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Journal of Chromatography A, 993 (2003) 179–185
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Determination of aluminum in environmental and biological samples by reversed-phase high-performance liquid chromatography via pre-column complexation with morin

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Received 28 October 2002; received in revised form 10 February 2003; accepted 14 February 2003

Abstract

Morin was used as a pre-column reagent for the determination of aluminum by RP-HPLC with fluorescence detection. This method has been successfully applied to direct determination of trace Al in environmental and biological samples. The response was linear from 6×10^{-9} to 6×10^{-5} *M* with a detection limit of 2×10^{-9} *M*. In addition, the different Al complexes with morin were separated by the proposed HPLC procedure and their coordination ratios were depicted by molar-ratio method. The results showed that 1:1 and 2:1 Al–morin complexes formed.

Keywords: Complexation; Aluminum; Morin; Quercetin; Pentahydroxyflavone; Flavones; Metal cations

1. Introduction

The ecological and biological effects of aluminum are now well recognized and gaining more and more interest [1,2]. Determination of Al in environmental and biological samples is very important. HPLC has been popularly used for this purpose in recent years. One major approach was to convert Al ions to chelates using various derivatizing reagents, which can be separated on reversed-phase (RP) columns with UV–Vis, fluorescence or electrochemical detection [3–7]. However, most of these methods have an unsatisfactory performance below the μg/l level for environmental and biological samples. Morin (3,5,7,2′,4′-pentahydroxyflavone) can selectively form highly colored and fluorescent complexes with Al, and has long been used for analysis of Al [8–10]. Hollman et al. [11] applied aluminum nitrate as a post-column reagent in RP-HPLC with fluorescence detection to determine flavonols including quercetin (3,5,7,3′,4′-pentahydroxyflavone), morin, and the like, in biological fluids. The comparative studies showed that the Al–morin complex had the strongest fluorescence intensity. Thus we thought that utilizing a pre-column reaction with morin would provide a

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00359-5

good procedure for trace Al determination by HPLC with fluorescence detection.

2. Experimental

2.1. Apparatus

HPLC was carried out on a Waters Alliance 2695 Separations Module (Waters, Milford, MA, USA). The detection system consisted of a Waters 996 UV–Vis photodiode-array detection (DAD) system in series with a Varian Fluorichrom fluorescence detector (Varian, Walnut Creek, CA, USA). The separation was controlled and the signal acquired from DAD was recorded by a Waters Millinium^{32} chromatography manager system. The signal from the fluorescence detector was recorded on a JS-3030 chromatographic working station (Johnsson Separation Science & Technology, Dalian, China). Four ODS (C_{18}) columns were studied: (a) Spherisorb ODS 2, 5 mm, 150×4.6-mm I.D. (Johnsson), (b) Nova-Pak C_{18}, 4 mm, 150×3.9-mm I.D. (Waters), (c) Kromasil C_{18}, 5 mm, 150 mm×4.0-mm I.D. (Huaiyin Institute for Fine Chemical Engineering, Huaiyin, China), and (d) LiChrosorb RP-18, 5 mm, 150×4.0-mm I.D. (Shanghai Institute of Materia Medica, The Chinese Academy of Sciences, Shanghai, China). A Mettler Toledo 320 pH meter equipped with a HA405-K2/120 combination electrode (Mettler-Toledo Instruments Shanghai, Shanghai, China) was used for pH measurement. All glassware and high-density polyethylene containers were carefully treated with 2.0 *M* nitric acid (guaranteed reagent) and rinsed with water (>18 MΩ·cm).

2.2. Reagents and chemicals

Morin (guaranteed reagent) was provided by the Kunming Branch Institute of Botany, Chinese Academy of Sciences, Kunming, China. Quercetin (biochemical reagent) was purchased from Shanghai Second Reagent Factory (Shanghai, China). Methanol (HPLC) was obtained from Hanbang Science & Technology (Huaiyin, China). All other reagents were of analytical grade unless stated otherwise.

2.3. General procedure

An aliquot of solution containing a suitable amount of Al was placed in a 25-ml volumetric flask; 1.25 ml of 1.0 *M* ammonium acetate–acetic acid buffer (pH 4.5) and an appropriate volume of 1.00×10^{-3} *M* morin in methanol were added, and the solution was made up to the mark with methanol or water so that the volume of methanol was 10 ml. The content of methanol was kept 40% (v/v) in order to maintain enough solubility of morin and its Al chelates. After sonication for 3 min, 10 ml of the solution were injected onto the Spherisorb 2 column. The typical mobile phase was methanol–water (30:70) adjusted to pH 1.0 with perchloric acid (guaranteed reagent). The other parameters were as follows: flow-rate, 1.0 ml/min; column temperature, 30 °C; wavelength for fluorescence detection, 418 nm (excitation) and 490 nm (emission). The analysis time including pre-column reaction and chromatographic measurement was less than 10 min. The blank test was necessary to surmount the possible Al at trace level in the reaction medium including chelating agents and acetate buffer, etc. Determinations of Al concentrations in samples were carried out by a calibration curve method using the peak height.

