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RESEARCH ARTICLE

Simultaneous Determination of Six Inorganic Anions in Animal Whole Blood Using Ion Chromatography With Suppressed Conductivity Detection

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ABSTRACT

A sensitive and robust ion chromatography method with suppressed conductivity detection (IC-CD) was developed and validated for the simultaneous quantification of six inorganic anions (fluoride, nitrite, bromide, nitrate, phosphate, and sulfate) in animal whole blood. The method employed a carbonate–bicarbonate eluent (4.5-mM Na_2CO_3, 0.8-mM $NaHCO_3$) at a flow of 1 mL/min and an anion-exchange column (4 × 250 mm, Dionex IonPac AS23 RFIC) to achieve baseline resolution of all analytes within a 30-min run. Sample preparation involved protein precipitation with ice-cold methanol followed by centrifugation, dilution, and filtration. The method demonstrated excellent linearity ($R^2 = 0.9974–0.9998$), with limits of detection (LODs) ranging from 0.27 to 2.96 mg/L. Accuracy was confirmed through recovery studies, yielding values between 99.4% and 118.2%. Intraday and interday precision, expressed as relative standard deviation (%RSD), was consistently below 10% for both peak areas and retention times. The developed IC-CD protocol offers several advantages over existing techniques, including minimal sample volume, simplified sample preparation, rapid analysis, and high reproducibility. This method provides a valuable application tool for assessing ionic balance, nutritional status, and environmental exposure in animal health monitoring and toxicological studies.

1 | Introduction

Trace analysis of inorganic anions such as fluoride (F^-), bromide (Br^-), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulfate (SO_4^{2-}) in biological matrices, particularly animal blood, is fundamental to understanding physiological status, diagnosing potential pathological conditions, and monitoring environmental and dietary exposures in animals (Bennekou et al. 2025; He et al. 2019; Kapinus et al. 2004). For example, fluoride at appropriate trace levels supports dental and skeletal health, yet chronic or elevated exposure can lead to dental and skeletal fluorosis as well as neurodevelopmental impairments in animals (Choubisa 2023, 2024; Devkishan et al. 2025; Veneri et al. 2023). Bromide, although not an essential nutrient, may accumulate from environmental or pharmacological sources and thus influence halide balance or produce neurological effects under excess conditions (Bennekou et al. 2025). Nitrite and nitrate function as intermediates in nitric oxide metabolism and may serve as biomarkers of vascular or environmental perturbation; yet, in domestic and wild animals, nitrate/nitrite toxicosis can occur with growth retardation, methemoglobinemia, and reproductive impairment (Aiello and Moses 2016; Bryan et al. 2012; Lundberg et al. 2008). Phosphate is critical for energy metabolism, nucleic acid and membrane synthesis, and bone mineralization, while

sulfate supports detoxification via sulfation pathways and structural sulfate biomolecules (Dordevic et al. 2023; Marks and Unwin 2022; Richardson et al. 2000).

Over recent decades, anthropogenic activities such as enhanced use of nitrogen-based fertilizers, industrial emissions, wastewater contamination, and groundwater pollution have increased the environmental burden of certain inorganic anions (Gontijo et al. 2017; Salim Moyel et al. 2024). These elevated exposures may influence animal health via contaminated water, feed, soil, and inhalation pathways, leading to oxidative stress, metabolic dysregulation, and organ damage (Bijay-Singh and Craswell 2021; Ren et al. 2025). Nutritional or functional deficiencies of some anions (e.g., phosphate and sulfate) may further compromise animal physiology, for instance, by impairing bone growth, energy metabolism, or detoxification capacity (Arai 2014; Kharl et al. 2025; Yadav et al. 2024). Thus, accurate monitoring of inorganic anions in biological matrices, and particularly whole blood, has emerged as a critical component of veterinary toxicology, environmental exposure assessment, and animal health surveillance (Gupta 2007; McClellan 2025).

Whole blood, unlike plasma or serum, encompasses both intracellular and extracellular components, providing a comprehensive assessment of systemic exposure and ionic homeostasis (He et al. 2019; Kage et al. 2008; Rahmé et al. 2014). However, its complex matrix comprising proteins, lipids, hemoglobin, and cellular material poses significant analytical challenges (Chapp et al. 2018; López-Ruiz 2000). Nitrite, in particular, is prone to rapid oxidation by hemoglobin, while proteins and salts contribute to coelution, baseline drift, and peak broadening in chromatographic analysis (Michalski 2009; Suzuki et al. 2005; Yan et al. 2016). Moreover, deproteinization or filtration steps, though necessary to minimize interference, can introduce variability or analyte loss (Kataoka 2017; Yan et al. 2016). The absence of certified whole-blood reference materials further complicates calibration and quantification. Thus, there is a critical need for robust, validated analytical techniques capable of quantifying trace anions in such complex matrices with high sensitivity and reproducibility.

One of the most powerful analytical techniques for ionic species is ion chromatography (IC) (López-Ruiz 2000; Michalski 2006). Prior to IC, inorganic anion determination often relied on gravimetry, titrimetry, photometry, or colorimetry approaches limited by sensitivity, selectivity, and interference (Gros and Gorenc 1997; López-Ruiz 2000; Mullins 1987). When coupled with suppressed conductivity detection (IC-CD), IC allows direct quantification of ions without derivatization, reduces sample handling, and lowers background noise, thereby increasing signal-to-noise ratio and enabling trace-level detection even in complex matrices (Chapp et al. 2018; He et al. 2019; Kapinus et al. 2004). Emerging reviews of IC and related ion-exchange techniques emphasize their growing applicability in biological, environmental, and forensic contexts (Buldini et al. 1997; Michalski 2009).

