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ORIGINAL PAPER

**Hong-zhen Lian · Yu-fen Kang · Shu-ping Bi
Arkin Yasin · Da-lin Shao · Yi-jun Chen · Le-mei Dai
Li-ching Tian**

Morin applied in speciation of aluminium in natural waters and biological samples by reversed-phase high-performance liquid chromatography with fluorescence detection

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Abstract A reversed-phase high-performance liquid chromatographic method with fluorescence detection for the determination of labile monomeric aluminium has been developed through pre-column complexation using morin as the analytical reagent. The highly fluorescent aluminium–morin complex (excitation wavelength 418 nm, emission wavelength 490 nm) was separated on a Spherisorb ODS 2 column with an eluent consisting of 30% methanol and 70% water (pH 1.0 with perchloric acid). The most remarkable point of this protocol was that only the most toxic aluminium species, that is, free aqua-aluminium ion and its monomeric hydroxo complex ions, selectively respond among various aluminium complexes. This strategy has been successfully applied to direct fractionation of the toxic aluminium in natural waters and biological samples without any pretreatment.

Keywords Aluminium · Speciation · HPLC · Morin

Introduction

It is well known that elevated aluminium concentration in natural waters is toxic to aquatic and terrestrial organisms [1]. Recently, available evidence pointed to the conclusion that uptake of Al by different routes, including in drinking water, might cause serious neurotic diseases such as Alzheimer's disease, Parkinson's disease and dialysis encephalopathy [2]. Al appears in natural and biological systems as different complexed species. Its toxicity, environmental and biological impacts are highly dependent on the existing forms. It has been generally recognized that "labile monomeric Al (operationally defined as Al_i)" including free aqua-ion and some inorganic complexes is the toxic form, of which the labile positively charged monomeric form (Al^{3+}, $Al(OH)^{2+}$ and $Al(OH)_2^{+}$) is the most harmful, while fluoro and organic complexes are thought to be less potent. Therefore, the development of methods for speciation of Al, notably analysis of toxic Al in environmental and biological samples, is of more important relevance than those for determination of total Al, and has received considerable attention worldwide [3, 4, 5, 6, 7, 8, 9, 10, 11].

The most frequently employed procedure during the last two decades for fractionation of Al_i in natural waters is the so-called "Driscoll" method [12] and its various modifications. The inorganic monomeric Al species present in most waters were assumed to be predominantly cationic and adsorbed by a cation exchanger. Conversely, organic species present in natural waters were assumed to be anionic and not adsorbed. This concept was fundamental to Driscoll's speciation methodology. The fraction recovered after passing through the column was operationally defined as Al_o. The concentration of Al_i was obtained as the difference between total monomeric Al and Al_o. This strategy, however, still has several main disadvantages:

1. The assumption that only Al_i is retained in a cation-exchange column limits this strategy to one which provides good approximation of Al_i.
2. Great error may be associated with the indirect determination of Al_i.
3. The most toxic species (Al^{3+}, $Al(OH)^{2+}$ and $Al(OH)_2^{+}$) cannot really be obtained because less harmful complexes, etc., were also included; and
4. the whole procedures such as complexation with 8-hydroxyquinoline (8-HQ) and extraction with methyl isobutyl ketone (MIBK) are time-consuming and labor intensive.

So developing some direct methods to specify Al_i is critical. Ion chromatographic methods have been developed for the determination and speciation of Al in natural wa-

