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Use of an amino acid in the mobile phase for the determination of ascorbic acid in food by high-performance liquid chromatography with electrochemical detection

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Abstract

The possibility of using monosodium L-glutamate (MSG) (20 m*M* MSG, pH 2.1) in the mobile phase for the determination of ascorbic acid (AA) in foods by high-performance liquid chromatography (HPLC) with electrochemical detection was examined. The hydrodynamic voltammogram of AA and the background current were also examined. The applied potential was set at 400 mV versus an Ag/AgCl reference electrode. It was demonstrated that MSG was a useful mobile phase for the determination of AA in foods. This paper also examines the stability of AA under various conditions in order to optimize HPLC conditions and the pre-run sample stabilization. The proposed method is simple, rapid (analysis time: ~6 min), sensitive (detection limit: ~0.1 ng per injection (5 μl) at a signal-to-noise ratio of 3), highly selective and reproducible (relative standard deviation: ~2.5%, $n=7$). The calibration graph of AA was linear in the range 0.1–50 ng per injection (5 μl). Recovery of AA was over 90% by the standard addition method.

Keywords: Mobile phase composition; Stability studies; Food analysis; Fruit juices; Ascorbic acid; Monosodium glutamate; Amino acids; Vitamins

1. Introduction

High-performance liquid chromatography (HPLC) with electrochemical detection (ED) is valuable for the highly selective and sensitive analysis of trace amounts of compounds in complex matrices. Usually a mobile phase containing inorganic salts such as sodium or potassium phosphate buffers with ethylenediaminetetraacetic acid disodium dihydrate (EDTA·2Na·2H_2O), which chelate with metal ions, is used for the determination of ascorbic acid (AA) in foods and biological fluids by HPLC–ED [1–12].

AA is easily oxidized in the presence of certain reagents (*e* halogens, hydrogen peroxide, heavy metal ions, especially Cu^{2+}, Fe^{3+}) and an alkaline pH [13]. It is known that Cu^{2+} oxidizes AA more strongly than Fe^{3+}. EDTA·–Cu chelate prevents Cu^{2+} from oxidising AA. However, an EDTA·–Fe complex oxidizes AA more strongly than Fe^{3+} alone. It is not always true that the addition of EDTA·2Na·2H_2O is effective.

Organic acid salts such as monosodium L-glutamate (MSG), sodium guanosine-5′-monophosphate and disodium succinate, which chelate with metal ions, have not generally been used in the mobile phase for the analysis of AA in foods by HPLC–ED.

It is crucial to optimize HPLC conditions and sample preparations for the pre-run sample stabilization to ensure that subsequent HPLC analysis is effective. Ref. [14] dealt with the relationship be-

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tween AA peak height and the number of repeat injections of standard AA solution to optimize the HPLC–ED conditions. We have injected the AA solution five times for the complete effect, prior to ordinary running.

Amino acids and peptides [15,16] have been used as column packings in gas chromatography for the separation of organic compounds. In HPLC [9,10,17–20], amino acids have been also used as internal standards for the determination of AA and amino acids in human plasma, reduction reagent for dehydroasorbic acid in foods and pre-analysis AA sample stabilization in real food samples.

AA is unstable under various conditions. However, its stability under the conditions tested to optimize HPLC, such as column temperature, pH of the mobile phase and injection volume, and the pre-run sample stabilization has not been examined carefully.

For the above reasons, this paper deals with the possibility of using MSG (20 m*M* MSG, pH 2.1), which is used for the umami substance in the cooking and chelates with metal ions [21,22], in the mobile phase for the determination of AA in foods by HPLC–ED set at 400 mV versus an Ag/AgCl reference electrode. To optimize HPLC conditions and sample preparation, this paper also deals with the stability of AA under various conditions and hydrodynamic voltammogram of AA and background current. In addition, we demonstrate the validity of the determination of AA in foods by HPLC–ED using MSG in a new mobile phase.

2. Experimental

2.1. Reagents and materials

The AA used in this study was purchased form Wako (Osaka, Japan). MSG was obtained from Ajinomoto (Tokyo, Japan). Other reagents were all of analytical grade. Nutrients, health foods and juices were commercially available. The membrane filters (HLC-Disk 25, 0.45 μm, polyvinyldifluoride) were purchased from Kanto Kagaku (Tokyo). Light-resistant brown volumetric flasks and glassware were used [13,23,24]. The volumetric flasks and other glassware were washed with tap water followed by a through rinsing with deionized water to eliminate cations, anions and residual chlorine [11].

2.2. Standard AA preparation

Standard AA (1–10 μg/ml) diluted with the mobile phase was freshly prepared in a brown volumetric flask prior to use. At the beginning of the AA analysis, standard AA (400 ng/ml) was injected into the HPLC system five times to obtain the optimized AA peak height [14].

2.3. Sample preparation

After samples were first diluted with the mobile phase to an estimated AA concentration of 1–10 μg/ml in a brown volumetric flask, this solution was filtered by a membrane filter (0.45 μm) and the filtrate (over 1 ml) was used for the determination of AA.

