# Analytical Methods

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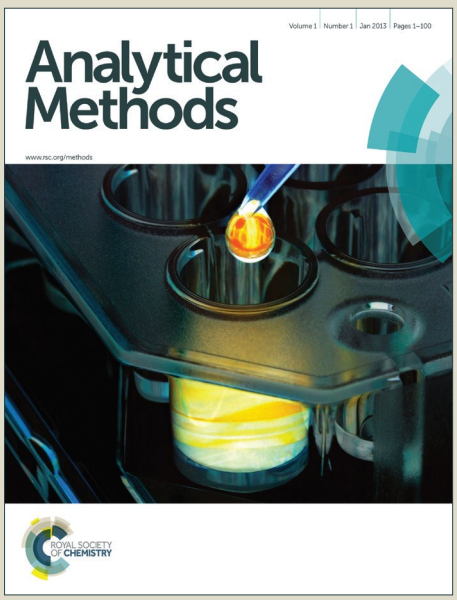


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ARTICLE

# Chlorine Speciation Analysis in Blood by Ion Chromatography-Inductively Coupled Plasma Mass Spectrometry

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At present chlorate salts are discussed as potential in-vivo disinfectants in food animals. The ongoing metabolism studies rely on robust and sensitive methods, though. Therefore a method for the determination of chlorine species in blood was developed. After their ion chromatographic separation, chlorate and chlorite were detected by single-quadrupole inductively coupled plasma mass spectrometry (IC-ICPMS). The suitability of the method was evaluated with spiking experiments. Estimated detection limits of ~0.1 mg Cl $L^{-1}$ chlorite and ~0.2 mg Cl $L^{-1}$ chlorate in measurement solutions (injection volume 20 µL) and ~0.5 mg Cl $L^{-1}$ chlorite and ~1.0 mg Cl $L^{-1}$ chlorate in blood were obtained. In order to overcome interfering $^{18}O^{16}O^{1}H^{+}$ and $^{36}Ar^{1}H^{+}$ ions, IC was also coupled to a triple-quadruple ICPMS (ICPQQQMS) operated in MS/MS mode with hydrogen as reaction gas. The signal-to-background ratios and, hence, the detection limits couldn't be improved, though, due to a constant chlorine background. However, with proper sample preparation the method is suited for the determination of anionic chlorine compounds in blood.

## 1. Introduction

In the last fifteen years chlorate salts, typically used as broadband herbicides[1] and as starting materials for the generation of chlorine dioxide,[2] have been discovered as potential in-vivo disinfectants for the food industry.[3] Several studies that investigated the antimicrobial effects of chlorate show that chlorate decreases effectively populations of specific nitrate-respiring pathogens like *E. coli* and *Salmonella* in the gastrointestinal tract of food-animal species. It is expected that chlorate may be administered to food animals right before slaughtering, in order to reduce hide or carcass contaminations by such pathogens and, consequently, human illnesses caused by the consumption of contaminated food.

An important step to get there is to investigate and understand the metabolism of chlorate salts in animals including their metabolites, excretion routes and bioaccumulation. Therefore sensitive, selective and robust analytical methods are necessary that enable the determination of chlorate and its metabolites like chlorite in the presence of complex biological matrices (blood, urine, faeces, tissue etc.).

Several papers have been published about chlorate metabolism studies in food animal species (cattle,[4-6] swine,[7] poultry,[8] sheep[9]). In works before 2011 test animals were administered radiolabelled sodium chlorate ($^{36}Cl$), which was determined by liquid scintillation counting (LSC). The methods achieved detection limits for the total radiochlorine of ~0.03-0.05 mg $kg^{-1}$ in liver, kidney, skeletal muscle and adipose tissue[5] and ~0.02 mg $kg^{-1}$ of radiolabelled chlorate in white skeletal muscle, dark skeletal muscle, skin and adipose tissue, gizzard, and liver.[8]

Smith and Taylor (2011) developed three methods for the determination of non-radiolabelled chlorate in different sheep matrices (blood serum, milk, urine, small intestine, cecum, colon and faeces).[10] Among these liquid chromatography (ion-exchange) coupled to electro-spray ionization mass spectrometry (LC-MS) achieved quantification limits of 0.011-0.21 mg Cl $L^{-1}$ in serum and milk despite sample dilution. However, the method required matrix matched calibrations and proper adjustments of the internal standard ($^{18}O$-labelled chlorate), and, therefore, was limited to samples with a chlorate content lower than 2 mg Cl $L^{-1}$. In urine, where higher chlorate concentrations were expected, ion chromatography with suppressed conductivity detection was used for the determination of chlorate after solid phase extraction. The quantification limits based on sample sizes of 100 or 25 µL ranged between 42.5 and 425 mg Cl $L^{-1}$, respectively.

Due to the complexity of the matrices dealt with, inductively coupled plasma mass spectrometry (ICPMS) seems to be an appropriate detection method for the determination of chlorate traces in biological samples because of its robustness and element-selectivity. ICPMS provides detection limits down to a few nanograms per litre for a large number of elements. Chlorine, however, is troubled by numerous polyatomic interferences, with the major interferences being $^{18}O^{16}O^{1}H^{+}$ and $^{36}Ar^{1}H^{+}$ on $^{35}Cl$ and $^{37}Cl$, respectively. Furthermore, due to its high first ionization potential (12.97 eV) it is ionized only partially (1 %) in the heat of the plasma.[11]

Pantsar-Kallio and Manninen[12] developed an IC-ICPMS method for the determination of disinfection by-products in drinking water, with detection limits of 0.5 mg Cl $L^{-1}$ for the chlorine species (chloride, chlorite, chlorate and perchlorate). Divjak and Goessler[13] demonstrated with aqueous multi-anion standards that IC-ICPMS equipped with an ultrasonic nebulizer

and membrane desolvator was capable of achieving detection limits of 0.004, 0.036 and 0.020 mg Cl $L^{-1}$ for chloride, chlorite and chlorate, respectively. Chloride and chlorate in water were determined by Fernández *et al.*[14] with IC-ICPMS detection limits of 0.3 and 0.2 mg Cl $L^{-1}$.

