw materials (25), each measured metal ion concentration in the testing solution was corrected by subtracting the amount detected in the negative control. All the samples were tested in triplicate and the relative standard deviation was within 0.5%, thus the average was utilized here.

Analysis of variance was used to explore the impact of the experimental factors on the metal ions. For each of the tested metals at their individual storage temperatures, a mathematical model was fitted to the experimental data. Formulation factors not included in the predictive equations were either not statistically significant or were concluded to be of far lesser importance than the included factors. The relative impact of the statistically significant factors over the range tested can be assessed by the magnitude and sign of their coefficients. The magnitude is the indication of their relative impact and the sign is the indication of the impact direction relative to that of variables. A positive sign indicates that the impact changed in the same direction as the change of the variables and the negative sign indicated the opposite trend.

The equation provides a quantitative prediction tool in assessing metal amounts of the untested formulations given the formulation components are within the specified testing ranges. The visual representations as well as the coefficients make it easy to compare the relative impact of the significant factors within the ranges tested. Given the need for higher protein concentration formulations and longer storage time, caution must be taken in applying the prediction model to the formulations outside of the tested ranges since the models may no longer hold true.

In each data analysis, adjusted R^2 and root mean square error (RMSE) are also provided. The adjusted R^2 is used to indicate the percentage of the total variation in the data explained by the model, adjusted for the number of terms in the model. The closer the adjusted R^2 is to 1.00, the better the model fits the experimental data. The RMSE is calculated from the difference between the experimental and predicted values (residuals). This number can be interpreted as the standard deviation of the residuals. For most of the models, we would expect about 95% of the observed values to lie within ±2 times the RMSE of the predicted values. The data on each storage condition are addressed individually.

Six Months Storage at −40°C

Over 6 months in contact with the coupons at −40°C, iron ions leaching into the testing solutions ranged from 3 to 276 ppb with a mean value of 49 ppb. Chromium ions ranged from below limit of quantitation to 17 ppb with a mean value of 5 ppb, while nickel ions increased from below limit of quantitation to 13 ppb with a mean value of 5 ppb. As expected, iron ions are the most abundant leachates among the three monitored metal ions, consistent with our previous findings (20).

A natural logarithmic transformation was applied to the data for iron, chromium, and nickel leachates as these data at 6 months were significantly higher and more variable compared to those at the initial time point. The prediction equations with the adjusted R^2 and RMSE (in natural logarithmic scale) for all three leachates are provided in Table II. The adjusted R^2, 70% with RMSE of 0.57% for iron leachates, 73% with RMSE of 0.45% for chromium, and 90% with RMSE of 0.35% for nickel, suggest that the models fit the experimental data reasonably well. The factors included in the models are only the statistically significant factors: metal chelator concentration, protein concentration, and contact time for iron leachates while protein concentration and contact time for chromium and nickel leachates. Other formulation factors, namely solution pH, the presence of methionine and polysorbate 80, and the metal chelator type (for iron leachates), or metal chelator concentration (for chromium and nickel) leachates, are not statistically significant. The coefficients of each significant factor for iron, chromium, and nickel leachates are positive, indicating that the amount of all three metal leachates increased accordingly with the increased amount of the significant factors. The impact of

Table II. Three Metal Ions of Iron, Chromium and Nickel Leaching Prediction Model along with Adjusted R^2 and Root Mean Square Error (RMSE) in Natural Logarithmic Scale

Storage temperature (°C)	Metal leachates	Prediction models	Adjusted R^2 (%)	RMSE (%)
−40°C	Iron	Ln (Fe)=1.827+0.0525×[protein conc.]+4.812×[metal chelator conc.]+0.251×contact time	70	0.57
	Chromium	Ln (Cr)=−0.0825+0.0628×[protein conc.]+0.195×contact time	73	0.45
	Nickel	Ln (Ni)=−0.364+0.0262×[protein conc.]+0.353×contact time	90	0.35
2–8°C	Iron	Ln (Fe)=1.754+0.0539×[protein conc.]+5.998×[metal chelator conc.]+0.657×contact time	82	0.51
	Chromium	Ln (Cr)=−0.0746+0.0621×[protein conc.]+0.410×contact time	77	0.42
	Nickel	Ln (Ni)=−0.493+0.0374×[protein conc.]+0.755×contact time	94	0.31
25°C	Iron	Ln (Fe)=1.476+0.0906×[protein conc.]+3.035×[metal chelator conc.]+1.164×contact time−0.0327×contact time×[protein conc.]+3.553×[metal chelator conc.]×contact time	96	0.34
	Chromium	Ln (Cr)=2.098+0.0887×[protein conc.]−0.404×pH−1.031×[chelator conc.]+0.901×contact time−0.0306×[protein]×contact time+3.064×[chelator conc.]×contact time	95	0.28
	Nickel	Ln (Ni)=−0.721+0.0601×[protein conc.]−0.649×[chelator conc.]+1.106×contact time−0.0217×[protein conc.]×contact time+1.170×[chelator conc.]×contact time	98	0.22

Unit for protein and metal chelator concentration, milligram pre millimeter; unit for contact time, month

significant factors on metal leachates is visually shown in the respective response surface plots: Fig. 1a, b for iron leachates, Fig. 1c for chromium, and Fig. 1d for nickel. Because no curvature and no interactions are observed among the significant factors, the models for all three metal leachates are additive. Consequently, the relative impact of the individual significant factor can be compared quantitatively over the range studied.

