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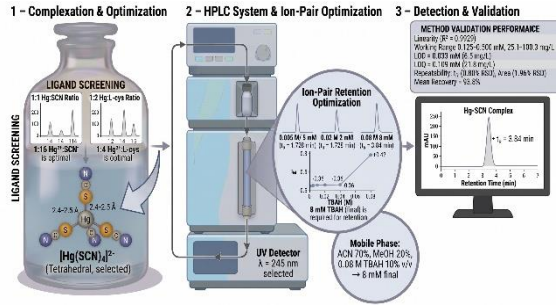
An RP-HPLC-UV Method for the Determination of Inorganic Mercury (II) via Sulfur-Donor Ligand Complexation and Ion-Pair Retention

Tahir Bakhsh[1,2]*, Nazhifa Jacinda Maheswari Abidin[1], Hermin Sulistyarti[1,2], Ani Mulyasuryani[1]

[1]Department of Chemistry, Faculty of Science, Technology and Mathematics, Universitas Brawijaya, Malang, Indonesia.
[2]LCAMIA: Research Center for Low Cost and Automated Method & Instrumentation Analysis, Universitas Brawijaya, Malang, Indonesia.

ABSTRACT



Mercury contamination in artisanal and small-scale gold mining regions requires accessible analytical methods, yet established techniques demand dedicated atomic spectrometry instrumentation. This study developed a reversed-phase HPLC-UV method for inorganic mercury(II) based on sulfur-donor ligand complexation and ion-pair retention on a conventional C18 column. Thiocyanate and L-cysteine were evaluated using tetrabutylammonium hydroxide (TBAH) as the ion-pairing reagent. Ultraviolet spectra recorded within the linear response range of the instrument placed the Hg-SCN absorption maximum at 240 nm, and detection was performed at 245 nm. A 1:16 Hg(II): SCN^- molar ratio and a final mobile-phase TBAH concentration of 8 mM (acetonitrile 70%, methanol 20%, aqueous TBAH 10% v/v) produced a dominant chromatographic peak. Against an experimentally measured void time of 2.70 min, retention changed regime rather than increasing progressively: the peak eluted before the void volume at 0.5 and 2 mM TBAH ($k' = -0.36$) and was retained at 8 mM ($k' = +0.42$). Calibration over 0.125-0.500 mM (25.1-100.3 mg/L), using seven independently prepared levels in triplicate, gave $R^2 = 0.9929$, an LOD of 0.033 mM (6.5 mg/L) and an LOQ of 0.109 mM (21.8 mg/L), the LOQ lying below the lowest calibration level. Retention-time and peak-area repeatability were 0.80% and 1.96% RSD, and mean recovery from spiked reagent water was 93.8%. Specificity and matrix effects were not assessed. The validated range lies four orders of magnitude above drinking-water guideline values, so the method is intended as a preliminary procedure for concentrated process and waste streams rather than for environmental monitoring or mercury speciation.

Keywords: *mercury (II); ion-pair RP-HPLC-UV; thiocyanate; tetrabutylammonium hydroxide; complexation*

**Corresponding Author:* *bakhshtahir24@student.ub.ac.id*
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INTRODUCTION

Mercury is a trace metal that is toxic and persistent, having serious health and environmental impacts [1], [2]. Inorganic mercury (Hg^{2+}) is a common component of industrial effluents and wastewaters, especially in the areas where artisanal and small-scale gold mining occurs [3] [4] [5] and its reliable determination in aqueous systems is analytically challenging [6]. Cold vapour atomic absorption spectrometry, cold vapour atomic fluorescence spectrometry and inductively coupled plasma mass

spectrometry are the established techniques and offer excellent sensitivity [7], [8] but require dedicated instrumentation that is not available in every analytical laboratory [9]. High-performance liquid chromatography with ultraviolet detection uses more widely used instrumentation, yet direct determination of inorganic mercury by RP-HPLC-UV is not straightforward [10], [11] Hg^{2+} lacks an intrinsic UV chromophore and is poorly retained on non-polar stationary phases [12], [13].

Complexation addresses both limitations. Sulfur ligands are well suited to this purpose because of the affinity of mercury for soft donors described by the hard-soft acid-base principle [14]. The resulting complexes frequently exhibit ligand-to-metal charge-transfer (LMCT) absorption in the ultraviolet, enabling UV detection [15]. Retention of the anionic complexes on a reversed-phase column is achieved with an ion-pair reagent, which associates with the charged analyte and increases its apparent hydrophobicity [16], [17]. Quaternary ammonium reagents such as tetrabutylammonium hydroxide (TBAH) have been applied to anionic metal complexes on C18 columns [18]. Retention and peak shape depend on complex charge, reagent concentration, and the ligand-to-metal ratio [19], [20].

