

1 **Selective Ultratrace Detection of Al(III) and Ga(III) Complexed with a Calcein**
2 **Isomer by Capillary Zone Electrophoresis with Laser-induced Fluorescent**
3 **Detection**

4
5 **Comparison of Emissive Polyaminocarboxylates as Derivatizing Ligands**

6
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1 **Abstract**

2 A highly sensitive and selective capillary zone electrophoresis with laser-induced fluorescent detection
3 (CZE-LIF) for Al³⁺ and Ga³⁺ was developed using a calcein 4',5'-isomer as a pre-capillary complexing
4 agent. Direct fluorescent detection without an agent added to the carrier buffer provided the notable
5 detection limits of 3.9×10^{-10} and 4.6×10^{-10} mol dm⁻³ for Al and Ga, respectively. By applying a river
6 water sample successfully to this system, its potential for practical applications was ascertained. A
7 comparison of three aromatic polyaminocarboxylate ligands related to the coordination structure and
8 the distance between the fluorophore and the chelating moiety, revealed some interesting factors
9 regarding their selectivity.

10 **Keywords.** Capillary zone electrophoresis, Laser-induced fluorescent detection, Calcein, Al, Ga,
11 kinetics

12

1 **1. Introduction**

2 When capillary zone electrophoresis (CZE) is employed, it is difficult to obtain high sensitivity
3 because of its short optical path length (typically 50-100 μm). For organic analytes, a laser-induced
4 fluorescent (LIF) detection technique is frequently employed with detection levels of 10^{-10} - 10^{-12} mol
5 dm^{-3} easily achievable with pre-capillary derivatization due to the covalent bonds of analytes with
6 emissive labeling agents. For metal ions, however, such trace levels of detection are not able to be
7 obtained if even when LIF is employed [1-7]. This is due to the customary addition of fluorescent
8 agents for detection in the carrier buffer solution, which also plays a role as a complexing agent for
9 separation. The emissive agents in the carrier give a substantially large baseline noise, and the signals
10 of the metal complexes are invariably superimposed on the large background. Although the complexing
11 agent can be omitted from the carrier buffer solution with pre-capillary complexing techniques, it
12 presents a problem in that kinetic stability on the dissociation reaction is essential for the detection of
13 metal ions [8-14]. The driving force to dissociate metal complexes arises when the bands of a metal
14 complex and a ligand are completely separated from each other in the capillary, which corresponds to
15 the concentration jump of the agent. At this point, the on-capillary complex equilibrium is no longer
16 maintained.

17 While several detection systems using CE and employing kinetically stable metal complexes with
18 pre-capillary complexation have been developed, fluorescent detection has been used sparingly; i.e.
19 UV/Vis detection methods are the most employed. We previously reported on a new aromatic
20 polyaminocarboxylate system with a fluorescein and a methyl-EDTA portion as a fluorophore and a
21 chelating moiety, respectively [15]. The CZE-LIF of those metal chelates was demonstrated to be a
22 highly sensitive, with 10^{-11} - 10^{-10} mol dm^{-3} level detection limits for Cu^{2+} , Zn^{2+} , Mn^{2+} , Ni^{2+} , Co^{2+} , Pb^{2+}
23 and Cd^{2+} ions. Interestingly, however, triply charged metal ions were not detected. On the other hand,
24 we also reported that a calcein isomer (4',5'-calcein, abbreviated as calcein or L or **1** in this paper) was
25 useful for the selective detection of Al^{3+} in reversed-phase HPLC with fluorescent detection, in which a
26 detection limit of 7.6×10^{-11} mol dm^{-3} (2.1 ng l^{-1}) was successfully achieved [16]. Judging from these
27 results, it is expected that L, which is a kind of aromatic polyaminocarboxylate ligand with two
28 iminodiacetate and a fluorescein portions, would be suited for detecting triply charged metal ions in
29 CE-LIF.

30 In this paper, we report on a highly sensitive CZE-LIF system for Al^{3+} and Ga^{3+} using L as a pre-
31 capillary complexing agent and discuss its selectivity, and compare L with the chemical structure of
32 antenna and the chelating moieties of other aromatic polyaminocarboxylate ligands.