3. Results and discussion

3.1. Chromatogram of Al–morin complexes

Fig. 1a shows a typical chromatogram of Al–morin by fluorescence detection. In order to clearly describe the chromatographic behavior of the complexes and to develop the optimal HPLC conditions, UV–Vis detection was achieved as well. As can be seen from the chromatogram at 415 nm, two peaks appeared, indicating that two complex species formed. The fluorescence detection is superior in sensitivity and selectivity to the UV–Vis detection.

3.2. Optimization of pre-column reaction

We added different amounts of morin to a solution with a fixed Al concentration (1.0×10^{-5} *M*). The results showed that when the molar proportion of

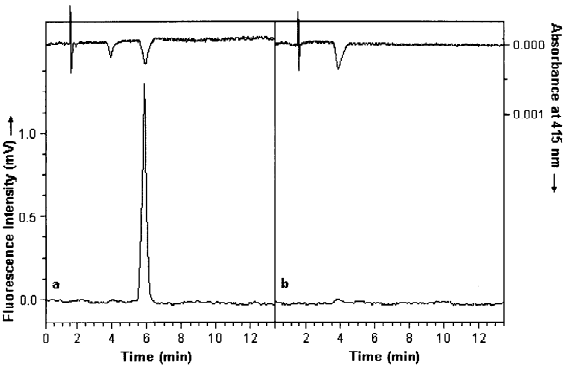

Fig. 1. Typical chromatograms of Al–morin (a) and Al–quercetin (b) chelates with fluorescence (λ_{ex} 418 nm, λ_{em} 490 nm) and UV detection (λ 415 nm). Column: Spherisorb ODS 2, 5 μm, 15-cm× 4.6-mm I.D. Column temperature, 30 °C; mobile phase, methanol–water (30:70) adjusted to pH 1.0 with perchloric acid; flow rate, 1.0 ml min^{-1}; injection volume, 10 μl.

morin–Al exceeded 100:1, the peak height of the chelate (fluorescence detection) reached a stable maximum. The highest limit of the molar ratio was not tested, but the peak shape was still constant when the ratio was up to 2000:1. This is because Al–morin was detected after being separated from excess ligand that may affect the response of the complex. To investigate the stability of Al–morin, the same solution, in which Al–morin was 1.0×10^{-5} *M*:200×10^{-5} *M*, was injected onto the column 20 times. The total time was 20 h. There was little difference in the peak heights of these serial injections in both UV–Vis and fluorescence detection. So it is concluded that chelation had completed in the general procedure.

It is well known that acetate buffer is suitable for the formation of Al–morin, and has been frequently used as reaction medium for the determination of Al by spectrophotometry and fluorimetry. So ammonium acetate–acetic acid buffer was chosen as the medium in the present work. The pH ranging from 3.5 to 6.5 and concentration ranging from 0.01 to 0.10 *M* did not give significant differences in the chromatographic behavior of Al–morin. Without buffer, however, the peak height of Al–morin became obviously smaller. In addition, when adding other buffers to the reaction medium, the most satisfactory peaks from fluorescence and UV–Vis detection appear to be given by the acetate buffer. Inversely, very poor chromatograms were obtained if perchloric acid (pH 1.0) was added probably due to no buffering effect. Potassium dihydrogenphosphate (0.025 *M*, pH 2.4) made even little peaks observable. This is because phosphate has a stronger ability to bind Al, so it results in a ligand-exchange reaction of Al complexes of organic ligands [12]. Finally, a 0.05 *M* ammonium acetate buffer of pH 4.5 was found to be favorable for Al–morin complex formation.

