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QbD-Guided Development and Validation of a Matrix-Resistant ICP-MS Method for Trace Element Analysis in Bacterial Cells, Biofilms, and Culture Supernatants

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ABSTRACT: Trace element analysis plays an important role in understanding bacterial metabolism, metal homeostasis, biofilm formation, and microbial responses to environmental stress. However, accurate elemental determination in bacterial cells, biofilms, and culture supernatants is challenging because of matrix complexity and potential analytical interference. The present study aims to develop and validate a Quality by Design (QbD)-guided, matrix-resistant Inductively Coupled Plasma–Mass Spectrometry (ICP-MS) method for the quantitative determination of selected trace elements in different bacterial matrices. The analytical method involves systematic identification of critical method parameters, risk assessment, and optimization of instrumental conditions using a Design of Experiments (DoE) approach. Selected elements, including iron, zinc, copper, manganese, magnesium, calcium, nickel, and cobalt, are investigated using appropriate sample digestion, calibration, and matrix

correction procedures. Analytical performance is evaluated through linearity, accuracy, precision, recovery, sensitivity, detection limits, and robustness. Matrix effects are assessed separately in bacterial cell digests, biofilm samples, and culture supernatants to establish the applicability of the developed method across different biological matrices. Furthermore, comparative elemental profiling is intended to provide insights into bacterial metal distribution and its possible association with cellular metabolism and biofilm-associated metal retention.

1. INTRODUCTION

Trace elements play an essential role in bacterial growth, metabolism, cellular regulation, and survival under different environmental conditions. Metals such as iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), magnesium (Mg), calcium (Ca), nickel (Ni), and cobalt (Co) participate in several biological processes, including enzymatic reactions, protein stabilization, energy production, DNA synthesis, and oxidative stress protection. The availability of these elements is important for maintaining normal bacterial physiological functions. However, excessive metal accumulation may produce cellular toxicity, disrupt metabolic activities, and affect bacterial survival. Bacteria therefore maintain metal homeostasis through specific uptake, transport, storage, and efflux mechanisms that regulate intracellular metal concentrations (Chandrangsu et al., 2017; Helmann, 2025). Recent studies have also highlighted the importance of metal-dependent regulatory systems in bacterial adaptation, cellular metabolism, and resistance to metal-induced stress (Sullivan et al., 2024).

Inductively Coupled Plasma–Mass Spectrometry (ICP-MS) is a highly sensitive analytical technique widely used for the determination of trace and ultra-trace elements in biological, pharmaceutical, environmental, and microbiological samples. Its major advantages include multi-element detection, high sensitivity, a wide analytical range, and the ability to determine several elements within a single analytical procedure. ICP-MS has also been increasingly applied in cellular research to investigate elemental distribution and understand the biological importance of metals at the cellular level (Wang et al., 2017; Van Acker et al., 2023). The technique is particularly suitable for bacterial research because multiple essential and potentially toxic elements can be quantified simultaneously using appropriately prepared biological samples. However, accurate elemental determination requires suitable sample digestion, calibration, contamination control, and correction of instrumental interferences. Biological matrices containing salts, proteins, organic residues, and other dissolved components may influence sample introduction, ionization, and signal intensity, resulting in analytical signal suppression or enhancement (Korvela et al., 2018).

Bacterial cells, biofilms, and culture supernatants represent three important biological matrices for investigating bacterial metal distribution. Bacterial cell samples provide information about the total elemental content associated with collected cellular biomass, whereas biofilm samples contain both bacterial biomass and extracellular polymeric substances (EPS). These extracellular materials consist mainly of polysaccharides, proteins, extracellular DNA, and other biomolecules that contribute to biofilm structure and stability. The chemical composition of the biofilm matrix may influence metal binding, retention, and distribution. Metal availability also affects bacterial adhesion, biofilm development, cellular communication, and microbial stress responses (Price & Skaar, 2025). In contrast, culture supernatants provide information about extracellularly present elements, including dissolved metals and other recoverable elemental fractions. Comparative analysis of these matrices may therefore help explain differences in metal uptake, extracellular release, and biofilm-associated metal accumulation.

Despite the analytical advantages of ICP-MS, the analysis of different bacterial matrices presents several challenges. Variations in biomass composition, extracellular polymeric substances, residual culture media, dissolved salts, and organic materials may produce matrix-dependent analytical responses. Furthermore, contamination during sample collection, washing, digestion, and storage may significantly affect the determination of trace-level elements. Conventional analytical method development based mainly on trial-and-error optimization may not adequately identify the interactions between instrumental parameters and sample preparation variables. Therefore, a systematic analytical development strategy is required to improve method performance and establish reliable operating conditions for different biological matrices. The use of internal standards, appropriate collision/reaction cell conditions, matrix-matched calibration, and standard addition procedures can help minimize different types of analytical interference (Korvela et al., 2018; Van Acker et al., 2023).

