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Research Article

Extraction of Chromium(III) from Urine and Water Samples Using Divalent Beta-diketonates Ligands and Determination with Reversed-Phase HPLC

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Abstract

Chromium is an element of interest due to its physiological properties in different oxidation states. Chromium (III) is believed to be beneficial in curbing effects of sugar diabetes in the body, and at the same time, chromium (VI) is carcinogenic. A lot of research has been conducted in an effort to conduct speciation to identify the Cr oxidation state available in a sample. Several methods have been used but with challenges. In this study, acetylacetonates and its derivatives was used to extract Cr(III) in urine and fresh water samples. It was observed that smaller ligands of acetylacetonate metal chelates gave high linear range, which decreased with increase in the size of the ligand. $Cr(AA)_3$, $Cr(DPM)_3$ and $Cr(DBM)_3$ gave linear range of 1 - 10000gn/ml, 1 - 1000 ng/mL and 1000 ng/mL respectively. The highest detection limit was observed when determining $Cr(DBM)_3$, which had benzene rings (0.84 ng/mL) as compared to the smaller ligand metal complex $Cr(AA)_3$ with 1.20 ng/mL. The use of ligand with benzene ring (DBM) further allowed for resolution of other metal complexes during the extraction of Cr(III), which avoided challenges of interferences. The method developed was applied successfully in real urine and fresh water samples using DBM as a chelating agent and obtained Cr(III) in the ranges 0.86 - 7.18 ng/mL and 5.94 - 8.99 ng/mL respectively. The percentage recovery was in the range 96 - 99% for fresh water and 99.8 - 105% for urine samples.

Keywords: Metal Chelates; Acetylacetonate;Dibenzoylmethane; Dipivaloylmethane; Chromium(III); High Performance Liquid Chromatography

Abbreviations

AA: Acetylacetonate; DBM:Dibenzoylmethane; DPM: Dipivaloylmethane

Introduction

The dependence of toxicity of elements on their oxidation state has prompted the development of analytical methods that could be used to obtain information about such speciation of many elements. Chromium is an element of interest in that it could be beneficial to humans or toxic depending on its oxidation state. The presence of Cr(III) in biological systems is believedto improve the responsiveness of insulin in controlling the blood sugar level and other metabolism reactions, such as the breakdown of fat, protein and carbohydrates [1-3]. As a result, many people take Cr(III) supplements as a way of controlling blood sugar level in diabetes type II patients or increasing the rate of fat metabolism in order to fight obesity [3-6]. The excretion of chromium from the body is primarily through urine [7]. Therefore, considerable research for determination of chromium is performed mostly upon urine with hopes of connecting certain diseases with lack of chromium in the body, correlated in turn to the Cr(III) ions found in urine. Water samples are also analyzed to determine environmental chromium to measure the extent of pollution.

In recent years, several methods have been developed to allow determination of chromium in its different oxidation states.

Selective extraction of chromium in a particular oxidation state (usually Cr(III)) with subsequent determination using element specific detection (atomic absorption spectroscopy, inductively coupled plasma mass spectrometry (ICPMS), inductively coupled atomic plasma emission spectrometry (ICPAES)) and chromatography is on the rise [8-12]. The use of a chelating agent is usually manipulated to involve preconcentration of the analyte prior to the determination of the chelated element, which is beneficial since in most cases as both Cr(III) and chromium(IV) are found in low concentrations in urine and non-polluted samples [8-10,13]. Hyphenation of liquid chromatography with specific element detection such as ICPMS, ICPAES and electrothermal atomic absorption spectrometry (ETAAS) has been possible but with problems of interferences [14]In ICPMS, the challenge is to correct for interferences for the most sensitive isotope $^{52}Cr^{+}$, due to ionization of argon, carbon (from liquid chromatography mobile phases) and chlorides [15]. Several methods have been developed to avoid these interferences. Charged and neutral resins have been used successfully to selectively adsorb and preconcentrate Cr(III) with subsequent determination by atomic absorption spectrometry thus avoiding the use of ICPMS [8,9,13,16,17]. During this process, chelating agents are used to functionalize the resins to enhance their selectivity and sorption power.Coprecipitation is another technique that has been used successfully in the separation and enrichment of trace heavy metals, including chromium [18]. This technique is believed to reduce the matrix effects found in urine samples from NaCl, KCl, $CaCl_{2,}$ urea and dextrose during the determination of chromium by atomic absorption spectrometry. Solid phase extraction and solid phase microextraction are techniques used in the extraction of chromium followed by atomic absorption spectrometry and gas chromatography [19,20].

In most of the techniques used in the speciation of chromium, chelating agents have been the most used to enhance selectivity and enrichment of Cr in a particular oxidation state. Some of the chelates that have been used in chromatographic analyses include divalent and tetradentate β ketoamines [21], β-diketonates [22], pyridylazo derivatives. Acetylacetone and some of its derivatives have been applied successfully in the chelation of Cr(III) in chromatography. These ligands form coordinatively saturated chelates with Cr(III) ions, enhancing the stability of the metal chelate.However, the work performed has been mainly qualitative. One of the earliest studies using β-diketonates chelates of Cr(III) was that conducted by Moshier., *et al.* with gas chromatography [23]. In their work, they successfully resolved β-diketonate metal chelates of aluminium(III), indium(III), chromium(III), rhodium(III), zirconium(IV) and hafnium(IV).The β-diketonate chelates containing fluorine were found to be suitable for gas chromatography because of their thermal lability [20,23,24]. The ability of fluorinated compounds (e.g. trifluoroacetylacetonates and thenoyltriflouroacetylacetonate metal chelates) to volatilize has since been applied to speciation of chromium using electrothermal absorption spectrometry [25-27]. In such studies, Cr(III) ions are chelated with a fluorinated ligand and then completely volatilized in the graphite furnace prior to atomization of Cr(VI). The difference between the total chromium and Cr(VI) gives the amount of Cr(III) in the sample. Trifluoroacetylacetonates and thenoyltrifluoroacetylacetonate are derivatives of the ligand, acetylacetone.Acetylacetone has been used applied widely in metal chelation followed by both qualitative and quantitative determination [28,29]. It has been used in extraction of chromium in sea water [30], leaves [31] and urine [32] samples using atomic absorption spectrometry, high performance liquid chromatography and gas chromatography techniques.In urine analyses, quantitative determination of chromium was performed by extracting the acetylacetonate chelate by supercritical fluid extraction.