Until now there are little examples for chlorine speciation analyses by IC-ICPMS, and most of them are dealing with aqueous solutions and water samples. The aim of the present work was to extend the scope of the chlorine speciation analysis towards complex biological sample matrices for the application in chlorate metabolism studies. Therefore a robust method was developed to determine chlorite and chlorate by IC-ICPMS in horse and human blood (whole blood, serum and plasma).

## 2. Experimental

### 2.1 Chemicals, standards and reagents

Purified water (18.2 MΩ cm, Milli-Q integral water purification system from Millipore GmbH, Vienna, Austria) was used for the dilution of the standard and stock solutions, and for the IC eluent generation. For the speciation analysis a chloride standard solution (1000 mg Cl $L^{-1}$ in water) was obtained from Roth (Carl Roth, Karlsruhe, Germany). Chlorite and chlorate stock solutions (1000 mg Cl $L^{-1}$) were prepared in water from $NaClO_2$ (80 % p.a.) and $KClO_3$ (≥ 99 % p.a.), respectively, purchased from Sigma-Aldrich (Steinheim, Germany). A solution of 0.9 % NaCl was prepared from NaCl (≥99.5 % p.a., Carl Roth, Karlsruhe, Germany). Acetonitrile (Rotisolv® HPLC) was purchased from Roth (Carl Roth, Karlsruhe, Germany). The IC-ICPMS/ICPQQQMS eluents were made with KOH from Roth (≥85 % p.a., treated like 100 %, Carl Roth, Karlsruhe, Germany). KOH from Sigma-Aldrich (semi-conductor grade, Steinheim, Germany) was used to prepare the calibration with the chlorine species. Lyophilised control serum (ClinChek® serum controls based on human serum, level I) was purchased from Recipe® (Munich, Germany) and stored at 4 °C. Human whole blood (stabilized with a citrate-phosphate-dextrose solution) and human plasma (via centrifugation of stabilized whole blood) were obtained from the Univ. Klinik für Blutgruppenserologie und Transfusionsmedizin (Graz, Austria) and stored in closed 50 mL polypropylene tubes (Cellstar®, Greiner Bio-One GmbH, Kremsmünster, Germany) at 4 °C. A chlorite and chlorate containing drug (stored at 4 °C) and frozen horse whole blood, plasma (via centrifugation of whole blood stabilized with Li-Heparin) and serum (via centrifugation of whole blood stabilized with EDTA) were provided by a cooperation partner. The horse blood samples were stored on dry-ice.

### 2.2 Instrumentation

**2.2.1 IC system for conductometric detection.** The ion chromatographic system with conductometric detection from Dionex (Dionex Corporation, Sunnyvale, USA) consisted of the ion chromatograph IC25, the eluent generator EG40 with a potassium hydroxide eluent generator cartridge (Dionex EGC III KOH from Dionex Corporation, Sunnyvale, USA) and a column oven LC25 with conductivity cell, an autosampler AS50 and the self-regenerating suppressor ASRS 300 (2 mm).

**2.2.2 IC-ICPMS.** For the coupling of ion chromatography with a single-quadrupole inductively coupled plasma mass spectrometer (IC-ICPMS) a high performance liquid chromatography (HPLC) system from Agilent (Agilent Technologies, Waldbronn, Germany) was used, which consisted of the degasser G1379A (Agilent 1100 Series), the capillary pump CapPump G1376A (Agilent 1100 Series), the autosampler ALS G1329A (Agilent 1200 Series) with the sample chiller ALSTherm G1330B (Agilent 1100 Series) and the column compartment COLCOM G1316A (Agilent 1100 Series).

The element-selective detection by ICPMS was performed on an Agilent 7500ce ICPMS (Agilent Technologies, Waldbronn, Germany) with ORS (octopole reaction system), equipped with a Micro Mist nebulizer (Glass Expansion, Melbourne, Australia), a Scott double pass spray chamber, a 2.5 mm ID quartz torch, a sampler cone made from copper with nickel tips and a skimmer cone made from nickel. For sample introduction a capillary (PEEK, 0.127 mm ID) was connecting the chromatographic column with the nebulizer on the spray chamber of the ICPMS. The ICPMS was tuned for suitable sensitivity and robustness using a solution containing 1 µg $L^{-1}$ Li, Cr, Fe, Co, Se, Y, Ce, and Tl in 2 % $HNO_3$. The masses (m/z) 7, 89 and 205 as well as the ratios of 156/140 (oxide ratio) and 70/140 (doubly charged ratio) were monitored. Typical values for $^{7}Li$, $^{89}Y$ and $^{205}Tl$ were $9 \times 10^4$, $8 \times 10^4$ and $7 \times 10^4$ cps, < 1.3 % oxide ratios and < 2.0 % doubly charged.