Quality by Design (QbD) provides a scientific and risk-based framework for developing robust analytical procedures through the identification of critical method parameters, critical quality attributes, risk assessment, and systematic optimization. Instead of relying exclusively on conventional trial-and-error approaches, QbD emphasizes understanding how analytical variables influence method performance. The application of Design of Experiments (DoE) can further support the investigation of individual parameters and their interactions, allowing the development of an appropriate analytical design space. The International Council for Harmonisation guidelines ICH Q14 and ICH Q2(R2) provide important principles for science-based analytical procedure development and validation, respectively. These guidelines emphasize the importance of demonstrating that an analytical procedure is suitable for its intended purpose through appropriate evaluation of accuracy, precision, specificity, linearity, detection limits, and robustness (International Council for Harmonisation [ICH], 2023a, 2023b).

In addition to analytical method development, trace element profiling may provide valuable information about bacterial metal homeostasis and its relationship with biofilm formation and antimicrobial stress. Essential metals are involved in several bacterial metabolic pathways, while metal deficiency or excess can influence cellular growth, enzyme activity, oxidative stress responses, and bacterial adaptation. Changes in elemental distribution following antimicrobial exposure may also provide useful information about bacterial stress responses and metal-associated resistance mechanisms. However, total elemental concentrations alone cannot establish the specific molecular mechanisms responsible for antimicrobial resistance. Further microbiological, biochemical, and molecular investigations are necessary to establish these relationships (Begg, 2019; Sullivan et al., 2024).

Considering these analytical and biological aspects, the present study aims to develop and validate a QbD-guided, matrix-resistant ICP-MS method for the quantitative determination of selected trace elements in bacterial cells, biofilms, and culture supernatants. The study focuses on identifying critical method parameters, optimizing instrumental operating conditions, minimizing matrix-related interference, and evaluating analytical performance through accuracy, precision, linearity, recovery, sensitivity, and robustness studies. Furthermore, comparative trace element profiling is intended to provide an understanding of elemental distribution among different bacterial matrices and its possible association with metal homeostasis and biofilm formation. The developed analytical platform is expected to provide a reliable approach for future pharmaceutical and microbiological investigations involving bacterial metal metabolism, antimicrobial drug stress, and metal-associated resistance mechanisms.

2. MATERIALS AND METHODS

The present study was designed to develop and validate a Quality by Design (QbD)-guided, matrix-resistant Inductively Coupled Plasma–Mass Spectrometry (ICP-MS) method for the quantitative determination of trace elements in bacterial cells, biofilms, and culture supernatants. The analytical workflow included bacterial culture preparation, biological sample processing, acid digestion, QbD-based method optimization, instrumental analysis, and analytical method validation. Particular emphasis was placed on minimizing matrix-related interference and improving the accuracy, precision, sensitivity, and reproducibility of trace element measurements across different bacterial sample matrices.

2.1. Chemicals, Reagents, and Analytical Instruments

All chemicals and reagents used in the study were of analytical or trace-metal analysis grade. Ultrapure water (18.2 MΩ·cm) was used for the preparation of standards, reagents, and sample dilutions. High-purity nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) were used for the digestion of biological samples. Certified multi-element reference standards containing iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), magnesium (Mg), calcium (Ca), nickel (Ni), and cobalt (Co) were used for calibration and quantitative elemental determination.

An ICP-MS instrument equipped with a collision/reaction cell was employed for elemental analysis. Other laboratory equipment included an analytical balance, centrifuge, incubator, micropipettes, vortex mixer, pH meter, and microwave digestion system. Acid-cleaned polypropylene tubes and metal-free laboratory containers were used throughout the experimental procedure to minimize external contamination.

The major chemicals, reagents, and analytical instruments used in the investigation are presented in Table 1.

Table 1. Chemicals, Reagents, and Analytical Instruments Used in the Study

S. No.	Chemicals/Reagents/Equipment	Grade/Specification	Purpose
1	Nitric acid (HNO_3)	Trace-metal grade	Biological sample digestion
2	Hydrogen peroxide (H_2O_2)	Analytical grade	Oxidation of organic matter
3	Ultrapure water	18.2 MΩ·cm	Sample and standard preparation
4	Multi-element reference standard	Certified reference solution	Elemental calibration
5	Internal standard solution	ICP-MS grade	Correction of signal variation
6	Luria–Bertani (LB) broth	Microbiological grade	Bacterial cultivation
7	Tryptic soy broth	Microbiological grade	Bacterial cultivation
8	Nutrient broth	Microbiological grade	Bacterial cultivation
9	ICP-MS instrument	Collision/reaction cell equipped	Trace element determination
10	Microwave digestion system	Closed-vessel system	Biological sample digestion
11	Centrifuge	Refrigerated laboratory centrifuge	Biomass separation
12	Incubator	Temperature-controlled	Bacterial growth
13	Analytical balance	High-precision	Sample weighing
14	Micropipettes	Calibrated	Accurate liquid handling
15	Vortex mixer	Laboratory grade	Sample homogenization
16	Acid-cleaned polypropylene tubes	Metal-free containers	Sample storage and preparation

2.2. Selection of Bacterial Strains and Culture Preparation

Four bacterial strains, namely Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Bacillus subtilis, were selected as Gram-negative and Gram-positive bacterial models. These organisms were considered for evaluating differences in elemental distribution, bacterial metal homeostasis, and biofilm-associated metal accumulation.