33

34 **2. Experimental**

35 **2.1. Apparatus**

1 The CE-LIF setup used in this study was assembled as follows. The voltage supply, the HCZE-30P
2 model, was purchased from Matsusada Precision (Shiga, Japan). An LIF detection system, ZETALIF
3 (Picometrics, Ramonville, France), equipped with a Model 163D 25 mW argon laser (Spectra-Physics,
4 CA, USA), was employed. The Bio-Focus 3000 CE system (Bio-Rad Laboratories Japan, Tokyo,
5 Japan) was employed to obtain electropherograms with UV/Vis absorbance detection. Fused-silica
6 capillary tubes (inner diameter of 50 μm , outer diameter of 375 μm , a total length of 60.0 cm, and an
7 effective length to the detection window of 46.5 and 55.7 cm for LIF and UV/vis absorbance detection,
8 respectively) were obtained from Scientific Glass Engineering (Austin, TX). New capillary tubes were
9 pretreated by rinsing with a 1 mol dm⁻³ solution of NaOH for 60 minutes and deionized water for 60
10 minutes. All buffer and rinsing solutions in the capillary tubes were exchanged under reduced pressure
11 by a vacuum pump. To avoid any serious contamination of Al³⁺ ions, perfluoroalkoxy (PFA) wares
12 were exclusively employed.

14 2.2. Chemicals

15 The reagents, 4',5'-bis[*N,N'*-bis(carboxymethyl)aminomethyl]fluorecein (calcein, >97% purity)
16 obtained from Fluka (Buchs, Switzerland) were dissolved in deionized water by the Milli-Q SP. TOC.
17 system (Millipore Co., Billerica, MA, USA) to give a concentration of 10⁻² mol dm⁻³. The standard
18 solutions of metal ions were prepared by dissolving the chloride salts (99.9 % purity, Wako Pure
19 Chemical Industries, Osaka, Japan) in deionized water with a few drops of concentrated hydrochloric
20 acid. The *o,o'*-dihydroxyazobenzene (DHAB) was obtained from Dojindo Lab. (Kumamoto, Japan).
21 The 0.1 mol dm⁻³ pH buffer solutions of 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
22 (HEPES)-NaOH and *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES)-NaOH (Dojindo Lab.,
23 Kumamoto, Japan) were prepared for the pH range 7.0-10.

25 2.3. CE-LIF procedure

26 The L and pH buffer solutions were added to the sample solution. The concentrations of the mixed
27 solution were typically 1 \times 10⁻⁶ mol dm⁻³ and 4 \times 10⁻² mol dm⁻³ for the L and pH buffer, respectively.
28 After 10 minutes, the sample solution was hydrodynamically injected into the capillary from the
29 cathodic end with Δ 5 cm in 72 s for LIF detection (an injection volume of 10 nL was calculated using
30 the Hagen-Poiseuille equation) and 5 psi \times s for UV/Vis detection (8 nL). Then voltage was applied at
31 15-20 kV with a constant voltage mode. A carrier buffer of 10⁻² mol dm⁻³ HEPES-NaOH (pH 7.50)
32 containing 5 \times 10⁻⁸ mol dm⁻³ of EDTA was typically employed. The power output of the laser was set
33 at 15.5 mW. The voltage of the photomultiplier in the LIF detection system was set at 570 V.

34 To avoid any serious Al³⁺ contamination from the reagents used, the chemical suppression technique
35 reported in our previous work was also examined [16]. The sample was prepared by the following

1 procedure; 50 μL of $1 \times 10^{-5} \text{ mol dm}^{-3}$ L stock solution containing $1.5 \times 10^{-5} \text{ mol dm}^{-3}$ of DHAB and
2 0.1 mol dm^{-3} of CHES-NaOH (pH 10.0) was added to the sample solution, and then 20 μL of 0.1 mol
3 dm^{-3} HEPES pH buffer solution containing $4.0 \times 10^{-6} \text{ mol dm}^{-3}$ of DHAB was added. The mixed
4 solution was made up to 500 μL with deionized water and injected to the CE-LIF after it had been left
5 to stand for 15 minutes.

