ELSEVIER

Journal of Chromatography A, 928 (2001) 139–144

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Enantiomeric purity determination of malic acid in apple juices by multi-beam circular dichroism detection

Atsushi Yamamoto[a,*], Nami Akiba[b], Shuji Kodama[a], Akinobu Matsunaga[a], Kayoko Kato[b], Hiroyuki Nakazawa[b]

[a]*Toyama Institute of Health, 17-1 Nakataikoyama, Kosugi-machi, Toyama 939-0363, Japan*
[b]*Faculty of Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*

Received 13 March 2001; received in revised form 7 June 2001; accepted 17 July 2001

Abstract

A multi-beam circular dichroism (CD) detector which is easily constructed by inserting inexpensive optics into a conventional photo-diode array detector has an advantage of simultaneous detection of the absorbance and CD. The enantiomeric purity determination of malic acid in beverages was performed by this detection system. Malic acid when complexed with Cu(II) was found to have an absorbance maximum at around 750 nm. The L-malic acid–Cu(II) complex showed a positive Cotton effect in its absorbance band and its anisotropy factor ($\Delta\varepsilon/\varepsilon$) was relatively large at about 1/170. This complex was retained on a reversed-phase column with the addition of racemic 2-hydroxy-3-methylbutyric acid to the mobile phase as the ligand. A plot of the relative peak areas between the CD and the absorption (Δabs/abs) versus optical purity showed good linearity with a correlation coefficient of 0.999, and the precision expressed as the relative standard deviation of the errors from the regression line was $\pm 2.7\%$ (2σ). The accuracy of the proposed method was assessed by capillary electrophoresis. Eight commercially available juice products were analyzed using this method. Five of them were thought to be adulterated with synthetic malic acid.

Keywords: Enantiomer separation; Multi-beam circular dichroism detection; Malic acid

1. Introduction

In Japan, a racemic mixture of malic acid is approved for use as a food additive and is mainly used as an acidulant. Malic acid naturally occurs only in the L-form and the racemate as a food additive is chemically synthesized from maleic acid. In Japan, food additives can be legally used in food products provided that they are declared on the label. Therefore, products that are labeled as 100% juice should not contain D-malate. Generally, juice products are produced by dilution of enriched juices. Adulterative addition of the racemic malate during the dilution process cannot be absolutely denied because of reduction of the production cost. Moreover, there is no limit to how much malic acid can be used in the food manufacturing process, so the amount that is consumed is not clear. For such food additives, it is important to determine the enantiomeric purity for the safety and quality control of food products. However, there is presently no convenient method for determining the enantiomeric

*Corresponding author. Tel.: +81-766-56-5506; fax: +81-766-56-7326.

E-mail address: lee07664@nifty.ne.jp (A. Yamamoto).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)01143-8

purity of malic acid in complex matrices such as food products.

In the past, the enantiomeric purity of malic acid in food products was obtained by determining total malate by a chromatographic method and then by enzymatically determining the amount of the L-enantiomer using L-malate dehydrogenase [1]. The amount of the D-enantiomer is then given by the difference. Several reports have shown that malic acid enantiomers can be resolved by HPLC with chiral stationary phase columns [2–5] or by the ligand-exchange mode on a normal column [6–8]. However, these studies mainly analyzed standard solutions and examined only a few food products. Doner and Cavender [9] successfully resolved malic acid enantiomers in apple juice, but their system required a synthetic ligand and a specific post-column reaction. Other studies [10,11] have reported that only the D-enantiomer in fruit juices could be quantitated by ligand-exchange HPLC with the usual reagents and a simple system. We have developed ligand-exchange ion chromatography for the simultaneous determination of D- and L-malic acids in apple juices [12]. However, the use of Cu(II) in one of the eluent buffers eventually degraded the ion-exchange capacity of the column due to absorption of Cu(II) onto the column surface.

We have developed a multi-beam circular dichroism (CD) detector [13]. It can detect a signal based on CD by inserting inexpensive optics (a prism and a retardation plate) into a conventional photo-diode array (PDA) detector. For further details of the apparatus, see Fig. 1 in Ref. [13]. This optical system detects a modulated CD wave superimposed on the absorption spectrum, which is produced by circularly polarized beams with opposite rotational senses at each adjacent quarter-wave of the retarder. An advantage of conventional CD instruments is simultaneous detection of absorbance and CD, thus making it possible to determine the optical purity without separating enantiomers [14]. The same applies to the multi-beam CD on a PDA detector. The purposes of this study were to establish a method for determining the optical purity of malic acid in beverages by multi-beam CD detection and to investigate the quantity of synthetic racemic malic acid used in commercial juice products.