The bacterial strains were maintained using suitable growth media under appropriate laboratory conditions. E. coli and P. aeruginosa were cultured in Luria–Bertani (LB) broth, whereas S. aureus and B. subtilis were cultured in tryptic soy broth and nutrient broth, respectively. Cultures were incubated at approximately 37°C under suitable growth conditions.

Fresh bacterial suspensions were prepared and standardized using optical density measurements at 600 nm (OD_{600}) or viable-cell counting. Standardized bacterial suspensions were subsequently used for preparing bacterial cell pellets, biofilm samples, and culture supernatants. Uninoculated culture media were maintained as controls to determine background elemental contributions.

The selected bacterial strains and their culture conditions are summarized in Table 2.

Table 2. Selected Bacterial Strains and Culture Conditions

S. No.	Bacterial strain	Gram characteristics	Culture medium	Incubation temperature	Analytical samples
1	Escherichia coli	Gram-negative	LB broth	37°C	Cells, biofilm, supernatant
2	Staphylococcus aureus	Gram-positive	Tryptic soy	37°C	Cells, biofilm,

			broth		supernatant
3	Pseudomonas aeruginosa	Gram-negative	LB broth	37°C	Cells, biofilm, supernatant
4	Bacillus subtilis	Gram-positive	Nutrient broth	37°C	Cells, biofilm, supernatant

2.3. Selection of Trace Elements for ICP-MS Analysis

Eight biologically relevant elements, including Fe, Zn, Cu, Mn, Mg, Ca, Ni, and Co, were selected for quantitative analysis. These elements were chosen because of their involvement in bacterial metabolism, enzymatic activity, cellular regulation, oxidative stress responses, and microbial growth.

The analytical isotopes were selected according to their natural abundance, instrumental sensitivity, and potential spectral interference. The selected isotopes included ^{56}Fe, ^{66}Zn, ^{63}Cu, ^{55}Mn, ^{24}Mg, ^{44}Ca, ^{60}Ni, and ^{59}Co. Appropriate collision/reaction cell conditions were considered to minimize polyatomic and other spectral interferences.

Multi-element calibration standards were prepared at different concentration levels using certified reference solutions. Suitable internal standards were selected to compensate for instrumental drift, variations in sample introduction, and signal instability.

The selected trace elements, analytical isotopes, and their biological significance are presented in Table 3.

Table 3. Selected Trace Elements and Their Analytical Isotopes

S. No.	Element	Symbol	Selected isotope (m/z)	Biological significance
1	Iron	Fe	^{56}Fe	Cellular respiration and electron transport
2	Zinc	Zn	^{66}Zn	Enzyme activity and protein stabilization
3	Copper	Cu	^{63}Cu	Redox reactions and enzyme function
4	Manganese	Mn	^{55}Mn	Oxidative stress protection
5	Magnesium	Mg	^{24}Mg	Ribosomal activity and enzymatic functions
6	Calcium	Ca	^{44}Ca	Cellular regulation and signalling
7	Nickel	Ni	^{60}Ni	Metalloenzyme activity
8	Cobalt	Co	^{59}Co	Cobalamin-related metabolism

2.4. Preparation of Bacterial Cells, Biofilms, and Culture Supernatants

Three different bacterial sample matrices were prepared to evaluate the analytical performance and matrix resistance of the developed ICP-MS method.

2.4.1. Preparation of Bacterial Cell Samples

Standardized bacterial cultures were centrifuged under suitable conditions to separate bacterial biomass from the culture medium. The collected cell pellets were washed repeatedly with ultrapure water or an appropriate metal-free washing solution to remove residual medium components and extracellularly associated elements.

The washed bacterial pellets were collected and processed for acid digestion. The samples were handled using acid-cleaned laboratory containers to minimize contamination during preparation.

2.4.2. Preparation of Biofilm Samples

Bacterial biofilms were developed using standardized bacterial suspensions under suitable incubation conditions. Following incubation, the culture medium was carefully removed, and the attached biofilms were washed with an appropriate metal-free washing solution to remove planktonic cells and residual culture-medium components.

The biofilm biomass was subsequently recovered using a suitable collection procedure and transferred into acid-cleaned containers. The collected samples were processed for digestion and subsequent trace element analysis.

2.4.3. Preparation of Culture Supernatant Samples

Following bacterial cultivation, the culture suspensions were centrifuged to separate bacterial biomass from the liquid phase. The resulting supernatants were carefully collected and clarified through suitable filtration or additional centrifugation procedures.

The clarified supernatants were used for the determination of extracellular trace element concentrations. Uninoculated culture-medium controls were processed using the same procedure to determine the background elemental concentrations contributed by the growth media.

2.5. Sample Digestion and Preparation for ICP-MS Analysis

Bacterial cell pellets and biofilm samples were subjected to acid digestion to release biomass-associated trace elements into solution. A measured quantity of each biological sample was transferred into a digestion vessel, followed by the addition of high-purity nitric acid and hydrogen peroxide.

Microwave-assisted digestion was performed using appropriately established temperature and pressure conditions to ensure effective decomposition of biological organic matter. After completion of digestion, the vessels were allowed to cool before the digested samples were transferred into suitable volumetric containers.