Despite extensive use of IC in environmental, food, clinical, and urine/plasma matrices (Kage et al. 2008), its application to whole blood remains less frequent largely due to the matrix complexity, high protein and cellular content, and potential interference or analyte loss (He et al. 2019; Hu et al. 2000; Yan et al. 2016). Nevertheless, recent advancements highlight that robust IC protocols can successfully quantify anions in whole blood of animals and humans with good reproducibility and minimal alteration of sample integrity (He et al. 2019). These developments underscore the potential of IC for toxicological, veterinary, physiological, and environmental exposure research (Weiss 2016; Yan et al. 2016).

The present study aims to develop and validate a sensitive, selective, and reproducible IC method with suppressed conductivity detection for the simultaneous quantification of six key inorganic anions (fluoride, nitrite, bromide, nitrate, phosphate, and sulfate) in animal whole blood. The validated method is expected to provide reliable anion profiling in biological matrices, facilitating studies in veterinary toxicology, animal physiology, environmental exposure assessment, and feed and food safety where trace determination of inorganic anions can serve as an important indicator of ionic imbalance, exposure burden, or early toxicological effect.

2 | Experimental

2.1 | Chemicals and Reagents

A certified mixed standard solution containing anions (fluoride, nitrite, bromide, nitrate, phosphate, and sulfate) (Thermo Scientific Dionex), already prepared in deionized water, was used in calibration and spiking experiments. Sodium carbonate (ACS reagent, anhydrous, ≥99.5%, powder or granules, calcined soda, carbonic acid disodium salt, Sigma-Aldrich) and sodium bicarbonate (ACS reagent, anhydrous, ≥99.7%, sodium hydrogen carbonate, Sigma-Aldrich). Methanol (hypergrade for LC–MS, Sigma-Aldrich) was used for sample preparation and protein precipitation. Ultrapure deionized water (resistivity ≥18.2 MΩ·cm) was used throughout the study for the preparation of eluents, reagents, and dilutions. Additionally, sterile Falcon tubes (Tarsons, SPINWIN centrifuge tube with conical bottom, PP with HDPE closure, 15 and 50 mL, Kolkata, India), IC-ready polyvials (Thermo Scientific, Dionex AS-DV Autosampler PolyVials with plain caps 5 mL, Massachusetts, USA), and 0.2-μm nylon syringe filters (Thermo Scientific, Target2 Nylon Syringe Filters, 0.2 μm, 17 mm, Massachusetts, USA) were utilized during sample processing and analysis to maintain sample integrity. All chemicals and reagents used were of high analytical grade to ensure accuracy and reproducibility.

2.2 | Instruments

Chromatographic analysis was performed on ion chromatograph (Thermo Scientific Dionex Inuvion) equipped with a Thermo Scientific Dionex AS-DV autosampler. Separation of anions was achieved using a Dionex IonPac AS23 RFIC analytical column (4 × 250 mm) protected by a Dionex IonPac AG23 RFIC guard column (4 × 50 mm). A Dionex ADRS 600 (4 mm) dynamically regenerated suppressor was employed for chemical suppression, and detection was performed using an Inuvion conductivity cell detector. A high-speed refrigerated centrifuge (Accumax, Neuation iFUGE UC02R, Gujarat, India)

was used for sample centrifugation during sample treatment. Ultrapure deionized water (Metrohm, ELGA, PURELAB Quest, Herisau, Switzerland) with resistivity not lower than 18.2 MΩ·cm was used throughout the analysis. Weighing of reagents was performed using an analytical balance (Mettler Toledo, Analytical Balance MX205DU, Selangor, Malaysia) with high precision.

2.3 | Preparation of Standard Solutions and Calibration

A certified Thermo Scientific Dionex anion standard solution containing fluoride (20 mg/L), nitrite (100 mg/L), bromide (100 mg/L), nitrate (100 mg/L), phosphate (150 mg/L), and sulfate (150 mg/L) was used for calibration. Working standard solutions were freshly prepared by appropriate dilution of the stock standard in ultrapure deionized water to obtain concentrations ranging from 0.2 to 37.5 mg/L. The concentration of each standard solution prepared for calibration standards is presented in Table 1.

Quantification of anions in whole-blood samples was performed using the external standard method. Calibration curves were constructed by plotting the peak area obtained from the ion chromatograph against the known concentrations of the working standard solutions (Table 1). Data acquisition and processing were performed using the Chromeleon chromatography data system (Version 7, Thermo Fisher Scientific). Peak integration was carried out using automatic baseline correction and peak detection with consistent parameters across all analyses. The integration settings included automatic smoothing (auto width), baseline noise range set to auto, and a tailing sensitivity factor of 0.1%. Calibration was performed using a linear regression model with offset (order 1, origin not forced). Global calibration settings were applied in total mode with normal curve fitting. All processing parameters were kept constant for calibration standards, quality control (QC) samples, and unknown samples to ensure reproducibility.

2.4 | Preparation of Eluent

The eluent used for the separation of anions consisted of 4.5-mM sodium carbonate (Na_2CO_3) and 0.8-mM sodium bicarbonate ($NaHCO_3$), pH 10.7 ± 0.01 at room temperature. Stock solutions of 500 mM each were prepared by dissolving 2.6 g of Na_2CO_3 and 2.1 g of $NaHCO_3$ separately in 50 mL of deionized water. Both stock solutions were prepared separately in precleaned Tarson tubes and sonicated for 10 min. From the stock solutions, 2 L of working eluent was prepared by dilution with ultrapure deionized water to achieve the desired concentrations of 4.5-mM Na_2CO_3 and 0.8-mM $NaHCO_3$. The eluent was filtered using a membrane filter and degassed prior to use to eliminate any entrapped air bubbles. During chromatographic analysis, the anion suppressor current was maintained at 25 mA, ensuring that the baseline conductivity remained between 18 and 20 μS at a flow rate of 1.0 mL/min, thereby providing optimal detection sensitivity and baseline stability.