For iron leachates, the impact (per unit) of metal chelator is 19-fold of that of contact time and 91-fold of that of protein concentration within all ranges tested. The overall impact of contact time was about twice as much as that of protein concentration and three times of that of metal chelator concentration as shown in Fig. 1a, b. Note these magnitudes are relative to ranges tested. Figure 1a shows the impact of contact time and protein concentration in the formulations without any chelating agent while Fig. 1b shows the effects in the formulations with 0.1 mg/mL metal chelator. The highest levels would be predicted at 6 months, for the highest protein concentration tested (20 mg/mL) and the highest chelator concentration (0.1 mg/mL). This is represented by the elevation of the surface towards the back on the right side in Fig. 1b.

For chromium leachates, the impact of contact time is three times of that of protein concentration in per unit term within the ranges tested. However, over the tested ranges, the overall impact of contact time is approximately equal to that of the protein concentration as shown in Fig. 1c. The highest amounts of chromium leachates would be predicted for 6 months in formulations containing 20 mg/mL protein, as represented by the elevated surfaces towards the back on the right side in Fig. 1c.

As with iron and chromium, the impact on leached nickel by contact duration of the solution with stainless steel is 13 times as much as that by protein concentration in per unit terms. In relative terms, the overall impact of contact time is about five times as high as that of protein over the ranges tested as shown in the response surface plot displayed in Fig. 1d. The highest amount of nickel leachates occurred in formulations containing the highest concentration of protein (20 mg/mL) over 6 months contact with stainless steel, represented by the elevated surface towards the back on the right side in Fig. 1d.

Compared to the iron leachates in the solutions, the concentrations of chromium and nickel were significantly lower, by about 1 order of magnitude. Considering all three metals, potentially migrating from stainless steel into the drug substance and/or product from the contact stainless steel, we see that contact time and protein concentration, as well as metal chelator concentration for iron leachates are the statistically significant factors. Their contribution to the total amount of metal leachates in the final products depends on the formulation compositions and contact duration. Ranked by the contribution per unit, milligram per milliliter for concentration and months for contact time, metal chelator concentration is the most significant factor, followed by contact time and protein concentration for leaching iron, while contact time followed by protein concentration is the most significant parameter for chromium and nickel leachates.

Three Months Storage at 2–8°C

The iron leachates ranged from 3 to 229 ppb with a mean of 69 ppb. The chromium ion leachates increased from below limit of quantitation to 17 ppb with a mean of 5 ppb and nickel increased from below limit of quantitation to 23 ppb with a mean of 5 ppb. The overall levels of all three metal leachates are higher than those at −40°C. As stated previously, due to higher and more variable values at the 3 months compared to initial time point, a natural logarithmic transformation was utilized for the data analysis.

The prediction equations with the adjusted R^2 and RMSE (in the natural logarithmic scale) for all three leachates at 2–8°C are also listed in Table II. The adjusted R^2, 82% with RMSE of 0.51% for iron leachates, 77% with RMSE of 0.42% for chromium, and 94% with RMSE of 0.31% for nickel are better than those observed at −40°C. The enhanced-adjusted

