

# Investigating matrix interference in the pharmacopeial limit test for aluminum in citric acid: a re-examination, for revision of the method

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## Abstract

In many pharmacopoeias, the limit test used for determining the level of aluminum in citric acid labeled for use in the manufacture of dialysate, is based on solvent extraction using 8-hydroxyquinoline and measurement of fluorescence. However, the fluorescence intensity (F.I.) readout from the extract of citric acid samples has been found to be highly dubious, showing low value, and even lower than that of a blank solution. The aim of this work therefore was to examine what effects the matrix has on the test. The comparison of the two standard curves of aluminum solutions in water, against those prepared in citric acid solutions revealed that they differed greatly in terms of slope and  $y$ -intercept. In addition, the F.I. values on the plot of the citric acid solution were much lower than that prepared in the water. In another experiment, a decrease in the F.I. of aluminum solution was clearly seen when the co-existing concentration of citric acid was increased. The results inferred that citric acid interfered with the test due to its acidity and metal-chelating capabilities. Based on this evidence, the pharmacopeial limit test for aluminum in citric acid should be revised; otherwise, it could yield results that underestimate aluminum levels and lead to inaccurate conclusions

## Keywords

aluminum, citric acid, interference, limit test, matrix effects

## Introduction

Aluminum contamination of the solutions used in dialysis has been found to cause toxicity such as osteomalacia, anemia, and dementia, in patients suffering from chronic renal failure who undergo long-term dialysis treatment (Parkinson et al. 1981; Tsai et al. 2018). For this reason, the levels of aluminum present in substances intended for

use in the preparation of dialysate must be checked to ensure that they do not exceed the permitted limit. For anhydrous citric acid and citric acid monohydrate (the substances used in citric acid-based dialysate, e.g., Citrasate), the maximum level of aluminum permitted is 0.2 ppm, as indicated in the monographs of most pharmacopoeias, including the United States Pharmacopeia (USP) and the National Formulary (NF) 2021 (The United States Phar-

macopeial Convention 2021), the European Pharmacopoeia (Ph. Eur.) 10.4 (The European Pharmacopoeia 2021) and the British Pharmacopoeia (BP) 2021 (The British Pharmacopoeia 2021). In each of these compendia, the analysis of aluminum levels is carried out using the same semi-quantitative assay method, based on the reaction of aluminum with 8-hydroxyquinoline. Following extraction of the complex formed using chloroform, the fluorescence intensity (F.I.) of the extracts are measured. The criterion for acceptance is that the F.I. of the sample solution should not exceed that of the standard aluminum solution.

Whilst the above-mentioned test is used to analyze the levels of aluminum in various substances such as sodium chloride and calcium phosphate, suspicious results are obtained when the method is applied to citric acid. In our experience and those reported by other laboratories, F.I. values measured from citric acid samples are low, and even lower than that of a blank solution. This can lead to underestimation of the concentration of aluminum and inaccurate conclusions. If such a batch of samples are allowed to be used for the manufacture of dialysate, safety concerns would arise.

In chemical analysis, the components of a sample other than the analyte of interest (referred to as the matrix) can affect the performance and validity of a test (Thompson and Ellison 2005). In the aforementioned limit test, the matrix in the citric acid-containing sample solution is different from that in the aluminum standard solution where citric acid is absent. Moreover, at the permitted level of aluminum (0.2 ppm), citric acid exists in the sample solution at a much higher concentration than the aluminum analyte ( $>10^6$  times). In such conditions, it is hypothesized that citric acid may account for the interference, thereby producing unreliable results. To test this hypothesis, the effects of the matrix on the compendial test were re-examined in this work by constructing and comparing the standard curves of aluminum solutions without citric acid, with those in which citric acid was. If there is no matrix effect on the test, both standard curves should be identical. However, if citric acid causes the interference, the slopes and/or y-intercepts of the curves would be different (Thompson and Ellison 2005). In this work, the studies were also conducted to gain insight on how citric acid affected the test if the interference was found. The study results would further call for the revision or improvement of the compendial method.