2.5 | Sample Collection

The whole-blood samples used for the validation were left over diagnostic samples from routine clinical care. All samples were collected from multiple animal species by veterinary doctors at Vantara, Jamnagar, into EDTA-coated vacutainer tubes. After collection, individual blood samples from each species available were pooled and thoroughly mixed to obtain a homogeneous composite sample representative of all the species. All samples were handled with care to prevent hemolysis and contamination. Proper labeling and documentation were maintained throughout the collection and pooling process. The pooled samples were immediately stored at the appropriate temperature (e.g., 2°C–8°C) until further processing and analysis. All procedures were conducted in accordance with institutional ethical guidelines.

2.6 | Sample Treatment

For sample preparation, 1 mL of deionized water was added to a clean 15-mL Falcon tube, followed by adding 1 mL of whole blood. To this, 2 mL of ice-cold methanol (stored at −20°C) was added to facilitate protein precipitation and extraction of anions. The mixture was vortexed for 30 s and subsequently centrifuged at 6000 rpm for 20 min at 4°C using a refrigerated centrifuge. After centrifugation, 2.5 mL of the supernatant was carefully transferred to a fresh Tarson tube and diluted to 7.5 mL with deionized water, followed by vortexing to ensure homogeneity.

TABLE 1 | Concentrations of mixed anion standard solutions for calibration at seven levels.

		Concentrations (mg/L) of calibration standards prepared from fixed volumes (mL) of mixed anion stock solution						
Anion	**Standard stock concentration (mg/L)**	**0.1**	**0.25**	**0.5**	**1**	**1.5**	**2**	**2.5**
Fluoride	20	0.2	0.5	1	2	3	4	5
Nitrite	100	1	2.5	5	10	15	20	25
Bromide	100	1	2.5	5	10	15	20	25
Nitrate	100	1	2.5	5	10	15	20	25
Phosphate	150	1.5	3.75	7.5	15	22.5	30	37.5
Sulfate	150	1.5	3.75	7.5	15	22.5	30	37.5

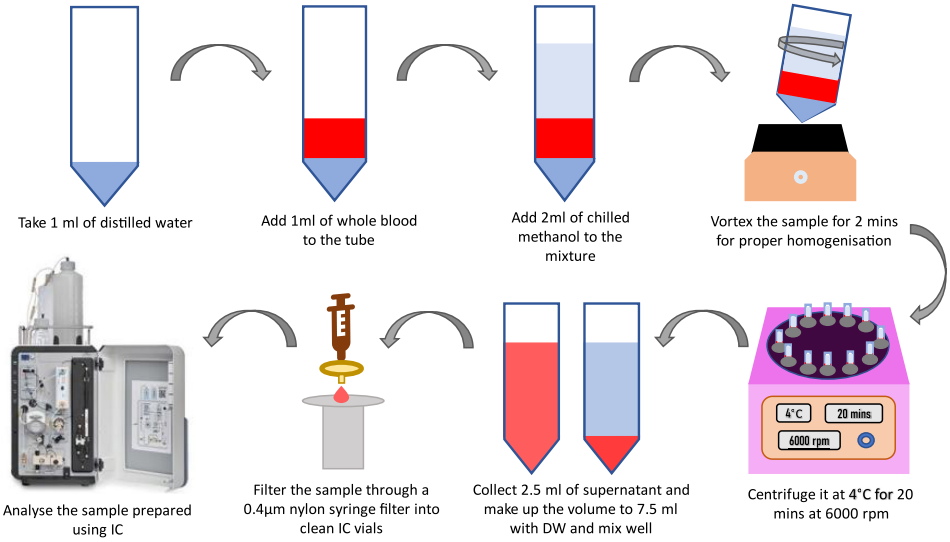

FIGURE 1 | Sample preparation procedure: Schematic representation of the sample preparation procedure for whole-blood analysis by ion chromatography.

The prepared sample was then filtered through a 0.2-µm nylon membrane filter and collected in IC-compatible polypropylene vials, which were capped prior to analysis, shown in Figure 1. All sample preparation steps were conducted in an ice box to maintain low temperature and minimize degradation or transformation of analytes (He et al. 2019).

2.7 | IC Conditions

Ion chromatographic analysis was carried out using the Inuvion IC system equipped with a conductivity detector, a suitable guard column, and an analytical column specific for anion separation. The eluent used was a carbonate–bicarbonate buffer composed of 4.5-mM Na_2CO_3 and 0.8-mM $NaHCO_3$, which facilitated the separation of anionic species. The flow rate of the mobile phase was maintained at 1.0 mL/min, in accordance with the specifications of the Inuvion instrument. The loop volume was maintained at 25 µL for all samples and standards while total delivery volume was 1500 µL, column temperature was controlled at 30°C, and suppressor current was set at 25 mA to ensure consistent retention times and peak shapes. Detection of the analytes was achieved using a suppressed conductivity detector to enhance sensitivity and reduce background noise. Other additional parameters for chromatographic conditions are attached in Table 2.

2.8 | QC Program

For the purpose of QC, all validation experiments were performed in triplicates. The QC validation parameters investigated were the limit of detection (LOD), limit of quantification (LOQ), linearity, precision, and accuracy. The LOD and LOQ were determined based on the standard deviation (σ) of replicate measurements obtained at low concentration levels. LOD and LOQ were calculated using the following expressions:

$$(\mathrm{LOD} = 3.3 \times \sigma) \text{ and } (\mathrm{LOQ} = 10 \times \sigma)$$

TABLE 2 | Instrumental and chromatographic conditions for analysis.