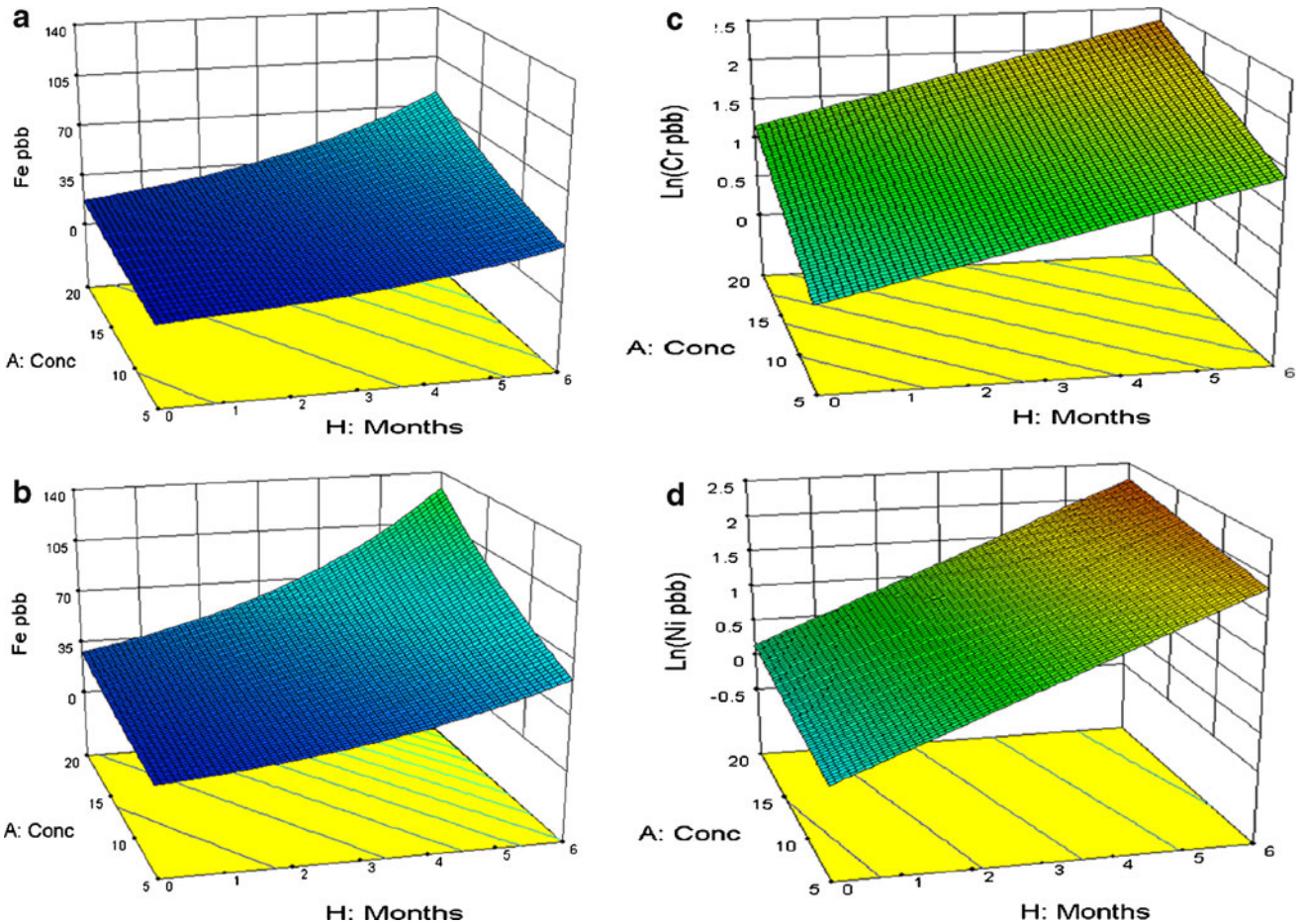

Fig. 1. The impact of the statistically significant formulation factors on metal ion leachates from stainless steel at the storage temperature of −40°C (**a** and **b** the impact on iron ion leachates: **a** for the formulations containing no metal chelator and **b** for the formulations containing 0.1 mg/mL metal chelator; **c** for the impact on chromium ion leachates in natural logarithmic scale and **d** for the impact on nickel ion leachates in natural logarithmic scale)

R^2 and RMSE suggest that the models fit experimental data very well. As discussed previously, only the statistically significant factors are included in the models. Other formulation factors are not statistically significant. The models for all three metal leachates of iron, chromium, and nickel are additive and the combined impacts of significant factors on metal leachates are presented in the response surface plots: Fig. 2a, b for iron leachates, Fig. 2c for chromium, and Fig. 2d for nickel.

As shown for the analysis of storage at −40°C, the significant effects for the three metal leachates at 2–8°C also arise from protein concentration and contact time, and includes metal chelator concentration for iron leachates. All three have positive coefficients: metal leachates increase as any of these factors and/or the combination thereof increases.

For iron leachates, as shown in Fig. 2a, b, the impact coefficient (per unit) of metal chelator concentration is about 110-fold of that of protein concentration and 9-fold of contact time. Over the ranges tested, the relative contributions for leaching iron decrease in the following order: contact time was about 2.5 times of that of protein concentration, and three times of that of chelator concentration. This is the same trend as observed for −40°C storage except that the magnitudes are higher. The highest amounts of ion leachates occurred in the formulation containing 20 mg/mL protein and 0.1 mg/mL metal chelator over 3 months contact with the stainless steel, as indicated by the elevation of the surface towards the back on the right side in Fig. 2b (note that Fig. 2a shows the impact of time and protein concentration in formulations containing no metal chelator while 2B shows the same effect in formulations with 0.1 mg/mL metal chelator.)

For chromium leachates, as shown in Fig. 2c, the impact (per unit) of contact time was about 7-fold that of protein concentration. Within the tested ranges, the overall impact of the contact time is 1.3 times that of protein concentration. The highest levels occurred at 3 months in the formulations containing 20 mg/mL protein, represented by the elevation of the surface towards the back on the right side in Fig. 2c.