H. Lian · Y. Kang · S. Bi (✉) · A. Yasin · D. Shao · Y. Chen
L. Dai · L. Tian
State Key Laboratory of Coordination Chemistry,
Department of Chemistry and Center of Materials Analysis,
Nanjing University,
22 Hankou Road, 210093 Nanjing, P.R. China
e-mail: bisp@nju.edu.cn

ters, soil solutions and other aqueous systems [3, 13]. Al was separated into several chemically defined fractions by use of a short, low capacity cation-exchange column coupled with post-column reaction with 8-hydroxyquinoline-5-sulfonate, and fluorimetric detection [14, 15]. An obvious problem of this fluorimetric detection method, for the analysis of complex natural samples, was the possible important interference from magnesium and zinc. Cation-exchange fast protein liquid chromatography (FPLC) with inductively coupled plasma atomic emission spectrometry (ICP–AES) or electrothermal atomic absorption spectrophotometry (ET–AAS) detection was reported to enable speciation of some positively charged monomeric Al, such as individual Al^{3+}, $Al(OH)^{2+}$ and $Al(OH)_2^{+}$, in environmental waters and soil extracts [16, 17], however, the coupling instrumentation was rather complicated and cost too much, and the separation was not very satisfactory. From the other point of view, since the toxic Al species are "labile monomeric", especially "the most toxic" forms, there is a need for a selective strategy for the species group rather than for each species [18]. A number of reports have published on reversed-phase high-performance liquid chromatographic (RP-HPLC) separation and determination of Al after pre-column complexation with 8-HQ [19], 2,2′-dihydroxyazobenzene [20], lumogallion [21], and the like. However, most of these reagents are excessively aggressive i.e. they sequester Al from non-toxic inorganic and organic complexes, and so overestimate the potential toxicity of a natural medium. On the other hand, they often form complexes with a large number of metal ions and reveal poor selectivity for Al.

It was indicated and demonstrated by Browne in cooperation with Driscoll and McColl [22, 23] that morin (3,5,7,2′,4′-pentahydroxyflavone) was a reagent with "minimized disturbance", the fluorescence from Al–morin measured was directly related to a parameter comprising Al^{3+} and Al–OH complexes. The procedure was rather lengthy though, with more than 30 min equilibration time. As the most familiar reagent among flavonoids, morin can selectively form a highly fluorescent complex with Al [24]. So it has been widely used as a reagent for fluorimetric as well as spectrophotometric analysis of Al for a long time [25, 26], although the targets of the determinations were not recognized to be associated with the most toxic Al. In order to increase the sensitivity, some preconcentration procedures have been developed. Hernandez and Escriche [27, 28] studied the optimum conditions for the extraction of Al–morin complex into MIBK and subsequently fluorimetric determination. The effects of non-ionic surfactants on the analysis of Al with morin were also investigated [29]. However, there were still some disadvantages associated with these methods:

1. the reaction takes at least 20 min at room temperature, and heating is often necessary;
2. the required preconcentration procedures and addition of sensitizer non-ionic surfactants are vulnerable to contamination and time-consuming; and
3. these "disturbing" procedures mentioned above cannot be used for Al speciation due to the poor stability of Al_i.

In 1996, Hollman and co-workers thoroughly developed the use of aluminium nitrate as a post-column reagent in RP-HPLC with fluorescence detection to determine flavonols, and indicated that Al–morin had the strongest fluorimetric intensity [30]. Consequently, pre-column reaction with morin followed by RP-HPLC method with fluorescence detection would offer much better selectivity for direct determination of the most toxic Al. When Browne et al. [22, 23] used morin to study the equilibrium of different Al species, the fluorescent signal originated from the whole reaction system. The amount of the analytical reagent should be minimized so as not to superimpose the toxic Al. In this HPLC method, Al–morin complex was separated from other fluorescent components and most interference was eliminated. In order to promote complexation addition of a slight excess of ligand was allowed. The toxic Al was determined with superior sensitivity to fluorimetry in a shorter time. Neither heating nor extraction procedure was needed.