Two sulfur-donor ligands were considered. Thiocyanate is an ambidentate ligand with strong affinity for soft metal ions [21]. For Hg^{2+} in aqueous solution, sulfur coordination has been established by X-ray diffraction, Raman and NMR measurements on tetrathiocyanato complexes. Stepwise formation proceeds from $Hg(SCN)^{+}$ to $[Hg(SCN)_4]^{2-}$, with constants determined potentiometrically in aqueous solution at an ionic strength of 0.2 mol dm^{-3} (KNO_3) and 25 °C [22]. A critical evaluation notes that determinations in nitrate media are complicated by competing nitrate coordination and bases its tentative value on perchlorate media [23]. Raman measurements have also been reported in dimethylformamide. None of these media corresponds to the 90% organic mobile phase used here, so the constants indicate the expected direction of complexation rather than providing a basis for calculating speciation under chromatographic conditions. The tetrahedral $[Hg(SCN)_4]^{2-}$ complex exhibits LMCT absorption near 240-260 nm [24]. L-cysteine is a biologically relevant thiol studied as a mercury-binding ligand [25], [26] and mercury shows high affinity for thiol groups generally [27]. The charge and coordination mode of Hg-cysteine complexes depend strongly on pH and on the ligand-to-metal ratio, and the species characterised in the literature were determined in alkaline aqueous solution [25], [28], which does not correspond to the unbuffered conditions used here; a single stoichiometry is therefore not adopted for this system.

Prior chromatographic work provides context. RP-HPLC-UV determination of mercury has been achieved with solid-phase preconcentration and dithizone derivatization, reaching nanogram detection limits with resolution of inorganic from

organomercury species [11] dynamically modified C18 monolithic columns have been used for lead, cadmium and mercury [12]; the retention of Hg^{2+} and organomercury species on C18 has been characterized directly [13]; and ion-pair HPLC coupled to ICP-MS has been applied to mercurial speciation [17]. Against this background, the combination examined here. Thiocyanate complexation with tetrabutylammonium ion-pairing on a conventional C18 column with UV detection, without preconcentration or derivatization by a strongly absorbing chelating agent. This has not, been reported.

Two limitations are stated at the outset. Thiocyanate is present in large excess and may displace ligands originally bound to mercury. The determination based on conversion to a common complex cannot distinguish inorganic mercury forms differing in their original coordination. Second, while atomic detectors are element-specific rather than species-specific when used alone, coupling them to a chromatographic separation provides species-specific determination, and hyphenated techniques such as HPLC–ICP-MS remain the established approach to mercury speciation [10], their instrumentation requirement, rather than any limitation of capability, is the gap addressed here.

This study evaluates thiocyanate and L-cysteine as complexing ligands for the determination of inorganic Hg(II) by ion-pair RP-HPLC-UV. The objectives are to establish the absorption maxima of both complexes and select detection wavelengths, to examine the effect of the ligand-to-mercury molar ratio, and to optimize the ion-pairing reagent concentration for retention of the Hg–SCN complex. The work characterizes inorganic Hg(II) only; no organomercury species were examined.

METHODS

1. Reagents and Materials

Mercury(II) chloride ($HgCl_2$, ACS reagent grade, ≥99.5%, Merck), potassium thiocyanate (KSCN, ACS reagent, ≥99.0%, Sigma-Aldrich), L-cysteine (reagent grade, Sigma-Aldrich) and tetra-n-butylammonium hydroxide (TBAH, 20% w/v in water, for synthesis, Sigma-Aldrich 818759) were used as supplied. HPLC-grade methanol and acetonitrile (Fisher Scientific) and deionized water (18.2 MΩ·cm) were used throughout. All glassware was soaked in 10% (v/v) nitric acid and rinsed thoroughly with deionized water before use to prevent mercury adsorption and contamination [29].

2. Instrumentation

Chromatographic analyses were performed on a Shimadzu HPLC system (LC-20AD pump, SPD-20AV UV-Vis detector; Shimadzu Corporation, Japan) with column oven, manual injection valve, and LC Solutions data acquisition software. The detector has a fixed spectral bandwidth of 8 nm, a 10 mm path length and a 12 µL flow cell; the detector response (time constant) was 1.0 s and the data acquisition rate 2 Hz. The pump was operated isocratically at 1.0 mL min^{-1} and the column oven at 40 °C. The column was a Fortis C18 (250 × 4.6 mm, 5 µm, 100 Å; part no. FT-F18-050905, Fortis Technologies, UK). Backpressure averaged

2.70 MPa (27 bar), ranging from 2.06 to 3.43 MPa. Ultraviolet spectra were recorded on a Shimadzu UV-1600 series spectrophotometer in absorbance mode using 1 cm quartz cells, scanning 200-400 nm at a sampling interval of 1.0 nm with a spectral bandwidth of 2.0 nm, at fast scan speed in single-scan mode, with deionized water as blank and for baseline correction.

3. Preparation of Stock Solutions

A 10.00 mM $HgCl_2$ stock solution was prepared by dissolving 271.5 mg of $HgCl_2$ in 100 mL of deionized water. Working solutions were prepared by direct dilution of the stock, each level being made independently. All stock and working solutions were prepared and held in glass volumetric flasks. Stock and working solutions were prepared fresh for each experimental session and were not stored between sessions. All solutions derive from a single gravimetrically prepared $HgCl_2$ stock; no independent second stock or certified reference standard was used. KSCN solutions of 4, 8 and 16 mM were prepared by dissolving appropriate amounts of KSCN in deionized water. L-cysteine solutions of 2, 4 and 8 mM were prepared freshly for each experimental session and protected from light; they were not purged of dissolved oxygen, and no stabiliser was used.