For nickel leachates, the contribution of the contact duration (per month) of the solution with stainless steel is about 20-fold that of protein concentration (per milligram per milliliter). Within the tested ranges, the overall impact of contact time is about four times of that of protein concentration as shown in the response surface plot in Fig. 2d. The formulation containing 20 mg/mL protein stored over 3 months exhibited the highest levels of nickel leachates, represented by the elevation of the surface towards the back on the right side in Fig. 2d.

Similar to the results at −40°C, iron leaching occurs to a much higher extent than the leaching of chromium and nickel. In the parameter ranges studied, the impact coefficient

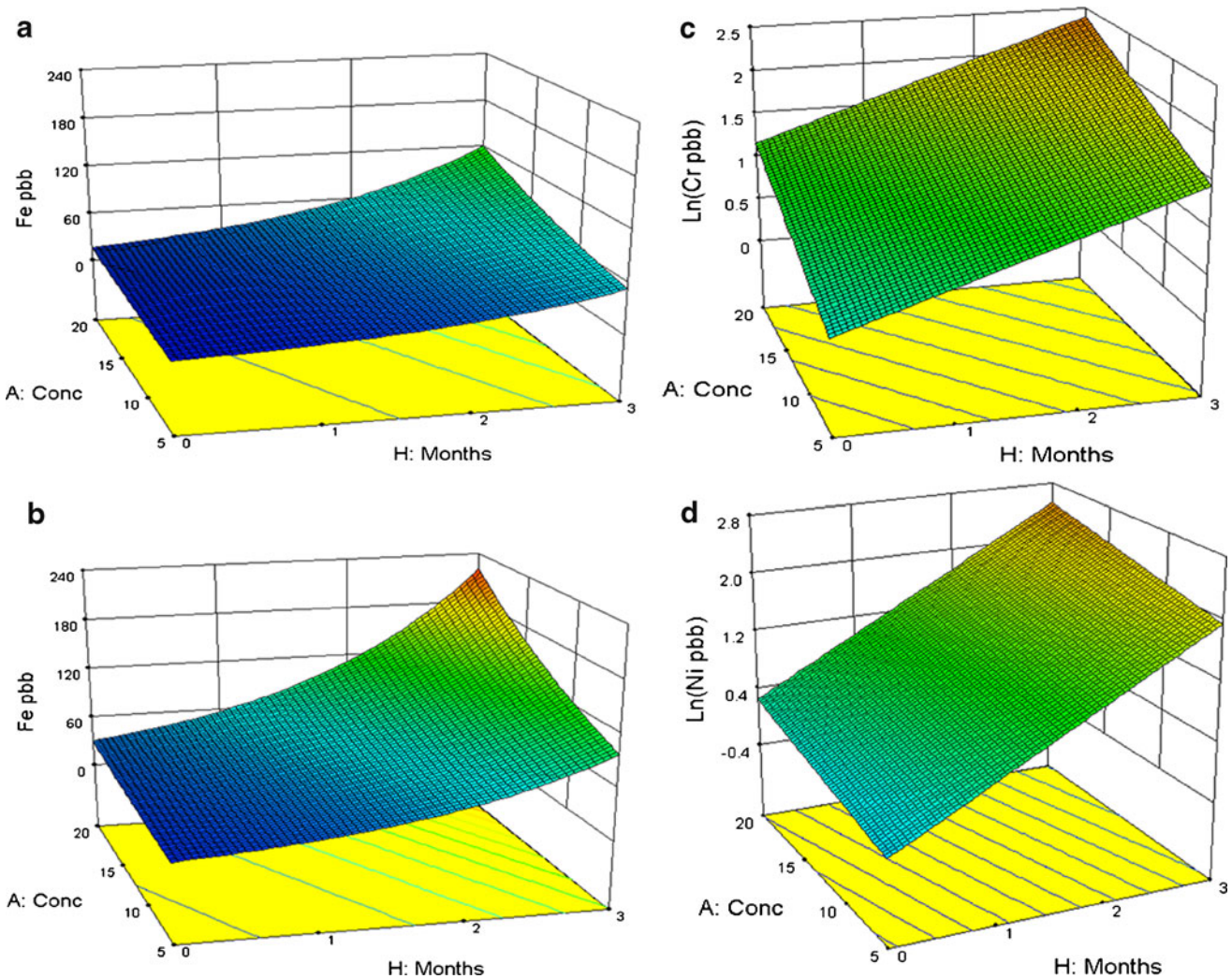

Fig. 2. The impact of the statistically significant formulation factors on metal ion leachates from stainless steel at the storage temperature of 2–8°C (**a** and **b** the impact on iron ion leachates: **a** for the formulations containing no metal chelator and **b** for the formulations containing 0.1 mg/mL metal chelator; **c** for the impact on chromium ion leachates in natural logarithmic scale; and **d** for the impact on nickel ion leachates in natural logarithmic scale)

(impact per unit) indicated that metal chelator concentration showed the most significant contribution for the leaching of iron from stainless steel, followed by contact time and then protein concentration, while contact time followed by protein concentration was most important for chromium and nickel leachates.