**2.2.3 IC-ICPQQQMS.** Coupling of ion chromatography with triple-quadrupole inductively coupled plasma mass spectrometer (IC-ICPQQQMS) was realized with a HPLC system from Agilent, consisting of the quaternary pump BioQuatPump G5611A (Agilent 1260 Series), the bio-inert high performance autosampler G5667A (Agilent 1260 Series) and the thermostatted column compartment G1316A (Agilent 1260 Series).

For the element-selective detection with an ICPQQQMS Agilent 8800 (Agilent Technologies, Waldbronn, Germany) a capillary (PEEK, 0.127 mm ID) was connecting the chromatographic column with the nebulizer on the spray chamber. The ICPQQQMS was equipped with a PFA inert sample introduction KIT with a Scott-type spray chamber (Savillex Corp.), a PFA micro-flow nebulizer (200 µL $min^{-1}$ self-aspiration rate at 1 L $min^{-1}$ Ar, Elemental Scientific Inc., Omaha, USA), a quartz torch with 2.5 mm sapphire injector, a sampler cone made from copper with nickel surface and a skimmer cone made from platinum. For sample introduction a capillary (PEEK, 0.127 mm ID) was connecting the chromatographic column with the nebulizer on the spray chamber of the ICPQQQMS. The ICPQQQMS was tuned using a multi-element solution and the autotune sequence of the instrument. Chlorine was detected in MS/MS mode with 4.5 mL $min^{-1}$ $H_2$ (gas purity > 99.9999 %) as reaction gas (35→37 and 37→39).

### 2.3 General chromatographic conditions

The ion-exchange chromatography was performed on the anion-exchange column IonPac® AS15 (2 x 250 mm) in combination with the precolumn IonPac® AG15 (2 x 50 mm) (Dionex corporation, Sunnyvale, USA) with KOH as mobile phase (isocratic or gradient) at a flow rate of 0.25 µL $min^{-1}$, an injection volume of 20 µL and 30 °C column temperature.

**2.3.1 IC with suppressed conductivity detection.** Chlorite and chlorate in spiked diluted reference serum were eluted isocratically with 30 mM KOH (generated online with the eluent generator, 19 mA suppressor current).

**2.3.2 IC-ICPMS and IC-ICPQQQMS.** The isocratic elution of chlorine compounds spiked into reference serum was realized with 40 mM KOH. For the gradient elution the mobile phase always was generated by the instrument with water and 100 mM KOH. The gradient time and the return to the initial conditions were optimized as follows:



 

Gradient time optimisation:

- *10-80 mM from **0-5 min** and 80-10 mM from 12.00-12.01 min*
- *10-80 mM from **0-4 min** and 80-10 mM from 12.00-12.01 min*
- *10-80 mM from **0-3 min** and 80-10 mM from 12.00-12.01 min*
- *10-80 mM from **0-1 min** and 80-10 mM from 12.00-12.01 min*
- *10-80 mM from **0.00-0.01 min** and 80-10 mM from 12.00-12.01 min*

Return period optimisation:

- *10-80 mM from 0.00-0.01 min and 80-10 mM from **12.00-12.01 min***
- *10-80 mM from 0.00-0.01 min and 80-10 mM from **10.00-10.01 min***
- *10-80 mM from 0.00-0.01 min and 80-10 mM from **8.00-8.01 min***
- *10-80 mM from 0.00-0.01 min and 80-10 mM from 6**.00-6.01 min***
- *10-80 mM from 0.00-0.01 min and 80-10 mM from **5.50-5.51 min***

After the gradient optimization the chlorine speciation analysis in blood samples was done with the following gradients:

- 15 mM to 80 or 90 mM in 0.00-0.01 min and 80 or 90 mM to 15 mM from 6.50-6.51 min (human blood samples).
- 10 mM to 90 mM in 0.00-0.01 min and 90 mM to 10 mM from 6.50-6.51 min (horse blood samples).

### 2.4 Preparation of reference serum and blood samples

**2.4.1 Reference serum.** Prior to the analysis the lyophilised reference serum samples were dissolved in 3 mL (volume given by the manufacturer) of a 40 mM KOH solution, agitated mechanically for 30 min and filtered through 0.2 µm Nylon® syringe filters (Markus Bruckner Analysentechnik, Linz, Austria) into 12 mL polystyrene (PS) tubes (Greiner Bio-One GmbH, Kremsmünster, Germany). The filtered samples were spiked with chlorite and chlorate, diluted 1+9 with water and pipetted into 0.7 mL polypropylene (PP) micro vials (VWR International GmbH, Vienna, Austria).

**2.4.2 Human blood samples.** Human whole blood and human plasma samples were spiked with a chlorite and chlorate containing drug at room temperature and kept at room temperature throughout the degradation studies. Immediately after spiking the time was recorded and the samples were carefully inverted 4-6 times. Aliquots were taken at varying intervals and prepared for the analysis. Whole blood aliquots (1 mL) were diluted 1+9 with water in 15 mL PP tubes (Cellstar®, Greiner Bio-One GmbH, Kremsmünster, Germany), centrifuged for 5 min at 4200 g and filtered through 0.2 µm Nylon® syringe filters into 0.7 mL PP micro vials. Precipitation was not observed in the plasma samples, therefore a less time-consuming sample preparation was chosen. Plasma aliquots (2 mL) were filtered through 0.2 µm Nylon® syringe filters into 12 mL PS tubes, diluted 1+9 with water and transferred into 0.7 mL PP micro vials. The vials were capped with polyethylene snap ring caps and placed in the HPLC autosampler. Approximately 14-17 min elapsed between spiking and the first injection of the whole blood samples, whereas the first plasma samples were injected 5-7 min after spiking.