Literature reveals that the determination of chromium chelated with organic ligands using newer chromatographic stationary phases such as the zirconia-based and improved silica columns is limited. The thermal and chemical stability of zirconia-based stationary phases tempts application to the determination of chromium as a chelate. Also evident from a literature survey was the lack of systematic study of acetylacetone and its derivatives to determine their limitations and advantages in the extraction of chromium. Acetylacetone and other β-diketonates are commercially available or relatively easy to prepare and they form very stable chelates with Cr(III) ions, giving possibilities of chromatographic measurements of Cr(III) chelates without degradation of the analytes on the column.

In this study the focus was on the following objectives:

- To compare the limitations and advantages of either silica or zirconia-based columns in the determination of Cr(III) chelated with acetylacetone and other β-diketones using HPLC.
- To analyze artificial urine and deionized water samples spiked with Cr(III) ions to determine the extraction efficiency, detection limits and linear range of chromium as a function of ligands and pH.

- To explore the possibilities of extracting and determining other metal ions such as Al(III), Cu(II) and Ni(II) simultaneously with Cr(III) ions using reversed-phase HPLC.
- To select the ligand that could be used for extraction of Cr(III) in real samples such as urine and water, based on extraction efficiency, linear calibration range and least interferences from other metal ions.

Chromium is an interesting element because of its physiochemical properties and improved and reliable extraction methods for Cr(III) in samples such as urine and water may lead to better understanding of Cr(III) in the human body and also the speciation of chromium in environmental samples.

Materials and Methods

Instrumentation

The chromatographic measurements were performed using model 2695 HPLC instrumentation with a Waters 2487 dual wavelength absorbance detector (Waters, USA). The data was acquired and processed with a Compaq Pentium III computer equipped with millennium 32 software (Waters, USA).Temperature of the columns was regulated using a model 7956 heater chiller, Jones Chromatography, U.K. pH was measured by a Corning 125 pH meter on aqueous samples before mixing with the organic modifier. The pH meter was calibrated with pH 4.0 and 7.0 standard solutions each time a buffer solution was prepared.

Samples were weighed using a Mettler AE 200 balance (USA). A Barnstead NANO pure II system was employed to prepare deionized ultra-pure water, which was used in all aqueous and mobile phase solutions.

Columns used were SymmetryShieldä C18 (150 x 3.9), J`sphere ODS H-80, C18 (150 x 4.6) both from Waters Inc., USA and DiamondBond C18 (150 x 4.6), ZirChrom-PS (150 x 4.6), manufactured by ZirChrom Separations, USA. A frit (0.5 µm stainless steel) from UpChurch Scientific and C-18 water reversed-phase sentry guard columns were connected between the injection port and the column to protect the column from damage by materials in the sample. The column temperature was set at 30°C and 60°C for silica-based and zirconia-based columns respectively unless stated in the discussion.

Chemicals

HPLC grade acetonitrile and chloroform were used as solvent or organic modifier (Fisher Scientific, USA). Monobasic potassium phosphate and phosphoric acid (HPLC grade), potassium hydroxide certified ACS were used to control pH (Fisher Scientific, USA). Artificial urine was prepared with sodium chloride and potassium chloride from EM Science, USA, calcium sulfate (W.A. Hammond Drierite Company, USA), magnesium sulfate heptahydrate, sodium sulfate, sodium dihydrogen phosphate and urea all from Fisher Scientific, USA at pH 2.15, 7.20, 12.15 following the procedure outlined by Gammelgaard and Jons[33]. Real urine samples were collected from volunteers and water samples from local lakes and ponds were analyzed within 24 hours.Chromium(III) nitrate (Fisher Scientific, USA) was used in preparing required solutions of Cr(III) ions for spiking of samples.

The ligands used in the extraction of Cr(III) from water and urine samples were acetylacetone (2,4-pentanedione), dibenzoylmethane (1,3-diphenyl-1,3-propanedione) (Eastman Organic Chemicals, USA) and dipivaloylmethane (2,2,6,6-tetramethyl-3,5-heptanedione) from Columbia Organic Chemicals, USA. The structures for these ligands are shown below in figure 1.

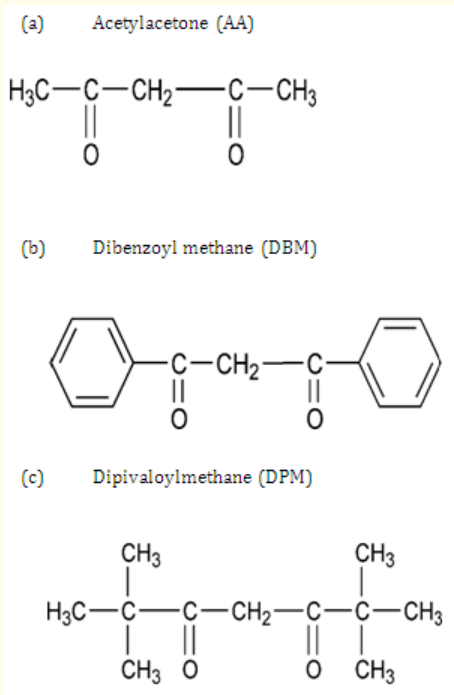

Figure 1: Showing the structures of (a) acetylacetone and its derivatives (b) dibenzoyl methane, and (c) dipivaloylmethane ligands used in the extraction of Cr(III) in both urine and water samples.

The respective metal chelate standards for these ligands were chromium(III) acetylacetonate ($Cr(AA)_3$), aluminum(III) acetylacetonate ($Al(AA)_3$, nickel(II) acetylacetonate ($Ni(AA)_2$), chromium(III) dipivaloylmethanate ($Cr(DPM)_3$), copper(II) dipivaloylmethanate ($Cu(DPM)_2$), nickel(II) dipivaloylmethanate ($Ni(DPM)_2$), chromium(III) dibenzoylmethanate ($Cr(DBM)_3$), aluminum(III) dibenzoylmethanate ($Al(DBM)_3$) and nickel(II) dibenzoylmethanate ($Ni(DBM)_2$) prepared earlier.