Three Month Storage at 25°C

As expected, iron ion leaching increased much more at 25°C compared to that at −40°C or 2–8°C. The iron leachates ranged from 3 to 550 ppb with a mean of 165 ppb, almost twice the amount of iron leachates at −40°C and 2–8°C. The chromium leachates increased from below the limit of quantitation to 27 ppb with a mean of 8 ppb, and nickel increased from below the limit of quantitation to 23 ppb with a mean of 8 ppb, a slight increase compared to the data at −40°C and 2–8°C. A natural logarithmic transformation was utilized for data analysis. The prediction equations along their R^2 and RMSE (in natural logarithmic scale) at 25°C are presented in Table II. The R^2, 96% with RMSE of 0.34% for iron leachates, 95% with RMSE of 0.28% for chromium, and 98% with RMSE of 0.22% for nickel suggest that the models fit experimental data with high accuracy.

As discussed previously, only the statistically significant factors are included in the models. Even though protein concentration, metal chelator concentration, and contact time of the tested solutions with the stainless steel are still the only three statistically significant factors within the testing parameter ranges, interactions between contact time and protein concentration, and metal chelator concentration are observed for all three metal leachates. The interactions greatly increased the prediction model complexity because of the inherent inadequacy due to the partial factorial design of $2^{(6-2)}$ (16 formulations) instead of the full factorial design of 2^6 (64 formulations). This partial DOE design was adopted to balance the required resources and the benefit. In this design, the individual contribution of the interacting factors cannot be separated from the combined effect as the interactions are present. In order to mathematically quantify the dependency of one factor on another, a more thorough investigation is needed which is outside the scope of the work

reported here. Therefore, in our data analysis, the interactions of the contact time with the protein concentration, and metal chelator concentration do not allow ranking of the relative contribution of the three major factors affecting three metal ion leachates.

For iron leachates, protein concentration, metal chelator concentration, and contact time are the three statistically significant factors and their individual impact coefficients are positive. However, interactions between metal chelator concentration and contact time, and between protein concentration and contact time are also observed. The combined effect of metal chelator concentration and contact time enhanced metal ion leaching, comparable to their individual effects. Thus, the overall effect of increased metal chelator concentration was to increase metal iron leachates in the solution, as shown in Fig. 3a, b. In contrast, the interaction between protein concentration and contact was opposite to the direction of their individual impact so that it is hard to quantify the individual final impact. However, the overall contribution of each parameter is qualitatively presented in Fig. 3a, b. The overall consequences of contact time were to increase metal ion leachates over the increased contact duration while no significant impact from protein concentration was observed. Despite the complexity of the interactions in the models, the overall impact of metal chelator concentration is more significant than that of contact time and protein concentration, that is, the amount of iron ion leachates increased much more dramatically in the formulations containing 0.1 mg/mL metal chelator (Fig. 3b) than the formulations containing no metal chelator (Fig. 3a). The highest amounts of iron leachates occurred in the formulations containing 5 mg/mL protein (lowest studied level) and 0.1 mg/mL metal chelator (the highest studied levels) over 3 months, represented by the elevation of the surface towards the front on the right side in Fig. 3b.

For chromium leachates, the model shown in Table II suggests that within the tested parameter ranges, besides protein concentration, metal chelator concentration and contact time as the significant factors, solution pH also played an important role. The lower pH of 5.5 increased chromium leaching more than pH 6.5. The interactions of contact time with protein concentration and with metal chelator concentration make it infeasible to quantify the individual contributions of protein concentration, metal chelator concentration, and contact time. However, over the tested ranges, an increased