4. Complex Formation

Complexation was carried out at a fixed ligand-to-mercury mole ratio. A 20.0 mL aliquot of the mercury working solution was taken. The volume of ligand solution required for the intended ratio was added to it, and the mixture was made up to a final volume of 40.0 mL with deionized water. For the thiocyanate molar-ratio study, 20.0 mL of 1.00 mM Hg(II) was combined with 20.0 mL of 4, 8 or 16 mM KSCN, giving a final mercury concentration of 0.500 mM at Hg(II):SCN^- ratios of 1:4, 1:8 and 1:16 respectively. For the L-cysteine study, the corresponding ligand solutions gave ratios of 1:2, 1:4 and 1:8 at the same final mercury concentration. For calibration, mercury working solutions of 0.25 to 1.00 mM were combined with 5.0 to 20.0 mL of 16 mM KSCN as required by the 1:16 ratio, giving final mercury concentrations of 0.125 to 0.500 mM. All mercury concentrations reported in this work are the concentrations of the solutions injected after complexation. Mixtures were stirred with a magnetic stirrer at room temperature for 15 minutes. Ultraviolet spectra of the free ligands, of mercury(II) chloride alone and of the mercury-ligand mixtures were recorded before chromatographic analysis, at a mercury(II) concentration of 0.10 mM with ligand concentrations set by the molar ratio.

5. Chromatographic Conditions

The column was maintained at 40 °C with a 40-minute equilibration before each set of injections. The mobile phase consisted of methanol (20%), acetonitrile (70%) and aqueous TBAH solution (10% v/v). Aqueous TBAH solutions of 0.005, 0.02 and 0.08 M were added at 10% (v/v), giving final mobile-phase TBAH concentrations of 0.5, 2 and 8 mM, respectively. The optimised mobile phase was methanol (20%), acetonitrile (70%), and 10% (v/v) aqueous 0.08 M TBAH, corresponding to a final TBAH concentration

of 8 mM. The mobile phase was filtered through a 0.45 µm membrane and degassed by sonication before use. The flow rate was 1.0 mL/min. Detection was at 245 nm for mercury-thiocyanate complexes and 225 nm for Hg–L-cysteine complexes. The ion-pairing reagent is a strong base. The aqueous 0.08 M working solution has a measured pH of 12.7. Diluted to 10% (v/v), the mobile phase contains a nominal hydroxide concentration of 7.66 mM, corresponding on an aqueous basis to approximately pH 11.9. The pH of the complete mobile phase is not reported: a glass electrode calibrated against aqueous buffers does not give a thermodynamically meaningful reading in a medium containing 90% organic solvent. The stationary phase is specified by its manufacturer for operation at both low and high pH and is tested to pH 12, so the mobile phase lies within its stated operating range, although near its upper limit. System suitability was established as follows. The void time was determined experimentally by injection of an unretained marker and found to be 2.70 min at 1.0 mL min^{-1}, corresponding to a void volume of 2.70 mL and a total column porosity of 0.650, consistent with a fully porous 5 µm, 100 Å packing of these dimensions. All retention factors reported in this work are calculated against this measured value; retention factors generated automatically by the chromatography data system are not reported, as the software assigns the void time to the first detected peak in each run, giving values that imply physically impossible column porosities. Under the validated conditions, across 21 injections, the mercury–thiocyanate peak eluted at 3.873 min with a retention factor of 0.43, a tailing factor of 1.128 ± 0.014, a plate count of 2,718 ± 126, retention-time repeatability of 0.80% RSD, peak-area repeatability of 1.96% RSD, and resolution from the nearest preceding peak of 3.94 (range 2.50-8.77). Efficiency measured at a retention factor of 0.43 is substantially depressed by extra-column dispersion, and a detector time constant of 1.0 s accounts for a further 5.5% of the observed peak variance, so the plate count characterises the system as configured rather than the packing.

6. Calibration and Quantification

Calibration standards for thiocyanate complexation were complexation were prepared at seven mercury(II) concentrations within the validated range: 0.125, 0.150, 0.200, 0.250, 0.300, 0.375 and 0.500 mM, corresponding to 25.1, 30.1, 40.1, 50.1, 60.2, 75.2 and 100.3 mg/L. Each was complexed with KSCN at the 1:16 Hg(II):SCN^- molar ratio. Three independent preparations were made at each level, giving 21 independently prepared solutions, each injected once. The replicates reported are therefore independent preparations, not repeated injections of a single solution. The limit of detection and limit of quantification were estimated as LOD = 3σ/S and LOQ = 10σ/S, where σ is the residual standard deviation of the calibration regression, and S is the slope, in accordance with ICH Q2(R2) [30] ICH Q14 was consulted for analytical procedure development [31]. The methodological basis of the σ/S approach is discussed further by Uhrovčík

[32]. The limits are calculated rather than experimentally confirmed, as the calibration does not extend to the calculated LOQ. Precision was evaluated from the calibration sequence, acquired within a single day following a single column equilibration; the figures reported therefore establish repeatability and not intermediate precision, and no inter-day precision assessment is reported. Accuracy was assessed by spike recovery at three levels (0.125, 0.250 and 0.500 mM Hg(II)), each in triplicate. Spiked samples were prepared by adding known amounts of Hg(II) to reagent water and eluent; no environmental, industrial or synthetic sample matrix was used.