Parameter	Description
Instrument	Thermo Scientific Dionex Inuvion ion chromatograph
Detector	Suppressed conductivity
Cell temperature (conductivity detector)	35°C
Autosampler	Thermo Scientific Dionex AS-DV
Analytical column	Dionex IonPac AS23 RFIC analytical column (4 × 250 mm)
Guard column	Dionex IonPac AG23 RFIC guard column (4 × 50 mm)
Column oven temperature	30°C
Eluent composition	4.5-mM Na_2CO_3 + 0.8-mM $NaHCO_3$
Eluent flow rate	1.0 mL/min
Suppressor	Dionex ADRS 600 dynamically regenerated anion suppressor (4 mm)
Suppressor current	25 mA
Baseline conductivity	18–20 µS
Injection volume	1500 µL
Run time	30 min
Elution mode	Isocratic
Maximum equilibration time	50 min
Flush factor	10

where σ represents the standard deviation of the analytical response (Bettencourt da Silva 2026; Kruezi et al. 2025; Lopez-Moreno et al. 2010). This approach is based on statistical estimation of analytical variability and is consistent with established analytical validation practices and guidelines established in the Eurachem/CITAC Guide (Bettencourt da Silva 2026). The calculated LOQ values were further evaluated to ensure acceptable analytical performance, with precision expressed as relative standard deviation (%RSD $\leq$ 20%) and accuracy within the range of 80%–120%.

3 | Analytical Method Validation

Analytical method validation included determinations of linearity, sensitivity, accuracy, precision, reproducibility, LOD, and LOQ. Method optimization and chromatographic performance parameters are summarized and discussed in detail below.

3.1 | Linearity, Sensitivity, and Detection Limits

The linearity, sensitivity, and precision of the method were systematically evaluated for all target anions. Calibration curves were constructed over the concentration ranges of 0.2–5 mg/L for fluoride; 1–25 mg/L for nitrite, bromide, and nitrate; and 1.5–37.5 mg/L for phosphate and sulfate. All analytes demonstrated excellent linearity within the tested ranges, with correlation coefficients (R^2) ranging from 0.9974 to 0.9998 (Table 3).

The LOD and LOQ were determined based on the standard deviation of replicate measurements at low concentration levels, which were in the range from 0.27 to 2.96 and 0.82 to 8.96 mg/L, respectively (Figure 2).

For certain analytes, the calculated LOQ exceeded the lowest calibration level, indicating that while lower concentrations were included in the calibration range (Figure 2), reliable quantification based on analytical variability and reproducibility was achieved only above the LOQ. Thus, the LOQ represents the true lower LOQ rather than the lowest calibration point.

3.2 | Method Optimization and Chromatographic Performance

Method optimization in the present study was primarily guided by previously reported IC methods and subsequently verified under the current experimental conditions. This approach was considered appropriate because IC with suppressed conductivity detection is a well-established technique for inorganic anion analysis, where key parameters such as eluent composition and suppressor conditions have already been extensively optimized and reported in the literature (López-Ruiz 2000; Michalski 2006).

For chromatographic separation, a carbonate–bicarbonate eluent system (4.5-mM Na_2CO_3 and 0.8-mM $NaHCO_3$) was selected based on its widespread use for multianion separation. This eluent system is known to provide a good balance between resolution and analysis time for common inorganic anions (Thermo Fisher Scientific Inc 2024). A flow rate of 1.0 mL/min was maintained in accordance with column specifications (Thermo Fisher Scientific Inc 2024). The suppressor current was set at 25 mA, which was selected based on the recommended empirical relationship between eluent concentration and flow rate for efficient chemical suppression in carbonate–bicarbonate systems. One such expression is given by

$$I = 2.5 \times \left[2 \times \left(C_{Na_2CO_3} + C_{NaHCO_3}\right) \times F\right]$$

where I is the suppressor current (mA), C represents the eluent concentration in mM, and F is the flow rate in mL/min.

This relationship is derived from the stoichiometric requirements for converting carbonate and bicarbonate ions into weakly conductive carbonic acid within the suppressor, along with practical efficiency considerations associated with electrolytic suppression systems. Under the selected conditions, stable baseline conductivity (18–20 μS), consistent retention times, and well-defined peak shapes were obtained.

Because whole blood is a complex matrix, containing proteins, cells, and other interfering components, most of the optimization effort was focused on sample preparation. Efficient protein removal is essential in such matrices to avoid column contamination, reduce baseline noise, and improve analyte response (Chapp et al. 2018; Kataoka 2017).

The two commonly used protein precipitation solvents, acetonitrile and methanol, were compared. When acetonitrile was used, relatively higher LOD (0.25, 3.17, 4.21, 3.05, 11.04, and 3.63 mg/L, respectively) with corresponding LOQ (0.76, 9.59, 12.76, 9.24, 33.46, and 10.99 mg/L) were observed for all six analytes (fluoride, nitrite, bromide, nitrate, phosphate, and sulfate),

TABLE 3 | Calibration range (mg/L), linearity (R^2), standard deviation (σ), and limit of detection and quantification (LOD and LOQ) for anions determined using the developed IC method.