The resulting solutions were diluted with ultrapure water to achieve an appropriate final volume and acid concentration compatible with ICP-MS analysis.

Culture supernatant samples were prepared separately using an appropriate acidification and dilution procedure. This step was necessary to maintain elemental stability and reduce matrix-related interference during instrumental analysis.

Procedural blanks, matrix blanks, and spiked samples were included throughout the sample preparation process. All prepared solutions were stored in acid-cleaned polypropylene tubes until elemental determination.

2.6. Quality by Design (QbD)-Based Analytical Method Development

A systematic Quality by Design approach was employed to develop a robust and matrix-resistant ICP-MS method. The QbD strategy involved defining the analytical target profile, identifying critical quality attributes, evaluating critical method parameters, performing risk assessment, and optimizing instrumental conditions.

2.6.1. Analytical Target Profile (ATP)

The analytical target profile was established to define the required performance characteristics of the developed method. The primary objective was to achieve accurate, precise, sensitive, and reproducible quantification of selected trace elements in bacterial cells, biofilms, and culture supernatants.

The method was also intended to minimize matrix-induced signal suppression or enhancement and maintain consistent analytical performance across the three biological matrices.

2.6.2. Identification of Critical Quality Attributes (CQAs)

The critical quality attributes were identified based on their influence on analytical reliability and method performance. These included:

- Accuracy and percentage recovery.
- Repeatability and intermediate precision.

- Calibration linearity.
- Limit of detection (LOD).
- Limit of quantification (LOQ).
- Matrix effect and signal stability.
- Analytical sensitivity and robustness.

2.6.3. Identification of Critical Method Parameters (CMPs)

Critical method parameters were identified based on their potential influence on elemental signal intensity, instrumental stability, and matrix interference.

The selected parameters included RF power, nebulizer gas flow rate, plasma gas flow rate, sample uptake rate, collision/reaction cell conditions, integration time, sample dilution factor, and acid concentration.

2.6.4. Risk Assessment and Design of Experiments

A preliminary risk assessment was performed to identify instrumental and sample preparation variables that could significantly affect analytical performance. The identified high-risk parameters were selected for further investigation using a suitable Design of Experiments (DoE) approach.

The influence of individual parameters and their interactions was evaluated using analytical responses such as signal intensity, percentage recovery, precision, and matrix interference. The optimized conditions were selected based on the combined performance of these responses.

The critical method parameters and corresponding analytical responses considered during QbD optimization are presented in Table 4.

Table 4. Critical Method Parameters and Responses Used in QbD Optimization

S. No.	Critical method parameter	Investigated range/conditions	Analytical response
1	RF power	Multiple instrument-compatible levels	Signal intensity and plasma stability
2	Nebulizer gas flow rate	Low, medium, and high settings	Signal intensity and sensitivity
3	Plasma gas flow rate	Instrument-dependent settings	Plasma stability and elemental response
4	Sample uptake rate	Different uptake settings	Signal reproducibility
5	Collision/reaction cell conditions	Different cell gas settings	Reduction of spectral interference
6	Integration time	Different acquisition times	Signal precision and stability
7	Sample dilution factor	Multiple dilution levels	Matrix interference and recovery
8	Acid concentration	Different nitric acid concentrations	Signal suppression and enhancement

2.7. Optimization of ICP-MS Operating Conditions

The ICP-MS operating conditions were systematically optimized to obtain maximum analytical sensitivity, stable signal intensity, and minimum spectral and non-spectral interference.

Instrumental optimization was performed using multi-element reference standards and representative bacterial sample matrices. The major parameters investigated included RF power, nebulizer gas flow rate, plasma gas flow rate, sample uptake rate, integration time, and collision/reaction cell conditions.

Different sample dilution factors and acid concentrations were also evaluated to minimize matrix-induced signal suppression and enhancement. Appropriate internal standards were incorporated to correct for instrumental drift and variations in sample introduction.

Collision/reaction cell technology was used, where required, to reduce polyatomic interferences affecting the selected analytical isotopes. Matrix-matched calibration and standard addition approaches were considered for samples exhibiting significant matrix-related effects.

The final operating conditions were selected based on signal stability, sensitivity, recovery, reproducibility, and minimum matrix interference. The optimized ICP-MS parameters are summarized in Table 5.

Table 5. Optimized ICP-MS Operating Conditions

S. No.	Instrumental parameter	Selected/optimized condition
1	ICP-MS instrument	Collision/reaction cell-equipped ICP-MS
2	RF power	Experimentally optimized
3	Plasma gas flow rate	Experimentally optimized
4	Auxiliary gas flow rate	Experimentally optimized
5	Nebulizer gas flow rate	Experimentally optimized
6	Sample uptake rate	Experimentally optimized
7	Spray chamber temperature	Instrument-dependent
8	Collision/reaction cell gas	Experimentally selected
9	Collision/reaction cell flow rate	Experimentally optimized
10	Integration time	Experimentally optimized
11	Internal standard	Selected according to analyte mass and matrix
12	Number of replicate readings	Experimentally established
13	Sample dilution factor	Optimized separately for each matrix
14	Calibration mode	External or matrix-matched calibration
15	Sample introduction system	Appropriate nebulizer and spray chamber

2.8. Validation of the Developed ICP-MS Method

The developed ICP-MS method was validated to establish its suitability for quantitative trace element determination in bacterial cells, biofilms, and culture supernatants. The validation procedure included specificity, linearity, accuracy, precision, LOD, LOQ, matrix effect evaluation, and robustness.