Results and Discussion

An ultraviolet-visible spectrum was obtained for 1000 ng/mL solutions of each of the metal chelates and substantial absorbances were observed at 275 nm and 340 nm for most of the chelates. These two wavelengths were both used in this study taking advantage of the capabilities of the Waters 2487 dual wavelength absorbance detector to monitor two wavelengths simultaneously. The sensitivities and effects of interferences from other species in the samples were compared for these two wavelengths as a function of ligands in the extraction of Cr(III) in our samples. The deionized water and artificial urine samples were each prepared at pH 2.15, 7.20 and 12.15 and then each sample was spiked with 10 ng/mL Cr(III) ions, to determine effect of Cr(III) extraction with respective ligands low, mid and high pH. A visible chromatographic peak was observed with 5 ng/mL concentration of all the metal chelates studied. However, 10 ng/mL Cr(III) ions spiked for recoveries would give a peak that had a better signal to peak ratio. Therefore, spiking samples with 10 ng/mL of Cr(III) ions was considered to be a compromise between a detectable concentration of chromium and a more realistic representation of the low concentrations of Cr(III) usually expected in real samples that are not polluted.

Extraction of Cr(III) from spiked deionized water and artificial urine samples using acetylacetone.

Chromatographic optimization to resolve the metal acetylacetonates chelates

J`sphere ODS H-80 and DiamondBond C18 were selected for this study based on results obtained from an earlier study [34]. When characterizing these columns, it was observed that J`sphere ODS H-80 displayed the best chromatographic properties compared to other silica-based columns. DiamondBond C18 on the other hand, had chromatographic properties very similar to those of silica-based columns and produced better chromatograms than the other zirconia-based columns.In general, there were no significant differences in the chromatograms between the two columns. The possibility of separating $Cr(AA)_3$, $Ni(AA)_2$ and $Al(AA)_3$ simultaneously was explored. The metal chelates, $Ni(AA)_2$ and $Al(AA)_3$ could not be separated from each other, even though there were both resolved from $Cr(AA)_3$. In addition, the nickel chelate had a substantial tailing peak. Different mobile phase ratios, temperatures, buffers and different organic modifiers did not resolve the Ni(II) and Al(III) metal chelates or improve the tailing of the nickel chelate. It was also observed that $Ni(AA)_2$ was decomposing on both the columns and that worsened with increase in pH.

The decomposition of $Ni(AA)_2$ has also been reported by Carr.,*et al.*[35] when studying chromatography of several beta-diketonates. This behavior is believed to be caused by the kinetic lability of $Ni(AA)_2$, leading to the decomposition and dissociation of this chelate on the chromatographic column [36]. In another study, Tollinche and Risby, could not resolve $Al(AA)_3$, $Cr(AA)_3$ and other beta-diketone metal chelates using reversed-phase HPLC, but could only separate these metal chelates with normal phase HPLC [37]. As a result the best chromatograms for both the columns were obtained with a mobile phase of 50% acetonitrile and 50% deionized water in the isocratic mode. However, the DiamondBond C18 produced slightly tailing peaks and relatively less sensitivity than the J`sphere ODS H-80.

Linear calibration range of $Cr(AA)_3$ standards and limit of detection

A linear calibration was obtained in the range 1-10^4 ng/mL, with R^2 values of 0.9998 and 0.9997 for J`sphere ODS H-80 and DiamondBond C18, respectively. The calibration curve passed through the origin which was an indication that the analyte was not lost during the chromatography. Different segments of the above range (1-100, 100-1000, 1000-10^4 ng/mL) were graphed to compare their slopes in order to ascertain the linearity obtained from 1-10^4 ng/mL). The calibration curve for the 1-10^4 ng/mL range obtained using the J`sphere ODS H-80 is shown below (Figure 2).

The slopes for the different segments (1-100, 100-1000, 1000-10^4 ng/mL) were not significantly different from each other for the above columns at 95% confidence limit.The R-squared values of the respective segments were all above 0.998. Based on these results it was concluded that the sensitivity for the different segments

was the same, and the linear range of 1-10^4 ng/mL was confirmed. Linear ranges of two orders of magnitude and 13-60,000 ng/mL have been reported for $Cr(AA)_3$ in earlier studies [32,36].

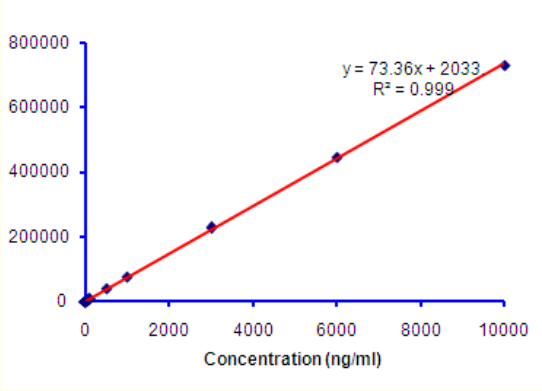

Figure 2: Calibration curve showing the linear calibration range of $Cr(AA)_3$ obtained on J`sphere ODS H-80, (50:50 v:v) acetonitrile:water mobile phase at 340 nm detector wavelength.

The limit of detection for $Cr(AA)_3$ was calculated as 3 times the standard deviation of the blank, divided by the slope of the calibration curve. The standard deviation of the blank was calculated from 10 replicates. The limit of detection obtained for $Cr(AA)_3$ was 1.20 ng/mL and 3.75 ng/mL for J`sphere ODS H-80 and DiamondBond C18 respectively. The limit of detection obtained using J`sphere ODS H-80 was better than that reported in literature (20 ng/mL) [32].

Recovery of Cr(III) ions from artificial urine and deionized water samples using acetylacetone as chelating ligand

Chromium(III) (10 ng/mL) was spiked in urine and deionized water samples at pH 2.15, 7.20 and 12.15. The highest recoveries obtained for both the deionized water and urine samples were 96 ± 2%and 106 ± 3% at pH 2.15 for J`sphere ODS H-80, respectively and 102 ±3% and 106 ± 3% for the DiamondBond C18, respectively. The recovery of the analytes worsened as pH was increased and minimal recovery of Cr(III) at pH 12.15 from both the deionized water and urine samples was obtained. The chromatography on the DiamondBond C18 and J`sphere ODS H-80 produced a baseline separation between the ligand and the analyte peak of $Cr(AA)_3$, therefore the excess concentration from the ligand added during the extraction process did not interfere with the $Cr(AA)_3$(Figures3a and 3b).