**2.4.3 Horse blood samples.** Horse whole blood, plasma and serum samples were thawed in a water bath (~60 °C) and carefully inverted 4-6 times. Aliquots of 0.5 mL were diluted 1+1+3 in 15 mL PP tubes with 0.5 mL acetonitrile and 1.5 mL water, agitated and centrifuged for 4 min at 2900 g. The supernatant was filtered through 0.2 µm Nylon® syringe filters into 0.7 mL PP micro vials. For a spike recovery test on the IC-ICPQQQMS 60 µL of chlorite and chlorate solutions (50 mg Cl $L^{-1}$ each) were mixed in 0.7 mL PP micro vials with 540 µL of a prepared sample.

## 3. Results and discussion

In drinking water anionic chlorine species like chlorite, chloride and chlorate are typically determined after their ion chromatographic separation by suppressed conductivity detection. The detection limits achieved can be below 10 µg Cl $L^{-1}$.[15] However, in order to obtain accurate results by suppressed conductivity detection a sufficient chromatographic separation of the analytes and the matrix is necessary.

The speciation analysis of chlorine anions is further gaining complexity when the scope of applications is extended towards complex biological samples like blood. In such a case either a more time-consuming chromatographic separation and/or an additional matrix removal step might be necessary to determine traces of the chlorine species by suppressed conductivity detection.

In order to demonstrate these difficulties a diluted and spiked (1.0 mg Cl $L^{-1}$ chlorite and chlorate) reference serum was injected onto an ion chromatographic system with suppressed conductivity detection (Fig. 1). The chromatograms reveal that chlorite cannot be resolved satisfyingly from the matrix, whereas chlorate is totally overlapped by matrix signals (Fig. 1B). Due to the low selectivity of the conductivity measurement the chromatographic separation has to be adjusted to the blood matrix in order to determine traces of chlorite and chlorate.

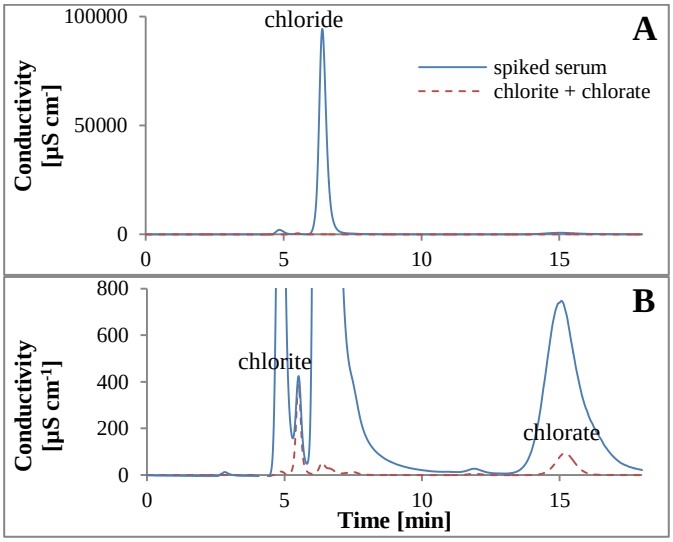


**Fig. 1:** IC chromatograms (30 mM KOH isocratic with suppressed conductivity detection) of a reference serum (1+9) spiked with 1.0 mg Cl $L^{-1}$ chlorite and chlorate and a standard of 1.0 mg Cl $L^{-1}$ chlorite and chlorate. **A:** Full-size serum chromatogram. **B:** Zoom in on the baseline.

ICPMS on the other hand does not need an extensive separation of the matrix. Regarding the chlorine speciation analysis in biological samples, the chromatography must allow a separation of chlorite from an excess of chloride. The IC-ICPMS chromatograms of a spiked serum sample (Fig. 2) clearly show that chlorite and chlorate are sufficiently separated and can be detected without interfering matrix compounds at m/z 35.

For convenience we decided to work without electrochemical suppression of the eluent during the IC-ICPMS measurements. Matrix elements like sodium and potassium are known to lead to signal suppression of analyte ions in the ICP, though.[16,17] Therefore we evaluated the effect of KOH on the signal of chlorine. Chloride standards were prepared in water and KOH (25 mM, 50 mM and 100 mM). The solutions were analysed quantitatively by ICPMS with a sample uptake of ~0.26 mL $min^{-1}$.

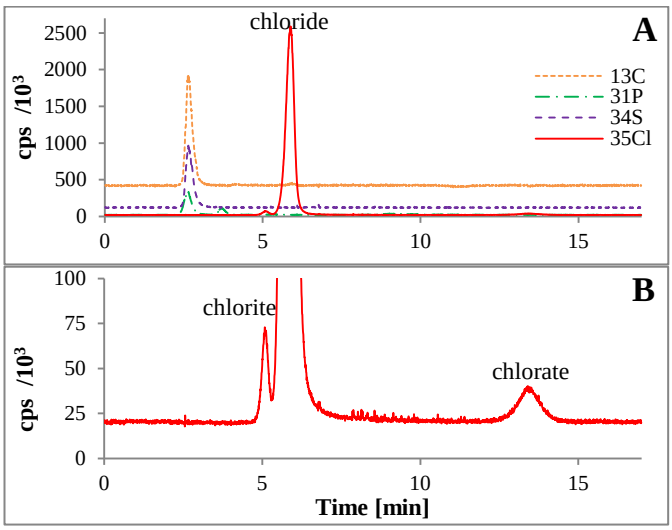


**Fig. 2** IC-ICPMS chromatograms (40 mM KOH isocratic) of a sample containing serum (1+9) spiked with 5.0 mg $L^{-1}$ chlorite and chlorate. Shown are the mass traces of carbon $^{13}C$, phosphorous $^{31}P$, sulfur $^{34}S$ (representing parts of the matrix) and chlorine $^{35}Cl$. **A:** All chromatograms. **B:** Zoom in on the chlorine baseline.