Experimental

Materials and reagents

Al stock solution (0.02 mol L^{-1}) was obtained by dissolving high-purity Al powder in 25 mL of 6.0 mol L^{-1} hydrochloric acid and diluting to 500 mL with water. Individual standard solutions of other metal ions were of ICP–AES grade. Standard solutions of all inorganic anions were obtained from their ammonium salts. Fulvic acid (FA) was extracted from black soil of Heilongjiang Province in China. Morin (guaranteed reagent) was purchased from Kunming Branch Institute of Botany, The Chinese Academy of Sciences, Kunming. Stock solution of morin was prepared in methanol to a concentration of 0.01 mol L^{-1}, stored at 4 °C; under these conditions it was stable for over three months. Methanol was HPLC grade. Perchloric acid (70–72%) and nitric acid were guaranteed reagents. All other reagents were of analytical-reagent grade. Water (>18 MΩ cm) used was prepared from Aquapro ultrapure water systems for the laboratory-scale (Ever Young Enterprises Development, Chongqing, PRC).

Instrumentation

High-performance liquid chromatographic experiments was performed using a Waters Alliance 2695 Separations Module (Waters, Milford, MA, USA) including a vacuum degasser, quaternary pump and autosampler. The column used was Spherisorb ODS 2, 5 μm, 150 mm×4.6 mm i.d. (Johnsson Separation Science and Technology Corporation, Dalian, PRC). The separation was controlled by a Waters Millennium^{32} chromatography manager system. The detection system consisted of a Varian Fluorichrom fluorescence detector (Varian, Walnut Creek, CA, USA) in series with a Waters 996 UV–Vis photodiode-array detector (PDA). The signals acquired from PDA and from fluorescence detector were recorded by Millennium^{32} and Model JS-3030 chromatographic working station (Johnsson), respectively. A Mettler Toledo 320 pH meter with a HA405-K2/120 combination electrode (Mettler-Toledo Instruments Shanghai, Shanghai) was used for pH measurement.

Procedures

Transfer an aliquot of Al ion solution into a 25-mL of volumetric flask. 1.25 mL of 1.0 mol L^{-1} NH_4Ac-HAc buffer (pH 4.5) and 2.5 mL of 1.00×10^{-3} mol L^{-1} morin solution in methanol were added. Then, methanol or water was added to the mark, making the methanol

volume 10 mL. The resulting mixture was sonicated 3 min. Both morin and its Al complex are sparingly soluble in water, so the content of methanol was maintained at 40% (v/v) in order to preserve their solubility enough in the resulting mixture. In addition, the sensitivity and selectivity of fluorescent reaction of Al with morin increases with the solvation effect of aqueous organic solvents containing oxygen, of which methanol is one of the most important sensitizers. About 5 mL of the mixture was filtered through a cellulose membrane with 0.45 µm micropore (Millipore, Bedford, MA, USA). Using an autosampler, 10 µL of the filtered solution was injected into the column immediately. The mobile phase consisted of methanol and water adjusted to pH 1.0 with $HClO_4$ (30+70). The flow rate was 1.0 mL min^{-1}, and column temperature 30 °C. For fluorescence detection, excitation and emission wavelength were 418 nm and 490 nm, respectively. The peak height was measured against a corresponding reagent blank. A calibration graph of peak height was used for unknown samples.

Results and discussion

Nature of Al–morin complex

Fluorimetric and spectrophotometric methods are generally used to investigate the equilibrium and kinetics of complexing reactions in solution. The progress of the reaction is followed by directly monitoring the entire reaction mixture. In most cases, controversial discussions have been made on the coordination ratio of Al to morin, so the ratio was only tentatively suggested [24]. The reason should be that different Al–morin complexes formed and unreactive ligand were not separated. In order to describe the mechanism of complexation between Al and morin, UV–Vis detection was also used in this study. Figure 1 shows a typical chromatogram of Al–morin generated at 350 nm. As can be seen, besides the unreacted morin peak two new peaks appeared, which implied that a pair of complexes was formed. The maximum absorption wavelengths obtained from PDA spectra (Fig. 1 inset) corresponding to these two chromatographic peaks were 395 and 415 nm,