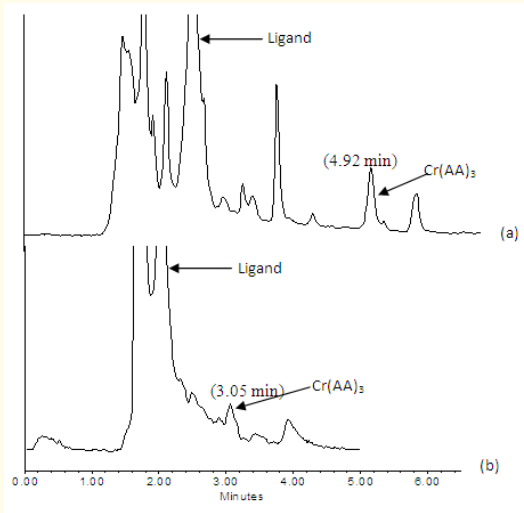

Figure 3a: Chromatogram of $Cr(AA)_3$ in deionized water spiked with 10 ng/mL Cr(III) ions using (50:50 v:v) acetonitrile: water mobile phase and 340 nm detection on (a). J`sphere ODS H-8 and (b) DiamondBond C18.

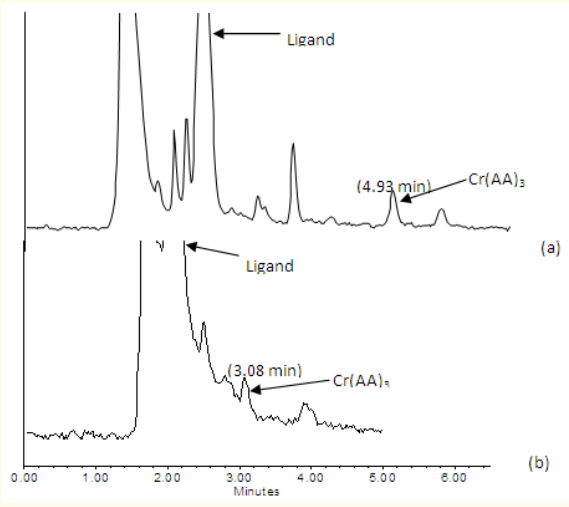

Figure 3b: Chromatogram of $Cr(AA)_3$ in artificial urine spiked with 10 ng/mL Cr(III) ions using (50:50 v:v) acetonitrile: water mobile phase and detected at 340 nm on (a). J`sphere ODS H-8 and DiamondBond C18.

For the two wavelengths (275nm and 340 nm), it was observed that their sensitivities were not significantly different from each other, at 95% confidence limit. However, the wavelength, 340 nm, which produced chromatograms with relatively fewer interfering peaks from the sample matrices would be preferred over 275 nm.

Extraction of Cr(III) ions with dipivaloylmethane from spiked deionized water and artificial urine samples

Dipivaloylmethane is a liquid at room temperature.In general, it seems to have had limited application to the extraction of metals for speciation analyses. Literature survey revealed that this ligand has been used successfully in the extraction of palladium and rhodium using supercritical fluid extraction with subsequent determination by HPLC [38]. The chromium chelate of this ligand, $Cr(DPM)_3$, was expected to be more hydrophobic than the $Cr(AA)_3$ because of the presence of two tertiary methyl groups, which could possibly cause problems of strong retention with the conventional silica columns. Samples were prepared as described for acetylacetone, by spiking Cr(III) ions into both the deionized water and urine samples at different pH. The miscibility of dipivaloylmethane with the aqueous samples was aided by warming the mixture for a few minutes. The effect of heating the mixture for different times (30 and 60 minutes) was explored to determine possible improvements in extraction efficiencies. Both 275 nm and 340 nm detection wavelengths were also used for this ligand.

Chromatographic optimization to resolve the metal dipivaloylmethane chelates

$Cr(DPM)_3$ was not eluted with 50% acetonitrile and 50% water as the mobile phase from J`sphere ODS H-80, as was with the case with $Cr(AA)_3$. An irreproducible peak of $Cr(DPM)_3$was obtained after increasing the concentration of acetonitrile in the mobile phase to 95% and 5% deionized water. The strong retention of $Cr(DPM)_3$ on the J`sphere ODS H-80 was expected since this column has very high hydrophobicity when compared to other silica-based columns [34]. Another column; SymmetryShieldä C18, which is slightly less hydrophobic than J`sphere ODS H-80 was evaluated with hopes that the retention of $Cr(DPM)_3$ would be reduced and also that there could be reduction in the content of the organic modifier in the mobile phase to produce more reproducible results. No improvement was observed however, as the SymmetryShieldä C18 also produced irreproducible chromatographic analyte peaks. Phosphate buffer at different pH or change in mobile phase and temperature did not improve reproducibility of the analyte peak for the silica-based columns. With buffer with high content of the organic modifier in the mobile phase, precipitation of organic modifier and the buffer was observed causing the HPLC system to lose pressure because of the clogging of the check valves.

Since no satisfactory results were observed with silica-based columns (due to hydrophobicity of $Cr(DPM)_3$) it was very unlikely that improvements could be achieved by using the DiamondBond, C18 column because of its similarities to the silica- based columns [34], especially the SymmetryShieldä C18 and J`sphere ODS H-80). Therefore, another zirconia-based column bonded with polystyrene, ZirChrom-PS, which is less hydrophobic than the DiamondBond C18 and silica-based columns [39] was evaluated with hopes that less organic solvent could be used with subsequent improvement in the reproducibility of the results. For this column, a reproducible peak of $Cr(DPM)_3$ was observed at about 2.7 minutes after optimizing the chromatographic conditions to a mobile phase of 60% acetonitrile and 40% water (without a buffer) at flow rate 1ml/min and column temperature of 60°C. A literature search revealed that polystyrene-divinylbenzene resins have been packed on a column and chelated with quinolin-8-ol for selective extraction and determination of Cr(III) ions from sea water [17], which confirms that a polystyrene substrate could be used to adsorb a chromium chelate and eluted as necessary.