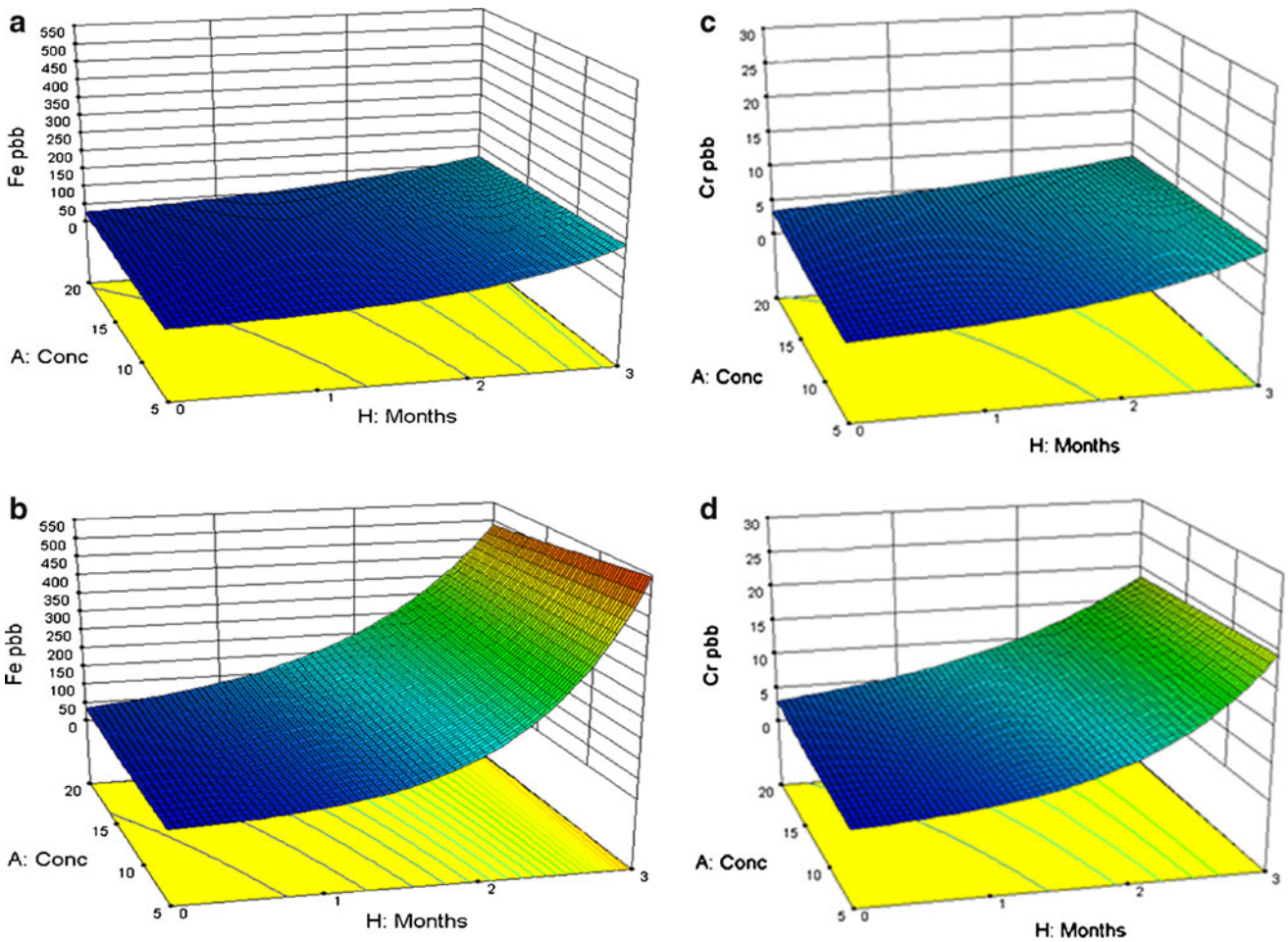

Fig. 3. The impact of the statistically significant formulation factors on metal ion leachates from stainless steel at the storage temperature of 25°C (**a** and **b** the impact on iron leachates: **a** for the formulations containing no metal chelator and b for the formulations containing 0.1 mg/mL metal chelator; **c**–**f** the impact on chromium ion leachates: **c** for the formulations at pH 6.5 containing no metal chelator, **d** for the formulations at pH 6.5 containing 0.1 mg/mL metal chelator, **e** for the formulations at pH 5.5 containing no metal chelator, and **f** for the formulations at pH 5.5 containing 0.1 mg/mL metal chelator; **g** and **h** the impact on nickel ion leachates: **g** for the formulations containing no metal chelator and **h** for the formulations containing 0.1 mg/mL metal chelator)