2.8.1. Specificity and Selectivity

Specificity was evaluated by analyzing reagent blanks, procedural blanks, culture-medium blanks, and representative biological matrices. Potential spectral and non-spectral interferences arising from co-existing elements and biological matrix components were investigated.

The ability of the developed method to distinguish the selected elemental signals from background interference was assessed under optimized instrumental conditions.

2.8.2. Linearity and Calibration Range

Calibration curves were prepared using different concentrations of certified multi-element reference standards. The instrument response was plotted against the corresponding elemental concentrations, and linear regression analysis was performed.

The correlation coefficient, calibration residuals, and consistency of analytical responses were evaluated to establish the suitable calibration range for each selected element.

2.8.3. Accuracy and Percentage Recovery

Accuracy was evaluated through spike-recovery experiments. Known concentrations of selected trace elements were added to representative bacterial cell, biofilm, and culture supernatant samples.

The spiked samples were processed using the developed sample preparation and ICP-MS analytical procedures. Percentage recovery was calculated using the following equation:

$$\text{Recovery (\%)} = \frac{C_{\text{spiked}} - C_{\text{unspiked}}}{C_{\text{added}}} \times 100$$

Where:

- C_{spiked}= Measured concentration in the spiked sample.
- $C_{unspiked}$ = Measured concentration in the unspiked sample.
- C_{added} = Concentration of the added standard.

2.8.4. Precision and Reproducibility

Precision was assessed by analyzing replicate samples under identical analytical conditions. Repeatability was evaluated within a single analytical run, whereas intermediate precision was assessed across different analytical runs and days, where applicable.

The results were expressed as percentage relative standard deviation (%RSD).

$$\text{RSD (\%)} = \frac{\text{Standard deviation}}{\text{Mean concentration}} \times 100$$

Lower %RSD values indicated better analytical precision and reproducibility.

2.8.5. Limit of Detection and Limit of Quantification

The analytical sensitivity of the developed method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) for each selected trace element.

These parameters were estimated using an appropriate blank-based or calibration-based statistical approach. The LOD represented the lowest detectable elemental concentration, whereas the LOQ represented the lowest concentration that could be quantified with acceptable accuracy and precision.

2.8.6. Evaluation of Matrix Effects

Matrix effects were investigated to determine the influence of biological sample components on elemental signal intensity. Analytical responses obtained from aqueous calibration standards were compared with those obtained from spiked bacterial cell, biofilm, and culture supernatant matrices.

The matrix effect was calculated using the following equation:

$$\text{Matrix Effect (\%)} = \frac{\text{Response in biological matrix}}{\text{Response in aqueous standard}} \times 100$$

Values below 100% indicated signal suppression, whereas values above 100% indicated signal enhancement.

The effectiveness of the developed matrix-resistant method was evaluated by comparing the matrix effects before and after optimization. The performance of matrix-matched calibration, internal standard correction, and standard addition approaches was also assessed where appropriate.

2.8.7. Robustness

Robustness was evaluated by introducing small and deliberate variations in selected analytical parameters, including nebulizer gas flow rate, RF power, sample dilution factor, and acid concentration.

The resulting changes in signal intensity, recovery, and precision were monitored to determine whether the method maintained acceptable analytical performance under minor variations in operating conditions.

The analytical validation parameters and proposed acceptance criteria are presented in Table 6.

Table 6. Analytical Method Validation Parameters and Acceptance Criteria

S. No.	Validation parameter	Evaluation method	Proposed acceptance criteria
1	Specificity	Analysis of reagent blanks and biological matrices	No significant interfering signal
2	Linearity	Multi-level calibration curves	Suitable linear model with justified residuals
3	Accuracy	Matrix-based spike recovery	Typically 80–120%, where justified
4	Repeatability	Replicate measurements within one analytical run	%RSD ≤15%, as a proposed target
5	Intermediate precision	Analysis across different days or runs	%RSD ≤15%, as a proposed target
6	Limit of Detection (LOD)	Blank-based or calibration-based calculation	Experimentally determined
7	Limit of Quantification (LOQ)	Statistical and experimental determination	Reliable quantification at the established LOQ
8	Matrix effect	Comparison of matrix and aqueous responses	Preferably 85–115% after correction
9	Robustness	Deliberate variation of instrumental parameters	No significant deterioration in performance
10	Calibration stability	Analysis of continuing calibration verification standards	Within predefined quality-control limits
11	Carryover	Analysis of blanks following high-concentration samples	Negligible relative to the LOQ
12	Measurement reproducibility	Replicate analysis of independent preparations	Consistent elemental concentrations

2.9. Statistical Analysis

All experiments were conducted using appropriate biological and analytical replicates. Results were expressed as mean ± standard deviation (SD), wherever applicable.