A higher sensitivity was observed at wavelength 340 nm than at 275 nm. A quantifiable peak was observed at 5 ng/mL concentration of the analyte, and a very small peak was also found for 1 ng/mL of $Cr(DPM)_3$.Baseline resolution was observed between the ligand dipivaloylmethane and the chromium(III)dipivaloylmethanate chelate.

Resolution of $Cr(DPM)_3$ from other metal DPM chelates (Cu and Ni)

Some research has indicated that the separation or chromatography of copper(II) and nickel(II) chelates of acetylacetone is very problematic [35,36]. Therefore, it was of interest to investigate the possibilities of chromatographing and perhaps separating these metals by chelating them with a bulkier and more hydrophobic derivative of acetylacetone; dipivaloylmethane and establishing if Cu(II) and Ni(II) could be determined simultaneously with Cr(III) through the formation of their respective metal chelates. The optimized experimental conditions used for $Cr(DPM)_3$ were also applied to the separation of the Cu(II) and Ni(II) dipivaloylmethanate.At detector wavelength 340 nm, it was observed that only the $Cr(DPM)_3$ was observed,

which could be beneficial for selective determination of Cr(III) in a sample containing Ni(II) and Cu(II) by reducing interferences, which might be caused by these metals.At wavelength 275 nm, all the three metal chelates were detected. However, there was no resolution between Ni(II) and Cu(II) dipivaloylmethanates under the above-optimized conditions, although the Cr(III) chelate was fully resolved from the other two metal chelates and the ligand.

Several efforts were made to resolve the Cu and Ni (II) dipivaloylmethane chelates through changes of the mobile phase organic content, but without any success. Different types of buffers at varying pH were also evaluated with hopes of manipulating the surface charge of the ZirChrom-PS for a possible multipoint interaction of the column substrate and the analyte as for a typical zirconia-based column [40]. It was hoped that a multipoint interaction site could affect separation of the analytes. Two different buffers were used at low pH, 2.15; phosphate buffer and trifluoroacetic acid. At this pH, the zirconia-substrate was expected to be relatively more positively charged for the trifluoroacetic acid. However, for the phosphate buffer, both negatively sites (from phosphate ion) and positively charged sites were expected to be present on the zirconia substrate, to interact with any dipole moments that may exist on the Ni(II) and Cu(II) dipivaloylmethanates [40,41]. At this pH, resolution was obtained between the three metal dipivaloylmethane chelates and it was not significantly different as a function of buffer type (Figure 4).The optimized mobile phase was (50:50 v:v) acetonitrile:water. However, the Cu(II) chelate co-eluted with the ligand peak and $Ni(DPM)_2$ very close to the system peaks.

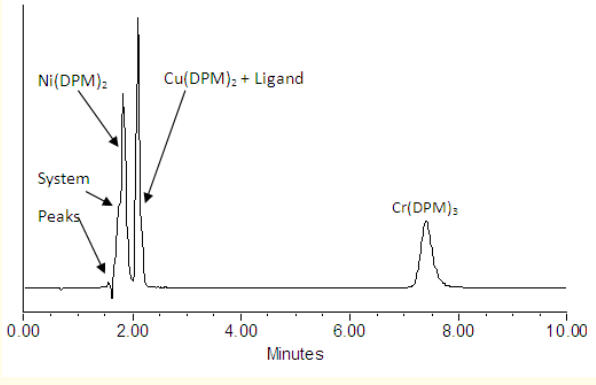

Figure 4: Separation of 1000 ng/mL Ni(II), Cu(II) and Cr(III) dipivaloyl chelates with (50:50 v:v) acetonitrile: phosphate buffer (pH 2.15), at detection 275 nm on ZirChrom-PS.

The power of the positive charge on the stationary phase of the ZirChrom-PS was reduced by increasing the pH to 4.75 using acetic acid buffer [41], but no improvement was observed in the resolution of the Cu(II) and Ni(II) dipivaloylmethanates. When the pH was increased to 12.15, it was observed that the $Ni(DPM)_2$ was decomposing and was almost unobserved at pH 12.15. It was concluded that the best results were those obtained at lower pH when the three metal chelates were resolved but with the Cu(II) and Ni(II) dipivaloylmethanates chelates co-eluting with the ligand and system peak, respectively. This method might be applied to qualitative determination of Cu(II) and Ni(II) dipivaloylmethane chelates, but not for quantitative analysis. However, at wavelength of 340nm, the Cr(III) dipivaloyl chelate can be determined safely without any interferences, since the other peaks were not absorbing, and hence no peak for them.

Linear calibration range of $Cr(DPM)_3$ standards and limit of detection

The $Cr(DPM)_3$ standard solutions with concentrations 1-10^4 ng/mL were chromatographed to determine the linear calibration range for this chelate. The chromatographic conditions were (60:40 v:v) acetonitrile:water as mobile phase at 340 nm detector wavelength. A linear calibration range was found to cover only the range 1-1000 ng/mL, with an R^2 value of 0.9976. The calibration curve passed through the origin (Figure 5). Non-linearity was observed above 1000 ng/mL.Different segments of the calibration range (1-100 ng/mL and 100-1000 ng/mL) were plotted and their slopes compared to determine linearity for the range 1-1000 ng/mL. The slopes of these curves were not significantly different at 95% confidence, indicating the same sensitivity from 1-1000 ng/mL, thereby confirming the working range.

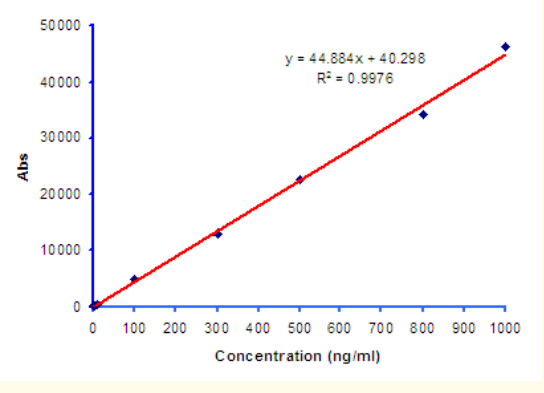

Figure 5: Calibration curve showing the linear calibration range of $Cr(DPM)_3$ obtained on ZirChrom-PS for 60/40 (v/v) acetonitrile/water at 340 nm detection.