The calibration curves show that increasing the KOH concentration reduces the chlorine signal. However, the suppression of the chlorine signal in 25 mM and 50 mM KOH was negligible (103 ± 2 % and 92 ± 2 % of the Cl signals in water). In 100 mM KOH the chlorine signal was decreased by almost a quarter (78 ± 1 % of the Cl signals in water).

In the present work the gradients for the IC-ICPMS measurements started at 10-15 mM KOH and were increased up to 90 mM KOH. At that concentration a chlorine signal suppression of less than 22 % was expected, affecting especially the late eluting chlorate. This suppression was tolerated in favour of a simpler experimental set-up without electrochemical suppression of the eluent.

For the evaluation of the stability of the separation, the repeatability of the IC-ICPMS method (40 mM KOH isocratic) was investigated. Therefore, a reference serum was diluted and spiked with chlorite and chlorate (5.0 mg Cl $L^{-1}$ each, total dilution 1+9). The sample was then injected 30 times over a period of 11 hours. The repeatability of the retention times and peak areas was satisfying, and the recovery rates determined with an external calibration in ultrapure water were within 95-101 % (Table 1).

There are other chromatographic columns, though, which would have provided a comparable or even better resolution of chlorite and chloride (e.g. the Shodex® IC SI-52 4E with carbonate buffer,[18] the Metrosep A Supp 1 with sodium hydroxide,[19] the Metrosep Anion Dual 2 with carbonate buffer,[20] the IonPac® AS11 and AS11HC with sodium hydroxide[21] and the IonPac® AS18 with potassium hydroxide[22]). However, in consideration of the elution order (chlorite before chloride) we found the combination of stationary and mobile phase sufficient for our analytical problem.

**Table 1** Recovery rates of the IC-ICPMS measurements of serum samples (1+9) spiked with different concentrations of chlorite and chlorate.

| n = 30 | Retention time [min] | Concentration [mg $L^{-1}$] | Recovery % |
|---|---|---|---|
| Chlorite | 5.08 ± 0.01 | 4.86 ± 0.07 | 97 ± 2 |
| Chlorate | 13.42 ± 0.04 | 4.97 ± 0.10 | 99 ± 2 |

Before working with blood as a complex biological matrix, we tried to further improve the chromatographic separation. The main aim was to increase the resolution between chlorite and chloride, while reducing the retention time and, thus, the peak broadening of chlorate. Developing a gradient elution seemed appropriate. Therefore, a solution was prepared containing 5.0 mg Cl $L^{-1}$ chlorite and chlorate in 0.09 % NaCl (~540 mg $L^{-1}$ chloride, corresponding to a tenfold diluted saline).

Different starting and end concentrations, and gradient runtimes were tested. After some trials, 10 mM and 80 mM KOH were chosen as starting and end concentration for the optimization of the gradient. The gradient time was successively reduced (Fig. 3), which lead to an improvement of the chlorite/chloride resolution, the chloride peak shape and the half width of the chlorate peak, compared to the isocratic conditions (Table 2). For further experiments step gradient elution was chosen with following settings (the variations were caused by day-to-day optimisations): 10 mM or 15 mM KOH were increased to 80 mM or 90 mM KOH in 0.00-0.01 min, followed by a reduction from 80 mM or 90 mM KOH to 10 mM or 15 mM KOH, either in 6.00-6.01 min or in 6.50-6.51 min.

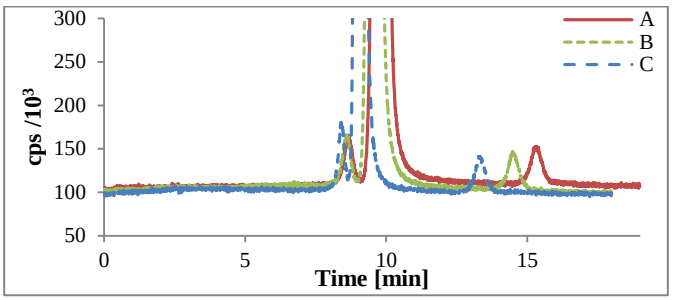


**Fig. 3** IC-ICPMS gradient time optimisation, detected at m/z 35. **A:** 10-80 mM KOH in 0-5 min. **B:** 10-80 mM KOH in 0-3 min. **C:** 10-80 mM KOH in 0.00-0.01 min.

In order to evaluate the suitability of the method for the analysis of real samples, the chlorine species were determined in human whole blood and human plasma spiked with a drug containing chlorite and chlorate. Aliquots were taken in varying intervals after spiking for the evaluation of the stability of the chlorine compounds in these matrices. The whole blood samples were diluted with water (1+9), centrifuged and filtered. The plasma samples were filtrated and diluted with water (1+9).

The recovery of chlorate was satisfying (Table 3). In average 90-105 % could be found of the initial spike. The relative standard deviation ranged between 5-20 %, though. The main cause of the fluctuations might have been that the chlorate content in the diluted samples was in the range of the quantification limit of ~0.7 mg Cl $L^{-1}$ (estimated with 10 × σ of the baseline noise, based on peak heights).