3.3. Conditions for separation

Different RP columns were tested under a variety of eluting conditions. An example was given with methanol–water (pH 1.0 with perchloric acid, 30:70) mobile phase and UV–Vis detection. With the Spherisorb 2 column, two Al–morin complexes would show the sharpest peak shapes and be best resolved chromatographically. Increasing the methanol content in the eluent caused an increase in detector response and a decrease in the retention of the Al–morin complexes. When the methanol content was over 40%, the separation of the two Al complexes was unsatisfactory. If the methanol content was lower than 20%, the peak widths of these two species were seriously increased and the peak shape changed randomly owing to the low solubility in the aqueous mobile phase. The perfect separation was obtained when the methanol content was 30%. Phosphate buffer, acetate buffer, and perchloric acid as the water phase portion of the eluent were studied. The peaks of the Al–morin complexes could be obtained only if the water phase was dilute perchloric acid. Another reason that perchlorate was chosen was an extremely low complexation power to Al [6]. The influence of the mobile phase pH on the separation was investigated (Fig. 2). The peaks were disrupted drastically when the pH was 2.0. Even no peak could be observed when the pH was up to 2.5. At lower pH (<1.5), the peak heights of Al–morin complexes were higher than those at higher pH values. This is most likely caused by the polyhydroxy groups of the complexes. A higher acidity could suppress their dissociation on the column. To investigate the effect of ion strength in the mobile phase on separation, an additional 0.025 *M* perchlorate was added as potassium perchlorate to perchloric acid

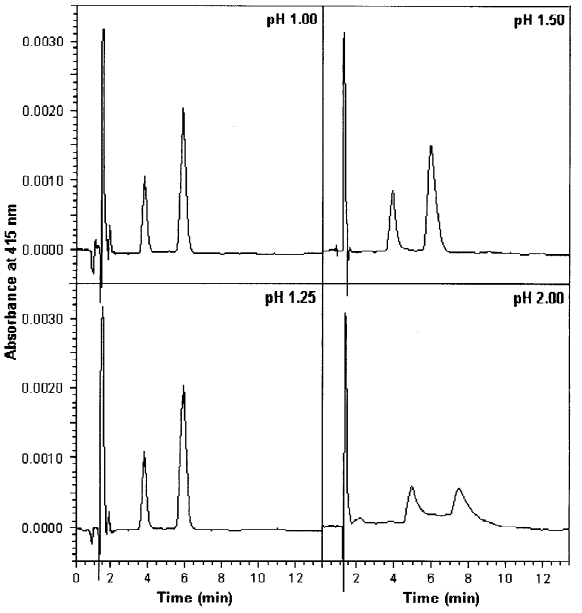

Fig. 2. Chromatograms of Al–morin chelates under different pH of mobile phase. Other conditions for HPLC were the same as in Fig. 1.

solution (pH 1.0). We did not find a distinct improvement in the ultimate peak shape.

3.4. Calibration graph and limit of detection

The peak height of the Al–morin complex was directly proportional to the Al concentration in the range from 6×10^{-9} to 6×10^{-5} *M*. The regression equation was $h(\text{cm})=0.9870+11.56C_{\text{Al}}$ with a correlation coefficient of 0.9987. The RSDs of peak height for six replicate determinations of 1.0×10^{-6} and 1.0×10^{-8} *M* Al were 1.2 and 1.8%, respectively. The detection limit, defined as the concentration where the peak height was three times the background was 2×10^{-9} *M* (5×10^{-13} g in the injection volume). This linear range and this detection limit were the widest and the lowest, respectively, found so far for the Al–morin system among various techniques for determining Al.

3.5. Analytical applications

The effects of foreign ions showed that more than 1000-fold excesses of Ca^{2+}, Co^{2+}, K^{+}, Na^{+}, Ni^{2+}, Zn^{2+}, MoO_4^{2-} or SiO_3^{2-} did not interfere. However, 1000-fold of Pb^{2+} or Sr^{2+}, 300-fold of Y^{3+} or Zr^{4+}, 200-fold of Mg^{2+}, 100-fold of Cd^{2+}, Fe^{3+} or Mn^{2+} interfered. Ga^{3+} and In^{3+} generated a significant interference (10-fold), but fortunately these ions are scarcely present in normal samples. Among common ions, the trouble resulted only from Cu^{2+} and Cr^{3+} may be relieved when the ion-to-Al molar ratio decreases below 50:1, which is greatly higher than the normal level of environmental and biological samples. Possible severe interference from F^{-} could be removed by acid digestion. The proposed method was applied to the determination of Al in some real samples. The results are given in Table 1 and in good agreement with those obtained by inductively coupled plasma atomic emission spectrometry (ICP-AES). The recovery was in the range 96.2–119.4%.