2.4. Apparatus and conditions

A model 655 A-11 high-performance liquid chromatograph (Hitachi, Tokyo) equipped with a Model ED 623 electrochemical detector (working electrode; glassy carbon, GL Science, Tokyo) was used. The applied potential was set at 400 mV versus an Ag/AgCl reference electrode. The samples were applied using a Rheodyne Model 7125 sample loop injector with an effective volume of 5 μl. HPLC was carried out on a 15×0.46 cm I.D. reversed-phase column Inertsil ODS-3 (5 μm) (1×0.46 cm I.D.) using 20 m*M* MSG (pH 2.1, adjusted with phosphoric acid) as the mobile phase. The flow-rate was 0.8 ml/min at room temperature.

3. Results and discussion

3.1. Stability of AA under various conditions

To optimize HPLC conditions and pre-run sample stabilization, the stability of AA was examined under various conditions. An evaluation of the stability of AA was made by comparing peak height ratio of a standard AA which was analyzed periodically by HPLC–ED.

3.1.1. Effect of pH values on the stability of standard AA

Firstly, we examined the stability (%) of AA (2.5 μ*M*) stored in brown flask, which was diluted with buffers of 50 m*M* potassium phosphate (pH 3–8.5) at both 25°C (room temperature) and 40°C (HPLC column temperature) after 1 h (Table 1). A desirable buffer keeps AA stable as long as possible. AA is known to be unstable in an alkaline pH solution. From the results in Table 1, it appears that the optimum conditions are an acidic mobile phase (pH 2.1) and a HPLC column temperature of 25°C, although analysis time of AA was ~6 min (see Fig. 7).

3.1.2. Effect of light on the stability of standard AA

Secondly, we examined the effect of daylight and UV light (265 nm) on the stability (%) of AA to optimize the choice of glassware for standard AA preparation and sample preparation. We examined the effect of daylight on the stability of standard AA aqueous solutions at pH 6 value, because most pH value in food sample aqueous solutions was around 6 except orange and tangerine juices. Ideally, the glassware must keep AA stable as long as possible. Periodic changes of stored AA in both a brown flask and a transparent flask, and AA in a free-plug transparent flask, which was exposed to UV light, at room temperature are shown in Fig. 1.

This demonstrates that AA was affected by daylight and UV light. After 1 h, the stabilities of AA stored under UV light, in a transparent flask and in a brown flask were 79.7, 84.2 and 95.6%, respectively. Thus, AA stored in a brown flask was most stable. From the above results, both standard AA and AA in sample aqueous solutions must be stored in a brown flask to optimize the analysis of AA.

Table 1
Effect of pH values on the stability of standard AA stored in brown flask at 25°C and 40°C after 1 h storage[a]

Temperature (°C)	pH					
	3	4.5	6.0	6.8	7.4	8.5
25	99.5	98.2	95.6	84.5	12.7	7.4
40	94.1	72.7	70.9	45.1	0	0

[a] Standard AA concentration, 2.5 μ*M*; buffer, 500 m*M* potassium phosphate.

3.1.3. Effect of temperature on the stability of standard AA

Thirdly, we also examined the effect of temperature on the stability of AA (Fig. 2). Periodic changes of AA stored in brown flask at pH 6 were tested at temperatures (25 to 80°C) to optimize the sample preparation and HPLC column conditions. The AA was stable for 1 h at 25°C. The HPLC column temperature must be kept low for the optimization of AA analytical data since AA is sensitive to temperature.

3.1.4. Effect of AA concentration on the stability of standard AA

Forthly, we studied the effect of AA concentration on the stability of AA (Fig. 3). A higher AA concentration gives greater stability of AA. HPLC–ED is highly selective and highly sensitive for the analysis of AA. There is a pitfall for the sample preparation. Although ED is excellent for AA analysis, lower AA concentration analyses do not always give good results, because the diluted AA in sample solution is not stable due to dissolved oxygen. Furthermore, the successive dilution procedure is tedious and time-consuming in routine work.

AA is more stable at higher concentrations in the sample solution; previously, we used a 20-μl loop for sample injection; the full loop fill injection method (20 μl) was employed. The sample was diluted a couple of times prior to injection in order to obtain an appropriate sensitivity. This dilution procedure was time-consuming and degraded the AA. The comparison of a 20- and a 5-μl injector shows that AA in a 5-fold concentrated sample could be analyzed by using a 5-μl injector which has the advantages of simpler sample preparation and increased stability of AA. Therefore, a 5-μl injector was used in this study.