## Materials and methods

### Materials

All chemicals were of reagent grade and were used without further purification. Citric acid samples were obtained from two different sources - Merck (Darmstadt, Germany) and BDH Chem (Poole, England). 8-Hydroxyquinoline was purchased from two manufacturers - Sigma-aldrich (St. Louis, MO) and Fluka (Munich, Germany). Chloroform (RCI Labscan, Thailand) and distilled water was used as solvents.

## Evaluation of the effects of the matrix

The effects of the matrix, which is produced as a result of the presence of citric acid, was examined by comparing the standard curves of aluminum solutions without citric acid, with those in which citric acid was present. For this purpose, aluminum standard solutions in the concentration range of 0–0.06 mg/ml, prepared using water and 0.2 g/ml citric acid solution as solvent were used. After the addition of acetate buffer (pH 6.0), the solutions were extracted with three successive portions of 0.5% w/v 8-hydroxyquinoline in chloroform, following the procedure described in the USP. The extracts from the chloroform phase were then subjected to fluorescence measurement, setting the excitation wavelength and emission wavelength at 492 and 518 nm, respectively. To obtain the F.I. data of the reagent blanks, chloroform was used to zero the instrument. The analyses were done in triplicate.

To ensure the reliability and reproducibility of the results, the analyses were carried out using citric acid obtained from two different sources, 8-hydroxyquinoline from two manufacturers, and two spectrofluorometer models i.e. RF-6000 and RF-1501 by Shimadzu (Japan). It should be noted that the instrument settings of the two spectrofluorometers were different. For RF-6000, the excitation and emission bandwidths were 3 nm and 3 nm, respectively, and the “high” sensitivity was set. For RF-1501, the excitation and emission bandwidths were 10 nm and 10 nm, respectively, and the “high” sensitivity was set. The contents of aluminum in the citric acid sourced from Merck and BDH Chem were 0.033 and 0.048 ppm, respectively, as determined by inductively coupled plasma-mass spectrometry (ICP-MS).

## Determination of aluminum by ICP-MS

The concentration of aluminum in the citric acid as well as in the aqueous phase before and after extraction was determined using an ICP-MS spectrometer (Model 7500ce, Agilent). Collision/reaction cells were adopted for removing spectral interferences in ICP-MS. The operating conditions were an ICP RF power of 1500 W, an ultrapure grade carrier (argon, 99.9995% pure) flow rate of 1.5 l/min, and a nebulizer pump speed of 0.1 rps. The signal intensities were compared to a calibration curve of aluminum which was prepared in the range of 0.5–100 mg/l. The data are presented as the average of triplicate determinations.

## Results and discussion

### Appearance and pH of the aqueous phase in different matrices

Prior to extraction, the pH of the aluminum solutions was measured. Even with the aid of a buffer added, the pH of the solution containing citric acid (pH 2.2) was very different from that in the water (pH 5.7). Once 8-hydroxyquinoline in chloroform was added and the solutions were shaken, the