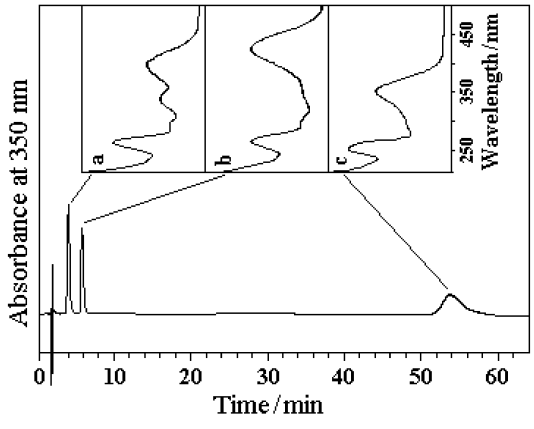

Fig. 1 Chromatogram of Al–morin (3.0×10^{-4} mol L^{-1} Al, 1.0×10^{-4} mol L^{-1} morin) with UV–Vis detection (λ 350 nm). Insets show UV–PDA spectra for (*a*) Al(morin), (*b*) Al_2 (morin), and (*c*) unreactive ligand. Column: Spherisorb ODS 2, 5 µm, 15 cm×4.6 mm i.d.. Column temperature: 30 °C. Mobile phase: 30:70 methanol/water adjusted to pH 1.0 with $HClO_4$. Flow rate: 1.0 mL min^{-1}. Injection volume: 10 µL

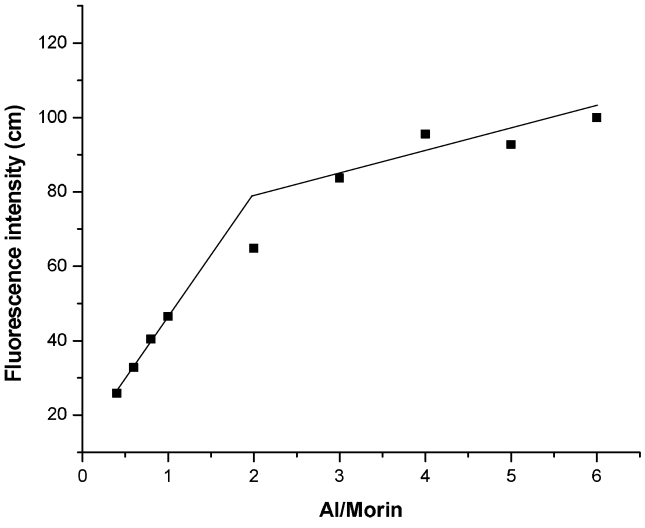

Fig. 2 Molar-ratio plot for the reaction of 1.0×10^{-4} mol L^{-1} morin with Al. Fluorescence detection wavelengths: λ_{ex} 418 nm, λ_{em} 490 nm. Other conditions for HPLC were the same as in Fig. 1

respectively. The coordination ratio of Al to morin was analyzed by plotting chromatographic peak height (H) at individual maximum UV–Vis wavelength versus Al/Morin. For the peak at 6 min in Fig. 1, molar-ratio plot at 415 nm revealed inflection at Al/Morin=2 (not shown), indicating the species Al_2 (morin), and a plot of peak height from fluorescence detector gave the same inflection (Fig. 2). For the first peak at 4 min, the molar-ratio plot at 395 nm displayed a turn at Al/Morin=1 (not shown), corresponding to a complex Al(morin), but very little peak of Al(morin) appeared when fluorescence detection was used. Therefore, both 1:1 and 2:1 complexes may form between Al and morin. The bathochromic effect of Al_2 (morin) was stronger than that of Al(morin). Meanwhile, for unreacted morin, a molar-ratio plot at 355 nm displayed a bend at Al/Morin=3:2, which gave the total complexing ratio. HPLC provides an alternative technique for tracking reaction of metal complexes; it enables not only the separation of the species under study but also the estimation of coordination ratio. Additionally, it is also found that the peak of Al_2 (morin) from fluorescence detector was much higher than that of Al(morin), indicating that the former makes a dominant contribution towards the fluorescent intensity.