3.1.5. Effect of metal ions on the stability of standard AA

Fifthly, we examined the effect of Cu^{2+} and Fe^{3+} on the stability of standard AA. The recoveries of AA diluted in mobile phases (pH 2.1 and 3) at 0, 30 and 60 min are shown in Table 2. The results

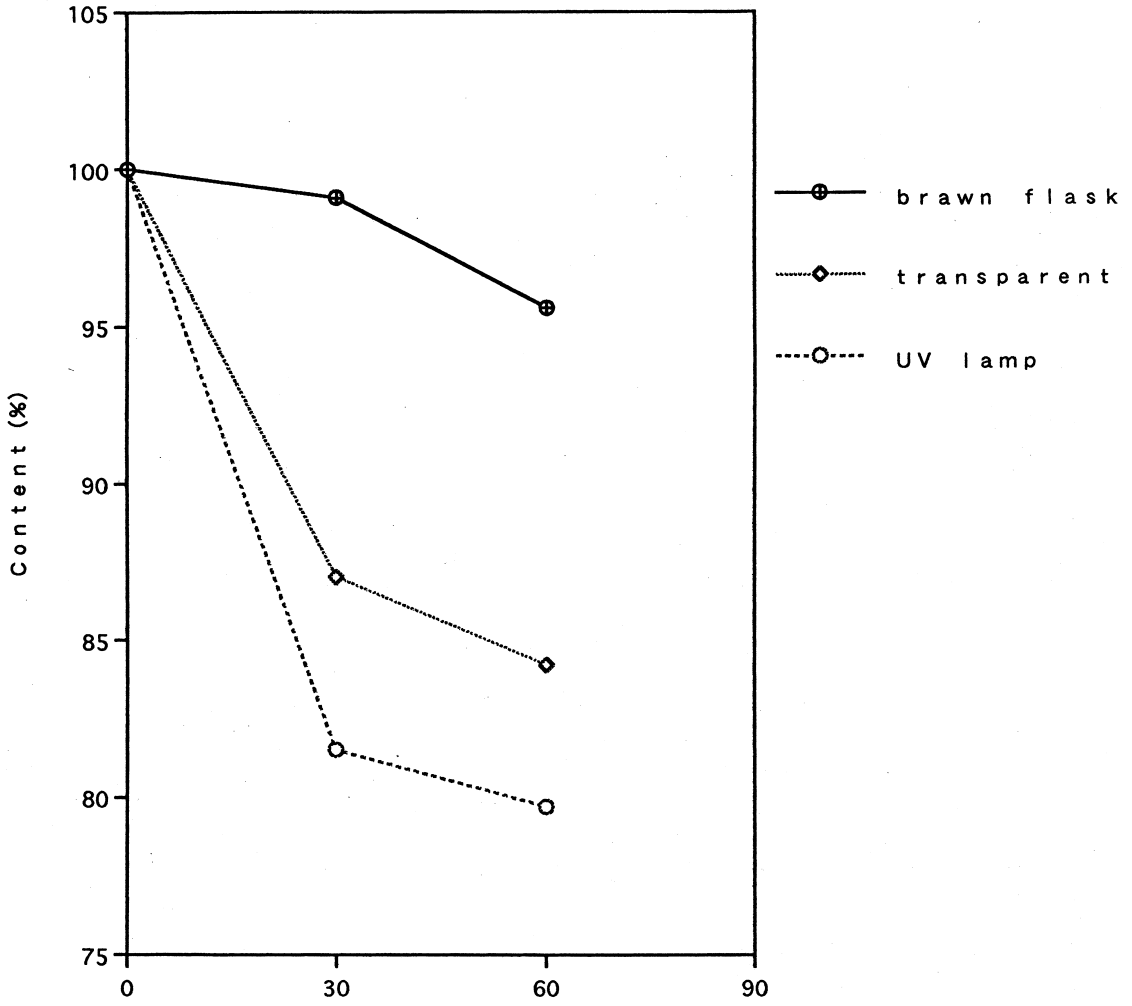

Fig. 1. Effect of daylight and UV light (265 nm) on the stability of standard AA at room temperature, standard AA concentration; 2.5 μ*M* buffer; 50 m*M* potassium phosphate (pH 6).

demonstrate that AA in the presence of Cu^{2+}, Fe^{3+}, EDTA–Cu, EDTA–Fe, MSG–Cu and MSG–Fe chelates is not oxidized under these conditions. Therefore, the proposed sample and standard AA preparation — dilution with the mobile phase (pH 2.1) — is suitable, because the metal ions and EDTA compounds do not interfere with the stability of AA.

In summary, daylight, pH value, temperature and AA concentration affected the stability of AA. To optimise the HPLC conditions, the AA in food samples must be prepared in acidic conditions and stored in a brown flask at lower temperature. The results are also useful for the optimization of sample preparation before HPLC AA analysis. AA in food samples must be prepared in acidic conditions and stored in a brown flask at lower temperature.