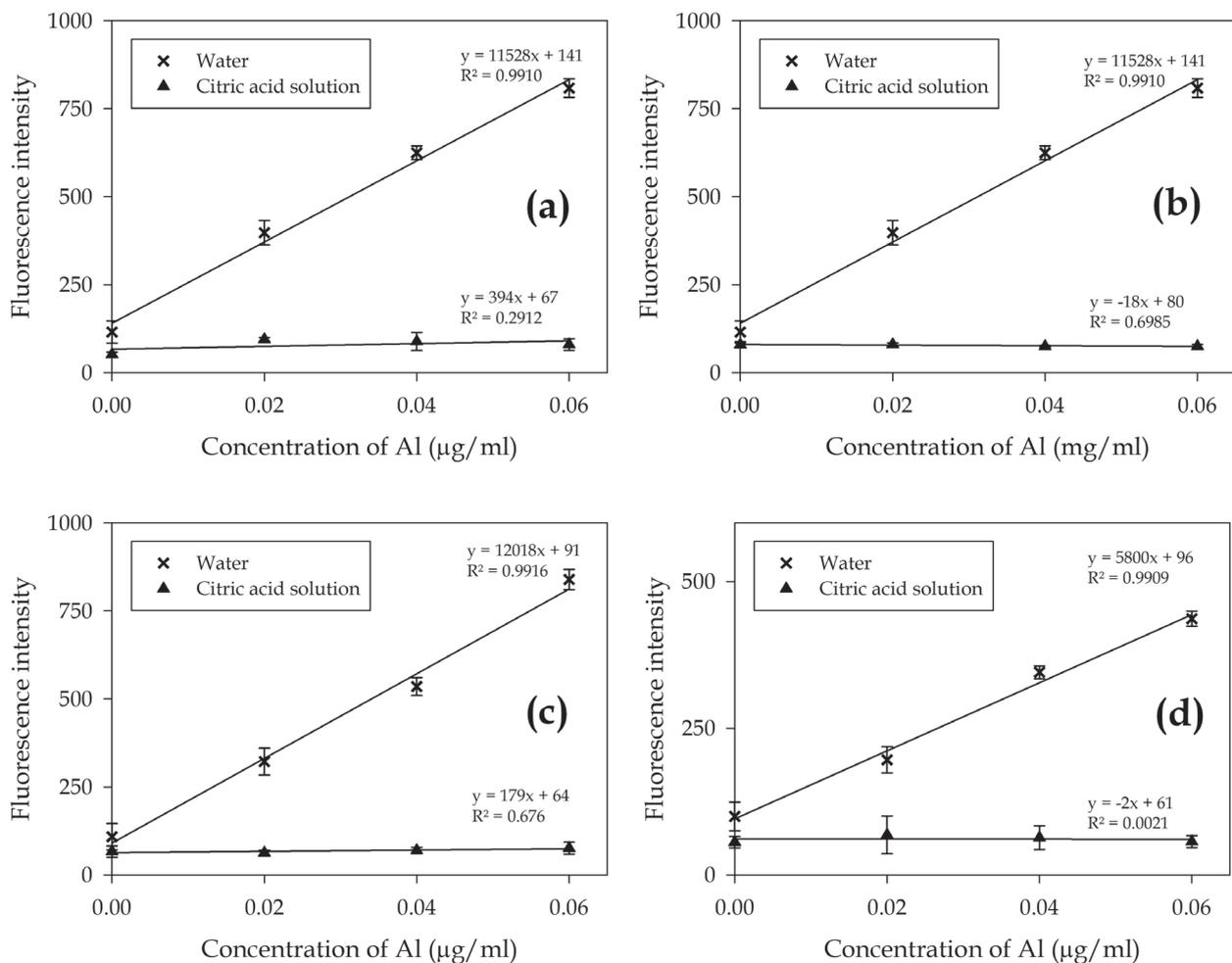
upper aqueous phase of the extraction of aluminum in the citric acid solution appeared to be more slightly yellow than that of the solution in the water. Since 8-hydroxyquinoline is insoluble in water, but soluble in chloroform and acidic aqueous medium (Marczenko and Balcerzak 2000), the low pH of the citric acid solution is likely to have promoted an increased distribution of 8-hydroxyquinoline into the aqueous phase, as seen by the more yellow color which it imparted, compared to the solution that was prepared in water.

