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Sequential fluorometric quantification of malic acid enantiomers by a single line flow-injection system using immobilized-enzyme reactors

Tadayuki Tsukatani[a,*], Kiyoshi Matsumoto[b]

[a] *Biotechnology and Food Research Institute, Fukuoka Industrial Technology Center, Kurume 839-0861, Japan*
[b] *Division of Food Biotechnology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School Kyushu University, Fukuoka 812-8581, Japan*

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Abstract

A method for the sequential enantiomeric quantification of D-malate and L-malate by a single line flow-injection analysis was developed using immobilized-enzyme reactors and fluorescence detection. An immobilized D-malate dehydrogenase (D-MDH) reactor and an immobilized L-malate dehydrogenase (L-MDH) reactor were introduced into the flow line in series. Sample and coenzyme (NAD^+ or $NADP^+$) were injected into the flow line by an open sandwich method. D-Malate was selectively oxidized by D-MDH when NAD^+ was injected with a sample. When $NADP^+$ was injected with a sample, L-malate was oxidized only by L-MDH. NADH or NADPH produced by the immobilized-enzyme reactors was monitored fluorometrically at 455 nm (excitation at 340 nm). Linear relationships between the responses and concentrations of D-malate and L-malate were observed in the ranges of 1×10^{-6}–1×10^{-4} M and 1×10^{-6}–2×10^{-4} M, respectively. The relative standard deviations for ten successive injections were less than 2% at the 0.1 mM level. This analytical method was applied to the sequential quantification of D-malate and L-malate in fruit juices and soft drinks, and the results showed good agreement with those obtained using conventional method (F-kit method).

Keywords: Flow-injection analysis; Immobilized-enzyme reactor; D-Malate; L-Malate; Fruit juice; Fluorometry

1. Introduction

A racemic mixture of malic acid is approved for use as a food additive and is used mainly as an acidulant. Although food additives can be legally used in food products provided that they are declared on the label, the possibility of adulterative addition of synthetic DL-malic acid cannot be absolutely ignored in the production of fruit juices such as apple juice. Of the two enantiomeric forms of malic acid, L-malic acid occurs naturally. On the other hand, D-malic acid is found in appreciable concentration only in the metabolism of some micro-organisms. Therefore, the detection of D-malic acid shows the adulteration with synthetic DL-malic acid [1].

Malic acid enantiomers have been simultaneously quantified by high performance liquid chromatography (HPLC) with chiral stationary phase columns [2–4] and the ligand-exchange mode on a normal column [5–7]. A method for quantification of malic acid enantiomers in apple juice by HPLC has been reported [8], but it requires a specific post-column reaction and is time consuming. As is well known, food is a complex mixture of many compounds, the concentrations of which are continuously changing. Therefore, high specificity and rapidity are required for the analysis of food. Enzymatic quantification is a suitable method to meet this demand. The combination of a biosensor and flow-injection analysis (FIA) constitutes a powerful technique for the quantification of food components with rapidity and specificity [9,10].

In most enzymatic assays of L-malate, the L-malate dehydrogenase (L-MDH) [EC. 1.1.1.37] reaction is used. However, the equilibrium lies very far in the direction of L-malate formation in the neutral pH region. Therefore, it is necessary to shift it in favor of NADH formation by removing the

* Corresponding author. Fax: +81 942 30 7244.
E-mail address: tukatani@fitc.pref.fukuoka.jp (T. Tsukatani).

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reaction products; oxalacetate is trapped with hydrazine or by the glutamate oxalacetate transaminase reaction [11]. Since such trapping reactions are relatively slow, their use is not recommended for FIA [12]. Contrastively, the $NADP^+$-specific L-malate dehydrogenase [EC. 1.1.1.40] reaction proceeds in the direction of pyruvate production in the neutral pH region [13]. Thus, we decided to use $NADP^+$-specific L-MDH for the quantification of L-malate. The D-malate dehydrogenase (D-MDH) reaction also proceeds in the direction of pyruvate formation in the presence of NAD^+ in weakly alkaline conditions [14]. It is thought that both enzymes can be applied to FIA for the quantification of D-malate and L-malate.