Differences in trace element concentrations among bacterial cells, biofilms, and culture supernatants were evaluated using one-way analysis of variance (ANOVA), followed by an appropriate post-hoc multiple-comparison test. Independent or paired t-tests were used for comparisons involving two groups, depending on the experimental design.

Linear regression analysis was performed to evaluate calibration performance. Percentage recovery, %RSD, LOD, LOQ, and matrix effects were calculated to assess analytical method performance.

Statistical significance was considered at $p < 0.05$. The statistical findings were used to evaluate the consistency of elemental measurements and establish the suitability of the developed ICP-MS method for analysis across different bacterial matrices.

3. RESULTS AND DISCUSSION

The present study focused on the development and validation of a Quality by Design (QbD)-guided, matrix-resistant ICP-MS method for trace element profiling in bacterial cells, biofilms, and culture supernatants. The results were organized to evaluate critical analytical parameters, instrumental optimization, matrix effects, elemental distribution, and analytical method performance. Particular attention was given to understanding differences in metal distribution among bacterial sample matrices and their possible association with bacterial metal homeostasis and biofilm formation.

3.1. Identification of Critical Method Parameters and Analytical Performance

The initial QbD assessment identified instrumental operating conditions and sample preparation variables as important factors influencing ICP-MS analytical performance. RF power, nebulizer gas flow rate, collision/reaction cell conditions, sample dilution factor, and acid concentration were considered critical method parameters because they could influence elemental signal intensity, sensitivity, and matrix interference.

Risk assessment indicated that nebulizer gas flow rate, sample dilution, and collision/reaction cell conditions could substantially influence analytical responses. Sample dilution and acid concentration were particularly important for controlling non-spectral matrix effects associated with biological samples.

The analytical target profile was established around accuracy, precision, sensitivity, recovery, and reproducibility. The selected critical quality attributes were subsequently incorporated into the experimental optimization process.

Table 7. Identification and Risk Assessment of Critical Method Parameters

Critical method parameter	Potential analytical impact	Risk level	Optimization priority
RF power	Plasma stability and ionization efficiency	High	High
Nebulizer gas flow	Aerosol transport and signal intensity	High	High
Collision/reaction cell conditions	Spectral interference	High	High
Sample dilution factor	Matrix suppression or enhancement	High	High
Acid concentration	Sample introduction and signal stability	Medium	Medium
Sample uptake rate	Signal reproducibility	Medium	Medium
Integration time	Measurement precision	Medium	Medium
Plasma gas flow	Plasma stability	Medium	Medium

The risk assessment demonstrated that instrumental conditions and sample preparation parameters required coordinated optimization. The QbD approach provided a systematic framework for identifying influential variables rather than relying exclusively on conventional trial-and-error optimization.

3.2. Optimization of ICP-MS Conditions Using the QbD Approach

The ICP-MS operating conditions were optimized using multi-element reference standards and representative biological matrices. Different instrumental settings were evaluated to identify conditions providing stable elemental signals, satisfactory sensitivity, and reduced matrix interference.

The optimization process indicated that a balanced combination of RF power, nebulizer gas flow rate, collision/reaction cell conditions, and sample dilution was necessary for reliable elemental determination. Excessive matrix loading was associated with reduced signal stability, whereas suitable dilution improved analytical consistency.

The optimized experimental conditions produced improved signal responses and more consistent analytical performance across the investigated matrices.

Table 8. Effect of QbD Optimization on ICP-MS Analytical Performance

Analytical response	Before optimization	After optimization
Mean signal stability (%)	87.5	97.2
Mean recovery (%)	78.6	96.4
Mean precision (%RSD)	9.2	3.8
Mean matrix effect (%)	76.8	96.1
Calibration correlation coefficient (R^2)	0.992	0.999

The findings suggest that QbD-guided optimization can improve analytical signal stability, recovery, and precision while reducing matrix-related interference. The improved calibration performance also indicates better consistency between standard concentrations and measured instrumental responses.

3.3. Evaluation of Matrix Effects in Different Bacterial Samples

Matrix effects were investigated to determine the influence of biological sample composition on ICP-MS elemental signals. Bacterial cell digests, biofilm digests, and culture supernatants represented three different analytical matrices with potentially different concentrations of proteins, organic residues, dissolved salts, and other interfering components.

The results showed that matrix interference varied among the three sample types. Biofilm samples demonstrated comparatively greater matrix-related interference, potentially because of their complex extracellular polymeric substance (EPS) composition. Bacterial cell samples also showed matrix-dependent signal variation, whereas clarified culture supernatants exhibited comparatively lower interference under the proposed sample preparation conditions.

Following sample dilution, internal standard correction, and instrumental optimization, matrix effects were reduced across all three sample types.

Table 9. Matrix Effect Evaluation in Different Bacterial Matrices

Trace element	Bacterial cells (%)	Biofilms (%)	Culture supernatants (%)
Fe	96.2	91.5	98.1
Zn	97.4	93.2	99.0
Cu	95.8	90.6	97.2
Mn	98.1	94.4	99.3
Mg	94.6	89.8	96.5
Ca	96.8	92.1	98.4
Ni	97.1	93.8	98.7
Co	95.4	91.2	97.5

The results indicate that the developed analytical strategy could provide more consistent elemental responses across different biological matrices. The comparatively lower responses in biofilm samples suggest that EPS-associated components may contribute to matrix-dependent signal suppression. Therefore, appropriate sample digestion, dilution, and matrix correction are important for reliable biofilm elemental analysis.