RESULTS AND DISCUSSION

1. Complexation Chemistry and Ligand Selection

Coordination of a sulfur-donor ligand to Hg(II) generates ligand-to-metal charge-transfer (LMCT) absorption in the ultraviolet, addressing the absence of an intrinsic chromophore in Hg^{2+}. For Hg^{2+} in aqueous solution, sulfur coordination has been established by X-ray diffraction, Raman and NMR measurements on complexes [33] Stepwise formation proceeds as:

$$Hg^{2+} + SCN^- \rightleftharpoons Hg(SCN)^+$$
$$Hg(SCN)^+ + SCN^- \rightleftharpoons Hg(SCN)_2$$
$$Hg(SCN)_2 + SCN^- \rightleftharpoons [Hg(SCN)_3]^-$$
$$[Hg(SCN)_3]^- + SCN^- \rightleftharpoons [Hg(SCN)_4]^{2-}$$

with cumulative constants (log β_n) of 9.1, 16.8, 19.6 and 21.8, determined in aqueous solution at I = 0.2 mol dm^{-3} (KNO_3) and 25 °C [22]. These conditions differ from the 90% organic mobile phase used here, so the constants indicate the expected direction of complexation rather than providing a basis for speciation calculation. The $[Hg(SCN)_4]^{2-}$ complex is tetrahedral with Hg-S bond lengths of 2.4-2.5 Å [33] For L-cysteine, charge and coordination mode depend on pH and ligand-to-metal ratio [28]. As the solutions used here were unbuffered and their pH not measured, the anionic character of the Hg-cysteine complex is assigned tentatively, and no stoichiometry is claimed. Chloride is present at twice the mercury concentration, the mercury source being $HgCl_2$, but reported formation constants for the thiocyanate system substantially exceed those for the corresponding chloride complexes and thiocyanate is present at a sixteen-fold excess, so thiocyanate coordination is expected to predominate.

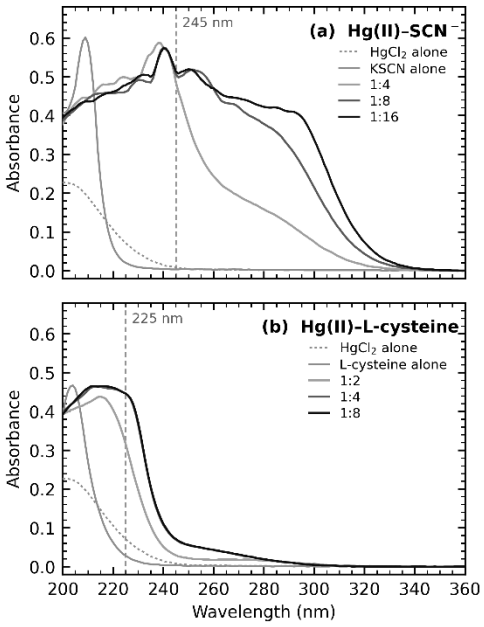

Figure 1. Ultraviolet absorption spectra of (a) the Hg(II)-thiocyanate and (b) the Hg(II)–L-cysteine.

Each spectrum includes $HgCl_2$ alone, free ligand alone, and Hg–ligand mixtures at the investigated ligand-to-metal ratios. The

grey and black spectra represent the individual components and mercury–ligand mixtures, respectively, while dashed lines indicate the selected detection wavelengths. The Hg(II) concentration was fixed at 0.10 mM, with ligand concentrations adjusted according to the examined molar ratios.

Spectra were recorded for each free ligand, for $HgCl_2$ alone and for the mercury–ligand mixtures (Figure 1), all within the linear response range of the instrument. $HgCl_2$ alone shows a monotonic decay with no absorption band, reaching baseline by 243 nm. Free thiocyanate absorbs below 230 nm with a maximum at 209 nm and returns to baseline by 229 nm; free L-cysteine decays to baseline by 231 nm. At 245 nm, $HgCl_2$ contributes 0.008 and thiocyanate 0.003 absorbance units against 0.513 for the 1:16 Baseline by 229 nm; free L-cysteine decays to baseline by 231 nm. At 245 nm, $HgCl_2$ contributes 0.008 and thiocyanate 0.003 absorbance units against 0.513 for the 1:16 mixture, so the measured absorbance arises almost entirely from complexation. The mixtures absorb where neither free component does, extending to 323, 333 and 337 nm at the 1:4, 1:8 and 1:16 ratios, consistent with an LMCT transition arising from sulfur coordination. Absorbance at 245 nm rises from 0.485 at 1:4 to 0.506 at 1:8 and 0.513 at 1:16, approaching a plateau. For the Hg–L-cysteine system, free L-cysteine contributes 0.028 and $HgCl_2$ 0.071 absorbance units at 225 nm against 0.446 for the 1:8 mixture.

The absorption maximum of the Hg–SCN system lies at 240 nm. Detection was performed at 245 nm, where the free components contribute less to the measured absorbance and, given the fixed 8 nm spectral bandwidth of the detector, the band-averaged response is approximately 97% of that at 240 nm. For the Hg–L-cysteine system the maximum lies at 212-215 nm within a broad band, and detection at 225 nm captures 96% of the maximum.