We developed a method for the sequential quantification of malic acid enantiomers by a single line FIA using immobilized-enzyme reactors and fluorescence detection. An immobilized-D-MDH reactor and an immobilized-L-MDH reactor were introduced into the flow line in series. The principle of this method for the sequential quantification of D-malate and L-malate makes use of the immobilized-enzyme reactors in a single line, the NADH or NADPH produced being monitored fluorometrically. D-Malate was selectively oxidized by D-MDH when NAD^+ was injected with a sample. When $NADP^+$ was injected with a sample, L-malate was oxidized only by L-MDH. This method for the sequential quantification of malic acid enantiomers is based on the following reactions:

$$\text{D-malate} + NAD^+ \xrightarrow{\text{D-MDH}} \text{pyruvate} + CO_2 + NADH + H^+ \quad (1)$$

$$\text{L-malate} + NADP^+ \xrightarrow{\text{L-MDH}} \text{pyruvate} + CO_2 + NADPH + H^+ \quad (2)$$

2. Experimental

2.1. Reagents

L-Malate dehydrogenase (L-MDH, oxalacetate-decarboxylating, EC 1.1.1.40, from chicken liver) and aminopropyl-controlled pore glass (APCPG, 80–120 mesh, mean pore diameter 700 Å) were bought from Sigma (St. Louis, MO, USA). D-Malate dehydrogenase (D-MDH, EC 1.1.1.83, from *Escherichia coli*) was obtained from Roche (Basel, Switzerland). NAD^+ and $NADP^+$ were purchased from Oriental Yeast (Tokyo, Japan). 4-(2-hydroxyethyl)-1-piperazinylethanesulfonic acid (HEPES) was bought from Dojindo (Kumamoto, Japan). All other chemicals were of analytical reagent grade and were used without further purification.

2.2. Preparation of immobilized-enzyme reactor

APCPG (0.12 g of dry weight) was used as the support for the enzyme immobilization. L-MDH (8 U) or D-MDH (7 U) was immobilized on APCPG as reported previously [15]. The enzyme-immobilized support was packed into a glass column (2.0 mm i.d. × 10 cm). The enzyme immobilization was carried out in 0.1 M phosphate buffer (pH 7.0). The enzyme reactor was stored in the coupling solution at 5 °C until use.

2.3. Flow system

A schematic diagram of the FIA system for the sequential quantification of D-malate and L-malate is shown in Fig. 1. The carrier solution in the reservoir was propelled by a micro-tube pump (MP-3, Tokyo Rikakikai, Tokyo, Japan) through an air damper, a sample injection valve (10-way switching valve, Select Pro, Alltech, KY, USA), a mixing coil (50 cm), the immobilized-enzyme reactors, then transported to a spectrofluorometer (Scanning Fluorescence Detector 470, Waters, Milford, MA, USA) with a flow-through cell connected to a recorder (FBR-251A, TOA, Tokyo, Japan) and finally to a waste tank. The sample flow system consisted of another micro-tube pump connected to sample injection valve (10-way switching valve) equipped with sample loops. The sample and the coenzyme (NAD^+ or $NADP^+$) were injected by an open sandwich method in order to save coenzymes. In this injection mode, sample (50 μl) and coenzyme (50 μl) were injected into zones next to each other [16,17] as shown in Fig. 2. Then, the sample and coenzyme were transported to the immobilized-enzyme reactors with being mixed. By a six-way switching valve (MPV-6, GL Science, Tokyo) in Fig. 1, the line for the coenzyme was switched between NAD^+ and $NADP^+$. Fluorescence intensity was measured at an excitation wavelength of 340 nm and emission wavelength of

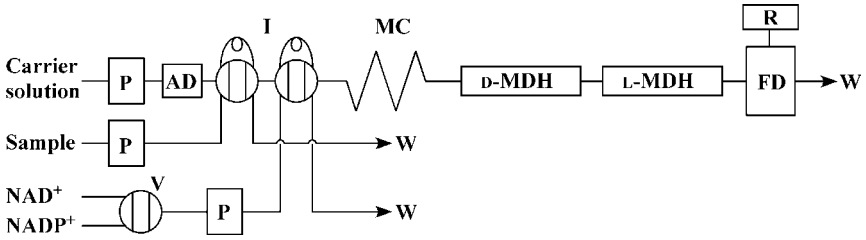

Fig. 1. Schematic diagram of the flow-injection system for the sequential quantification of D-malate and L-malate. P: micro-tube pump (carrier solution, 1.5 ml min^{-1}); AD: air damper; I: injector (10-way switching valve); MC: mixing coil (50 cm); D-MDH: immobilized D-MDH reactor (2.0 mm i.d. × 10 cm); L-MDH: immobilized L-MDH reactor (2.0 mm i.d. × 10 cm); V: switching valve (six-way switching valve); FD: fluorescence detector; R: recorder; W: waste. Inner diameter of tube is 1.0 mm.