The limit of detection for $Cr(DPM)_3$ was found to be 2.26 ng/mL with ZirChrom-PS, which was better than that obtained for $Cr(AA)_3$ using another zirconia-based column, DiamondBond C18 for the Cr(III)acetylacetonates chelates (3.75 ng/mL), but not for the J`sphere ODS H-80, whose limit was 1.20 ng/mL for $Cr(AA)_3$.

Recovery of Cr(III) ions from artificial urine and deionized water samples with dipivaloylmethane as chelating ligand

The deionized water and artificial urine samples were spiked with 10 ng/mL of Cr(III) and samples at different pHs prepared as in the case of $Cr(DPM)_3$The detection wavelength used was 275 nm and with a mobile phase composition of 60/40 (v/v) acetonitrile and water with no buffer. Heat was found to aid the dissolution of the ligand, which could make it more labile and extract the Cr(III) ions. To determine the effect of heat on the extraction of Cr(III) from both the urine and deionized water samples, the respective mixtures of the ligand and the samples were heated at 60°C for 30 and 60 minutes. After the respective times elapsed, the solutions had turned cloudy, and a white precipitate collected at the bottom of the test tubes after cooling to room temperature. The white precipitate was suspected to be the decomposed $Cr(DPM)_3$ and that was confirmed by filtering the precipitate out of the solution, subsequent analyses of the solution showing no chromatographic peak for $Cr(DPM)_3$. To solve this problem, fresh samples were prepared and warmed for few minutes to aid miscibility of the ligand and the deionized water or artificial urine samples. It was essential not to warm the samples for extended periods to avoid formation of the white precipitate. Thus extended heating of the samples with the ligand was not necessary for the extraction of Cr(III) from deionized water and artificial urine samples with dipivaloylmethane.

Baseline resolution from $Cr(DPM)_3$was observed from the ligand and analyte for both the deionized water and artificial urine (Figure 6). The resolution between the ligand and analyte was such that quantitative analysis was feasible even at low concentrations of Cr(III) ions without fear that the ligand peak would superimpose on the analyte peak.

Very low recoveries (which were below 5%) were obtained when Cr(III) extraction was performed at low pH (2.15) for both urine and deionized water samples. There was little difference in the recoveries obtained at mid and high pHs, 7.20 and 12.15. The Cr(III)recovery for the deionized water sample was 95 ± 2% and 108 ± 3% at pH 7.20 and 12.15,respectively. For the artificial urine sample, it was 106 ± 4% and 104 ± 3% at pH 7.20 and 12.15, respectively. It was observed that all the extractions for Cr(III) obtained at pH 12.15 with this ligand were above 100% for both the deionized water and urine samples.v It was also observed that at 95% confidence level, percentage recoveries of Cr(III) from both water and urine samples obtained as dipivaloylmethanate were not significantly different from those obtained for the acetylacetonate. However, dipivaloylmethane works best at pH around 7 and lower pH for acetylacetone.

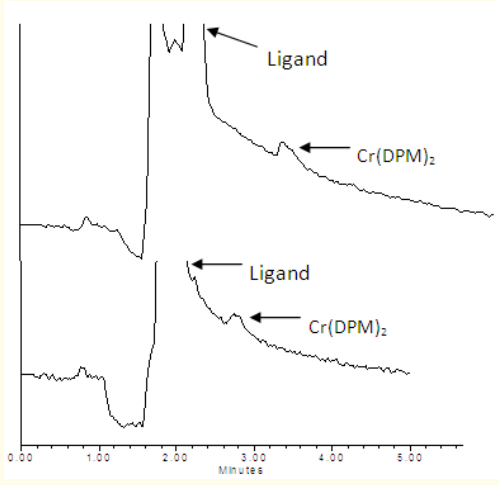

Figure 6: Chromatogram of $Cr(DPM)_3$ in (a) deionized water and (b) artificial urine spiked with 10 ng/mL Cr(III) ions using (60:40 v:v) acetonitrile: water mobile phase and 340 nm detection on ZirChrom-PS.

Extraction of Cr(III) from spiked deionized water and artificial urine samples using dibenzoylmethane (DBM) ligand

The combination of chromium(III) ions and dibenzoylmethane could possibly have health benefits to human beings; chromium is known to be involved in promoting metabolism in the body and on the other hand dibenzoylmethane is believed to have an inhibitory effect against cooked food mutagens and prostate cancer [42,43]. The successful chromatography of $Cr(DBM)_3$ in body fluids and urine could be a stepping stone in understanding the chemistry of this compound in the body if such a study could be undertaken. Dibenzoylmethane as a chelating agent has been used to derivatize depleted uranium in urine samples with determination by reversed-phase HPLC [44]. From the literature it seems there

is limited chromatographic study of dibenzoylmethane as an extraction ligand in metal speciation or chelation.

Dibenzoylmethane is a yellow crystalline solid at room temperature that readily dissolves in acetonitrile. A stock solution of this ligand was prepared and small aliquots added to the deionized water and urine samples to extract spiked Cr(III) ions. The deionized water and urine samples were prepared as described for acetylacetone and dipivaloylmethane. The presence of benzene rings at the ends of the dibenzoylmethane suggested that $Cr(DBM)_3$ would be hydrophobic and could require a high organic content in the mobile phase to elute.

Chromatographic optimization to resolve the metal dipivaloylmethane chelates

Three columns were used in the study of $Cr(DBM)_3$; J`sphere ODS H-80, DiamondBond C18 and ZirChrom-PS. The elution of the chelate was achieved with a mobile phase containing 10% acetonitrile and 90% water without a buffer on both J`sphere ODS H-80 and DiamondBond C18. The wavelength 275 nm produced results with higher sensitivity than 340 nm. The shape of the analyte peak was more symmetrical with J`sphere ODS H-80, whereas the sensitivity of the analyte was slightly better with DiamondBond C18. The ZirChrom-PS was used with hopes of eluting the $Cr(DBM)_3$ with a lower content of the organic modifier in the mobile phase. Elution of $Cr(DBM)_3$ with baseline resolution from the ligand was achieved with 50% acetonitrile and 50% water as mobile phase on ZirChrom-PS. However, the peaks were very broad and unsymmetrical making quantification difficult. When the organic modifier content in the mobile phase was increased with hopes of improving the shape of the peaks, the resolution between the analyte and the ligand was lost. On the other hand, reduction in the content of the organic modifier in the mobile phase worsened broadening and tailing of the peaks. The use of different buffers and temperatures did not improve the results obtained with ZirChrom-PS.The silica-based column, J`sphere ODS H-80 was preferred over the other columns based on the quality of the chromatogram and the feasibility of utilizing (90:10 v:v) acetonitrile:water as mobile phase at 275 nm.