2. Optimization of the Ligand-to-Mercury Molar Ratio

The molar ratio of Hg(II) to ligand governs both the species distribution in solution and the resulting chromatographic behaviour. Both ligand systems were examined under the optimisation conditions described in Methods Section 2, with detection at 245 nm for Hg-SCN and 225 nm for Hg–L-cysteine. Selectivity evaluation was initially performed using blank injections and individual reagent solutions under the calibration conditions. Mobile-phase and injection-solvent blanks produced single peaks before the void volume at 2.40 and 2.30 min, respectively. $HgCl_2$ at 1.00 mM without ligand produced a single peak at 2.475 min and showed no response at the analyte retention time. KSCN at 16 mM without mercury produced peaks at 2.583, 3.017, 4.533, and 5.300 min, whereas TBAH at 8 mM without mercury or ligand produced peaks at 1.633, 1.733, 2.033, and 2.450 min, together with a peak at 3.842 min that coincided with the analyte response. Comparison with the calibration chromatograms indicated that several recurring peaks originated from reagent, ligand, or solvent contributions. The peak

near 4.5 min increased with mercury concentration and was considered ligand-derived; its correlation with mercury was attributed to the fixed ligand-to-mercury ratio applied during calibration.

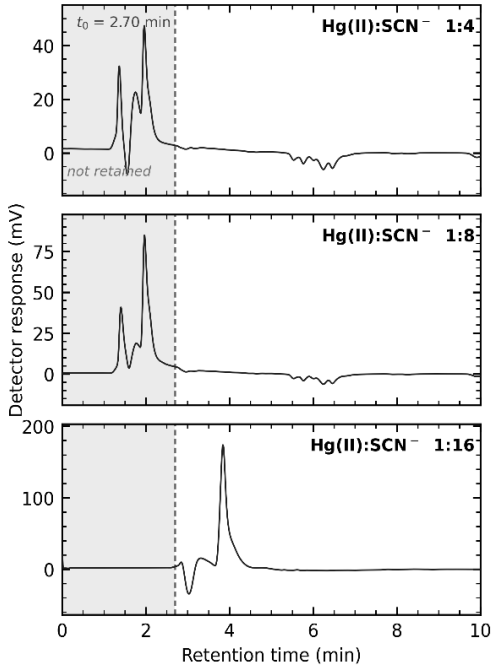

Figure 2. Chromatograms of Hg(II)-thiocyanate at (a) 1:4, (b) 1:8 and (c) 1:16.

The effect of Hg(II):SCN^- molar ratio was evaluated at 1:4, 1:8, and 1:16, as presented in Figure 2. These measurements were obtained in two analytical sessions and were not designed as a matched series; therefore, peak areas were interpreted qualitatively. At 1:4 and 1:8 ratios, the dominant peaks appeared at 1.959 and 1.965 min, respectively. Both peaks eluted before the measured void time of 2.70 min, corresponding to an apparent retention factor of −0.27, indicating that the detected species were not retained by the stationary phase under these conditions. The peak areas remained almost unchanged when the thiocyanate concentration was increased from the 1:4 to 1:8 ratio (1,130,134 and 1,157,800 area units). In contrast, the 1:16 ratio produced a retained peak at 3.841 min with k′ = +0.42. The retention time and peak area obtained from three injections were 3.832 ± 0.020 min and 4,085,612 ± 144,036 area units, respectively. The higher thiocyanate excess therefore produced the most suitable chromatographic response under the investigated conditions. However, the detected signal was not assigned to a specific mercury thiocyanate species because peak-purity analysis and orthogonal structural confirmation were not available. Consequently, the Hg(II):SCN^- ratio of 1:16 was selected for subsequent analytical procedures.

The Hg(II):L-cysteine system was evaluated at molar ratios of 1:2, 1:4, and 1:8, as shown in Figure 3. All three conditions produced dominant retained peaks at 3.937 min (area 10,620,269), 4.592 min (area 15,160,942), and 4.121 min (area 19,969,785), respectively. The calculated retention factors were +0.46, +0.70, and +0.53 relative to the measured void time. Although each condition produced a single dominant chromatographic peak, this observation does not confirm the formation of a single chemical species because complexes with similar charge and hydrophobicity may coelute under the applied chromatographic conditions. Therefore, the results were interpreted as differences in chromatographic behaviour rather than definitive identification of individual Hg–L-cysteine complexes. These measurements

were obtained from single injections and therefore do not include replicate uncertainty. The 1:4 ratio was selected for further analysis because it provided the highest peak area while maintaining suitable retention behaviour. L-cysteine oxidation to cystine may compete with mercury complexation; therefore, solutions were prepared immediately before analysis without oxygen removal. The absence of a free ligand absorption band in Figure 1 supports the presence of intact thiol groups under the investigated conditions.