2. Experiment

2.1. Apparatus

The LC system consisted of a Shimadzu (Kyoto, Japan) LC-10ADvp pump, a Rheodyne (Cotati, CA, USA) 7725i injector with a 100-μl loop, a Shimadzu SPD-M10Avp PDA detector, a Shimadzu CTO-10Avp column oven. Chromatographic separation was performed on a 25 cm×4.6 mm I.D. reversed-phase column (CAPCELL PAK C_{18} UG120; Shiseido, Tokyo, Japan) maintained at 40°C. To detect CD waves, a Gran-Taylor prism (Sigma Koki, Hidaka, Saitama, Japan, 1 cm cube) was placed in the compartment for the wavelength calibrating filter and a quartz plate (Five Lab., Kawasaki, Japan, 2.18 mm thick) was attached to the incident light side of the flow-cell in the PDA detector. The principal axis of the quartz plate was inclined by 45° from the polarizing plane. A mixture of 7 m*M* copper(II) acetate+3.5 m*M* 2-hydroxy-3-methylbutyric acid was used as the mobile phase at a flow-rate of 1.0 ml/min. The PDA data were processed with a spreadsheet program (MS Excel; Microsoft Corp., Redmond, WA, USA).

Absorption spectra were measured by a Shimadzu UV-1600pc spectrometer and CD spectra were measured by a Jasco (Hachiouji, Tokyo, Japan) J-600C CD spectrophotometer.

A capillary electrophoresis system (Agilent Technologies, Palo Alto, CA, USA) was used for the electrophoretic experiments. The separations were performed in a polyvinyl alcohol-coated capillary of 56 cm×50 μm I.D. maintained at 30°C. 1 m*M* $CuSO_4$+1 m*M* L-tartaric acid (pH 5.1) was used as the background electrolyte. The power supply was operated in the constant voltage mode at −20 kV. The sample was injected by applying a pressure of 50 mbar for 2 s. The detection wavelength was set at 280 nm.

2.2. Reagents

L(−)-Malic acid, D(+)-malic acid, copper(II) acetate monohydrate, sodium hydroxide and acetonitrile were all of analytical grade and were purchased from Wako (Osaka, Japan). Racemic 2-hydroxy-3-methyl-

butyric acid was purchased from Sigma Aldrich Japan (Tokyo, Japan). Water was purified with a Milli-Q system (Nippon Millipore, Tokyo, Japan).

2.3. Preparation of sample solutions

Commercially available apple juices and sour drinks were used for the experiment. The only pretreatment of the sample was centrifugation (3000 rev./min, 5 min) and dilution of the supernatant with purified water, followed by filtration through a cartridge (Oasis HLB 60 mg; Waters, Milford, MA, USA) conditioned by means of 3 ml of acetonitrile followed by 10 ml of purified water.

3. Results and discussion

CD is defined as the difference in absorbance between right and left circularly polarized beams at the Cotton band of a chiral compound. The sensitivity of HPLC-CD detection is dependent upon the bandwidth of the observed Cotton effect of the analyte. Conventional CD detectors often use a large spectral bandwidth of the incident light to increase the total light intensity. The multi-beam CD, where the applicable wavelengths are restricted to around 1/4 wavelengths of the retarder, requires a broader Cotton band to enhance the peak intensities. Unfortunately, malic acid has no absorption band in the visible and UV part of the spectrum. Lu et al. have reported that hydroxy acids form a complex with Cu(II) that has an absorption band in the UV region and HPLC is possible with this complex [15]. But an absorption band does not necessarily show CD. The CD and absorbance spectra of Cu(II)–L-malic acid are shown in Fig. 1. This complex shows a very broad spectra and has an absorbance maximum at around 750 nm and a positive CD maximum at around 720 nm. The anisotropy factor ($\Delta\varepsilon/\varepsilon$) [16] is relatively large (about 1/170). This means that multi-beam CD would be applicable to the optical purity determination of malic acid in this wavelength range.