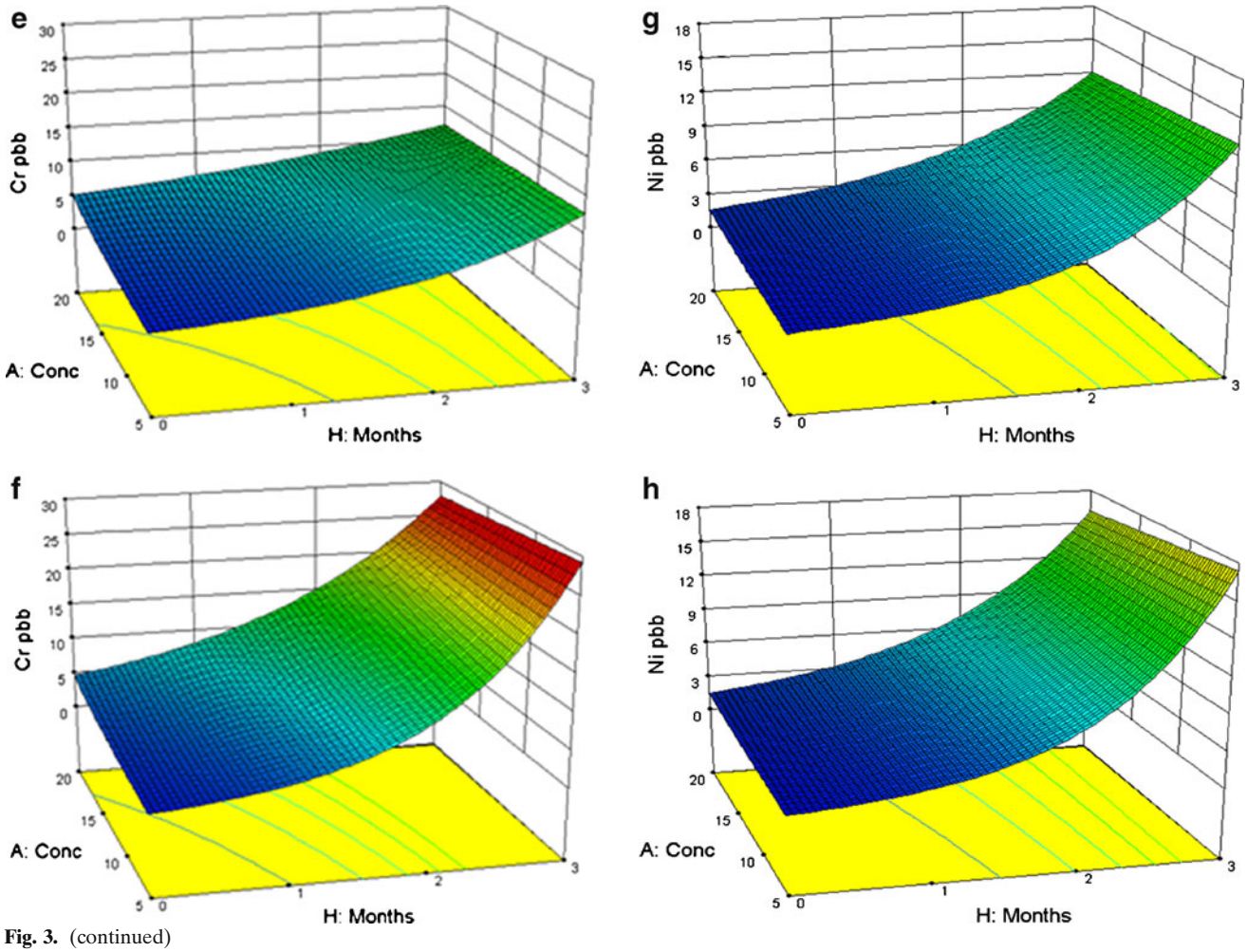

Fig. 3. (continued)

contact time and metal chelator concentration increased chromium leachates as shown in Fig. 3c–f. As a consequence of the combined effects, the amount of chromium leachates increased most significantly in the formulations at pH 5.5 (lowest tested pH) containing 0.1 mg/mL metal chelators (highest tested level), as shown in Fig. 3f. This indicates that a decrease of pH and an increase in metal chelator concentration increase the capacity of the formulation for leaching chromium from stainless steel. The highest amount of chromium leachates occurred in the formulations at pH 5.5 containing the lowest protein concentration of 5 mg/mL protein, and 0.1 mg/mL metal chelator over 3 months, represented by the elevation of the surface towards the front on the right side in Fig. 3f, the worst case scenario. The chromium leaching pattern observed at 25°C is dramatically different from that observed at lower storage temperatures of −40°C and 2–8°C.

For nickel ion leachates, protein concentration, metal chelator concentration, and contact time are still the three statistically most significant factors. As for iron and chromium leachates, the complicated interactions between the contact time and protein concentration, and the interaction with metal chelator concentration make it infeasible to quantify each individual impact. However, the overall impact of the significant factors as exhibited in Fig. 3g, h, the increased contact duration and metal chelator concentration increased nickel leachates in the formulations. The nickel leachates changed more significantly in the formulations containing 0.1 mg/mL metal chelators as shown in Fig. 3h which indicates that the metal chelator plays an important role. The highest amount of nickel leachates occurred in the formulations containing the lowest protein concentration of 5 mg/mL protein, and highest metal chelator concentration of 0.1 mg/mL over 3 months, represented by the elevation of the surface towards the front on right side in Fig. 3h. Analogous to the data for chromium leachates, the observed nickel leaching pattern from stainless steel at 25°C is also dramatically different from that observed for lower storage temperatures of −40°C and 2–8°C.

Compared to the leaching patterns of iron, chromium, and nickel at −40°C and 2–8°C, more complex metal leaching patterns were observed at 25°C, which indicates that temperature plays an important role. Regardless of the interactions, under the three tested storage conditions, metal chelator concentration, protein concentration, and contact time are the three major statistically significant factors for leaching iron, chromium, and nickel from stainless steel. The statistically significant and insignificant formulation components affecting metal ion leachates at storage temperature of −40°C, 2–8°C, and 25°C are summarized in Table III.