Chlorite showed a different behaviour. Although the first aliquots were taken right after spiking, chlorite couldn't be detected in any of the whole blood samples. The degradation – most probably to chloride – in whole blood must had been so fast that the chlorite concentration fell below the detection limit (~0.1 mg $L^{-1}$ in diluted samples, estimated with 3 × σ of the baseline noise, based on peak heights) during the sample preparation, even though the initial spike was higher than that of chlorate (20-22 mg Cl $L^{-1}$ whole blood). When one of the blood samples was spiked additionally with 100 mg Cl $L^{-1}$ whole blood of chlorite, more than two thirds of it had been degraded before the measurement. That amount was not sufficient to observe a significant increase of chloride, though, which was found in concentrations of ~2500 mg $L^{-1}$ whole blood (semi-quantitative value).

**Table 2** Optimisation parameters of the chromatographic conditions (mobile phase KOH): resolution R,* peak asymmetry factor $A_s$,** half-width $W_{1/2}$ and net retention time $netR_t$***.

| Chromatography | R chlorite/ chloride | $A_s$ chloride | $W_{1/2}$ chlorate [s] | $netR_t$ chlorate [min] |
|---|---|---|---|---|
| isocratic 40 mM**** | 1.8 | 0.75 | 45 | 9.4 |
| 10-80 mM in 0-5 min | 2.3 | 0.55 | 29 | 11.3 |
| 10-80 mM in 0-3 min | 2.2 | 0.75 | 24 | 10.5 |
| 10-80 mM in 0.00-0.01 min | 1.9 | 1.11 | 24 | 9.3 |

*Calculated with the retention times ($R_t$) and the half-widths ($W_{1/2}$): $R = 2 (R_{tB}-R_{tA})/[1.7 (W_{1/2A}+W_{1/2B})]$

**Peak asymmetry factor ($A_s$) as the quotient of the width at 10 % of the maximal height of the back half (b) and the front half (a) of the peak: $A_s$= b/a. $A_s < 1$ peak fronting, $A_s > 1$ peak tailing.

***$netRt_{analyte} = Rt_{analyte} - Rt_{mobile\ pase}$

****Reference serum diluted and spiked with chlorite and chlorate.

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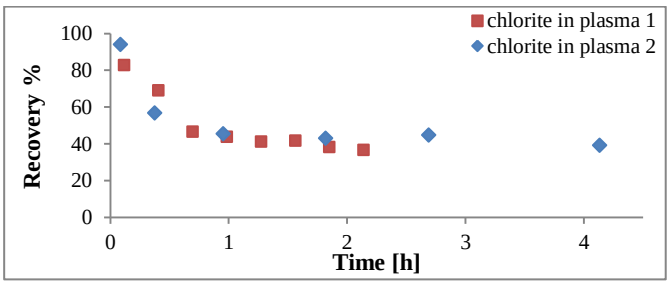


**Fig. 4** Degradation of chlorite in human plasma. The time was recorded right after spiking. The recovery rates were plotted against the IC-ICPMS injection time.

The spiking experiments with human whole blood and human plasma revealed that the IC-ICPMS method is principally suited for the determination of anionic chlorine compounds in complex biological matrices. However, one has to keep in mind that some analytes might not be stable enough under such conditions, as was shown for chlorite.

While one reason that hampers the chlorine detection by ICPMS cannot so easily be overcome, namely the incomplete ionization, triple-quadrupole ICPMS enables effective interference removal, thus reducing the background signal. It was shown for phosphorus and sulfur that these elements can be detected quasi interference free

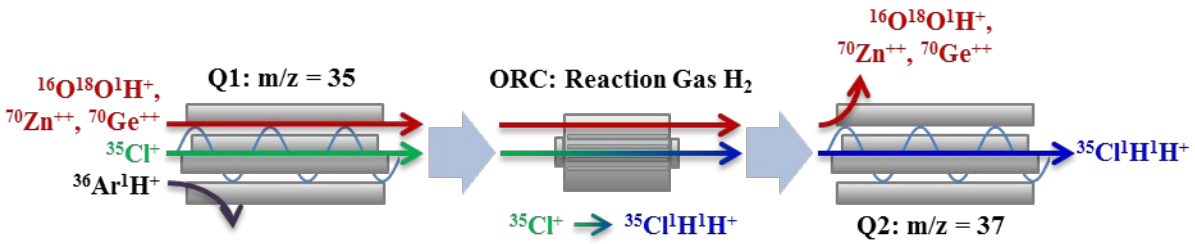


**Fig. 5** Chlorine determination by ICPQQQMS in MS/MS mode with $H_2$ as reaction gas.

**Table 3** Recovery rates for chlorate in whole blood and plasma.

| Sample Matrix | Chlorate (mg Cl $kg^{-1}$) | Expected (mg Cl $kg^{-1}$) | Recovery % |
|---|---|---|---|
| Whole blood (n = 8) | 6.0 ± 0.6 | 6.6 | 91 ± 9 |
| Whole blood (n = 6) | 6.2 ± 0.9 | 7.0 | 89 ± 13 |
| Plasma (n = 8) | 6.7 ± 1.2 | 6.4 | 104 ± 19 |
| Plasma (n = 6) | 6.5 ± 0.4 | 7.0 | 93 ± 6 |

However, a slow degradation of chlorite could be observed in spiked plasma (Fig. 4), with a half-life between 40-44 min. These results are in good agreement with results from Hakk *et al.*[23] who observed a similar degradation of chlorite fortified into bovine serum (~2 h half-life) and rat urine (~5 h half-life). They argue that chlorite might be reduced to chloride in the presence of physiological reductants such as thiols (e.g. cysteine), ferrous iron in haemoglobin and ascorbic acid. The different behaviour of chlorite observed in whole blood and plasma supports this hypothesis.

as oxide ions in MS/MS mode after the reaction with oxygen.[24] The manufacturer published that chlorine measurements could be improved using hydrogen as cell gas in MS/MS mode.[25] In short, chlorine ions and on-mass interferences pass the first quadrupole (Q1) and enter the octopole reaction cell (ORC), where the chlorine ions react with $H_2$, forming $ClH_2^+$ ions (Fig. 5), which are then detected after passing the second quadrupole (Q2) on m/z 35+2 or 37+2 (mass-shift).