Analyte	Calibration range (mg/L)	R^2	σ	LOD (mg/L)	LOQ (mg/L)
Fluoride	0.2–5.0	0.9986	0.08	0.27	0.82
Nitrite	1–25	0.9998	0.52	1.73	5.25
Bromide	1–25	0.9988	0.17	0.58	1.76
Nitrate	1–25	0.9974	0.41	1.38	4.17
Phosphate	1.5–37.5	0.9987	0.89	2.96	8.96
Sulfate	1.5–37.5	0.9985	0.28	0.95	2.89

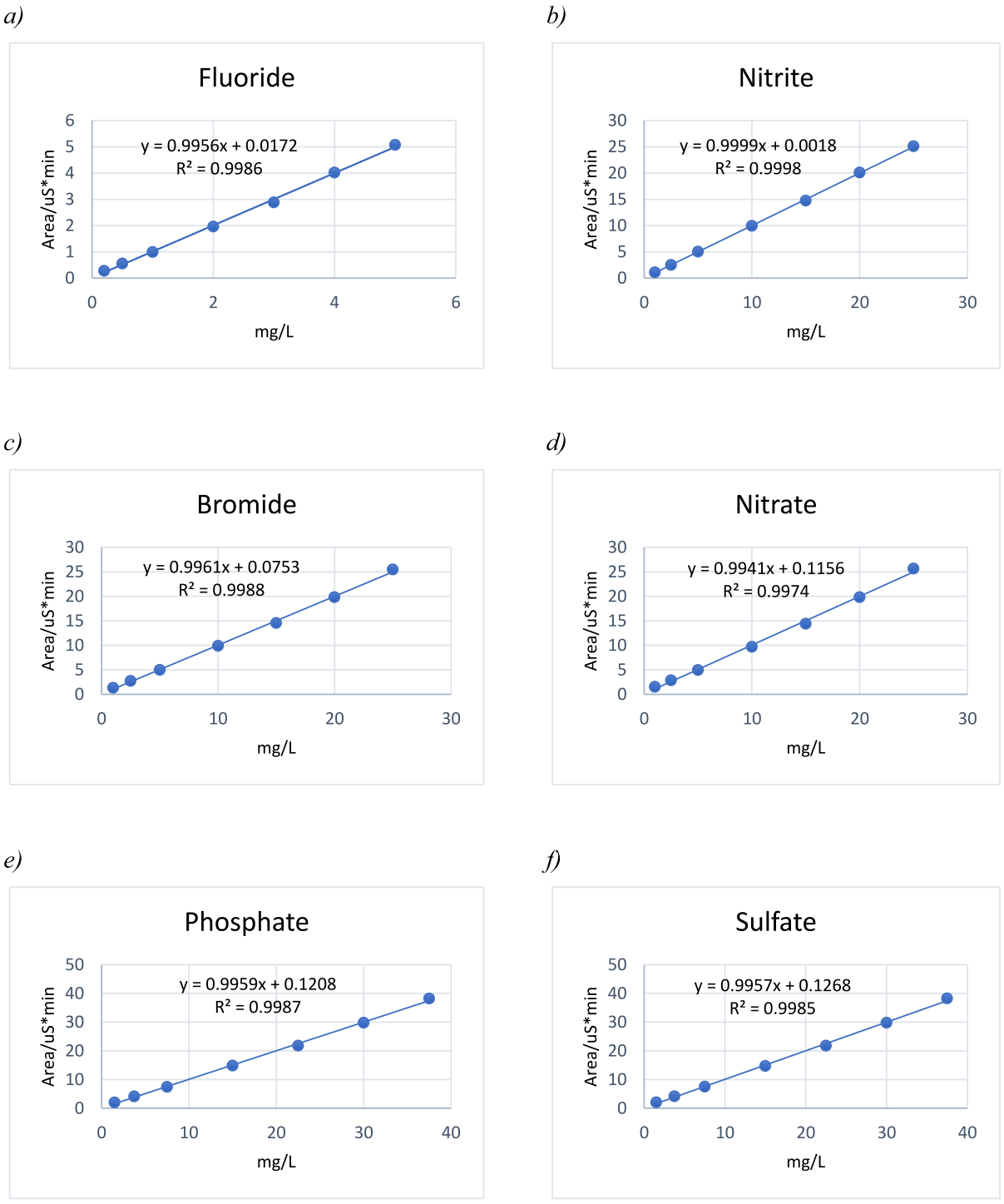

FIGURE 2 | Representative calibration curves of six anions: (a) F^- (0.2–5 mg/L), (b) NO_2^- (1–25 mg/L), (c) Br^- (1–25 mg/L), (d) NO_3^- (1–25 mg/L), (e) PO_4^{3-} (1.5–37.5 mg/L), and (f) SO_4^{2-} (1.5–37.5 mg/L).

indicating reduced sensitivity and suggesting incomplete matrix removal and residual interferences (Table 4).

In contrast, the use of ice-cold methanol (−20°C) resulted in noticeably improved analytical performance. Methanol is known to be effective for protein precipitation due to its ability to disrupt protein structure through hydrogen bonding interactions, leading to better matrix cleanup compared with acetonitrile (Capello et al. 2007). In addition, performing the extraction at low temperature improves precipitation efficiency and helps reduce enzymatic activity in the sample (Sitnikov et al. 2016; Tan and Yiap 2009). This becomes particularly important for analytes such as nitrite, which are unstable in whole blood and can undergo oxidation or transformation if not properly stabilized. Furthermore, from a green chemistry perspective, alcohol-based solvents such as methanol are often preferred due to their relatively lower toxicity, higher biodegradability, and reduced environmental persistence compared with many conventional organic solvents (Capello et al. 2007; Moniz and Sarraguça 2026). In the present study, the use of ice-cold

methanol likely minimized these transformations, resulting in improved signal intensity and lower detection limits.

Methanol has also been reported to provide better recovery of small polar and ionic compounds in biological matrices, while reducing coextraction of interfering substances (Chapp et al. 2018; He et al. 2019). This is consistent with the observations in this study, where all six inorganic anions, fluoride, nitrite, bromide, nitrate, phosphate, and sulfate were baseline-resolved within a 30-min run as mentioned in Table 2.

The order of elution followed the expected trend of increasing ionic size and affinity for the stationary phase:

$$F^- \rightarrow NO_2^- \rightarrow Br^- \rightarrow NO_3^- \rightarrow PO_4^{3-} \rightarrow SO_4^{2-}$$

The standard peak of all measured six anions is represented through their chromatogram in Figure 3 in order of their elution through the column. Blank blood extracts displayed no significant interference peaks, confirming the selectivity of the method for all analytes in the complex whole-blood matrix.