Effect of morin quantity

For a fixed Al ion concentration (1.0×10^{-5} mol L^{-1}) solution, different amounts of morin were added. The fluorescence intensity of Al–morin complex ceased to increase after the molar proportion of morin:Al exceeded 50:1 (Fig. 3). Moreover, when the ratio was up to 2000:1 the peak shape was still constant. Because too much excess ligand may lead to overestimation of the most toxic Al due to the competitive reaction with Al present in fluoro and organic complexes, the highest limit of the molar ra-

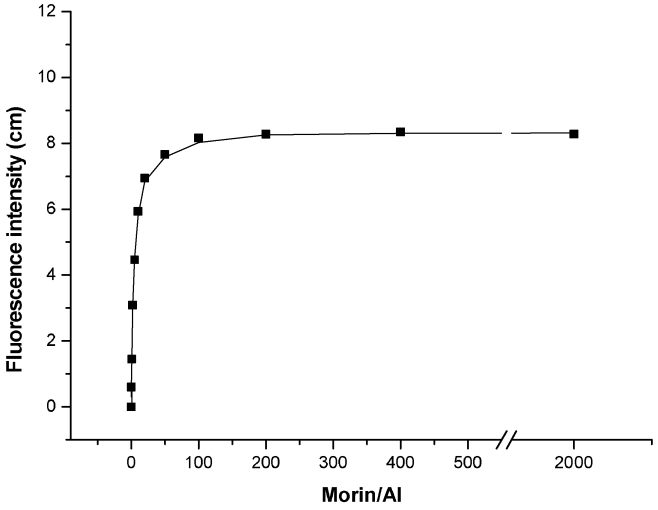

Fig. 3 Dependence of fluorescence intensity on morin:Al molar ratio in pre-column reaction medium. Al concentration: 1.0×10^{-5} mol L^{-1}.Conditions for HPLC were the same as in Fig. 2

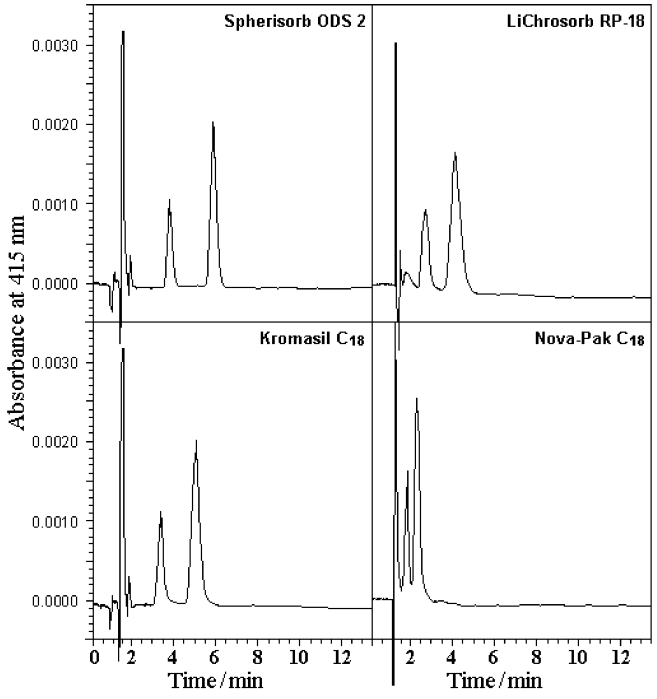

Fig. 4 Chromatograms of Al–morin complexes on different ODS columns with UV–Vis detection (λ 415 nm). Other conditions for HPLC were the same as in Fig. 2

tio was not tested. The molar ratio should not exceed 200:1 in the later experiments.

Effect of pH and buffer concentration in pre-column reaction medium

When diluted perchloric acid was test as reaction medium, very weak fluorescent signal was observed because of no buffering ability. Phosphate buffer is capable of binding Al, so could not be utilized here. Acetate buffer solutions were prepared by mixing NH_4Ac with HAc to give various concentrations and pH. The fluorescence response of Al–morin (1.0×10^{-5} mol L^{-1}) was measured in a range of concentration from 0.01 to 0.10 mol L^{-1} and pH from 3.5 to 6.5. The peak height did not change significantly over the ranges investigated. Finally, a 0.05 mol L^{-1} NH_4HAc-HAc buffer of pH 4.5 was chosen to avoid violent change of labile Al species.