With that in mind, the chlorine compounds were determined in spiked horse blood (whole blood, serum and plasma) by single- and triple-quadrupole ICPMS. The sample preparation was similar to the preparation of the human whole blood samples, with one additional step. To minimize loading of the column with proteins, whole blood, serum and plasma samples were mixed with one part acetonitrile prior to the dilution with water (total dilution 1+4). Precipitation was observed, and the resulting suspensions were centrifuged and filtered before the measurements.

For the IC-ICPQQQMS analysis in reaction gas mode the $H_2$ gas flow was optimized. Against our expectations the mass-shifts 35→37 and 37→39 didn't remove the entire background. The highest mass-shift signals (at a $H_2$ flow of 3.0-3.5 mL $min^{-1}$) went hand in hand with high background signals. In order to improve the signal-to-noise ratio (at the expense of sensitivity) the measurements were made with 4.5 mL $H_2$ $min^{-1}$.

In contrary to the single-quadrupole ICPMS the mass-shift on the triple-quadrupole ICPMS made it possible to reduce the argide interference $^{36}Ar^1H^+$ significantly, which enabled the simultaneous detection of $^{35}Cl$ and $^{37}Cl$. Therefore the chlorine compounds could

be determined on both chlorine isotopes, which is shown in Fig. 6 (5.0 mg Cl $L^{-1}$ standard solution **A** and horse plasma spiked with 5.0 mg Cl $L^{-1}$ chlorite and chlorate **B**; signals on the main y-axis). Against our expectations a significant part of the chlorine background was shifted, too. The mass-shift 35→37 resulted in detection limits of ~0.1 mg Cl $L^{-1}$ chlorite and ~0.3 mg Cl $L^{-1}$ chlorate in the diluted samples (estimated with 3 × σ of the baseline noise, based on peak heights). The single-quadrupole IC-ICPMS analysis of horse blood yielded detection limits of ~0.1 mg Cl $L^{-1}$ chlorite and ~0.2 mg Cl $L^{-1}$ chlorate in the diluted samples (estimated with 3 × σ of the baseline noise, based on peak heights). Hence, the mass-shift method didn't improve the chlorine detection.

A closer look at the signal ratios of the mass shifts (Fig. 6 secondary axis) reveals that a significant part of the m/z 35 background must have been chlorine contaminations. According to the relative isotopic abundance of $^{35}Cl$ (75.78 %) and $^{37}Cl$ (24.22 %)[26] the natural isotopic ratio $^{37}Cl/^{35}Cl$ is 0.32. The signal ratios of the mass-shifts found in the standard and plasma samples were 0.40 ± 0.02, which accounts for 126 ± 7 % of the natural isotopic ratio ($^{37}Cl/^{35}Cl$ signal ratios obtained by single-quadrupole ICPMS are typically >> 1).

The slightly elevated experimental isotopic ratio may be a result of the mass discrimination during the atomization, ionization, ion extraction and transmission processes, of different reaction rates of the chlorine isotopes with hydrogen, and of co-reacting $^{36}Ar^{1}H^{+}$, which could partially contribute to the m/z 37→39 background signal as $^{36}Ar^{1}H_{3}^{+}$. However, the fact that the signal ratios from the parts of the chromatograms where chlorine compounds elute are equal to the 35→37/37→39 baseline signal ratios indicates that the background signal was caused mostly by chlorine contaminations, not polyatomic interferences.

The influence of some of the potential contamination sources was evaluated prior to the analysis. In order to exclude contaminations from the eluent, the KOH was replaced by ultrapure water (18.2 MΩ cm). The ICPQQQMS measurements with and without HPLC system showed that there was a constant background on m/z 35→37 and 37→39 independent from the set-up. Rinsing of the vials, the use of a quartz and an inert PFA sample introduction kit, as well as different nebulizers and cones didn't reduce the background. Bottled HPLC grade water even had higher chlorine signals than the ultrapure water used throughout the experiments. Nevertheless, all efforts to further improve the chlorine determination by ICPQQQMS were without success.

In the end the detection limits by IC-ICPMS (estimated with 3 × σ of the baseline noise, based on peak heights) were ~0.1 mg Cl $L^{-1}$ chlorite and ~0.2 mg Cl $L^{-1}$ chlorate in prepared samples and – depending on the dilution (1+4 or 1+9) – ~0.5 or 1.0 mg Cl $L^{-1}$ chlorite and ~1.0 or 2.0 mg Cl $L^{-1}$ chlorate in blood. Probably 2-4 times better detection limits could be achieved simply by increasing the injection volume. Additionally, the samples could be diluted less during the sample preparation. But it has to be considered that in both cases more chloride from the biological samples would be injected onto the column. This might call for a better chromatographic separation of chlorite and chloride, or an additional sample preparation step to remove chloride. The effect of electrochemical suppression should be little, though, because the estimated suppression of the chlorine signal is less than 22 %.