6

7 **3. Results and discussion**

8 **3.1. Electrophoretic behavior**

9 The metal complexes with L were injected into the CE with UV/Vis detection at 488 nm after pre-
10 capillary chelation. Only the distinct peaks of Al^{3+} and Ga^{3+} were observed as shown in Figure 1. None
11 of the other metal complexes (Ca^{2+} , Mg^{2+} , Fe^{2+} , Cu^{2+} , Zn^{2+} , Ni^{2+} , Co^{2+} , Mn^{2+} , Pb^{2+} , Cd^{2+} and Hg^{2+})
12 showed any trace peaks with simultaneous addition. Regarding In^{3+} , an extremely broadened peak was
13 observed near the peak of the ligand, quantification was impossible due to overlap. The selectivity of
14 Al^{3+} and Ga^{3+} over the other metal ions seems to be mainly based on the effect of the kinetic
15 characteristics. Only the kinetic stable complexes which survived during migration were detected, and
16 the kinetic active complexes decomposed; i.e. the dissociation kinetics controls the detection selectivity.
17 The water exchange rate constants, $k_{\text{H}_2\text{O}}$, for all undetected metal ions in the CE are more than 1×10^4
18 s^{-1} , while a metal ion with a large $k_{\text{H}_2\text{O}}$ value frequently tends to form labile complexes on ligand-
19 exchange reaction [17]. On the other hand, the values of $k_{\text{H}_2\text{O}}$ of the triply-charged group IIIA metal
20 ions are 1.29, 4.0×10^2 and $4.0 \times 10^4 \text{ s}^{-1}$ for Al^{3+} , Ga^{3+} and In^{3+} , respectively [17]. Judging from these
21 values, this complexing system seems to be sufficiently inert to be detected in the CE only for metal
22 ions with $k_{\text{H}_2\text{O}}$ of less than 10^4 s^{-1} . The In^{3+} complex on the borderline between inert and labile was
23 detected as a half-dissociated broad peak. No interference for the detection of Al^{3+} and Ga^{3+} was
24 observed at the concentration range of $2.0 \times 10^{-5} \text{ mol dm}^{-3}$ of foreign metal ions owing to the kinetic
25 selectivity.

26 A similar migration behavior to that using UV/Vis detection was observed using the LIF detection
27 system as shown in Figure 2. Paramagnetic (Fe^{2+} , Cu^{2+} , Ni^{2+} , Co^{2+} and Mn^{2+}) and heavy metal ions
28 (Pb^{2+} , Cd^{2+} and Hg^{2+}) are generally known to significantly quench the ligand-centered emission and to
29 be difficult to detect with the fluorescence detection. Meanwhile, the Al^{3+} - and Ga^{3+} -L complexes were
30 distinctly detectable with fluorescence detection since these metal ions were closed-shell metal ions,
31 which did not quench the ligand-centered emission. Other closed-shell metal complexes (Ca^{2+} , Mg^{2+}
32 and Zn^{2+}) were not detected due to the on-capillary dissociation processes as described above.

33 In Figure 2, the peak of the Al complex is about ten times higher than that of the Ga complex. This is due
34 to contaminant Al ions ($4.2 \times 10^{-9} \text{ mol dm}^{-3}$). Since the masking reagent, EDTA, was added to the
35 carrier buffer solution, the contaminant Al in the carrier should have been suppressed. Therefore, the

1 source of the contamination most likely originated from the reagents used for the pre-capillary
2 complexing process; L and the pH buffer solutions. Accordingly, a contaminant suppression technique
3 for Al was employed [16]. This simple technique is based on the combination of the kinetics and
4 thermodynamics of Al complexes; by simply adding of DHAB to used reagents, all contaminant Al in
5 the reagents used is converted into an inactive DHAB complex on the pre-capillary complexation
6 process. On the other hand, sample Al ions are derivatized into the L complexes with fast complex
7 formation kinetics. When this technique was employed, the peak of contaminant Al successfully
8 decreased by a factor of fifteen. While over 99.9 % of the contaminant Al theoretically was suppressed
9 by the formation of DHAB complexes, an Al peak of about 10 ng l^{-1} ($3.7 \times 10^{-10} \text{ mol dm}^{-3}$) was still
10 observed. Such behavior was also observed in HPLC experiments, where it was expected that the Al
11 originated from the instruments. This technique was successfully employed for the determination of Al
12 but not for Ga. The L complexes with Ga ions seemed to dissociate due to a fast ligand-exchange
13 reaction in the pre-capillary process ($\text{Ga-L} + \text{DHAB} \rightarrow \text{Ga-DHAB} + \text{L}$; fast). This suggests that the
14 Ga-L complex is far more labile than the Al-L complex, which is supported by the $k_{\text{H}_2\text{O}}$ values.