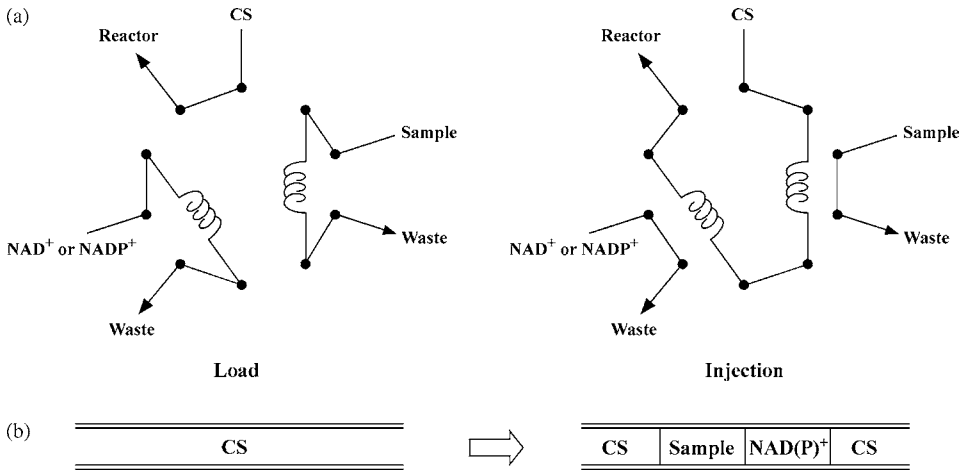

Fig. 2. (a) Arrangement of 10-way switching valve at load and injection position and (b) the arrangement of each solution in the tube before and after injection. CS: carrier solution; reactor: immobilized-enzyme reactor.

455 nm with a spectrofluorometer. The flow system was operated at room temperature.

2.4. F-kit Method

F-kits (determination kits using an enzymatic, spectrophotometric method, no. 139068 for L-malate and no. 1215558 for D-malate) were purchased from Roche (Basel, Switzerland). The F-kit method for L-malate quantification is an enzymatic, spectrophotometric method using L-MDH and NAD^+ as coenzymes in a free state. The F-kit for D-malate quantification consists of D-MDH and NAD^+. The analytical procedures were performed according to the manufacturer's manual.

3. Results and discussion

3.1. Optimization of FIA system

The effect of pH values on the responses to D-malate or L-malate at the 0.1 mM level was studied using 0.1 M HEPES buffer (pH 7.0–9.0). Responses for both D-malate and L-malate increased with increasing pH. HEPES buffer (0.1 M, pH 8.0) was used considering the optimum pH of free D-MDH (about 8.5) and free L-MDH (about 7.5).

NAD^+ is required as a coenzyme in the oxidation of D-malate by D-MDH. On the other hand, L-malate is converted to pyruvate in the presence of $NADP^+$. D-Malate or L-malate was analyzed by the immobilized-enzyme reactor in the presence of NAD^+ or $NADP^+$, respectively, in the range of 0.1–10 mM in order to determine the optimal concentration of coenzymes. The coenzymes were injected into the flow line by the open sandwich method for economy. The effect of coenzyme concentrations on the responses to D-malate or L-malate at the 0.1 mM level was investigated. Both responses increased with increasing concentration and became almost constant above 1.0 mM. Therefore, coenzymes were used at the concentration of 1.0 mM for economy.

Both D-MDH and L-MDH require Mg^{2+} as an activator [13,14]. We studied the effect of the concentration of Mg^{2+} on the response to D-malate or L-malate at the 0.1 mM level. Both responses increased with increasing Mg^{2+} concentration and became almost constant above 10 mM. Thus, the Mg^{2+} concentration of 10 mM was selected for the carrier solution. In addition, the active sites of D-MDH and L-MDH are thought to be a sulfhydryl group and, therefore, 0.5 mM dithiothreitol was also added to the carrier solution in order to protect such groups.