3.6. Formation of Al complexes of morin

Fig. 3a shows chromatograms relative to a synthetic solution of Al–morin generated at 350 nm by UV–Vis detection. As can be seen, besides the unreacted morin peak appeared at 54 min, there are two peaks emerged at the time of 4.0 and 6.0 min. The maximum absorption wavelengths from DAD spectra (Fig. 3a, insert) corresponding to these two

Table 1
Determination of Al in real samples[a]

Sample	Determined	RSD (%)	ICP-AES
Lebaishi water	1.02×10^{-6} *M*	5.3	1.11×10^{-6} *M*
Wahaha water	5.30×10^{-7} *M*	6.4	5.56×10^{-7} *M*
Tea infusion	5.55×10^{-5} *M*	11.7	5.18×10^{-5} *M*
Fetal bovine serum	7.75×10^{-5} *M*	4.3	7.41×10^{-5} *M*
Human hair	11.3×10^{-6} g/g	3.7	12.2×10^{-6} g/g

[a] Fluorescence detection method.

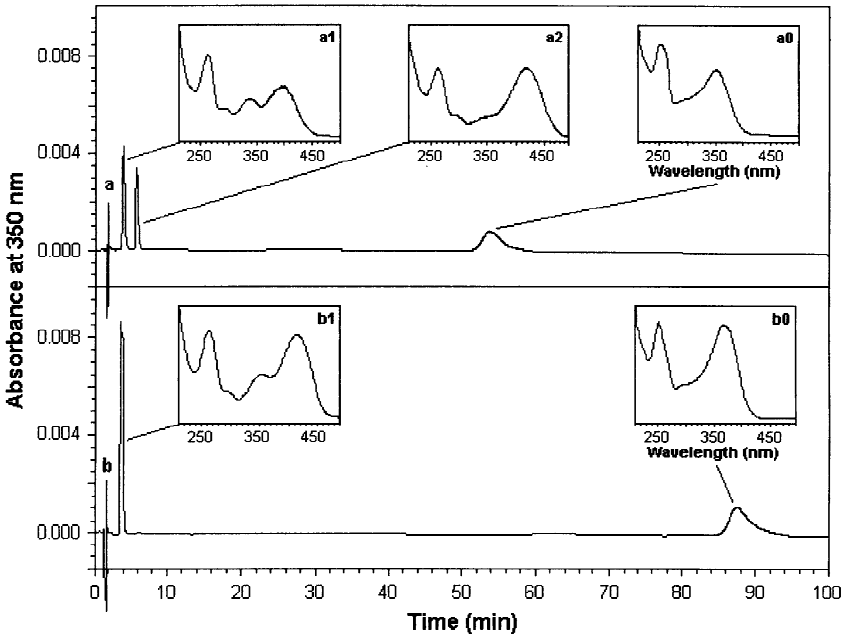

Fig. 3. Chromatograms for (a) Al–morin (3.0×10^{-4} Al:1.0×10^{-4} *M* morin) and (b) Al–quercetin (3.0×10^{-4} Al:1.0×10^{-4} *M* quercetin) with UV detection at 350 nm. Conditions for HPLC were the same as in Fig. 1. Inserts show DAD spectra for the chelates and unreactive ligands.

chromatographic peaks are 395 and 415 nm, respectively. Addition of Al to morin caused a reduction in the intensity of band I (355 nm), and growth of two new spectral peaks with appreciable bathochromic shift [8], which further verified the above conclusion that two Al–morin species formed. For quercetin, only a new peak occurred and gave an absorption apex at 415 nm (Fig. 3b), which meant quercetin formed a single complex under these conditions. The coordination ratios of Al to morin or quercetin were analyzed by plotting chromatographic peak height (*h*) at individual wavelength versus the molar ratio of Al to ligand (Al/L). It is shown that, for the former peak in Fig. 3a, a molar-ratio plot displayed an inflection at Al/L=1 corresponding to complex Al(morin), however, for the second peak, the plot revealed an inflection at Al/L=2 indicating species Al_2(morin). The bathochromic effect of Al_2(morin) was stronger than that of Al(morin). Meanwhile, for unreacted morin, a molar-ratio plot displayed an inflection at Al/L=3:2, which was the total complexing ratio (Fig. 4a). Accordingly, Al(quercetin) was the only species formed for single inflection at Al/L=1 for both the formed complex and unreacted ligand (Fig. 4b). This complex exhibited a similar spectrum shape (Fig. 3b, insert b1) to Al(Morin) (Fig. 3a, insert a1) although with a different maximum. This is the first experiment elucidating the coordination ratios of Al to morin or quercetin by HPLC. Morin and quercetin possess two possible chelating sites, 3-hydroxy-4-oxo and 5-hydroxy-4-oxo systems. Other authors [13,14] have indicated that Al is dominantly bond to 3-OH (Fig. 5a,b, aqua ligands are not shown) by its partly aromatic character, although the flavones containing only a free 5-OH form 1:1 complexes with Al. For quercetin, such sole 1:1 complex was formed because there is no evidence that 3′,4′-dihydroxyflavone forms a complex in acid solution [15,16]. For morin, however, an additional 2:1 complex is formed as presumably schemed in Fig. 5c. The 3,2′-dihydroxy system in morin is a potential chelating site, which is so placed that a seven-membered chelate ring is formed on binding Al ion. An Al ion bridges 2′- and 3-hydroxy groups rather than 4-keto- and 3-hydroxy groups, and makes another Al ion binding with