16 **3.2. Detection limits and an application to a river water sample**

17 Ultrasensitive detection of metal-L complexes was considered highly likely since the fluorescein
18 fluorophore with an absorbance maximum at around 490 nm was suitable for the excitation with an
19 argon laser ($\lambda_{\text{ex}} = 488 \text{ nm}$). The calibration curves for Al^{3+} and Ga^{3+} were obtained. Their linear ranges,
20 correlation factors and detection limits were 1×10^{-8} - 1×10^{-6} and 5×10^{-10} - $1 \times 10^{-8} \text{ mol dm}^{-3}$, $R^2 =$
21 0.993 and 0.998 , and 4.2×10^{-9} (42 atto mole as amount basis) (based on 3σ) and $4.6 \times 10^{-10} \text{ mol dm}^{-3}$
22 (4.6 atto mole as amount basis) (based on $S/N = 3$) for Al^{3+} and Ga^{3+} , respectively. The comparatively
23 poor detection limit of Al to Ga was due to the fluctuation of the contaminant Al peak. When the
24 masking technique was employed for Al, the linear ranges, the correlation factor of the calibration
25 curve and the detection limit based on 3σ were 1×10^{-9} - $1 \times 10^{-7} \text{ mol dm}^{-3}$, $R^2 = 0.9938$ and 3.9×10^{-10}
26 mol dm^{-3} (10 ng l^{-1} , 3.9 atto mole as amount basis). These detection limits are the most sensitive among
27 the CZE methods for metal ions.

28 The determination of Al in a river water sample (JC0031) was carried out as an example of its
29 application to real samples (Figure 3). Some unknown peaks appeared with low reliability as shown in
30 Figure 2 (25-27 min) and Figure 3 (32-36 min). Since these peak heights changed depending on the lot
31 number of the agent and exposure to extremely low pH solutions, the origin of the peaks seemed to be
32 from impure substances. However, the reliability of the peaks of Al^{3+} , Ga^{3+} and L were sufficiently
33 high for quantitative determination. The value determined by our method ($14 \mu\text{g l}^{-1}$) was similar to the
34 certified value ($13 \mu\text{g l}^{-1}$). No Ga peak was observed, which indicated that the contaminant level of Ga
35 lay at levels lower than $10^{-10} \text{ mol dm}^{-3}$.

1 3.3. Comparison of three ligands regarding selectivity and chemical structure

2 We have previously reported on some CE and HPLC systems with fluorescence detection using three
3 kinds of aromatic polyaminocarboxylate ligands as pre-column derivatizing reagents [15,16,18]. A
4 comparison of these systems with this study is informative and useful with respect to the molecular
5 design of fluorescent ligands for analytical separation systems (summarized in Table 1).