### Effects of the matrix on the test

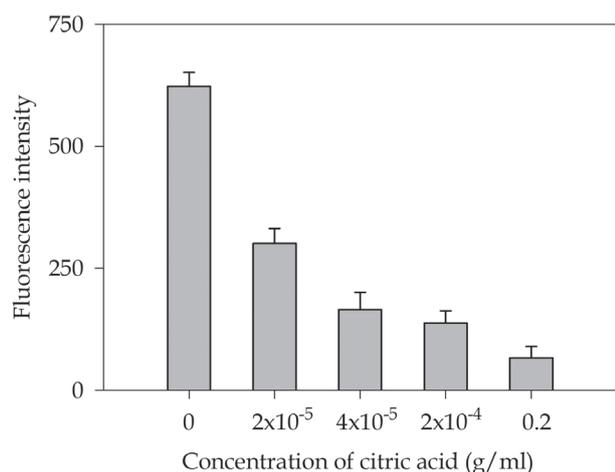
Over the range (0–0.06 mg/ml) that covered the concentration of the aluminum standard solution mentioned in the compendial method (0.04 mg/ml), the standard curves obtained from the aluminum solutions prepared in 0.2 g/ml citric acid solution and those prepared in water, differed greatly in terms of both slope and y-intercept (Figure 1), indicating the interference effects caused by citric acid. In addition, the F.I. values on the plot belonging to the citric acid solution were much lower than those of the water. Conducting the experiments using chemicals

and instruments from different manufacturers produced the same results. In another experiment, in which varying concentrations of citric acid were added to 0.04 mg/ml aluminum solutions, a decrease in the F.I. was clearly seen when the co-existing concentration of the citric acid increased (Figure 2). From these findings, it can be concluded that the sample matrix, i.e. citric acid, interfered with the test by dramatically lowering the analytical signals. Consequently, the decision of acceptance, which is made by comparing the F.I. of a sample solution with that of a standard solution, as described in the pharmacopeias, can be seen to be misleading and result in the underestimation of the aluminum content in samples.

As previously mentioned, the pH of the aluminum solution containing citric acid was much lower than that in water. Therefore, it was investigated to determine whether the interference was attributable to the acidity of citric acid. By adjusting the pH of the citric acid solution from pH 2.2 to 4.0, the F.I. increased (Figure 3). However, further increasing the pH of the citric acid solution to 5.7 did not give an equal F.I. to that of the aluminum solution in water. These results imply that while the acidity of citric



**Figure 1.** Standard curves of aluminum in water and in citric acid solutions, prepared using chemicals and instruments of different manufacturers; (a) citric acid of Merck/8-hydroxyquinoline of Sigma-aldrich/ spectrofluorometer RF-1501, (b) citric acid of BDH Chem/8-hydroxyquinoline of Sigma-aldrich/spectrofluorometer RF-1501, (c) citric acid of Merck/8-hydroxyquinoline of Fluka/spectrofluorometer RF-1501, (d) citric acid of Merck/8-hydroxyquinoline of Sigma-aldrich/spectrofluorometer RF-6000.

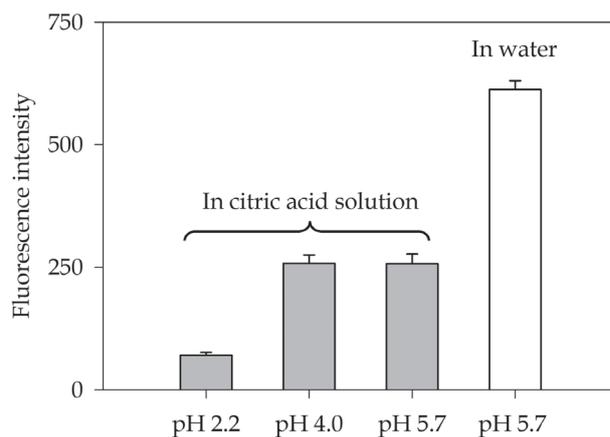


**Figure 2.** Effect of the concentration of citric acid co-existing in 0.04 mg/ml aluminum solution on the fluorescence intensity of the extract as measured using RF-6000 spectrofluorometer (Shimadzu).