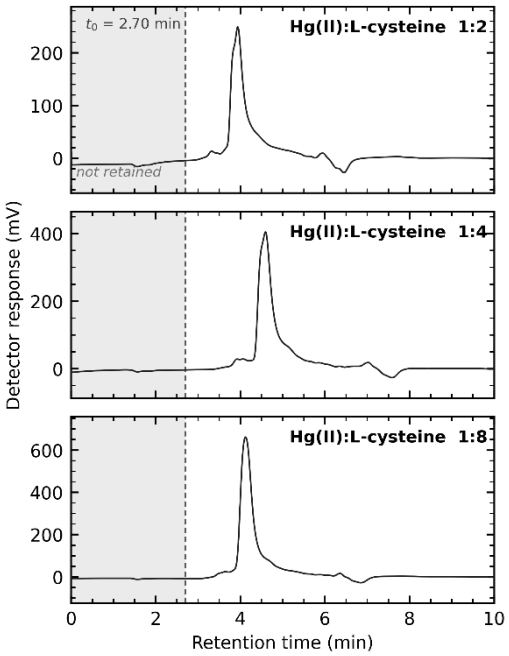

Figure 3. Chromatograms of Hg(II)–L-cysteine at (a) 1:2, (b) 1:4 and (c) 1:8.

3. Comparative Evaluation of Ligands and Selection of Thiocyanate

Under the optimized conditions, the chromatographic behaviour of the Hg-SCN and Hg–L-cysteine systems differed markedly. Thiocyanate was selected for validation. The $[Hg(SCN)_4]^{2-}$ complex is well characterised, with established geometry and spectroscopic properties [22], [33] and its detection wavelength of 245 nm lies clear of the absorption of free ligand, which returns to baseline by 229 nm (Figure 1). Detection at 225 nm for Hg–L-cysteine lies closer to the solvent cut-off and to the ligand's own absorption, and $HgCl_2$ contributes measurably there. Potassium thiocyanate is chemically stable and requires no special handling, whereas L-cysteine is prone to oxidation. The higher peak areas obtained for Hg–L-cysteine are not directly comparable, the two systems being monitored at different wavelengths against different spectral backgrounds. The 1:16 Hg(II): SCN^- ratio with a final TBAH concentration of 8 mM was therefore adopted for all calibration and analytical performance studies.

4. Optimization of TBAH Concentration for Ion-Pair Retention

The anionic mercury-thiocyanate complex is hydrophilic and would be expected to elute near the void volume without an ion-pairing reagent. Tetrabutylammonium is strongly hydrophobic [19] and association between the two yields a less polar species with greater affinity for the C18 phase. Three final TBAH concentrations were examined at the 1:16 ratio (Table 1, Figure 4), with retention factors calculated against the measured void time of 2.70 min. At 0.5 and 2 mM the peak appeared at 1.727 and 1.729 min, before the void time, at apparent retention factors of −0.36; the complex is therefore not retained under these conditions, most probably through exclusion of the doubly charged anion from part of the intraparticle pore volume by electrostatic

repulsion from ionised surface silanols. At 8 mM the peak appeared at 3.842 min with k′ = +0.42 and is genuinely retained. The transition is abrupt rather than gradual, retention changing character between 2 and 8 mM rather than increasing progressively. As a working model, this is interpreted as tetrabutylammonium adsorption reaching a coverage sufficient to reverse the effective surface charge, converting a surface that repels the anionic complex into one that retains it electrostatically, consistent with the dynamic ion-exchange description of ion-pair retention [34]. The dominant mechanism depends on analyte, stationary phase, organic fraction, pH, , and reagent concentration [20] and only three widely spaced concentrations were examined, which cannot establish a threshold position or a retention model. Peak area increases approximately threefold across the series at nominally constant mercury concentration; such a change cannot be attributed to retention alone, and the areas in Table 1 are reported as comparative rather than quantitative.

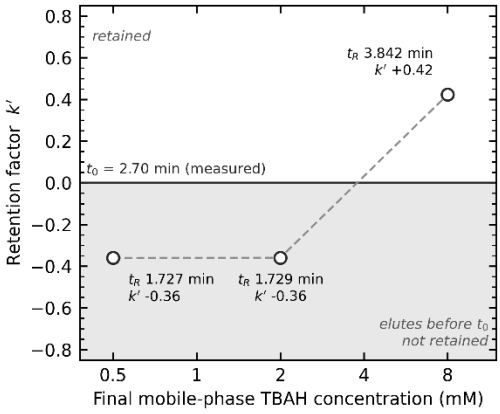

Figure 4. Retention factor of the Hg-SCN peak against final mobile-phase TBAH concentration.

Table 1. Effect of final mobile-phase TBAH concentration on the retention and peak area of the Hg-SCN complex at the 1:16 molar ratio

Aqueous TBAH (10% v/v)	Final TBAH	tR (min)	k′	Peak area
0.005 M	0.5 mM	1.727	−0.36	1,330,176
0.02 M	2 mM	1.728	−0.36	1,896,471
0.08 M	8 mM	3.842	+0.42	3,931,696

Note: TBAH = tetrabutylammonium hydroxide; tR = retention time; k′ = retention factor.

5. Calibration and Analytical Performance

Calibration was performed at seven Hg(II) concentrations within the validated range, with each level independently prepared in triplicate (n = 21) and analyzed using a 10 µL injection under the 7-min chromatographic program. Ordinary least-squares regression yielded the equation Area = (3.168 ± 0.128) × 10^6C + (9.23 ± 3.82) × 10^4, where C represents Hg(II) concentration in mM and the uncertainties represent 95% confidence intervals. The calibration curve exhibited a coefficient of determination (R^2) of 0.9929 and a residual standard deviation of 3.44 × 10^4 area units, as shown in Figure 5. The residuals were normally distributed (Shapiro–Wilk, p = 0.62) and homoscedastic (Levene, p = 0.60), supporting the use of ordinary rather than weighted least-squares regression.