Choice of chromatographic column

The chromatograms obtained from different columns with UV–Vis detection at 415 nm are recorded in Fig. 4. Among the four columns tested, Spherisorb 2 gave the best peak shape and resolution of two Al–morin complexes. Thereby, all subsequent data reported refer to this column.

Choice of mobile phase

When methanol content of the mobile phase was above 40% (v/v), two Al–morin species could not be separated. If the content was less than 20%, peaks became lower and broader probably owing to the low solubility of the complexes in the eluent lacking in methanol. The satisfactory result was obtained when methanol content was 30%. Phosphate buffer, acetate buffer, and perchloric acid as the water phase portion of the mobile phase were compared. The peak of Al–morin could be observed only when dilute $HClO_4$ was used. Additionally, $HClO_4$ has extremely low power to complex Al ion [31]. This mobile phase did not decrease the column efficiency in daily use within over nine months. The influence of pH on the separation was investigated. At a lower pH (<1.5), the peak height of Al–morin was higher than that at a higher pH. There are several OH groups on the skeleton of Al–morin complexes. Stronger acidity could suppress their dissociation, whereas higher pH gave side effect on the peak shape. Once pH of the mobile phase exceeded 2.0, the peak was disrupted drastically. No peak occurred if the pH was over 2.5. The effect of the ionic strength of the mobile phase on the separation was also studied by adding 0.025 mol L^{-1} $KClO_4$ to $HClO_4$ solution (pH 1.0). The result showed that additional salt could not make the peak shape better. Although acetate buffer (pH 4.5) was used as the best medium for complexation of Al–morin, it was unsuitable eluent for HPLC here. Phosphate buffer cannot be used as the eluent because it could react with Al ion [32].

Interferences

The effects of 18 metal cations, four inorganic anions, one phenol, seven phenolic and organic acids, and one humic substance on the determination of 1.0×10^{-6} mol L^{-1} Al ion were examined. We measured the fluorescence of Al–morin

Table 1 Tolerance limits of foreign substances[a]

Cations	
Ca^{2+}	>1000
Cd^{2+}	100
Co^{2+}	>1000
Cr^{3+}	50
Cu^{2+}	50
Fe^{3+}	100
Ga^{3+}	10
In^{3+}	10
K^{+}	>1000
Mg^{2+}	800
Mn^{2+}	100
Na^{+}	>1000
Ni^{2+}	>1000
Pb^{2+}	1000
Sr^{2+}	1000
Y^{3+}	300
Zn^{2+}	>1000
Zr^{4+}	300
Inorganic anions	
F^{-}	0.5
MoO_4^{2-}	>1000
PO_4^{3-}	50
SiO_3^{2-}	>1000
Phenols, phenolic acids, and organic acids	
Catechol	50
Caffeic acid	100
Citric acid	0.5
Gallic acid	5
Oxalic acid	0.3
Salicylic acid	100
Tannic acid	10
Tartaric acid	3
Humic substances	
Fulvic acid	80[b]

[a]Fluorimetric detection for 1.0×10^{-6} mol L^{-1} Al was performed
[b]TOC (mg L^{-1})