3.2. Effect of MSG concentration on the retention time and AA peak height

At the beginning of the work, the effect of the concentration of MSG on the retention time and AA peak height was examined for the suitable mobile phase concentration. Table 3 demonstrates that MSG concentration (15–50 m*M*) gave the almost same retention time (~4.7 min) and AA peak height (~7

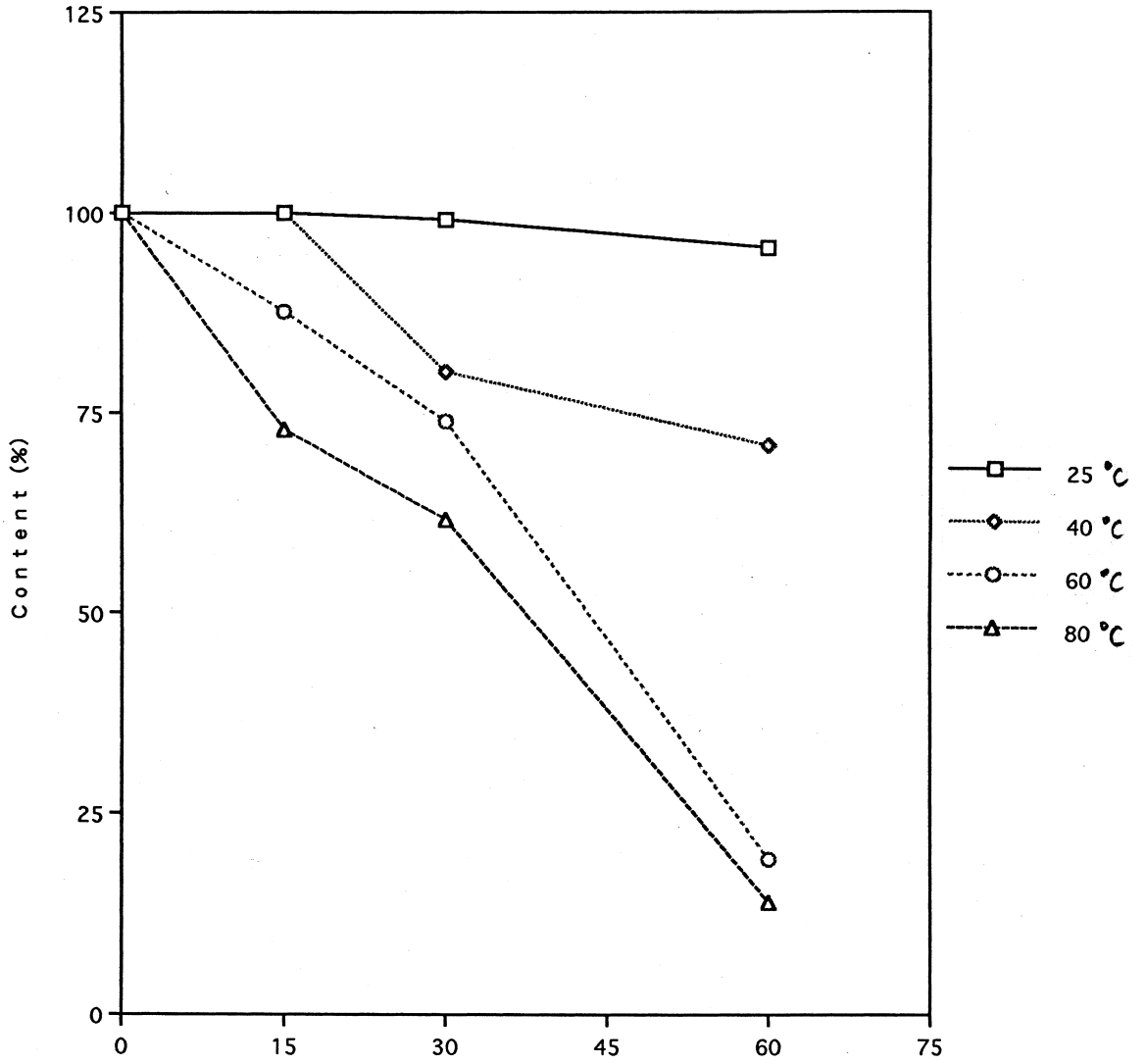

Fig. 2. Effect of temperature on the stability of standard AA stored in brown flask, standard AA concentration; 2.5 μ*M* buffer; 50 m*M* potassium phosphate (pH 6).

cm). In the present paper, 20 m*M* MSG was employed.

3.3. Hydrodynamic voltammogram and background current

The relationship between applied potential and sensitivity of AA and electrochemical active uric acid (UA) was examined. A typical hydrodynamic voltammogram is illustrated in Fig. 4. The background current of mobile phase (20 m*M* MSG (pH 2.1) and 20 m*M* KH_2PO_4 (pH 2.1)) is shown in Fig. 5. The current (peak height) at each applied potential was divided by the current at the most possible potential to obtain the relative current ratio. This value was plotted against the applied potential. the detector gave a linear response up to +900 mV versus an Ag/AgCl reference electrode for AA.