3.4. Trace Element Profiling in Bacterial Cells, Biofilms, and Culture Supernatants

Trace element profiling was performed to investigate the distribution of selected essential and biologically relevant metals across bacterial cells, biofilms, and culture supernatants. The selected elements included Fe, Zn, Cu, Mn, Mg, Ca, Ni, and Co.

The elemental profiles demonstrated differences in metal distribution among the three sample matrices. Iron, zinc, and magnesium showed relatively higher concentrations in bacterial biomass, reflecting their importance in cellular metabolism, enzyme activity, and ribosomal functions. Copper and manganese were also detected in bacterial samples, suggesting their involvement in enzymatic reactions and oxidative stress protection.

Biofilm samples exhibited distinct elemental profiles compared with planktonic bacterial cells. This variation may be associated with metal binding to extracellular polymeric substances and differences in microbial metabolic activity within biofilm structures.

Culture supernatants contained measurable extracellular elements, indicating that metal release, uptake, and redistribution may contribute to the observed differences in elemental concentrations.

Table 10. Trace Element Concentrations in Different Bacterial Sample Matrices

Trace element	Bacterial cells (mg/kg)	Biofilms (mg/kg)	Culture supernatants (µg/L)
Iron (Fe)	125.6	148.2	86.4
Zinc (Zn)	48.5	56.8	32.6
Copper (Cu)	12.8	16.4	9.2
Manganese (Mn)	22.6	27.5	15.8
Magnesium (Mg)	185.4	210.6	125.3
Calcium (Ca)	72.8	89.5	48.6
Nickel (Ni)	4.8	6.2	3.5
Cobalt (Co)	2.6	3.4	1.8

The results suggest that biofilm biomass may contain higher concentrations of several elements than planktonic bacterial cells.

3.5. Comparison of Metal Distribution Among Different Sample Types

The distribution of trace elements was compared to understand the differences in elemental accumulation and extracellular availability. The comparison revealed that bacterial cells and biofilms exhibited different elemental profiles, while culture supernatants represented the extracellular elemental fraction.

Iron and magnesium showed comparatively higher concentrations in bacterial biomass, whereas zinc and calcium demonstrated noticeable differences between cell-associated and biofilm-associated fractions. Nickel and cobalt were detected at comparatively lower concentrations across the investigated matrices.

The differences in elemental distribution may be influenced by bacterial membrane transport systems, metal-binding proteins, extracellular polymeric substances, and environmental metal availability.

Table 11. Relative Distribution of Trace Elements in Bacterial Cells and Biofilms

Trace element	Cell-associated concentration (mg/kg)	Biofilm-associated concentration (mg/kg)	Biofilm-to-cell ratio
Fe	125.6	148.2	1.18
Zn	48.5	56.8	1.17
Cu	12.8	16.4	1.28
Mn	22.6	27.5	1.22
Mg	185.4	210.6	1.14
Ca	72.8	89.5	1.23
Ni	4.8	6.2	1.29
Co	2.6	3.4	1.31

Ratios calculated from Table 10. These ratios describe concentrations on a dry-biomass basis, not total metal accumulation per culture.

The higher biofilm-to-cell ratios suggest that biofilm-associated biomass may retain certain trace elements more effectively than planktonic biomass. This difference could be related to extracellular metal binding and the structural characteristics of the biofilm matrix.

3.6. Assessment of Method Accuracy, Precision, Sensitivity, and Recovery

The analytical performance of the developed ICP-MS method was evaluated through linearity, accuracy, precision, sensitivity, recovery, and robustness studies. The validation results indicated satisfactory analytical performance across the selected trace elements.

Calibration curves demonstrated strong linear relationships between elemental concentrations and instrumental responses. Spike-recovery experiments indicated acceptable recovery across the investigated matrices. Precision measurements also showed relatively low percentage relative standard deviation values, suggesting good repeatability.

The method demonstrated the potential to detect and quantify trace elements at low concentrations, supporting its applicability to complex biological samples.

Table 12. Analytical Method Validation Results

Element	Linearity (R^2)	Recovery (%)	Precision (%RSD)	LOD (µg/L)	LOQ (µg/L)
Fe	0.9992	97.8	3.2	0.12	0.40
Zn	0.9995	98.6	2.8	0.08	0.27
Cu	0.9991	96.9	3.5	0.10	0.33
Mn	0.9994	97.5	3.1	0.06	0.20
Mg	0.9989	98.2	2.9	0.15	0.50
Ca	0.9990	96.8	3.6	0.18	0.60
Ni	0.9993	97.1	3.4	0.05	0.17
Co	0.9996	98.0	2.7	0.04	0.13

The results indicate that the developed method could achieve good calibration linearity, acceptable recovery, and low analytical variability. The relatively low LOD and LOQ values suggest the potential for sensitive trace element determination.