Despite the overall linear response, the lack-of-fit test was significant, F(5,14) = 10.17, p = 0.0003. Addition of a quadratic

term did not significantly improve the regression (p = 0.87), indicating that the observed deviation was not attributable to curvature. Instead, the mean responses at individual calibration levels deviated from the fitted line to a greater extent than predicted by within-level variation, corresponding to differences of up to 5.5% between nominal and delivered concentrations. Because all calibration solutions were derived from a single gravimetrically prepared stock solution, any systematic error associated with preparation of that stock could not be detected from the replicate measurements. Nevertheless, the calibration established the linear response and analytical slope within the investigated concentration range. The back-calculated concentrations ranged from 94.5% to 103.5% of the nominal values, with no individual determination falling outside ±15%.

The regression intercept differed significantly from zero (p = 0.0001) and corresponded to approximately 23% of the signal observed at the lowest calibration level. Regression based on peak height produced a comparable concentration offset, differing by less than 3%, while the area-to-height ratio remained constant across the calibration levels. These observations suggest that the offset behaved as a co-eluting component of relatively constant magnitude rather than as a baseline artefact. This interpretation is consistent with the reagent-derived peak observed at approximately 3.842 min in the blank injections.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the relationships LOD = 3σ/S and LOQ = 10σ/S, giving values of 0.033 mM (6.5 mg/L) and 0.109 mM (21.8 mg/L), respectively. The calculated LOQ was below the lowest calibration level of 0.125 mM. Therefore, the LOD and LOQ should be regarded as calculated estimates rather than experimentally verified limits. The validated working range of the method was consequently established as 0.125–0.500 mM Hg(II), corresponding to 25.1–100.3 mg/L.

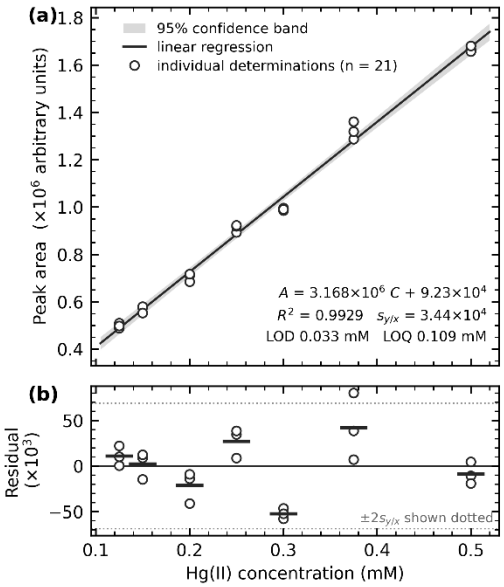

Figure 5. Calibration over 0.125-0.500 mM Hg(II). (a) Peak area against concentration for seven levels prepared independently in triplicate (n = 21), with the regression line and its 95% confidence band. (b) Residuals; horizontal dashes mark level means, dotted lines ±2s(y/x).

Precision was assessed from the calibration sequence acquired within a single day following one column equilibration (Table 2). Retention-time repeatability was 0.80%

RSD, while the pooled peak-area repeatability across the 21 injections was 1.96% RSD. Because each independently prepared solution was injected only once, these values incorporate both preparation and injection variability and therefore represent repeatability rather than intermediate precision. All 21 solutions were prepared before the analytical sequence and injected in three passes over approximately four hours. Relative to the first pass, the mean normalized responses were 1.0025 and 1.0022 for the second and third passes, respectively, neither of which differed significantly from unity. This finding indicates that the mercury–thiocyanate complexes remained stable over the working period. In addition, injection of the mobile phase immediately after the highest calibration standard produced no detectable peak at the analyte retention time, indicating no observable carryover under the tested conditions.

Table 2. Precision of the optimised Hg–SCN method

Hg(II) (mM)	Hg(II) (mg/L)	Mean tR (min)	Peak area RSD (%)
0.125	25.1	3.89	2.18
0.150	30.1	3.89	2.60
0.200	40.1	3.88	2.47
0.250	50.1	3.87	1.79
0.300	60.2	3.87	0.57
0.375	75.2	3.85	2.78
0.500	100.3	3.83	0.72
All levels	—	**3.873 (0.80% RSD)**	**1.96**

Note: tR = retention time; RSD = relative standard deviation

Table 3. Spike recovery of Hg(II) from reagent water at three levels, three replicates per level.