When the applied potential was set at +900 mV versus an Ag/AgCl reference electrode, not only AA,

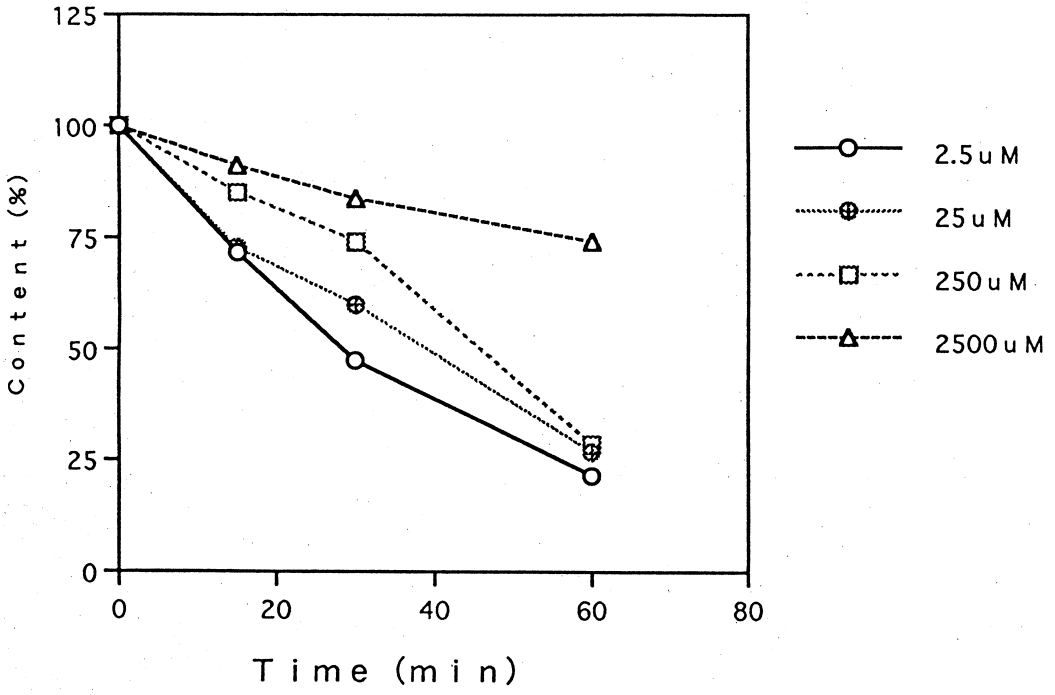

Fig. 3. Effect of AA concentration on the stability of standard AA stored in brown flask, buffer; 50 m*M* potassium phosphate (pH 6.8).

but also another electrochemically active compounds such as UA, cysteine and tyrosine [11] were detected and their dilution of sample solution was needed, because of higher sensitivity. Further dilution was not suitable for routine work because it was tedious, time-consuming and degraded the AA. Further dilution resulted in poor RSD values. Thus, the applied potential was set at lower +400 mV versus an Ag/AgCl reference electrode and smaller injection volume (5 μl) was used.

Comparison of background current (nA) of 20 m*M* KH_2PO_4 (pH 2.1) and 20 m*M* MSG (pH 2.1) indicated that both mobile phases showed the almost the same current at 400 mV and the former showed

Table 2
Effect of Cu^{2+} and Fe^{3+} on the stability of AA[a]

pH	Metal ions	AA recovery (%)		
		0 min	30 min	60 min
3	None	100	100	99.5
	Cu^{2+}	100	100	96.6
	Fe^{3+}	100	100	98.8
	EDTA–Fe	100	100	98.3
	EDTA–Cu	100	100	99.6
	MSG–Fe	100	100	99.1
	MSG–Cu	100	100	99.2
2.1	None	100	100	99.6
	Cu^{2+}	100	100	98.7
	Fe^{3+}	100	100	99.7

[a] AA; 2.5 μ*M*, metal ion; 0.5 μ*M*.

Table 3
Effect of MSG (mobile phase) concentration on the retention time and AA peak height[a]

MSG conc. (m*M*)	AA peak height (cm)	
	Retention time (min)	Peak height (cm)
15	4.71	7.1
20	4.69	6.9
25	4.71	7.2
40	4.69	6.9
50	4.70	7.2

[a] Flow rate; 0.6 ml/min.