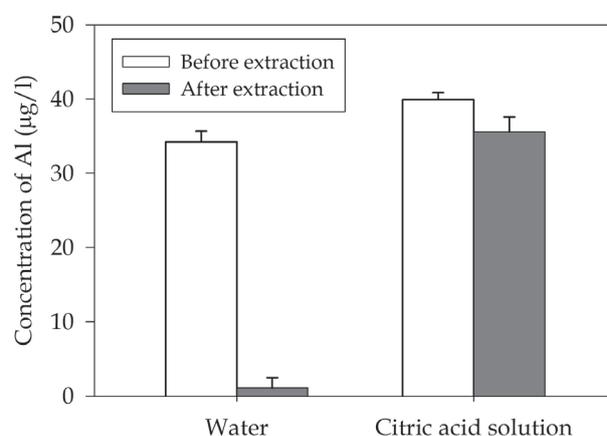
acid may affect the distribution of amphoteric 8-hydroxyquinoline and/or the extractability of the aluminum complex into chloroform which occurred to a greater extent at neutral pH (Marczenko and Balcerzak 2000), it was not the only reason for the interference.

Citric acid is capable of chelating metals including aluminum (Feng et al. 1990; Karbouj 2007; Asemave 2018). Therefore, it is possible that the citric acid which was present in the aqueous phase of the test competed with 8-hydroxyquinoline in reacting with the aluminum, resulting in the partition of aluminum species into the aqueous phase. As hypothesized, the quantitation of aluminum in the aqueous phases using ICP-MS revealed that following the extraction of aluminum in the citric acid solution, the majority of aluminum remained present in the aqueous phase (Figure 4), probably due to the formation of water-soluble complex with citric acid. In contrast, in the solution without citric acid, aluminum was almost completely extracted into the organic phase, since the complex of aluminum and 8-hydroxyquinoline is soluble in chloroform. Based on these results, it is evidenced that citric acid causes interference in the test owing to its acidity and metal-chelating capabilities.

In the view of the pharmaceutical quality control and manufacturing, the tests of active pharmaceutical ingredients which are affected by matrix interference may cause inaccurate conclusions, and it can become a safety hazard for consumers. Consequently, the revision of the test and/or the investigation of more reliable assays which overcome the matrix effects are recommended. For this purpose, the limit test based on atomic absorption spectroscopy as described in some pharmacopeias for the determination of aluminum in certain raw materials e.g. potassium chloride, sodium acetate and sodium carbonate (The United States Pharmacopeial Convention 2021), or those reported in some research articles (Woolfson and Gracey 1988; Sharma et al. 2015) may be used. Alternatively, selective and sensitive assays relying on atomic emission spectroscopy such as ICP-MS (Li et al. 2017) as well as spectrop-



**Figure 3.** Effect of pH of 0.04 mg/ml aluminum solution prepared in 0.2 g/ml citric acid solution, on the fluorescence intensity of the extract, compared with that of a solution prepared in water. The measurements were done using RF-6000 spectrofluorometer (Shimadzu).



**Figure 4.** Concentration of aluminum in the aqueous phase in the extraction of 0.04 mg/ml aluminum solution prepared in water and in the solution containing 0.2 g/ml citric acid. Phase.

hotometry (Ahmed and Hossan 1995; Kamino et al. 2005) and fluorimetry (Raggi et al. 1999; Hossain et al. 2020) previously proposed for the determination of aluminum in pharmaceutical and biological samples may be interesting choices. Prior to use for the replacement of the current compendial test, the method validation and investigation of matrix interference caused by citric acid must be carried out to ensure that reliable results will be obtained.

## Conclusion

In the compendial limit test of aluminum in citric acid, which relies on the extraction of aluminum as a complex with 8-hydroxyquinoline into the organic phase, the sample solution is dramatically affected by the matrix i.e. citric acid. From the experiments carried out, it was evidenced that the more acidic condition of a citric acid sample solution favored the dissociation and distribution of 8-hydroxyquinoline into the aqueous phase. In addition, citric

acid was found to compete with 8-hydroxyquinoline in chelating with aluminum, thus lowering the concentration of the complex of 8-hydroxyquinoline and aluminum in the chloroform. This interference resulted in lower F.I values of the citric acid sample, than that of the standard solution when they were compared. Since this occurrence can cause inaccurate conclusions, it is recommended that this compendial test should be revised or a new method should be investigated for the replacement of the current method, in order to ensure the safety of patients.

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## Conflict of interest statement

The authors declared no conflict of interest.

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