6 First, regarding emissive characteristics, the distance between the fluorophores and the center metal
7 ions seems to control the quenching process by paramagnetic and heavy metal effects. The
8 paramagnetic metal ions (Fe^{2+} , Cu^{2+} , Ni^{2+} , Co^{2+} , Mn^{2+}) quench the ligand-centered emission of **1** and
9 ABEDTA (**2**) complexes, while other metal ions, including the heavy metal ions (Cd^{2+} , Pb^{2+} and Hg^{2+}),
10 retain the emissive characteristics of these complexes. However, the intensity of the ligand-centered
11 emission of the Pb^{2+} -**1** and -**2** complexes was half of that of the free ligands (data not shown).
12 Furthermore, it was reported that the closed-shell lanthanide ions, La^{3+} and Lu^{3+} , quench the ligand-
13 centered emission of **1** [19]. Therefore, a heavy atom effect seems to be at work in certain cases. On the
14 other hand, the ligand-centered emissions of the FTC-ABEDTA (**3**) complexes with paramagnetic and
15 heavy metal ions retain their emissive characteristics. An approximation of the structures of the
16 complexes was made to estimate the distances between the fluorescent moiety and the metal ion as
17 shown in Figure 4. The MM/MD simulation was made for Ca^{2+} complexes since there was no
18 parameter set for Al^{3+} available in the software employed. It is noted that the aim of the calculation is
19 merely to estimate the distance between a center-metal and a fluorophore for the complexes with
20 typical metal ions in aqueous solution, and is not to obtain precise chemical structures. Nevertheless,
21 reasonable calculated values were obtained for the distance between carboxylate O atoms and Ca^{2+}
22 (2.22-2.23 Å), and imino N atoms and Ca^{2+} (2.48-2.55 Å) in the complexes with **2** and **3**, which values
23 were comparable to those of EDTA obtained from the crystal structure ($\text{Ca}\cdots\text{O}$, 2.37-2.45 Å; $\text{Ca}\cdots\text{N}$,
24 2.62-2.71 Å) [20]. The distances between the fluorophore and the metal ion for **1**, **2**, and **3** complexes
25 are approximately 3, 6, 11-12 Å, respectively. This fact suggests that the fluorescent characteristics of
26 the ligands can be controlled by the distance modulation between the fluorescent and the chelating
27 moiety. Hence, **1** selectively works as a pre-column derivatizing reagent for closed-shell and non-heavy
28 metal ions, as described in this study, whereas the simultaneous detection of multiple metal ions is
29 achieved by **3** since all metal complexes have strongly emissive nature.

30 Secondly, the chemical structure of the chelating moiety, such as the coordination number or cavity
31 size, has a substantial influence on selectivity based on the decomposition processes in the separation
32 column. The methyl-EDTA frame in the **2** and **3** ligands provides kinetic stability for metal complexes,
33 with the exception of the triply charged metal ions. While the same coordination structures of **2** and **3**
34 provide different selectivity, this is due to the differences in the experimental conditions (at pH 7.5 for
35 **2** complexes and pH 12 for **3** complexes) and of the charges of the complexes (2- for $[\text{M}^{2+}\text{-2}]$; 3- or 4-
36 for $[\text{M}^{2+}\text{-3}]$ complexes). It is well known that the pH value substantially affects the rate of acid
37 dissociation processes [21], and the charge of the complex frequently affects the rate of ligand-

1 exchange processes with attacking nucleophilic ligands [22]. On the other hand, the **1** ligand provides
2 kinetic stability selective to only triply charged metal complexes. This may be a result of the cavity size
3 in the coordination sphere; the ligand, **1**, seems to have a smaller coordination cavity than **2** and **3**
4 ligands. The coordination structure of the Al-**1** complex remains unknown, yet at least it has been
5 reported that the composition of the Al-**1** complex is one to one [16]. In the present work, it is
6 interestingly revealed that a boundary of $k_{\text{H}_2\text{O}}$ allows an inert complex to form with the **1** ligand at
7 around 10^{-4} s^{-1} . The selectivity in our CE system is probably related with the mechanism of the
8 dissociation process. It was reported that the solvent exchange reaction of Al^{3+} and Ga^{3+} occurs with a
9 very similar mechanism (both were dissociation mechanism). However, the association mechanism of
10 In^{3+} was quite different [17]. In contrast to **1**, the larger cavity size in **2** and **3** complexes seems to be
11 suited to doubly charged metal ions. The kinetic stability of the complexes with these ligands seems not
12 to be interrelated with the $k_{\text{H}_2\text{O}}$ values; (the order of $k_{\text{H}_2\text{O}}$: $\text{Al}^{3+} < \text{Ga}^{3+} < \text{Ni}^{2+} < \text{Mg}^{2+} < \text{Co}^{2+} < \text{Fe}^{2+} <$
13 $\text{Mn}^{2+} < \text{Zn}^{2+} < \text{Cd}^{2+} < \text{Hg}^{2+} < \text{Cu}^{2+} < \text{Pb}^{2+}$). This suggests that the dissociation kinetics of the **2** and **3**
14 complexes is rather complicated to be involved in solvolysis, acid-assisted dissociation and ligand-
15 exchange dissociation processes. It is emphasized that the inertness of the complex is controlled by the
16 nature of the metal ion for the **1** complex and controlled by the characteristics of the ligand for the **2**
17 and **3** complexes. Modulating the distance between fluorophores and the chelating moieties, and also
18 controlling the chelating structure can provide different selectivity for various metal ions.