Protein concentration ranges vary widely in biopharmaceutical products. Our observation that protein concentration controls the capacity for metal leaching from stainless steel is

Table III. Summary Presentation of Statistically Significant and Insignificant Factors Impacting Metal Leachability at Storage Temperatures of −40°C, 2–8°C, and 25°C

Storage temperature (°C)	Leachables	Significant factors	Insignificant factors
−40°C	Iron	Protein conc., metal chelator conc. and contact time	pH, metal chelator type, anti-oxidant and surfactant
	Chromium Nickel	Protein conc. and contact time	pH, metal chelator conc./type, anti-oxidant and surfactant
2–8°C	Iron	Protein conc., metal chelator conc. and contact time	pH, metal chelator type, anti-oxidant and surfactant
	Chromium Nickel	Protein conc. and contact time	pH, metal chelator conc./type, anti-oxidant and surfactant
25°C	Iron	Protein conc., metal chelator conc. and contact time	pH, metal chelator type, anti-oxidant and surfactant
	Chromium	Protein conc., metal chelator conc., contact time and pH	Metal chelator type, anti-oxidant and surfactant
	Nickel	Protein conc., metal chelator conc. and contact time	Metal chelator type, pH, anti-oxidant and surfactant

consistent with previous findings (26–28). It was reported that the presence of proteins had a significant impact on the passivation behavior of the metals and alloys (26–28). Acting as complexing agents for dissolved metal ions, the protein stimulated the dissolution rate of a base metal in a structure-dependent manner, and, consequently, suppressed the formation of the protective oxide layer (27). Brown and Merritt reported that the presence of protein produced pitting corrosion on the surface of stainless steel (26). The same phenomenon was also observed by us during protein drug product storage in 316 L stainless steel minitanks. Woodman demonstrated that in the presence of protein, the leached nickel and chromium predominately formed metallo-organic complexes with the protein (28). In addition, serum protein *in vivo* and *in vitro* fluids also showed a significant capacity at increasing cobalt, chromium and nickel leaching from 316 L stainless steel (28) and the leaching pattern was quantitatively fitted into a mathematical model.

In approved biotherapeutic drug products, metal chelators such as Na_2EDTA and DTPA are present at relatively low concentrations. EDTA was reported to facilitate metal leaching from contact stainless steel into solution (20,27), consistent with the present study. However, our observation of no significant difference between Na_2EDTA and DTPA is different from previous observations when the leaching capacity was investigated only in 20 mM histidine buffer (20).

The impact of contact time was reported to play a significant role in leaching metal ions from stainless steel when the impact of individual component of buffer species, and metal chelator in 20 mM histidine was investigated (20), a similar phenomenon as observed in this study.

We note here that this study was performed with histidine buffer which is commonly used in biotherapeutics drug products. Histidine buffer has a significant temperature coefficient ($-0.022\ K^{-1}$) such that formulations prepared at ambient room temperature (~25°C) will experience a pH approximately 0.4 units higher at 2–8°C (29). Thus samples at 2–8°C actually underwent leaching at a slightly higher pH than stated. However, this represents exactly the situation that would occur in practice.

In the current competitive market, the need for high concentration biotherapeutic drug products has increased dramatically to improve dosing and storage convenience. A protein concentration of 100 mg/mL and higher has been seen in some approved biotherapeutic products. In most cases, protein concentration in drug substance has to be even higher than the finished drug product due to the dilution effect of excipients which must be added afterwards. Also, the storage duration of the drug substance can be over 24 months. Therefore, the overall impact of protein concentration, metal chelator concentration, and contact time needs to be carefully evaluated with respect to the formulation composition and the storage duration. We expect that if different ranges of the factors had been studied, the relative magnitudes of impact would not be the same. However, it can be generally expected that the presence of metal chelators and long contact time at higher temperatures will always contribute to more leaching of metal ions.

CONCLUSIONS

Biotherapeutic formulation factors of protein concentration, metal chelator concentration/type, solution pH, the presence of methionine and surfactant of polysorbate 80, and contact time were studied for their capacity to promote metal leaching from contact stainless steel in a defined formulation matrix. Among them, metal chelator concentration, protein concentration, and contact time are the three statistically most significant parameters affecting metal leaching from the contact with 316 L stainless steel at storage temperatures of −40°C, 2–8°C, and 25°C. Furthermore, temperature is an important factor affecting metal leaching pattern. Increased temperature dramatically increased metal leaching amount, probably simply because of higher mobility of ions and reduced viscosity of the disaccharide solutions. The higher mobility enables leached ions to be transported away from the metal surface and thus maintains a higher driving force for leaching compared to that at lower temperatures. Regardless of the interactions among factors, increased metal chelator concentration and contact duration enhanced metal leaching into the solution for all three tested conditions. However, the complexity caused by the interactions makes the impact of protein concentration more complicated at storage temperature of 25°C. At −40°C

and 2–8°C, the increased protein concentration increased the metal leachates. Within the tested pH range of 5.5–6.5, solution pH played a minor role for facilitating chromium leaching from stainless steel into the product only at 25°C. No statistically significant impact is observed for anti-oxidant (methionine) and the surfactant (polysorbate 80). During biotherapeutic product development, each formulation component, and potential storage duration and temperature must be carefully evaluated for its impact to reduce the risk, consequently, to optimize the biotherapeutics formulation to achieve the target drug product shelf life with acceptable quality.

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