There are two modes, i.e. ion-pair and ligand-exchange, to retain and separate the Cu(II)–hydroxy acid complexes on a reversed-phase column. In the ion-pair mode, hydrophobic alkyl sulfates or quaternary ammonium salts provided good chromatograms for the Cu(II)–malate complex. However, the amount of malate that could be injected was limited. Injection of a large amount resulted in a deformation of peak shape. More malate could be injected with the ligand-exchange mode than with the ion-pair mode. When using a hydroxy carboxylic acid as a ligand, the retention of malic acid increased as the number of carbons on the ligand increased. 2-Hydroxy-3-methylbutyric acid, having five carbons, gave a measurable peak for malic acid. It should be noted that 2-hydroxy-3-methylbutyric acid, like malic acid, has a chiral carbon, so the use of one enantiomer of this acid enabled the chiral separation of malic acid on a normal column. However, this system gave poor separation of malic acid enantiomers from the other organic acids and the turbulence in the baseline made it difficult to apply this method to actual samples.

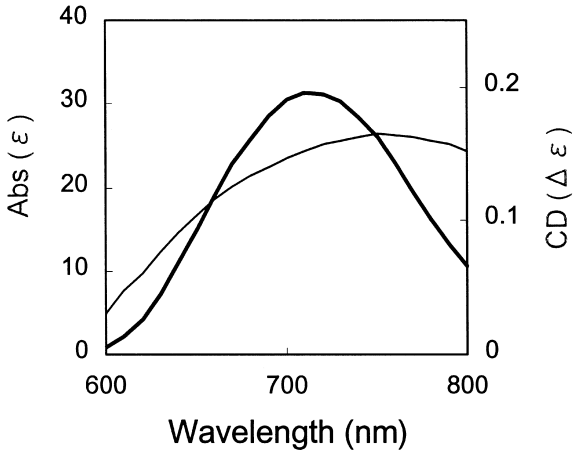

Fig. 1. CD (thick line) and visible absorption (thin line) spectra of Cu(II)–malate complex. Sample solution: 10 m*M* Cu(II) acetate/5 m*M* L-malic acid (pH 5), reference solution: 10 m*M* Cu(II) acetate (pH 5).

The CD chromatograms were prepared from actual PDA data as described previously [13]. The CD wave was extracted with first differential processing of the absorption spectrum. Because the first differential spectrum of Cu(II)–malate complex was almost linear, we connected nodes of the CD wave by straight lines to form a baseline. First differentia-

tion alters the node of the CD wave to the values at 1/4 wavelength of the quartz plate. The absolute values of the CD waves at 1/2 wavelength, which correspond to the tops and troughs of the waves, were added up in the region of 690 to 750 nm, and the aggregate was used for the vertical axis of the CD chromatogram. To obtain normal chromatograms with photometric detection, 752 nm was chosen because this wavelength corresponds to 1/2 wavelength and is independent of the enantiomeric composition. The absorption and CD chromatograms obtained under the preceding procedures are shown in Fig. 2. Deservedly, the responses of malate peaks in the absorption chromatograms were invariable by altering their enantiomeric composition, but the peak of the enantiomers were inverted in the CD chromatograms. Furthermore, the peak strengths depended on the enantiomeric purity. Because the straight lines that connected the values at 1/4 wavelengths, which were used for the baseline, did not completely coincide with the first differential absorption spectrum of the racemate, a slight negative peak was observed in the racemate. But it did not affect the enantiomeric purity determination. The negative peaks in Fig. 2 are referred to as the system peaks [17] derived from the components in the mobile phase. They did not interfere with the quantification of the malate peak.

The ratio of the peak intensities between CD and absorbance is used for the optical purity determination. To determine it with a high accuracy, the CD peak must be precisely quantified because of its relatively small intensity. Many small deviations appear on the baseline when expanding the CD chromatograms in Fig. 2. Consequently, the CD peak areas remarkably depend on the manner of the baseline drawing. In multi-beam CD detection, the shapes of the CD and absorbance peaks should agree precisely because both were made from absorbance spectra obtained from the same flow-cell. Then the slopes of the start and end points of the CD peak can be guessed from the shape of the absorbance peak. A spline curve, which is defined as a series of polynomial expressions that pass through arbitrary points and is differentiable for all intervals, can be drawn using the CD values and their slopes at both points. The CD peak area was calculated by using this curve as the baseline. The linear plot of peak area ratios of the CD to the absorbance versus optical purity is shown in Fig. 3. It showed good linearity with a correlation coefficient (r^2) of 0.999. When using the standard error from this regression line for the confidence interval of this method, the enantiomeric purity could be determined with an accuracy of $\pm 2.7\%$ (2σ). In other words, a juice product in which the added synthetic acid is over the 5.4% level can be classified as adulterated.