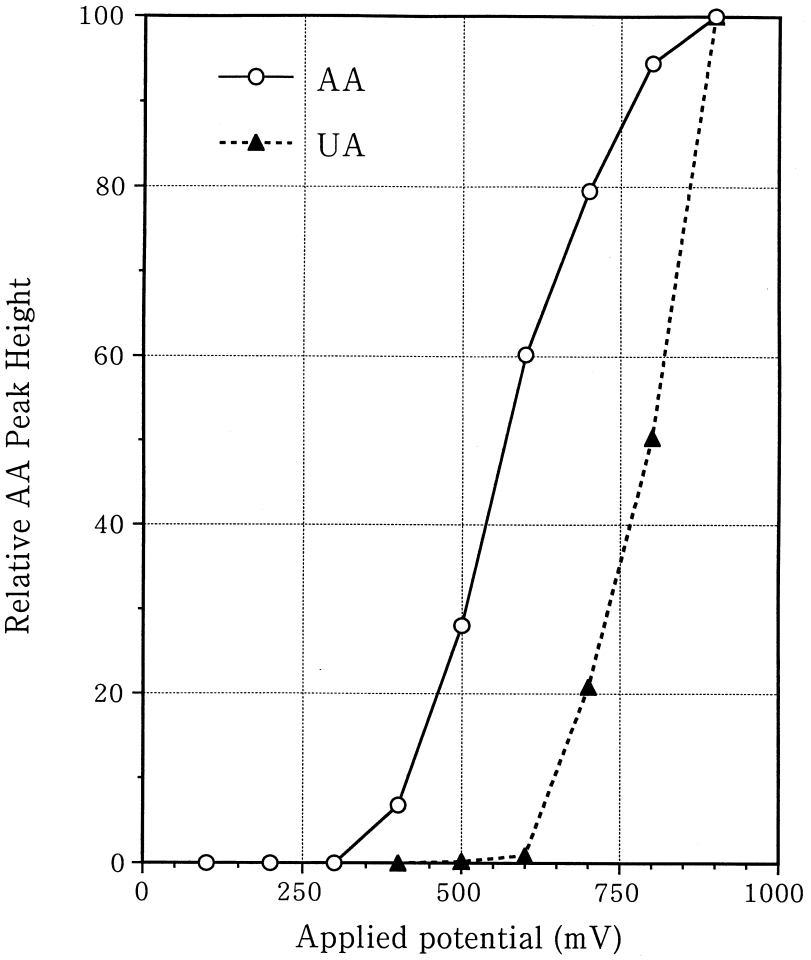

Fig. 4. Hydrodynamic voltammogram of AA and uric acid (UA).

the relatively lower current at 500–1000 mV. In the present work, MSG was the suitable mobile phase for the determination of AA at lower 400 mV.

3.4. Chromatography

The next effort was focused on the chromatograms of AA in foods. Chromatograms of AA in athlete food, nutritional supplement and orange juice diluted in mobile phase are shown in Fig. 6. The highly selective and rapid detection of AA (analysis time: about 6 min) in the presence of many kinds of compounds is possible with ED set at 400 mV versus an Ag/AgCl reference electrode. This allows analysis without the need for sample clean-up. These chromatograms demonstrate that the application of the proposed method to the determination of AA in many kinds of foods is possible. The limit of detection from Fig. 6A was ~0.1 ng per injection (5 μl) at a signal-to-noise ratio of 3:1.

3.5. Determination of AA

The calibration graph for AA was constructed by plotting the peak height of AA against the amount of AA. Satisfactory linearity was obtained in the range

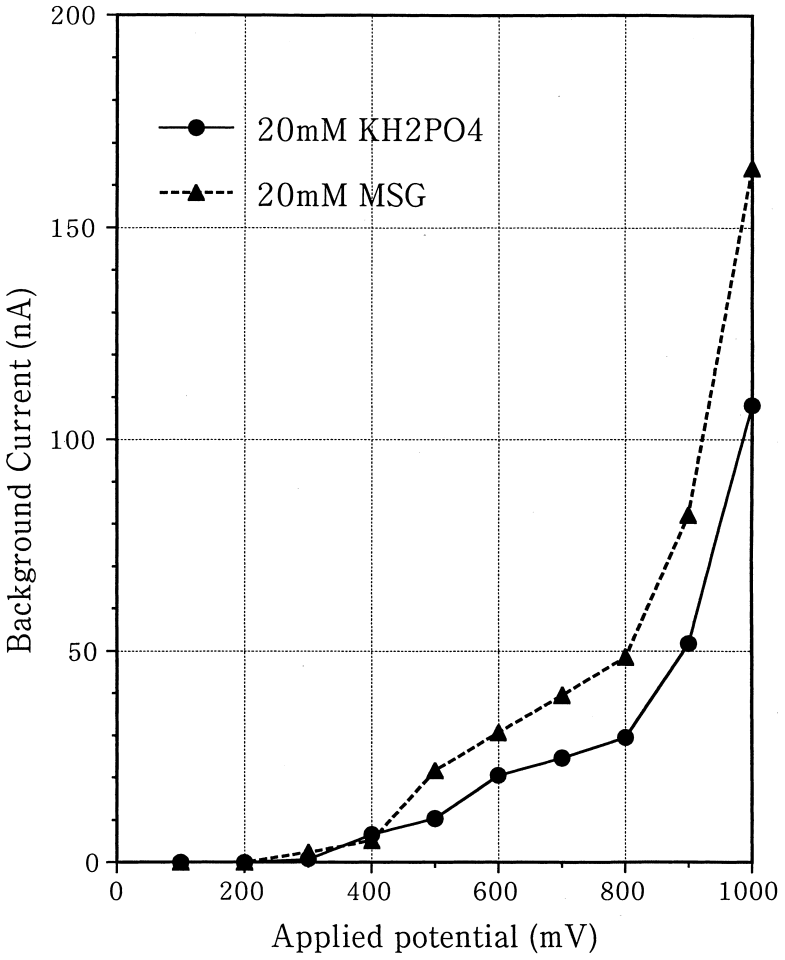

Fig. 5. Background current of mobile phase (20 m*M* MSG (pH 2.1) and 20 m*M* KH_2PO_4 (pH 2.1).