Resolution of $Cr(DBM)_3$ from other metal DBM chelates (Al and Ni)

J`sphere ODS H-80 and DiamondBond C18 columns were evaluated in an effort to resolve $Cr(DBM)_3$, $Al(DBM)_3$ and $Ni(DBM)_2$. Two detection wavelengths, 275 nm and 340 nm were used in this study. As noted earlier, Ni(II)acetylacetonate is very difficult to chromatograph[35]. This study was to establish if dibenzoylmethane, a derivative of acetylacetone, could produce a stable chromatogram which does not co-elute with the other metals chelates, as was the case with the acetylacetone and dipivaloylmethane Ni(II) chelates, respectively. Standard solutions containing 1000 ng/mL of $Cr(DBM)_3$, $Al(DBM)_3$ and $Ni(DBM)_2$ were prepared and determined using 90% acetonitrile and 10% water as mobile phase, with no buffer at 275 nm. Under these chromatographic conditions the three metal chelates were separated with baseline resolution on J`sphere ODS H-80 (Figure 7), but not with the DiamondBond C18, where the Al(III) and Ni(II) dibenzoylmethanate chelates lacked baseline resolution. When using the 340 nm wavelength the $Ni(DBM)_2$ chelate could not be detected, but only $Al(DBM)_3$ and $Cr(DBM)_3$ were detectable. From the above results, at detection wavelength 275 nm and (60:40 v:v) acetonitrile and water as mobile phase, it was possible to determine the $Ni(DBM)_2$ and $Cr(DBM)_3$ chelates with baseline resolution.

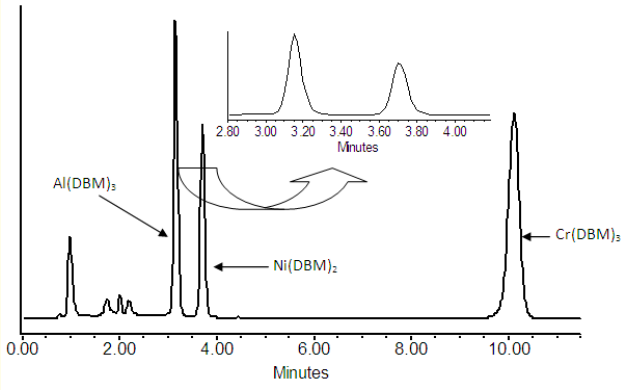

Figure 7: Separation of 1000 ng/mL Al(III), Ni(II) and Cr(III) dibenzoylmethanate chelates with (90:10 v:v) acetonitrile/water mobile phase system at 275 nm detection on J`sphere ODS H-80.

Linear calibration range of $Cr(DBM)_3$ standards and limit of detection

$Cr(DBM)_3$ standards in the range $1\text{-}10^4$ ng/mL were used to determine the linear range for both J`sphere ODS H-80 and DiamondBond C18 columns. A linear calibration range 1-1000 ng/mL was observed for both the columns, which was similar to that obtained using $Cr(DPM)_3$, suggesting that perhaps larger metal

chelates (from dipivaloylmethane and dibenzoylmethane) produce narrow linear calibration range when compared to relatively smaller metal chelates such as those of the acetylacetonates. Non-linearity was observed above 1000 ng/mL. The calibration curve based on the 1-1000 ng/mL range passed through the origin with an R^2 value of 0.9993 (Figure 8). There was no significant difference in the slopes obtained for the calibration ranges 1-100 ng/mL, 100-1000 ng/mL and 1- 10000 ng/mL, indicating similar sensitivity of $Cr(DBM)_3$ for the entire range of 1-1000 ng/mL. The limit of detection for $Cr(DBM)_3$ was found to be 0.84 ng/mL when using J`sphere ODS H-80 and 1.25 ng/mL using DiamondBond C18, which is the best limit of detection obtained when comparing data obtained from the three ligands in this study.

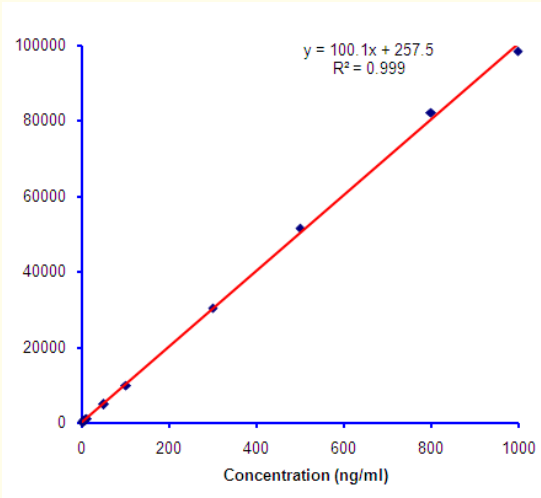

Figure 8: Calibration curve showing the linear range of $Cr(DBM)_3$ obtained on J`sphere ODS H-80 with (90:10 v:v) acetonitrile: water as mobile phase system at 275 nm detection.

Recovery of Cr(III) ions from artificial urine and deionized water samples on dibenzoylmethane as chelating ligand

The deionized water and urine samples were prepared as described above for samples extracted with acetylacetone. The ligand was added in excess to the urine samples prepared at various pHs. When the ligand was added to both the deionized water and urine samples some cloudiness was observed, which disappeared in less than five minutes of warming the mixture. An analyte peak was observed for both the samples, which were extracted at pH 7.20 and nothing at low pH (2.15) and with very little extracted at high pH (12.15) (Figure 9).The extraction efficiencies of deionized water and urine samples were 97 ± 3% and 105 ± 4%, respectively.