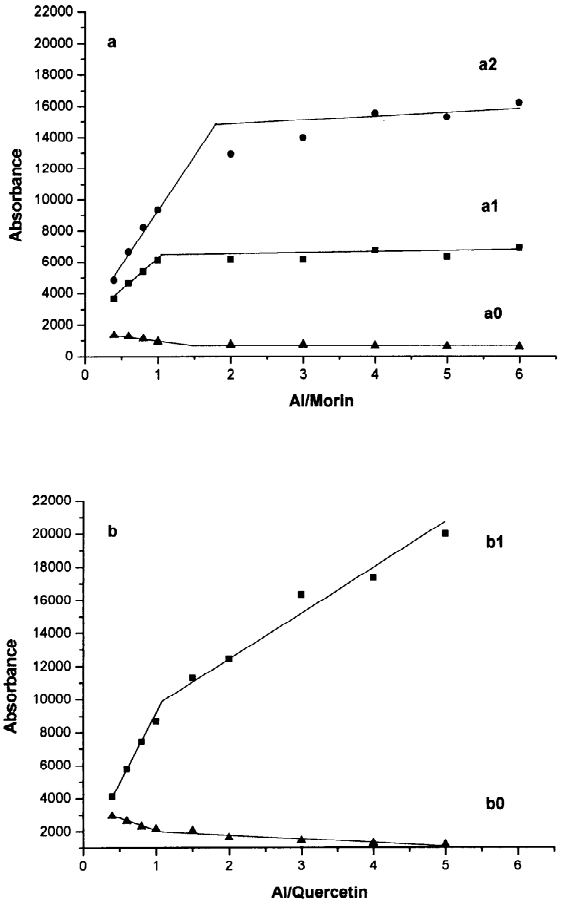

Fig. 4. Molar-ratio plots for the reaction of 1.0×10^{-4} *M* morin (a) and quercetin (b) with Al. Plot of the data at 355 nm (a0), 395 nm (a1), 415 nm (a2), 365 nm (b1) and 415 nm (b2). Conditions for HPLC as in Fig. 1.

5-hydroxy-4-oxo system possible. For this reason, a condensed ring coordination complex consisting of two benzenes and three heteroatom rings appears. The only difference between morin and quercetin is the 2′-OH. If this hydroxy group were not involved in complexation, quercetin would form 2:1 chelate with Al like morin. It can be seen from Fig. 1 that the peak of Al_2(morin) is much higher than that of Al(morin), indicating that Al_2(morin) makes a dominant contribution towards the fluorescent intensity of Al–morin presumably owing to the much wider electron conjugation. In contrast, very little peak of Al(quercetin) appeared because of no 2:1 complex formation (Fig. 1b).

Acknowledgements

This project is supported by the National Natural Science Foundation of China (No.20075011), Research Fund of the State Education Ministry of China for Young Teachers in Key Universities, and Analysis & Test Fund of Center of Materials Analysis, Nanjing University. We are extremely grateful to Professor Hong-yuan Chen and Xiao-zeng You for their encouragement and help.

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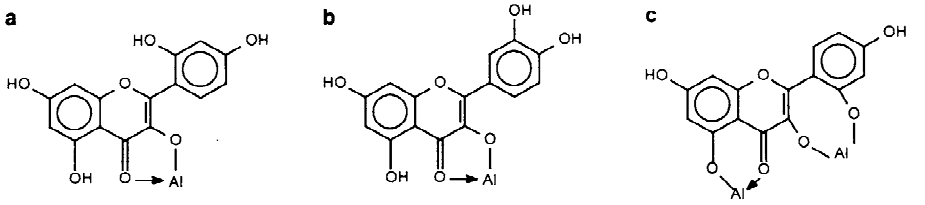

Fig. 5. Possible structures for Al–morin and Al–quercetin chelates. (a) Al(morin), (b) Al(quercetin), (c) Al_2(morin).

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