20 **4. Conclusion**

21 It is noteworthy that sub nano molar level detections are successfully achieved using CZE. These
22 detection limits are comparable to those obtained by instrumental analytical methods. Although CE is
23 considered a low sensitivity method even when LIF detection is employed [4-7], the researches we
24 have carried out [15] have overthrown the belief that it is impossible to achieve high sensitivity without
25 any pre-concentration techniques. The relationship between the coordination structure in
26 polyaminocarboxylate ligands and the selectivity in the CE is not completely clear at this stage.
27 However, it is clear that powerful chemical systems for CE will be achieved once we understand the
28 total design of a ligand based on the combination of emissive and kinetic characteristics.

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7

1 Legends for Figures

2 **Figure 1.** Typical electropherogram of metal-L complexes with UV/Vis detection. Sample, $C_L = 5.0 \times$
3 $10^{-4} \text{ mol dm}^{-3}$, $C_{\text{metal}} = 2.0 \times 10^{-5} \text{ mol dm}^{-3}$ (metal = Ca^{2+} , Mg^{2+} , Fe^{2+} , Cu^{2+} , Zn^{2+} , Ni^{2+} , Co^{2+} , Mn^{2+} ,
4 Cd^{2+} , Hg^{2+} and Pb^{2+}), $C_{\text{Al}} = 2.5 \times 10^{-5} \text{ mol dm}^{-3}$, $C_{\text{Ga}} = 7.0 \times 10^{-5} \text{ mol dm}^{-3}$, $C_{\text{HEPES}} = 1.0 \times 10^{-2} \text{ mol dm}^{-3}$.
5 Carrier buffer, $C_{\text{HEPES}} = 2.0 \times 10^{-2} \text{ mol dm}^{-3}$ (pH 7.5), $C_{\text{EDTA}} = 5.0 \times 10^{-8} \text{ mol dm}^{-3}$. Applied voltage,
6 15 kV.

7 **Figure 2.** Typical electropherogram of Al^{3+} and Ga^{3+} -L complexes with LIF detection. Sample, $C_L =$
8 $1.0 \times 10^{-6} \text{ mol dm}^{-3}$, $C_{\text{Al}} = C_{\text{Ga}} = 1.0 \times 10^{-8} \text{ mol dm}^{-3}$, $C_{\text{HEPES}} = 4.0 \times 10^{-3} \text{ mol dm}^{-3}$. Carrier buffer,
9 $C_{\text{HEPES}} = 4.0 \times 10^{-3} \text{ mol dm}^{-3}$ (pH 7.5), $C_{\text{EDTA}} = 5.0 \times 10^{-8} \text{ mol dm}^{-3}$. Applied voltage, 15 kV.

10

11 **Figure 3.** Typical electropherogram of a river water sample (JAC0031). Sample, $C_L = 1.0 \times 10^{-6} \text{ mol}$
12 dm^{-3} , $C_{\text{HEPES}} = 2.0 \times 10^{-2} \text{ mol dm}^{-3}$, river water (JAC0031) was diluted by a factor of 10. Carrier buffer,
13 $C_{\text{HEPES}} = 4.0 \times 10^{-3} \text{ mol dm}^{-3}$ (pH 7.5), $C_{\text{EDTA}} = 5.0 \times 10^{-8} \text{ mol dm}^{-3}$.

14

15 **Figure 4.** Possible structures of the Ca^{2+} complexes with aromatic moiety-containing
16 polyaminocarboxylate ligands: (a) Ca^{2+} -1, (b) Ca^{2+} -2, (c) Ca^{2+} -3. The **solid arrows** indicate the
17 distances between fluorescent moiety and Ca^{2+} . These models and distances with standard deviation
18 were obtained from the Monte Carlo Multiple Minimum (MCMM) procedure [23] and Stochastic
19 Dynamics simulations [24] (simulation time: 500 ps, time step: 1 fs, 300 K, SHAKE: applied to all
20 bonds involving hydrogen atoms), respectively. All calculations were made with the OPLS2003 force
21 field and GB/SA treatment water, as implemented in the Macromodel 9.0 package [25]. In the case of
22 Ca^{2+} -3 complex, two stable structures were obtained.