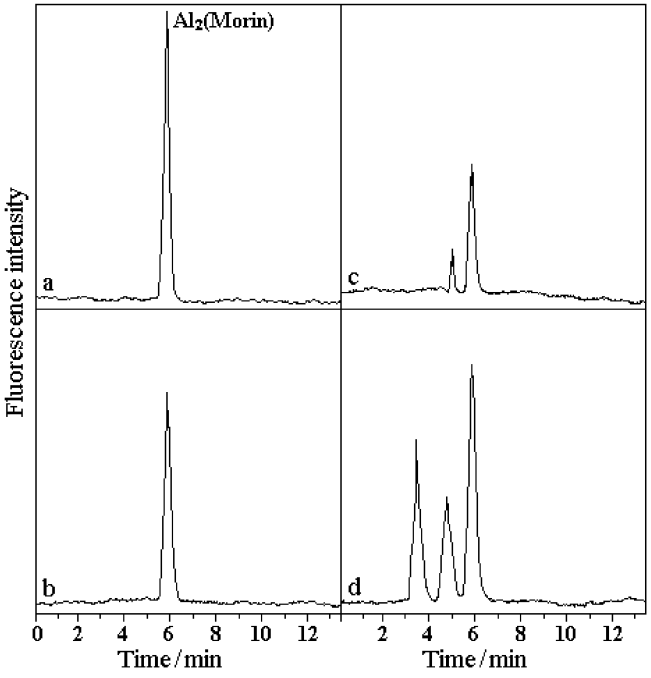

Fig. 5 Chromatograms of Al–morin complex at pH 4.5. (*a*) 1.0×10^{-6} mol L^{-1} Al^{3+}; (*b*) river water; (*c*) serum; (*d*) urine. Real samples were processed without digestion. Conditions for HPLC were the same as in Fig. 2

without and with foreign substances added. The tolerance limit was expressed as the maximum foreign substance-to-Al molar ratio to be determined within an error of 5%. The results are shown in Table 1. Fluoride, citric, gallic, oxalic, tannic and tartaric acids generated severe negative interference. It was indicated that there is a competitive complexation of Al ion between the weak analytical agent and organic matter, fluoride, phosphate, etc. Morin is a weak invasive reagent, and under acidic condition Al complexes of citric, oxalic, fulvic acids and fluoride cannot be substituted by morin. This provided a hint for our speciation idea that we can determine the most toxic Al in the form of morin complex by RP-HPLC. On the basis of a study of the influence of these inorganic and organic ligands it was found that employing the proposed method positively charged monomeric aquo-, and hydroxy-Al species plus the complexes with weak phenolic ligands such as salicylic and caffeic acids were determined, but no Al-F complexes were included. Additionally, since salicylic and caffeic acids concentration in natural water and biological sample are generally very low, the labile inorganic monomeric Al was obtained dominantly. Moreover, the reaction with morin excluded the $[AlF_n]^{(3-n)+}$ species, which are not thought to be toxic, from the measured labile monomeric Al fraction. Such a procedure would provide a more reasonable estimate of the most toxic Al fraction. In fact, it is these "interferences" which make the analysis of the most toxic Al using morin possibly operational without sample digestion.

Linear range, precision and detection limit

Under the proposed experimental conditions, the linear relationship of peak height of Al–morin complex versus Al ion concentration was obtained from 6×10^{-9} to 6×10^{-5} mol L^{-1}, which was the widest linear range found so far for Al–morin system. This is because it was detected after being separated from unreactive morin that may adversely affect the fluorescent response of the complex. The regression equation was H (cm)=0.54+12.79C with correlation coefficient of 0.9949. The repeatability expressed in relative standard deviation (RSD) in peak height was 1.75% for four replicate analyses of 1.0×10^{-6} mol L^{-1} Al ion. The detection limit, taken as the concentration equivalent to three times the standard deviation of the bank, was 2.0×10^{-9} mol L^{-1}. This extreme low detection limit was very suited to achieve the direct determination of the most toxic Al.