These results align closely with the detection capabilities reported by Michalski (2009) and He et al. (2019) for biological matrices, underscoring the high analytical sensitivity of IC-CD. The wide linear dynamic range ensures applicability for both physiological and environmentally elevated exposure levels in animals.

TABLE 4 | Analytical performance comparison (LOD and LOQ) of six anions following protein precipitation with ice-cold methanol versus acetonitrile.

	Ice-cold methanol		Acetonitrile	
Analyte	**LOD (mg/L)**	**LOQ (mg/L)**	**LOD (mg/L)**	**LOQ (mg/L)**
Fluoride	0.27	0.82	0.25	0.76
Nitrite	1.73	5.25	3.17	9.59
Bromide	0.58	1.76	4.21	12.76
Nitrate	1.38	4.17	3.05	9.24
Phosphate	2.96	8.96	11.04	33.46
Sulfate	0.95	2.89	3.63	10.99

3.3 | Precision, Accuracy, and Reproducibility

The precision and reproducibility of the method were evaluated according to Eurachem/CITAC recommendations (Bettencourt da Silva 2026). The developed method demonstrated excellent reproducibility in both intraday and interday analysis as represented by chromatograms in Figure 4.

Precision was assessed as intraday (six replicates on Day 1) and interday (six replicates over 3 days) variability and expressed as %RSD. Precision was calculated as (Standard deviation/Mean concentration) × 100. All values were consistently below 10% (Table 5), suggesting an acceptable instrumental and method precision.

Accuracy was estimated using the recovery study, where blank-extracted whole-blood samples were spiked with a known concentration of analytes and analyzed under similar conditions. The recovery (%) was calculated as follows: Accuracy (%) = (measured − unspiked)/spiked × 100, with a mean recovery between 99.4% and 118.2%, shown in Table 5, well within the acceptance range (80%–120%). These findings confirm that the sample preparation approach did not introduce significant analyte loss or matrix suppression. Comparable reproducibility has been reported in previous IC methods applied to serum and plasma (He et al. 2019; Rahmé et al. 2014; Yan et al. 2016), but the current protocol extends such robustness to the more challenging whole-blood matrix.

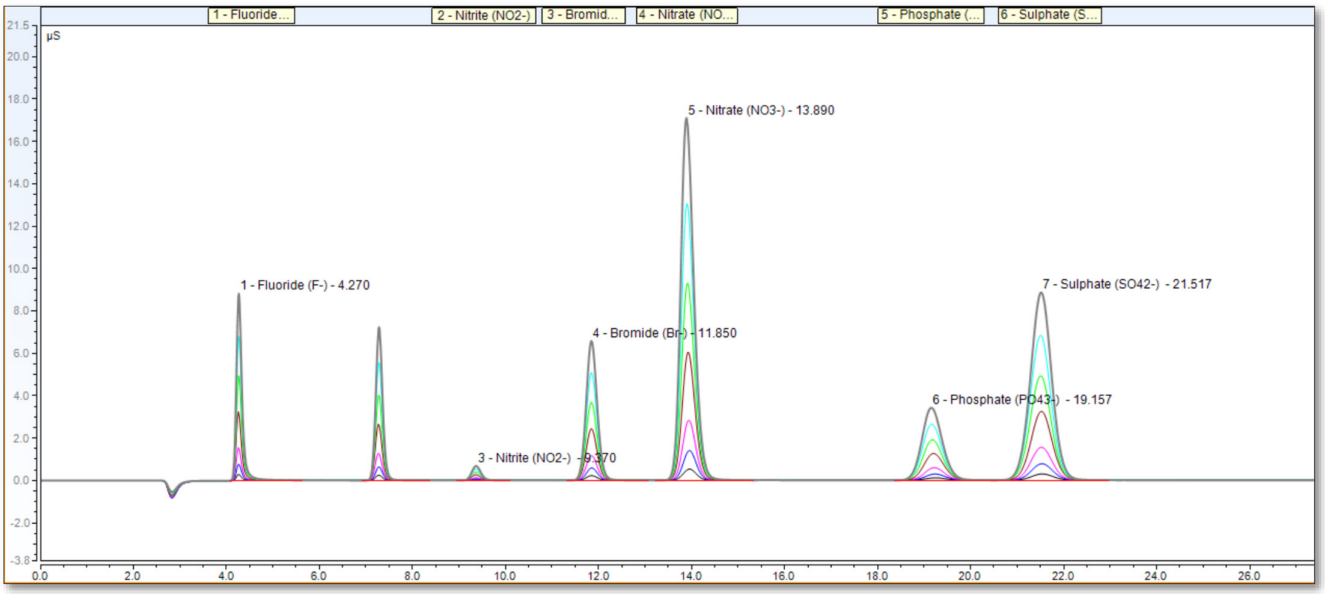

FIGURE 3 | Representative anion chromatogram from IC containing F^-, Br^-, NO_2^-, NO_3^-, PO_4^{3-}, and SO_4^{2-}.