The enantiomeric purity of malic acid in commercially available juice products was determined by using this regression line. Samples used here were a laboratory-made apple juice and eight commercial samples having apple juice contents in the range 20–100%. Chromatograms of these samples are shown in Fig. 4. Even with simple pretreatment, no

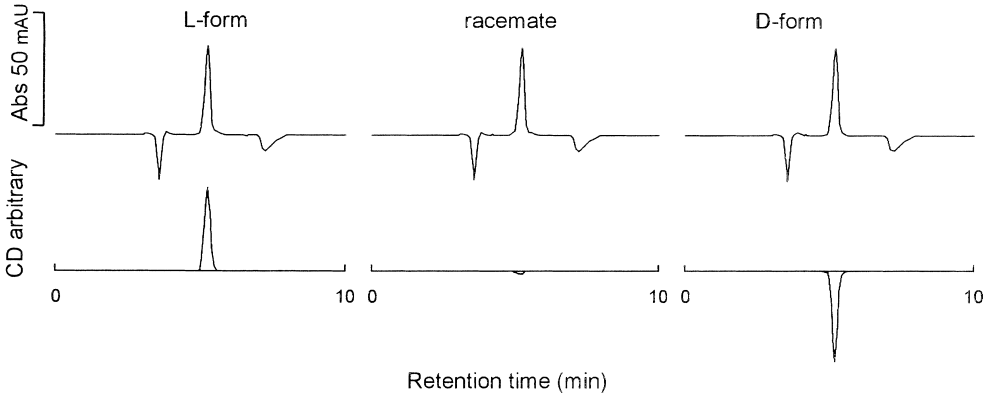

Fig. 2. CD (lower trace) and visible (upper) chromatograms of the two isomers of malic acid with an injection of 0.125 μmol of each.

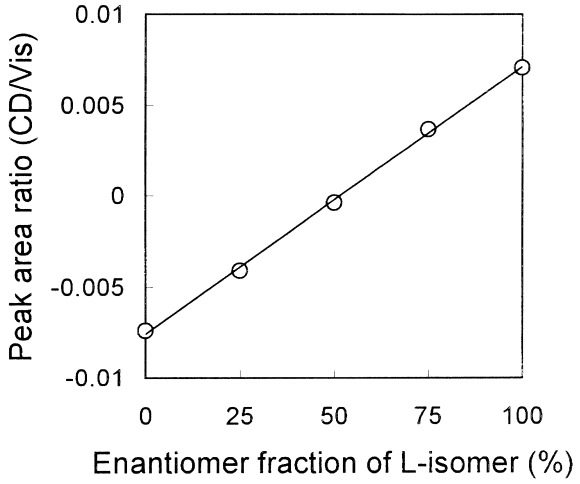

Fig. 3. Linear plot of peak area ratios versus composition of the enantiomers for malic acid at 0.125 μmol injection level.

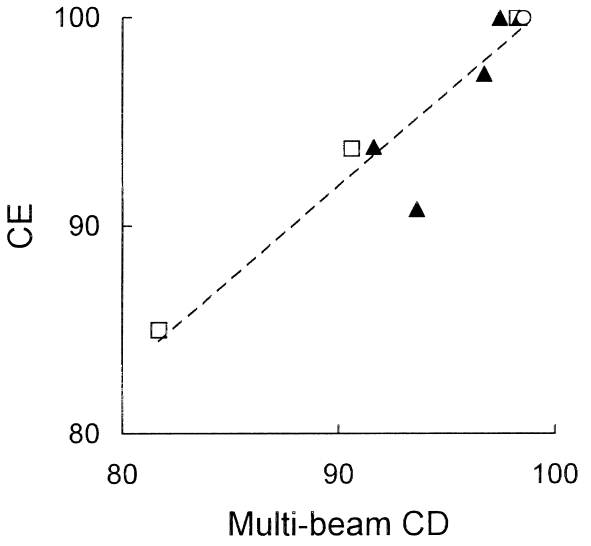

Fig. 5. Comparison of the results obtained for the enantiomeric purity by multi-beam CD and CE. ○, Laboratory-made juice; ▲, fruit juice (apple juice content of 100%); □, fruit soft drink (20–30%).

interfering peaks were observed on the visible chromatograms. Validation of the results obtained with this system was performed by comparison with those obtained by capillary electrophoresis (CE) [18]. Fig. 5 compares the results for the enantiomeric purity obtained by both methods. There was good agreement with the values for enantiomeric purity ($r^2 = 0.892$). As mentioned above, samples whose enantiomeric purity is lower than 97.3% were classified as adulterated. Five samples were assigned to this category, and this was confirmed by CE. Furthermore, three of these five samples were labeled as