Table 2 Determination of the most toxic Al in real samples (n=3)

No. Sample	Determined (mol L^{-1})	RSD (%)	ICP–AES (mol L^{-1})	Driscoll's method (mol L^{-1})	Added (mol L^{-1})	Found (mol L^{-1})	RSD (%)	Recovery (%)
1 Yangzhou canal water	7.55×10^{-6}	0.7	1.13×10^{-5}	7.82×10^{-6}				
2 Xinan river water	3.44×10^{-6}	4.0	6.31×10^{-6}	3.42×10^{-6}				
3 Yingtan stream water	4.18×10^{-6}	4.5	7.52×10^{-6}	4.32×10^{-6}				
4 Xiantai cave water	3.99×10^{-6}	2.2	5.45×10^{-6}	4.01×10^{-6}				
5 Zhenjiang pond water	1.51×10^{-6}	8.5	2.96×10^{-6}	1.69×10^{-6}				
6 Zhenzhu spring water	0.78×10^{-6}	1.6	5.56×10^{-6}		0.50×10^{-6}	1.25×10^{-6}	13.7	96.2
7 Nongfu lake water	0.45×10^{-6}[a]	6.0	1.22×10^{-6}		0.25×10^{-6}	0.78×10^{-6}	6.4	117.8
8 Cerebrospinal fluid	2.83×10^{-6}	6.7	5.93×10^{-6}		1.50×10^{-6}	4.88×10^{-6}	8.6	119.4
9 Urine	7.92×10^{-6}	1.1	3.33×10^{-5}		1.00×10^{-6}	8.52×10^{-6}	0.8	94.9
10 Fetal bovine serum	5.88×10^{-6}[b]	1.0	7.41×10^{-5}		3.50×10^{-6}	1.04×10^{-5}	3.0	117.3
11 Human serum	4.27×10^{-6}	2.2	3.11×10^{-5}					
12 Tea infusion	ND[c,d]		5.18×10^{-5}		1.50×10^{-6}	ND		

[a,b,c]Al concentrations with digestion are 1.25×10^{-6}, 7.75×10^{-5}, 5.55×10^{-5} mol L^{-1}, respectively
[d]ND indicates "not detected"

Speciation analysis

Figure 5 showed a typical chromatogram relative to a standard solution of 1.0×10^{-6} mol L^{-1} Al ion and those from several sample solutions by fluorescence detection with morin. By comparing the experimental results conducted with and without digestion before analysis, it is found that the results obtained with digestion as described by Ahmed and Hossan [26] were consistent with those of ICP–AES (Table 2 footnote a, b and c), whereas the results obtained without digestion were often less than those of ICP–AES. Why did this phenomenon happen? The "mild" ability of morin to catch Al as indicated by Browne et al. [22, 23], and the existence of natural organic matters (NOM) and fluoride are the main reasons (Table 1). Morin is a weak competing reagent, and could not compete with these substances for Al ion, and is able to sequester Al only from Al^{3+}, $Al(OH)_2^+$ and $Al(OH)^{2+}$ in natural waters and biological samples. Except filtration with 0.45 μm membrane, any treatment to samples was unnecessary so as not to disturb the existing form of the most toxic Al of interest. The most toxic Al in tea infusion was not detected, because all of Al in tea binds to high molecular weight (HMW) hydrolyzable polyphenols irrespective of the origin of the tea, and morin could not compete with the powerful natural organic ligands for free Al [33, 34]. The main carriers of Al in serum were demonstrated to be transferrin (80–90%), citrate and phosphate (10–20%) and a very little Al exists freely [35, 36]. The composition of cerebrospinal fluid is relative simple, in which there are rich adrenergic neurotransmitters [37, 38], so important Al forms are complexes of these neurotransmitters with Al. The remaining free form detected by this method was close to 50% of the total Al.

Conclusion

A new strategy for the determination of the labile monomeric Al has been described on the basis of the selective reaction of morin and this group of Al species. The analysis was performed by RP-HPLC coupled with fluorescence detection after pre-column reaction. The proposed procedure combined the advantages of RP-HPLC, i.e. powerful separation, good reliability and wide calibration curve, with the advantages of pre-column complexation with morin, i.e. special reactivity, minimized disturbance and high sensitivity. The results obtained from natural waters and biological samples by this method were in satisfactory agreement with those by Driscoll's speciation method. From our results it clearly indicates that the present study offered a simple, highly selective and ultra-sensitive method for fractionation of the most toxic Al. Morin is a general one, and other naturally occurring flavonols are expected to show the same feature.

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