The effects of the flow rate on the response to D-malate or L-malate at the 0.1 mM level and the peak width (time) for achieving 10th ($t_{w1/10}$) of the maximum peak height were investigated in the range of 0.5–2.0 ml min^{-1}. The response to D-malate decreased remarkably with increasing flow rate up to 1.2 ml min^{-1}. The value of $t_{w1/10}$ rapidly decreased with increasing flow rate up to 1.5 ml min^{-1}. On the other hand, a broad profile of the dependence of the flow rate on the response to L-malate was obtained. The choice of flow rate involves a compromise between sensitivity and sample output rate. A flow rate of 1.5 ml min^{-1} was used in this experiment, considering its relatively high response and the short sample output time. The baseline reversion time was about 2.0 min at this flow rate.

3.2. Calibration and reproducibility

Standard mixtures containing D-malate and/or L-malate were measured sequentially under optimal conditions. FIA responses increased rapidly just after injection of the sample and returned to baseline within about 2.0 min as shown in Fig. 3. The determination frequency was about 30 tests per hour. The response peak for D-malate was selectively obtained when NAD^+ was injected as a coenzyme with a

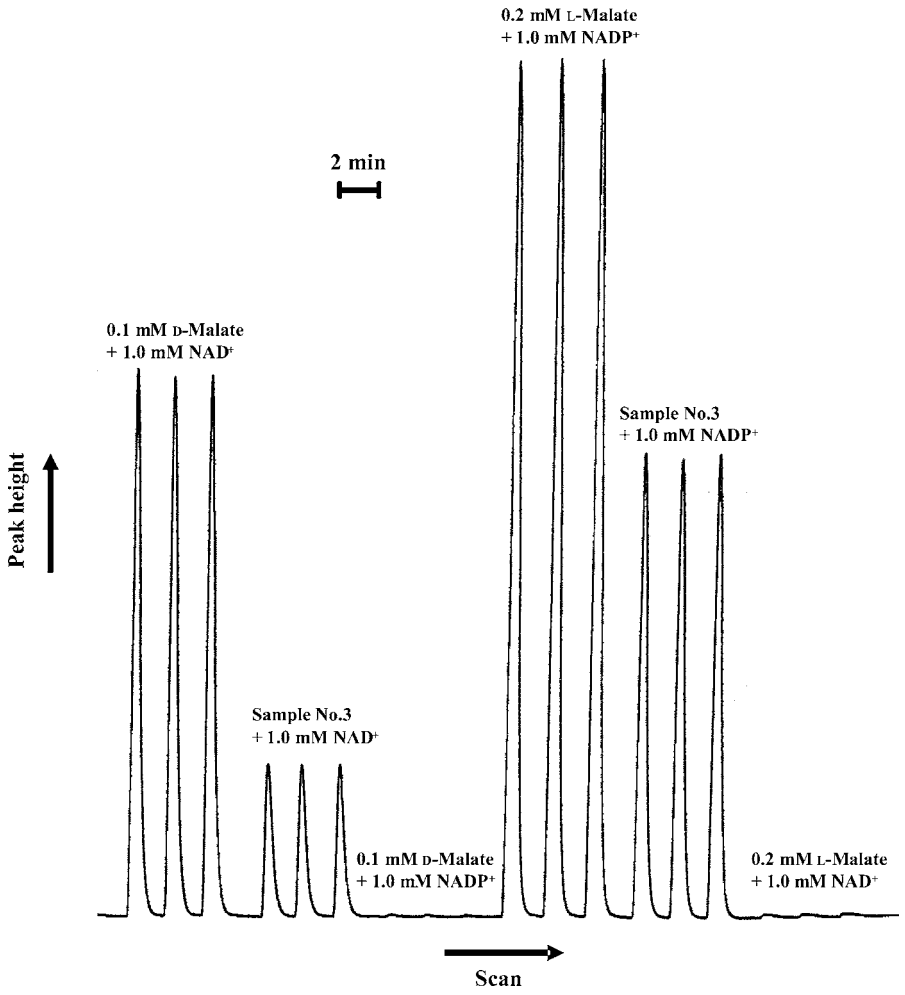

Fig. 3. Typical FIA peaks for sequential quantification of D-malate and L-malate.

sample. When $NADP^+$ was injected with a sample, only the response peak for L-malate was observed.