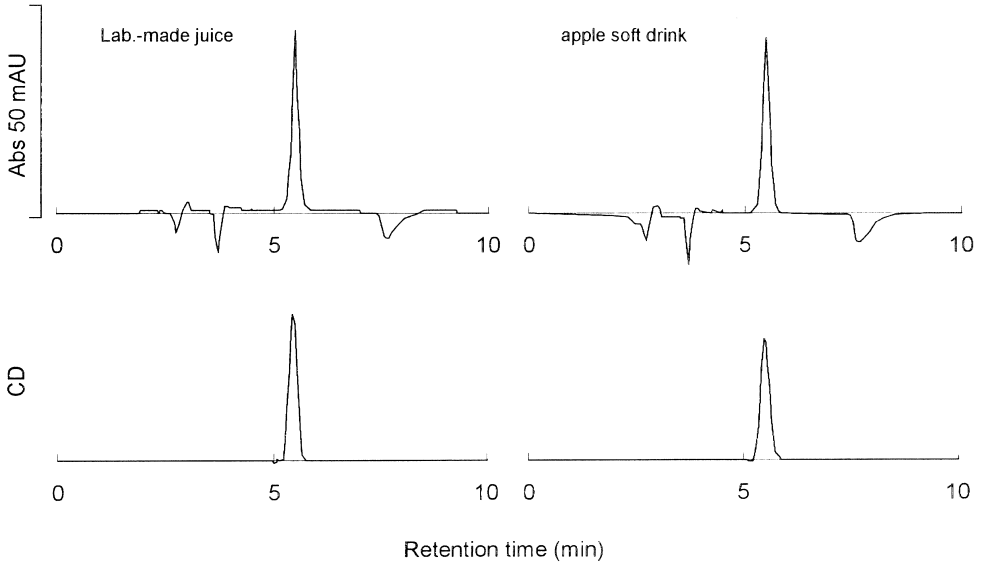

Fig. 4. CD (lower trace) and visible (upper) chromatograms of a laboratory-made juice (left) and a commercial juice (right).

100% apple juice and no acidulant was listed in their ingredients. These samples appear to be illegal products with false labeling.

In conclusion, this detection system makes it possible to make routine analyses of enantiomeric purity. This method requires no chiral separation and can be easily installed in laboratories by using inexpensive optics. The only piece of equipment that is needed is a PDA detector.

References

[1] E.R. Elkins, J.R. Heuser, J. AOAC Int. 77 (1994) 411.
[2] H. Katoh, T. Ishida, S. Kuwata, H. Kiniwa, Chromatographia 28 (1989) 481.
[3] H. Katoh, T. Ishida, Y. Baba, H. Kiniwa, J. Chromatogr. 473 (1989) 241.
[4] N. Oi, H. Kitahara, R. Kira, J. Chromatogr. 592 (1992) 291.
[5] M. Brightwell, M. Pawlowska, J. Zukowski, J. Liquid Chromatogr. 18 (1995) 2765.
[6] I. Benecke, J. Chromatogr. 291 (1984) 155.
[7] R. Horikawa, H. Sakamoto, T. Tanimura, J. Liquid Chromatogr. 9 (1986) 537.
[8] G. Gübitz, S. Mihellyes, G. Kobinger, A. Wutte, J. Chromatogr. A 666 (1994) 91.
[9] L.W. Doner, P.J. Cavender, J. Food Sci. 53 (1988) 1898.
[10] T.A. Eisele, J.R. Heuser, J. Food Sci. 55 (1990) 1614.
[11] T.A. Eisele, J. AOAC Int. 79 (1996) 50.
[12] A. Yamamoto, A. Matsunaga, E. Mizukami, K. Hayakawa, M. Miyazaki, J. Chromatogr. 585 (1991) 315.
[13] A. Yamamoto, S. Kodama, A. Matsunaga, K. Hayakawa, M. Kitaoka, Analyst 124 (1999) 483.
[14] A.F. Drake, J.M. Gould, S.F. Mason, J. Chromatogr. 202 (1980) 239.
[15] D.S. Lu, W.Y. Feng, D.H. Ling, W.Z. Hua, J. Chromatogr. 623 (1992) 55.
[16] P. Salvadori, C. Bertucci, C. Rosini, Chirality 3 (1991) 376.
[17] S. Levin, E. Grushka, Anal. Chem. 59 (1987) 1157.
[18] S. Kodama, A. Yamamoto, A. Matsunaga, T. Soga, K. Hayakawa, Electrophoresis, in press.