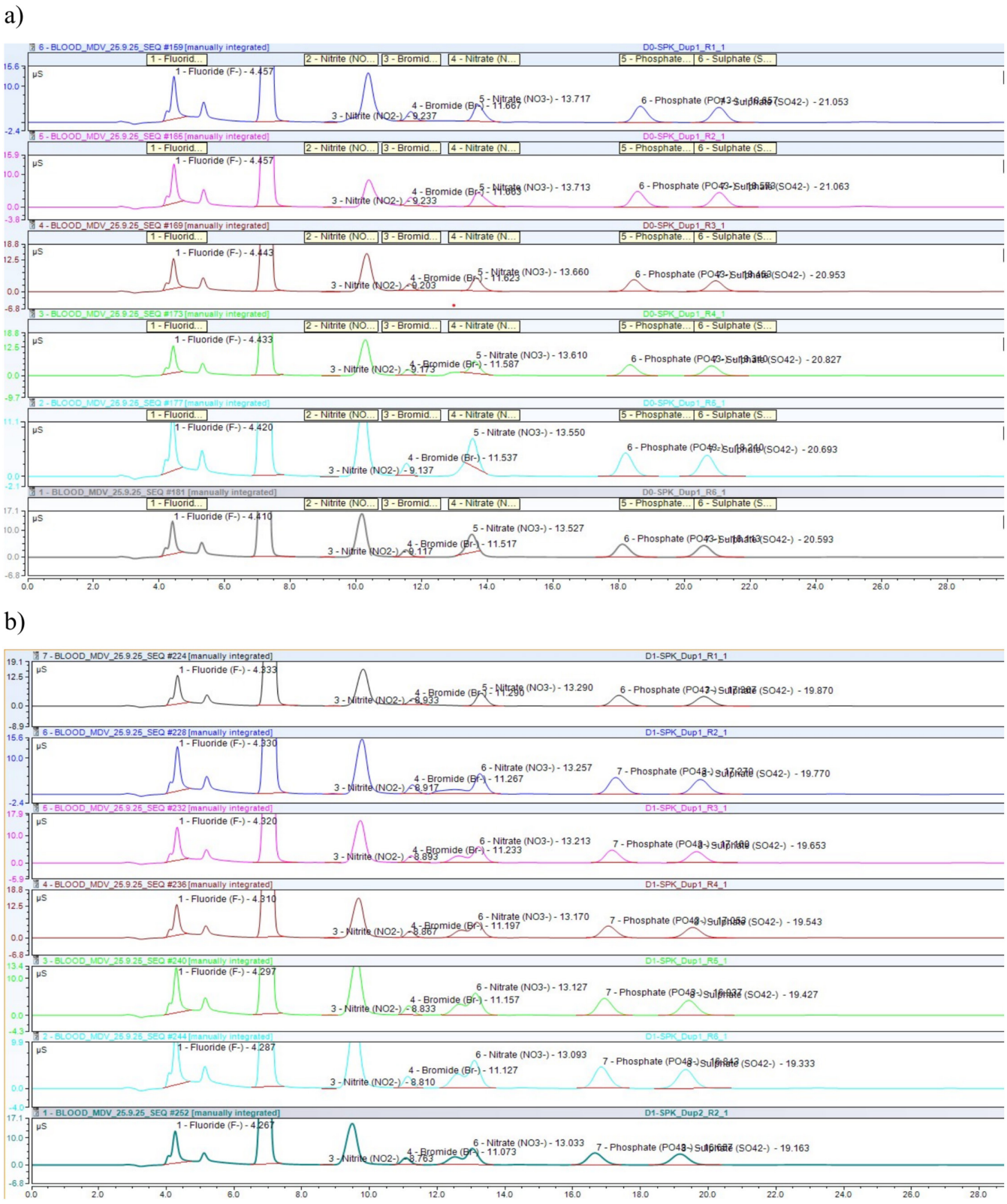

FIGURE 4 | Chromatograms representing intraday and interday recovery for all six measured anions. (a) Intraday and (b) interday.

4 | Application of the Method

Application of the validated method to the whole-blood samples revealed the presence of all six target anions (Figure 5). The quantitative results obtained under the present experimental conditions indicated that sulfate was detected at comparatively higher concentrations, while other anions, such as nitrite and fluoride, were present at trace levels. These observations are based on measured data and demonstrate the method's capability to analyze analytes across a broad concentration range

within a complex biological matrix. While consistent trends were observed within the analyzed samples, broader physiological interpretation is constrained by the absence of well-defined reference ranges for inorganic anions in animal whole blood and potential interspecies variability. Accordingly, the results are reported within the scope of the current dataset and should not be generalized across species without further validation.

Nitrite was detected at comparatively lower concentrations, consistent with its transient nature and susceptibility to oxidation by hemoglobin (Aiello and Moses 2016; Bryan et al. 2012; Ward et al. 2005). The low-temperature methanol extraction effectively stabilized nitrite, allowing its reliable detection, an outcome that emphasizes the importance of immediate enzymatic quenching in biological analyses (Sitnikov et al. 2016). These results suggest that the developed IC method enables simultaneous quantification of both major and trace-level anions within a single analytical framework, supporting its applicability for complex matrices such as whole blood.

TABLE 5 | Accuracy and precision of six anions in whole blood.

		% RSD	
Analyte	**Recovery %**	**Intraday**	**Interday**
Fluoride	104.3	4.7	3.8
Nitrite	101.0	6.2	6.2
Bromide	104.6	2.0	3.2
Nitrate	99.5	5.0	2.8
Phosphate	118.2	6.1	2.6
Sulfate	99.4	2.3	0.6

Recent studies employing IC in plasma, serum, urine, or environmental samples have demonstrated good linearity, acceptable reproducibility, and moderate detection limits; however, their application to whole blood has been constrained by matrix complexity and analyte instability (Chapp et al. 2018; Huang et al. 2024). For example, Chapp et al. (2018), He et al. (2019), and Michalski (2009) reported successful anion determination in serum but noted that protein and cellular interference presented major challenges when whole blood was attempted, often necessitating laborious deproteinization and multistep extraction protocols (Salas-Auvert et al. 1995).

Over previously reported methods, our study offers a streamlined and robust analytical approach for simultaneous quantification of inorganic anions directly in animal whole blood. The protocol involves a simplified protein precipitation without multistep cleanup, rapid analysis within 30 min, excellent linearity and high reproducibility without derivatization.