Linear relationships between the responses and concentrations of each compound were observed between 1×10^{-6} and 1×10^{-4} M for D-malate and 1×10^{-6} and 2×10^{-4} M for L-malate with correlation coefficients lager than 0.999 (n = 8), respectively. The detection limit, determined as the concentration that gave twice the peak height of the background signal, was 5×10^{-7} and 2×10^{-7} M for D-malate and L-malate, respectively. The relative standard deviations for ten successive injections were less than 2% at the 0.1 mM level. The life times of the D-MDH reactor and the L-MDH reactor were evaluated for one month. Both response peaks decreased gradually to about 80% of the initial value when ten samples were analyzed every day.

3.3. Interference study

Fruit juices contain various kinds of organic acid that may affect the D-MDH and L-MDH reactions. The influence of D-malate, L-malate, citrate, succinate, D-lactate, L-lactate, acetate, L-tartrate and L-ascorbate, as co-existing components in fruit juices, on the quantification of D-malate or L-malate was investigated (Table 1). The addition of these components had no influence on the quantification of L-malate at the 0.1 mM level. On the other hand, these organic acids did not have any influence on the quantification of D-malate at the 0.1 mM

Table 1
Influence of organic acids on the quantification of D-malate and L-malate

Interferent	Concentration (mM sample solution)	Recovery (%)	
		D-Malate	L-Malate
D-Malate	0.1	–	99.5
	1.0	–	99.0
L-Malate	0.1	99.1	–
	1.0	98.8	–
Citrate	0.1	99.3	99.2
	1.0	95.5	101.2
Succinate	0.1	99.9	99.2
L-Lactate	0.1	101.4	100.8
D-Lactate	0.1	97.2	98.8
Acetate	0.1	97.8	102.8
L-Tartrate	0.1	97.6	100.2
	1.0	91.3	100.4
	2.0	87.9	97.3
L-Ascorbate	0.1	102.3	99.6

Table 2
Recovery of D-malate and L-malate in apple juice spiked with standard solutions

D-Malate			L-Malate		
Spiked (mM)	Found (mM)	Recovery (%)	Spiked (mM)	Found (mM)	Recovery (%)
0.020	0.020 ± 0.001	100.0	0.020	0.020 ± 0.001	100.0
0.040	0.042 ± 0.000	105.0	0.040	0.041 ± 0.001	102.5
0.080	0.079 ± 0.002	98.8	0.080	0.084 ± 0.001	105.0

level except for L-tartrate at a relatively high concentration. D-MDH is known to catalyze the oxidation of L-tartrate as a secondary activity in addition of the oxidation of D-malate, although at a much lower rate. Therefore, L-tartrate in foodstuffs, such as wines and grape juices, may have an influence on the quantification of D-malate by D-MDH. Although L-tartrate is rarely found at a high concentration in fruit juices, such as apple juice, it is necessary to eliminate L-tartrate to quantify D-malate in wine and grape juice in which it is found at a high concentration. It is thought that L-tartrate can be removed according to the precipitation procedure reported previously [18]. The elimination of L-tartrate for wine analysis is under investigation.

3.4. Application to fruit juice and soft drink

To test the validity of the method, recovery for the quantification of D-malate or L-malate in an apple juice was investigated. The mean recoveries for a commercial apple juice were 101.3% for D-malate and 102.5% for L-malate, respectively, as shown in Table 2.

Table 3 shows the results for the sequential quantification of D-malate or L-malate in commercial fruit juices and soft drinks using this system. Fruit juices and soft drinks were filtered with a membrane filter (0.45 μm) and diluted 50–200-fold with distilled water, then subjected to FIA measurement. The self-fluorescence of a sample was measured without the coenzymes. Fruit juices and soft drinks gave a slightly blank signal. The blank signals were less than about 5% of the fluorescence signal observed using the immobilized-enzyme reactors. The peak height corresponding to the D-malate or L-malate concentration in fruit juices and soft drinks was calculated by subtracting the blank signal from the response signal, and the concentration was estimated from the calibration curve obtained by the present method. The results obtained by the present method (y) were compared with those obtained by the F-kit method (x). A straight line, defined by the equation $y = 1.026x + 0.002$, with a standard deviation of the slope $Sm = 0.026$ and of the intercept $Sb = 0.011$, and a standard error of the estimate $Sy,x = 0.012$, correlation coefficient $r = 0.9980$ ($n = 8$), was obtained in the quantification of D-malate. In the quantification of L-malate, a straight line, defined by the equation $y = 0.986x + 0.018$, with a standard deviation of the slope $Sm = 0.015$ and of the intercept $Sb = 0.019$, and a standard error of the estimate $Sy,x = 0.008$, correlation coefficient $r = 0.9987$ ($n = 13$), was obtained. The correlation of the results is highly satisfactory, and the values found fall within the ranges reported as normal for fruit juices and soft drinks. Of the 13 samples, D-malate was detected in eight samples. One of five apple juices in which pure apple juice alone was indicated, contained D-malate. In soft drinks, it is thought that D-malate and L-malate were detected at the same concentrations because synthetic DL-malate was added only as an acidulant. The present method for the sequential quantification of D-malate and L-malate with the immobilized-enzyme reactors described herein is useful in the detection of the adulteration of fruits juices with synthetic DL-malate.