0.1–50 ng ($y = 0.258x + 0.113$, y = peak height, x = amount of AA in ng). A known amount of AA was added to the athlete food and overall recoveries were estimated by the standard addition method. AA recovery was over 90% by the standard addition method. The relative standard deviation (RSD) was 2.5% ($n = 7$) with no addition of AA (Table 4).

Application of the proposed method to the determination of AA in other foods was also studied (Table 5). There was a good agreement between the concentration indicated and that found. The method established here could be applicable to the other foods.

Comparison of recovery and RSD obtained by proposed method and published methods demonstrated that the former's recovery and RSD were >90% and 2.5% and the latter's were >90% and 2.9, 3.3, 3.2 and 2.8% (Table 6). So there were no difference in recoveries and RSD values between the methods, despite different food samples, dissolving solution, mobile phase, injection volume, AA concentration and applied potential.

The present work indicates that it is possible to replace traditionally employed inorganic salts like phosphate buffers with EDTA as mobile phase by an organic salt (MSG). In routine work, it is of benefit

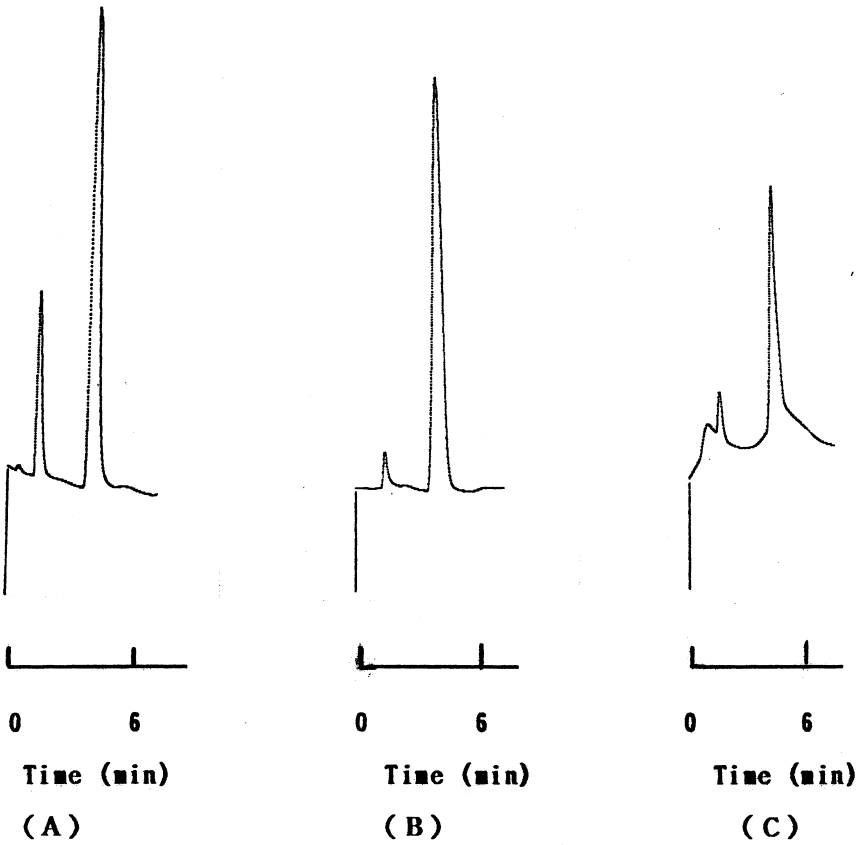

Fig. 6. Chromatograms of AA in foods diluted with mobile phase by HPLC–ED set at 400 mV versus an Ag/AgCl reference electrode. (A) Athlete food (B) nutritional supplement (C) orange juice. HPLC was carried out on a 15×0.46 cm I.D. column of Inertsil ODS-3 (5 μm) with guard column of Inertsil ODS-3 (5 μm) (1×0.46 cm I.D.) using 20 m*M* MSG (pH 2.1, adjusted with phosphoric acid) with no EDTA·2Na·2H_2O as a mobile phase at a flow-rate of 0.8 ml/min under an ambient conditions. Peak 1=AA.

that EDTA·2Na·2H_2 does not have to be prepared. Since MSGT contains sodium and it also chelates the metal ions it eliminates the need for inorganic salt and EDTA·2Na·2H_2O in the mobile phase.