Spike level (mM)	Replicate recoveries (%)	Mean (%)	RSD (%)	95% CI (%)
0.500	95.1, 92.9, 98.7	95.6	3.0	88.3–102.8
0.250	86.0, 101.6, 96.1	94.6	8.4	74.9–114.2
0.125	82.9, 90.8, 99.7	91.2	9.2	70.3–112.0
Overall (n = 9)	—	**93.8**	**6.7**	**88.9–98.6**

Note: RSD = relative standard deviation; CI = confidence interval

Accuracy was evaluated through spike-recovery experiments in reagent water at three Hg(II) concentrations, each analyzed in triplicate (Table 3). Mean recoveries were 95.6%, 94.6%, and 91.2% at 0.500, 0.250, and 0.125 mM, respectively, with an overall mean recovery of 93.8%. However, because only three replicates were analyzed at each concentration, the corresponding 95% confidence intervals were relatively wide. At the two lower concentration levels, the lower limits of the confidence intervals fell below the 80% acceptance criterion. Thus, although the mean recovery met the acceptance criterion

at all tested levels, the available data were insufficient to demonstrate with 95% confidence that the true recovery remained within the specified range at the two lower levels. Furthermore, recovery was evaluated only in reagent water rather than in environmental or industrial samples; therefore, potential matrix effects and accuracy in real sample matrices remain unassessed.

The validated concentration range of 25.1–100.3 mg/L Hg(II) is approximately four orders of magnitude higher than drinking-water guideline concentrations. Accordingly, the method is not suitable for direct environmental surveillance or trace-level mercury determination. Its more appropriate application is the analysis of concentrated process and waste streams containing mercury at tens to hundreds of milligrams per litre, such as amalgamation effluents from artisanal gold processing, spent chlor-alkali brines, and leachates from mercury-containing wastes, including lamp and battery recycling residues. Samples with Hg(II) concentrations above the validated range would require volumetric dilution, whereas samples below the lower limit would require a preconcentration step, which was not investigated in the present study.

6. Comparison with Other Methods for Mercury Determination

Table 4 compares the proposed method with established techniques for mercury determination in aqueous matrices. The comparison methods were selected because they are also used for mercury analysis in water, allowing the detection limits to be assessed on a broadly comparable basis. The difference in sensitivity is substantial: the present method has an LOD of 6.5 mg/L, which is several orders of magnitude higher than those achieved by atomic spectrometric techniques. Therefore, the method should not be regarded as an alternative to these techniques for trace-level mercury determination. The comparison with reversed-phase HPLC-UV is more relevant from an instrumental perspective. Solid-phase preconcentration followed by dithizone derivatisation has been reported to achieve nanogram-level detection limits and to resolve different mercury species using the same general class of chromatographic instrumentation [11]. The improved sensitivity of that approach is primarily associated with the preconcentration step and the use of a strongly absorbing chromophore, neither of which was incorporated into the present method.

The method developed in this study provides a simpler alternative for inorganic Hg(II) determination at milligram-per-litre concentrations using conventional isocratic HPLC-UV instrumentation without dedicated mercury-specific equipment or additional solid-phase extraction procedures. This simplified configuration reduces instrument requirements and sample preparation complexity, although with a corresponding decrease in analytical sensitivity. Therefore, the method is more suitable for laboratories equipped with routine liquid chromatography systems but without access to cold-vapor atomic spectrometric instrumentation. The proposed approach is particularly applicable

for concentrated samples, such as process solutions or waste streams, where mercury concentrations are substantially higher than environmental trace levels.

Table 4. Comparison of the present method with established techniques for mercury determination in aqueous matrices

Method	Matrix	Sample preparation	LOD	Recovery	Instrumentation
This work	Reagent water	Complexation, 15 min	6.5 mg/L	93.8%	Conventional HPLC-UV
RP-HPLC-UV with SPE [11]	Aqueous	SPE, dithizone derivatisation	0.14 ng	—	Conventional HPLC-UV
FI-CV-AAS [35]	Wastewater	On-line reduction	20 µg/L	—	Dedicated
CV-AFS [36]	Water	Oxidation, purge and trap	1.8 ng/L	77–123%	Dedicated
CV-ICP-MS [37]	Natural water	Reduction	0.7 ng/L	CRM-verified	Dedicated

Note: LOD = limit of detection; SPE = solid-phase extraction; CV = cold vapor; AAS = atomic absorption spectrometry; AFS = atomic fluorescence spectrometry; ICP-MS = inductively coupled plasma mass spectrometry; CRM = certified reference material.

CONCLUSION

An ion-pair reversed-phase HPLC-UV method was developed for the determination of inorganic mercury(II) via sulfur-donor ligand complexation. Complexation with thiocyanate and ion pairing with tetrabutylammonium allow inorganic mercury(II) to be determined on a conventional C18 column with ultraviolet detection. The retention behaviour observed here is the principal chemical finding: measured against an experimentally determined void time, the mercury-thiocyanate complex is excluded from the stationary phase at low reagent concentration and retained at 8 mM, a change of regime rather than a gradual increase, consistent with a dynamic ion-exchange contribution to retention.

The method quantifies mercury between 25.1 and 100.3 mg/L with a detection limit of 6.5 mg/L, using HPLC-UV and requiring neither dedicated mercury instrumentation nor a preconcentration step. Its value therefore lies in accessibility rather than sensitivity, and its natural application is to concentrated process and waste streams. Several weaknesses limit wider use: recovery was established only in reagent water and not against an independent method; no interference study was performed, and peak identity was not confirmed by orthogonal detection. Future work should address matrix validation, a denser series of reagent concentrations to locate the retention threshold, and orthogonal confirmation of peak identity; extension to mercury speciation would also require evidence of species preservation and resolution of at least one organomercury species.

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