Table 3
Comparison of the results obtained by the proposed method (FIA) with those obtained by F-kit (enzymatic, batch) method

Sample (description)	Sample number	D-Malate (g/l)			L-Malate (g/l)		
		FIA (A)	F-kit (B)	Bias (A–B)	FIA (A)	F-kit (B)	Bias (A–B)
Apple juice	1	nd	nd	nd	3.71	3.66	0.05
(Juice = 100%)	2	nd	nd	nd	3.89	3.77	0.12
	3	0.71	0.69	0.02	2.90	2.88	0.02
	4	nd	nd	nd	4.05	4.11	−0.06
	5	nd	nd	nd	4.23	4.47	−0.24
Apple juice	6	0.27	0.28	−0.01	1.17	1.21	−0.04
(Juice = 20–30%)	7	nd	nd	nd	0.68	0.66	0.02
	8	0.16	0.14	0.02	0.99	0.99	0.00
	9	0.34	0.32	0.02	1.34	1.36	−0.02
Fruit soft drink	10	0.27	0.28	−0.01	0.38	0.33	0.05
(Juice = 20–30%)	11	0.39	0.38	0.01	0.42	0.42	0.00
Soft drink	12	0.57	0.54	0.03	0.57	0.57	0.00
(Juice = 0%)	13	0.43	0.41	0.02	0.40	0.42	−0.02

References

[1] V.K. Agarwal, J. Assoc. Off. Anal. Chem. 71 (1988) 466.
[2] H. Katoh, T. Ishida, S. Kuwata, H. Kiniwa, J. Chromatogr. 473 (1989) 241.
[3] N. Oi, H. Kitahara, R. Kira, J. Chromatogr. 592 (1992) 291.
[4] M. Brightwell, M. Pawlowska, J. Zukowski, J. Liq. Chromatogr. 18 (1995) 2765.
[5] I. Benecke, J. Chromatogr. 291 (1984) 155.
[6] R. Horikawa, H. Sakamoto, T. Tanimura, J. Liq. Chromatogr. 9 (1986) 537.
[7] G. Gubitz, S. Mihellyes, G. Kobinger, A. Wutte, J. Chromatogr. A 666 (1994) 91.
[8] L.W. Doner, P.J. Cavender, J. Food Sci. 53 (1988) 1898.
[9] K. Matsumoto, T. Tsukatani, S. Higuchi, Sens. Mater. 7 (1995) 167.
[10] K. Matsumoto, H. Kamikado, H. Matsubara, Y. Osajima, Anal. Chem. 60 (1988) 147.
[11] H. Mollering, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, vol. 3, second ed., Academic Press, New York, 1974, p. 1589.
[12] R. Puchades, M.A. Herrero, A. Maquieira, J. Atienza, Food Chem. 42 (1991) 167.
[13] S. Suye, N. Yoshihara, S. Inuta, Biosci. Biotech. Biochem. 56 (1992) 1488.
[14] H.O. Beutler, B. Wurst, Deutsche Lebensmittel-Rundschau 86 (1990) 341.
[15] K. Matsumoto, K. Waki, Anal. Chim. Acta 380 (1999) 1.
[16] J. Toei, Anal. Lett. 20 (1987) 1951.
[17] K. Matsumoto, H. Matsubara, M. Hamada, T. Doi, Y. Osajima, Agric. Biol. Chem. 55 (1991) 1055.
[18] H.O. Beutler, B. Wurst, Deutsche Lebensmittel-Rundschau 86 (1990) 386.