Table 4
Recovery of AA added to athlete food

Added (mg/g)	Found (mg/g)	Recovery	
		(mg/g)	%
0	23.7[a]	–	–
6.2	29.5	5.8	93.9
12.5	35.4	11.7	93.6
25	47.2	25.5	94.0

[a] RSD; 2.5% (n=7).

Table 5
Content of AA in foods

	AA concentration	
	Indicated	Determined
Athlete food (mg/g)		
A	25.0	23.7
B	25.0	24.1
Nutritional supplement (mg/100 g)		
A	0.35	0.34
B	0.95	0.91
Infant milk (mg/100 g)	50	51.7
Grated daikon[a] (Japanese radish)	[b]	0.13
Orange juice[a] (mg/ml)	[b]	0.22

[a] Freshly prepared just prior to use.
[b] Not described.

Table 6
Comparison of RSD obtained by proposed method and published methods

	RSD (%)				
	Proposed method	Published methods			
		[14]		[9]	[10]
Sample	Athlete food	Japanese tea	Human urine	Orange juice	Human plasma
Dissolving solution	20 m*M* MSG (pH 2.1)	100 m*M* KH_2PO_4	(pH 3) with EDTA	Deionized water	10 m*M* KH_2PO_4 (pH 6.8 with Cys)
Mobile phase	20 m*M* MSG (pH 2.1)	100 m*M* KH_2PO_4	(pH 3) with EDTA	100 m*M* KH_2PO_4	(pH 3) with EDTA
Injection volume	5 μl	20 μl	20 μl	20 μl	20 μl
AA concentration	1~10 μg/ml	100~800 ng/ml	100~800 ng/ml	10 μg/ml	10 μg/ml
Applied potential	400 mV	400 mV	400 mV	300 mV	300 mV
Recovery (RSD)	>90% (2.5%)	>90% (2.9%)	>90% (3.3%)	>90% (3.2%)	>90% (2.8%)

4. Conclusion

In conclusion, it was possible to replace an inorganic salt with EDTA·2Na·$2H_2O$ by MSG in the mobile phase for the determination of AA in foods. The stability of AA under various conditions was also examined in order to optimize HPLC conditions and pre-run sample stabilization using MSG in a new mobile phase. The proposed method was applied to the determination of AA in foods.

References

[1] K. Iriyama, M. Yoshiura, T. Iwamoto, Y. Ozaki, Anal. Biochem. 141 (1984) 238.
[2] M. Shiura, T. Iwamoto, K. Iriyama, Jikeiika Med. J. 32 (1985) 21.
[3] W.A. Behrens, R. Madere, Anal. Biochem. 165 (1987) 102.
[4] R.R. Howard, T. Peterson, P.R. Kastl, J. Chromatogr. 414 (1987) 434.
[5] W.A. Behrens, R. Madere, J. Liq. Chromatogr. 15 (1992) 753.
[6] W.D. Grahham, D. Annette, J. Chromatogr. 594 (1992) 187.
[7] L.S. Liau, B.L. Lee, A.L. New, C.N. Ong, J. Chromatogr. 612 (1993) 63.
[8] H. Iwase, J. Chromatogr. 505 (1992) 277.
[9] H. Iwase, I. Ono, J. Chromatogr. A 654 (1993) 215.
[10] H. Iwase, I. Ono, J. Chromatogr. B 655 (1994) 195.
[11] H. Iwase, I. Ono, J. Agric. Food Chem. 45 (1987) 4664.
[12] H. Iwase, I. Ono, J. Chromatogr. A 806 (1998) 361.
[13] A.P. De Leenheer, W.E. Lambert, H.J. Nelis, in: Modern Chromatographic Analysis of Vitamins, 2nd ed., Marcel Dekker, New York, 1992, p. 235.
[14] H. Iwase, in preparation.
[15] A. Murai, Y. Tachikawa, J. Chromatogr. 14 (1964) 100.
[16] H. Iwase, J. Chromatogr. 93 (1974) 233.
[17] D.B. Dennison, T.G. Brawley, G.L.K. Hunter, J. Agric. Food Chem. 29 (1981) 927.
[18] H. Iwase, I. Ono, J. Chromatogr. B 663 (1995) 15.
[19] N. Nimura, T. Kinoshita, J. Chromatogr. 352 (1986) 169.
[20] A.I. Eledterov, M.G. Kolpachnikova, P.N. Mesterenko, O.A. Shpignn, J. Chromatogr. A 769 (1997) 179.
[21] J.P. Greenstein, M. Winitz, in: Chemistry of the Amino Acids, Wiley, New York, 1961, p. 1929.
[22] R. Teranishi, I. Hornatein, K.-H. Engel (Eds.), Food Reviews International, Vols. 2 and 3, Marcel Dekker, New York, 1998.
[23] The United States Pharmacopeia, 23rd Revision, US Pharmacopeia Convention, Rockville, MD, 1995, p. 130.
[24] The Pharmacopeia of Japan, 12th ed., Hirokawa, Tokyo, 1991, pp. C–37.