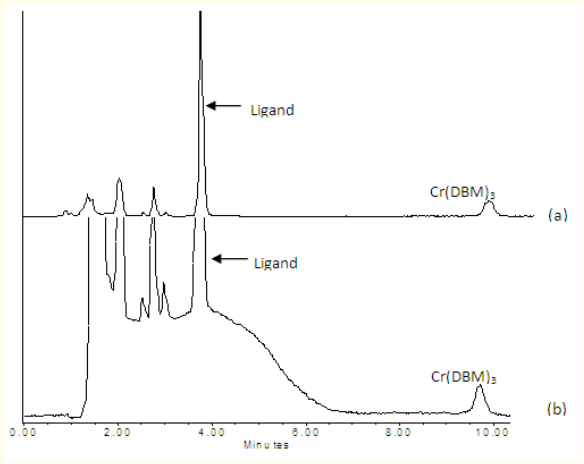

Figure 9: Chromatogram of $Cr(DBM)_3$ in spiked (1000 ng/mL Cr(III)) deionized water and artificial urine samples using (90:10 v:v) acetonitrile: water mobile phase at 340 nm detection with J`sphere ODS H-80.

The chromatographic peak of $Cr(DBM)_3$ has a reasonable retention time just far away enough from where most of the matrix peaks from water and urine samples are eluted, suggesting of less interferences and reliable quantitative analysis (Figure 9). This ligand was the only one for which Ni(II)ions could be determined simultaneously with Cr(III) ions. The best limit of detection was also observed for dibenzoylmethane as a chelating ligand for Cr(III). Even though, this ligand has a limited linear calibration range when compared to that obtained when using acetylacetone, it should be noted that most of the concentrations of Cr(III) in water and urine samples lie well below the 1000 ng/mL allowing dibenzoylmethane to be used successfully for quantitative determination of Cr(III) samples typically within the range 1-1000 ng/mL.As a result, dibenzoylmethane was selected as the ligand for the extraction of Cr(III) in real samples.

Extraction of Cr(III) in real water and urine samples with dibenzoylmethane as chelating ligand

In preparation for sample collection, polypropylene bottles were soaked in a (50:50 v:v) water:nitric acid bath. The bottles were rinsed thoroughly with deionized water and filled with 10% nitric

acid until the time of use. Water samples were collected from local fresh water sources; the campus pond, Puffers pond and Lake Wyola in Amherst, Massachusetts, USA. At the sampling site polypropylene bottles were rinsed with the fresh water before sampling. To collect urine samples, healthy volunteers were requested to give urine in polypropylene bottles prepared as described above. All samples were analyzed within 24 hours of sampling or collection to avoid interconversion of Cr(VI) to Cr(III). Previous studies recommended determination of Cr(III) in water samples to be performed as soon as possible to avoid such interconvention [8,15]. All the samples were prepared at pH 7.20 since from the previous studies it was established that extraction of Cr(III) is dependent on the pH of the sample. The ligand dibenzoylmethane was then added to the samples and the mixture shaken vigorously to extract any Cr(III) ions that may be present. The samples were analyzed using the optimized conditions obtained for deionized water and artificial urine samples and the results are shown in table 1 for five replicates. The detection limit of Cr(III) for this method has been established to be 0.84 ng/mL.

Samples	Concentration Cr(III) found (ng/mL)	Concentration of samples spiked with 100 ng/mL of Cr(III)	Percentage Recovery (%)
Campus Pond	5.94 ± 0.36	105.1 ± 2.17	99 ± 2.2
Puffers Pond	8.82 ± 0.19	107.7 ± 2.61	99 ± 2.6
Lake Wyola	8.99 ± 0.30	105.7 ± 2.12	96 ± 2.1
Urine 1	4.22 ± 0.22	109.2 ± 3.75	105 ± 3.8
Urine 2	7.18 ± 0.25	110.6 ± 1.96	103 ± 2.0
Urine 3	0.86 ± 0.14	103.8 ± 3.02	104 ± 3.0
Urine 4	ND	99.8 ± 4.78	99.8 ± 4.8

Table 1: Extraction of Cr(III) ions in water and urine samples with dibenzoyl methane as a chelating ligand with (90:10 v:v) acetonitrile: water mobile phase at 275 nm detection on J`sphere ODS H-80.

The percentage recovery for water samples was from 96-99% and that for urine samples from 99-105%. The concentration range obtained from the water samples was 5.94-8.99 ng/mL, which is within the range expected for fresh water [8]. For urine samples, Cr(III) concentrations over 29 ng/mL have been reported for diabetic patients and about 12.3 ng/mL for healthy patients [32]. In this study, the highest concentration was 7.18 ng/mL.

Conclusion

In general, there is no significant difference in the chromatograms obtained with J`sphere ODS H-80 and a zirconia-based column, DiamondBond C18. Variation of the charge on the substrate by using buffers and changes in temperature did not improve the results. The advantage of chemical and thermal stability of the DiamondBond C18 was not beneficial in this case. However, it should be noted that ZirChrom-PS may be useful for very hydrophobic species and can bring benefits of reducing analysis duration and amount of organic modifier required to elute the hydrophobic species, which would otherwise be strongly retained on the silica-based and DiamondBond C18 reversed-phase columns. The use of smaller ligands such as acetylacetone can bring about a better linear calibration range, but produces shorter elution time of the chelate, when the metal chelate peak may be affected by peaks from the matrices of the samples compromising the quality of quantitative determination. For the diketone ligands, it was observed that introduction of benzene rings improves the detection limit with the ultraviolet-visible detector. Also upon increasing the size of the extraction ligand, the retention time of the chelate increases, so as to be reasonably far from where most of the sample matrix peakselute (shorter retention times), thus improving the quality of quantitative analysis, with less interference from the sample matrices peaks. No studied metals were found to co-elute or interfere with the peaks of Cr(III) acetylacetonate and its derivatives. For nickel(II) chelates, chromatographic stability improved with the increase in the size of the diketone ligand, being best for dibenzoylmethane, which has aromatic rings. As the ligand increased in size, the resolution between the chelates of the metals studied and the Cr(III) chelate increased. Overall, divalent ligands containing aromatic rings may be preferred based on their moderate retention time for metal chelate elution, avoiding lower retention regions where the majority of the sample matrices elute. The divalent ligands with benzene rings also improve detection with the ultraviolet-visible detector, which is the most common detector in HPLC.

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Conflict of Interest

The author does not have any conflict of interest in the publication of this research work.

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