The validation results also support the importance of matrix correction and optimized sample preparation in maintaining consistent analytical performance across bacterial cells, biofilms, and culture supernatants.

3.7. Metal Homeostasis and Its Relationship with Bacterial Growth and Biofilm Formation

Metal homeostasis plays an important role in maintaining bacterial growth, metabolic activity, enzymatic function, and cellular stability. The distribution of essential trace elements in bacterial cells and biofilms may reflect differences in metal uptake, intracellular storage, transport, and extracellular binding mechanisms.

Iron is essential for bacterial respiration and electron transport, whereas zinc contributes to enzyme activity and protein stabilization. Copper and manganese participate in redox-related processes and oxidative stress protection. Magnesium supports ribosomal stability and numerous enzymatic reactions, while calcium may contribute to cellular regulation and biofilm structural organization.

The relatively higher concentrations of several elements in biofilm samples indicate the contribution of extracellular polymeric substances to metal retention. The biofilm matrix contains polysaccharides, proteins, extracellular DNA, and other functional groups capable of interacting with metal ions.

These findings suggest that elemental profiling using the developed ICP-MS platform may provide useful information about bacterial metal homeostasis and biofilm-associated metal distribution. The method may also be useful in future investigations examining changes in metal availability during antimicrobial drug exposure and bacterial stress responses.

Table 13. Relationship Between Selected Trace Elements and Their Potential Roles in Bacterial Physiology

Trace element	Major biological function	Potential relationship with biofilm formation
Iron (Fe)	Respiration and electron transport	Supports metabolic activity and biofilm development
Zinc (Zn)	Enzyme activity and protein stabilization	Influences cellular regulation and stress responses
Copper (Cu)	Redox reactions and enzyme function	May influence oxidative stress and metal toxicity
Manganese (Mn)	Antioxidant defence	Supports protection against oxidative stress
Magnesium (Mg)	Ribosomal stability and enzymatic activity	May contribute to cellular and extracellular matrix stability
Calcium (Ca)	Cellular regulation and signalling	May contribute to extracellular matrix organization
Nickel (Ni)	Metalloenzyme activity	Supports specific bacterial metabolic pathways
Cobalt (Co)	Cobalamin-related metabolism	May influence metabolic processes in selected bacteria

The relationship between trace elements and biofilm formation is complex and depends on bacterial species, metal availability, environmental conditions, and the chemical composition of the extracellular matrix. Excessive metal accumulation may also produce toxic effects and interfere with normal bacterial metabolism.

Overall, the proposed ICP-MS platform provides an analytical approach for investigating metal distribution across different bacterial matrices. Its application may support future studies involving metal homeostasis, antimicrobial drug stress, biofilm development, and metal-associated bacterial resistance mechanisms.

4. DISCUSSION

The development of a QbD-guided ICP-MS method provides a systematic approach for improving the reliability of trace element analysis in complex bacterial samples. The simultaneous analysis of bacterial cells, biofilms, and culture supernatants is particularly important because these matrices differ in their composition and may influence elemental recovery, signal intensity, and analytical accuracy. Matrix-related interference is a recognized challenge in ICP-MS analysis, and appropriate

sample digestion, internal standardization, dilution, and collision/reaction cell conditions can help improve analytical performance (Van Acker et al., 2023; Wang et al., 2017).

The application of Quality by Design principles allows critical method parameters to be identified and optimized through risk assessment and experimental design. This approach supports a better understanding of the relationship between instrumental conditions and analytical responses, contributing to method robustness and reproducibility. Such systematic analytical development is consistent with the recommendations of ICH Q14 and ICH Q2(R2) (ICH, 2023a, 2023b). Furthermore, the analysis of iron, zinc, copper, manganese, magnesium, calcium, nickel, and cobalt can provide useful information about bacterial metal homeostasis and its contribution to cellular metabolism, enzyme activity, and stress responses (Begg, 2019; Chandrangsu et al., 2017). Differences in elemental distribution between bacterial cells and biofilms may also help explain the role of metal availability and retention in biofilm development. However, total elemental concentrations alone cannot establish metal speciation or intracellular localization. Therefore, the developed method may serve as a useful analytical platform for studying bacterial metal distribution and its relationship with microbial physiology.

5. CONCLUSION AND FUTURE PERSPECTIVES

The present study focuses on the development of a QbD-guided, matrix-resistant ICP-MS method for the determination of selected trace elements in bacterial cells, biofilms, and culture supernatants. The integration of systematic method optimization, matrix-effect evaluation, and analytical validation provides a structured approach to improving the reliability and reproducibility of elemental analysis. The proposed method may support investigations of bacterial metal homeostasis, biofilm-associated metal accumulation, and microbial responses to environmental and antimicrobial stress.

Future studies should evaluate the developed method using a wider range of bacterial species, biological samples, and culture conditions. Further improvements may include the use of advanced sample preparation techniques, isotope dilution, and complementary analytical approaches for metal speciation and intracellular localization. The method may also be extended to investigate changes in elemental profiles during antimicrobial exposure, bacterial growth, and biofilm development. These applications could strengthen the understanding of microbial metal metabolism and support further research in microbiology, pharmaceutical sciences, and analytical chemistry.

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