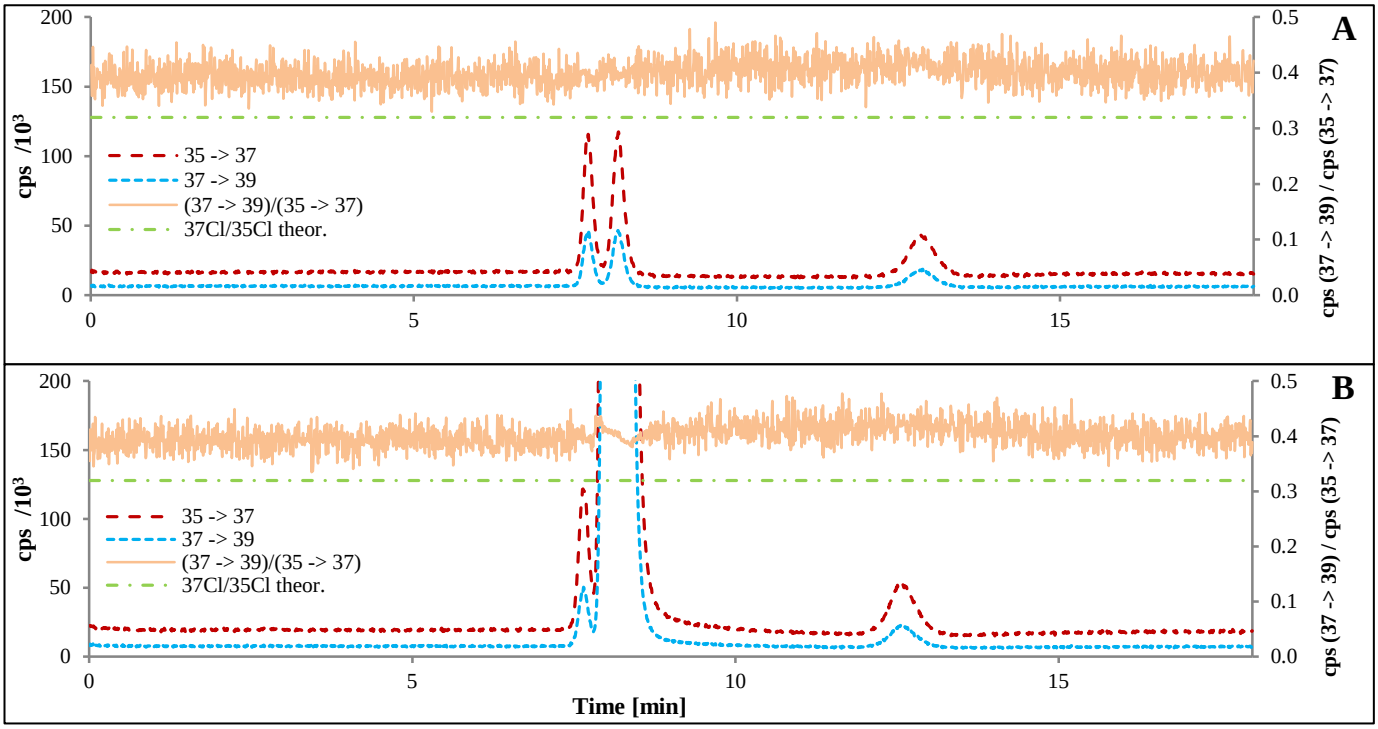


**Fig. 6** Chlorine speciation analysis by IC-ICPQQQMS. **A:** Standard solution (5.0 mg Cl $L^{-1}$) **B:** Horse plasma spiked with chlorite and chlorate (5.0 mg Cl $L^{-1}$). Recovery of chlorite 93 % and of chlorate 104 %.

## 4. Conclusion

In the present work we have successfully developed a method for the determination of anionic chlorine compounds in whole blood, serum and plasma by IC-ICPMS. The detection limits (estimated with 3 × σ of the baseline noise, based on peak heights) were ~0.1 mg Cl $L^{-1}$ chlorite and ~0.2 mg $L^{-1}$ chlorate in the prepared samples and ~0.5 mg Cl $L^{-1}$ chlorite and ~1.0 mg $L^{-1}$ chlorate in blood matrices (1+4 dilution). However, the investigations of the stability of chlorite and chlorate showed that chlorite is degraded fast in whole blood (beyond the detection limit within 14-18 min from the first spiking

to the injection) and slower in plasma. Chlorate on the other hand was stable in whole blood and plasma over periods of 2-4 hours. The attempt to improve the chlorine detection with a triple-quadrupole ICPMS in mass-shift mode with hydrogen was hampered by a constant chlorine background. Due to the relatively stable $^{37}Cl/^{35}Cl$ signal ratios we assume that chlorine contaminations were a problem during the measurements. Despite our efforts the contamination source couldn't be identified, yet. A focus of future work will be to evaluate probable chlorine contamination sources like the plasma gas or the ambient air. Further, the scope of the method should be extended to biological matrices other than blood like tissue or urine.

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## Notes and references

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## Graphical Abstract

The present work describes the development of a robust method for the determination of traces of the anionic chlorine species chlorite and chlorate in blood matrices (estimated detection limits ~0.5-1.0 mg Cl $L^{-1}$) by ion chromatography coupled to inductively coupled plasma mass spectrometry.