The validated IC method provides a sensitive, selective, and reproducible tool for the simultaneous quantification of multiple inorganic anions in complex biological matrices such as whole blood. While this study is focused on method development and validation, it establishes a robust analytical foundation for future applications in veterinary toxicology, feed safety, and environmental biomonitoring.

The major advantage of this method is its application to whole blood, a complex and underexplored matrix that integrates both intracellular and extracellular compartments, thereby providing a more comprehensive representative of anion distribution. It is particularly advantageous in veterinary context where interspecies variability in size and blood volume can limit serum availability, especially in small animal. Moreover, some anions demonstrate difference in their distribution in plasma and cellular fractions,

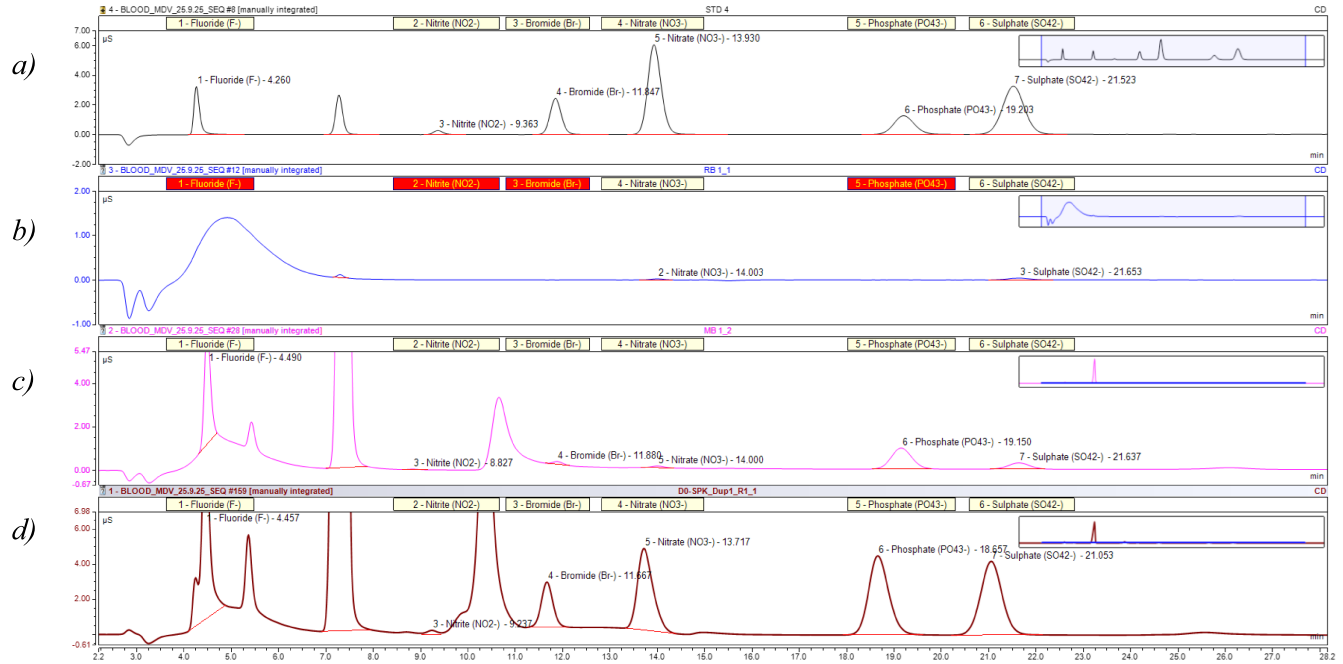

FIGURE 5 | Chromatograms representing (a) standard, (b) reagent blank, (c) matrix blank, and (d) spiked whole blood.

reinforcing the value of whole-blood analysis for accurate ionic profiling. Methodologically, the use of methanol as a precipitating agent not only simplifies sample preparation and minimizes analyte loss but also aligns with green chemistry principles by offering a safer and more environment favorable alternatives to acetonitrile (Capello et al. 2007; Moniz and Sarraguça 2026).

Future investigations may focus on applying the method to species-specific datasets with larger sample populations, evaluating temporal variations in ionic composition, and coupling the technique with mass spectrometric detection (IC-MS) for enhanced selectivity and trace-level confirmation of analytes. Overall, the established protocol represents a significant advancement towards comprehensive and reliable ionic profiling in animal whole blood, particularly for analytes spanning diverse concentration levels. The major advantage of this method is its application to whole blood, a complex and underexplored matrix that integrates both intracellular and extracellular compartments, thereby providing a more comprehensive representation of anion distribution. It is particularly advantageous in veterinary context where interspecies variability in size and blood volume can limit serum availability, especially in small animals. Moreover, some anions demonstrate differences in their distribution in plasma and cellular fractions, reinforcing the value of whole-blood analysis for accurate ionic profiling.

5 | Conclusion

The IC-CD procedure developed here provides a practical, validated approach for simultaneous quantification of six important inorganic anions in animal whole blood with performance characteristics appropriate for both physiological monitoring and many exposure assessment contexts. When combined with well-defined preanalytical procedures, matrix-aware calibration strategies, and species-specific reference intervals, the method can significantly strengthen veterinary toxicology and environmental exposure investigations. Continued method refinement (internal standards, stability testing) and broader validation across species will increase confidence for diagnostic and surveillance applications.

Author Contributions

Babita Dhanik: conceptualization, writing – review and editing, methodology, investigation, visualization, supervision. **Pallavi Khandelia:** writing – review and editing, methodology, investigation. **Aakanksha Paliwal:** writing – review and editing, methodology, investigation. **Ketan Patil:** writing – review and editing, supervision.

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Ethics Statement

Ethical concerns are not needed because all samples were leftover diagnostic samples from routine clinical care and no additional blood samples or volumes were collected specifically for this study. Also, all biosafety measures and protocols were followed to ensure the safety of personnel conducting laboratory analysis.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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