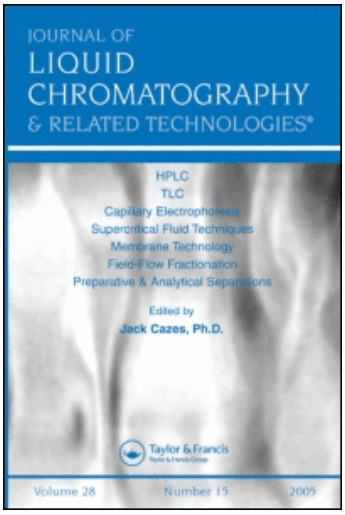

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DETERMINATION OF DIKETOPIPERAZINE IN SOFT DRINKS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Analytical method of diketopiperazine (5-Benzyl-3,6-dioxo-2 piperazineacetic Acid: DKP), a major degradation product of aspartame (APM), in soft drinks was developed by means of high performance liquid chromatography (HPLC). A sample was purified using Bond Elut SCX connected to Bond Elut C8 with 20 % acetonitrile as the eluent. A Nucleosil 5-C_{18} column was employed for the HPLC with 10 mM potassium dihydrogenphosphate and acetonitrile (85+15, v/v) adjusted pH to 4.0 as the mobile phase. The calibration curve was rectilinear in the range of 0.5 to 10.0 μg/ml for DKP. The average recoveries were 99.8 % and 96.0 % for DKP added to soft drinks at the level of 10 μg/ml and 2.5 μg/ml, respectively. The DKP was found in six commercial samples in the range of 9.5 to 26.0 μg/ml.

INTRODUCTION

APM is a dipeptide sweetener specified as a food additive in 1983 for Japan, and has been used in any diet foods such as soft drinks because APM has about 200 times more sweetness per calorie as sugar. It was reported that APM in aqueous solution was partially decomposed depending on its pH and temperature eventually forming DKP (1). Since DKP does not have sweetness, APM lose the sweetness from this conversion. Therefore, there was a need to develop of a method to determine DKP in order to control the quality and evaluate the safety of soft drinks.

The use of gas chromatography (GC) and HPLC was reported for the determination of DKP. However, the GC method (2) was time consuming for the derivatization of DKP; therefore, it was not used to analyze commercial soft drinks. As for the HPLC method (3), samples were loaded directly into the HPLC column without any purification. Therfore, the chromatograms were influenced by other additives.

This paper proposes a simple and rapid HPLC method for the determination of DKP in APM-sweetened soft drinks.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile and methanol were of HPLC grade (Wako Pure Chemical Industry, Japan). Water was glass distilled and deionized. All other chemicals were of reagent grade and used without further purification.

DKP was prepared according to the methods (1,3,4) as follows. A 100 mg APM (Tokyo Kasei Kogyo, Japan) in a closed vessel was heated at 150℃ for 6 hr in a sand bath. The contents were then dissolved with approximately 8 ml hot water. After decolorizing with an

adequate amount of activated charcoal, the solution was filtered. The filtrate was then allowed to cool. The DKP eventually crystallized as a white amorphous product. The DKP was also recrystallized from water. This procedure gave DKP in approximately 50 % yield. The structure of the obtained DKP was confirmed by means of mass spectrometry (MS), infrared absorption spectrometry, and elemental analysis (anal. Calcd. for $C_{13}H_{14}N_2O_4$: C, 59.54; H, 5.38; N, 10.68. Found : C, 58.49; H, 5.39; N, 10.59).

A DKP standard solution was prepared by dissolving 10 mg of DKP in 10 ml water to produce a stock solution of 1000 μg/ml. Working standards were prepared by diluting the stock solution with water to appropriate concentrations.

A HPLC mobile phase was prepared by mixing 10 mM potassium dihydrogenphosphate solution and acetonitrile (85+15, v/v) and then acidifying pH to 4.0 with phosphoric acid.

The clean up column was a Bond Elut SCX cartridge connected to a Bond Elut C8 cartridge (Analytichem International,USA) with a polyethylene tube (as shown in Figure 1). The column was conditioned by eluting with 3 ml methanol and then 3 ml water prior to use.

Apparatus

The HPLC was carried out using a Shimadzu LC 6A system equipped with a SPD 6A (Shimadzu Seisakusho, Japan) spectrophtometer. The column was a Nucleosil 5 C_{18} (4.6 mm I.D. × 250 mm, Nagel, GFR). The mobile phase was run isocratically at ambient temperature at a flow rate of 0.7 ml/min. The wavelength of detection was 210 nm and the sensitivity was 0.08 AUFS.

Mass spectra were obtained with a Shimadzu QP-1000A mass spectrometer, which was employed in electron impact mode having an ionization energy of 70 eV and ion source temperature of 250 ℃.

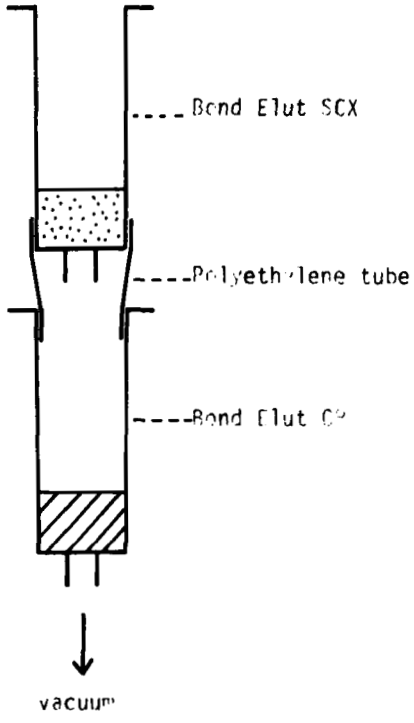

FIGURE 1. Clean up column with Bond Elut.

Sample Preparation

Soft drinks were degassed in an ultra sonic bath. A 2 ml sample was applied to the clean up column and the column was eluted at a flow rate of 1 ml/min. After the column was washed with 1 ml of water, the SCX cartridge was removed. The C8 cartridge was washed with 1 ml of water and then with 0.5 ml of 20 % acetonitrile. The C8 cartridge was then eluted with 1.5 ml of 20 % acetonitrile. The eluate was diluted to 4 ml with water. A 10 μl sample was injected into the HPLC.

Confirmation of DKP by MS

DKP was preparatively fractionated by HPLC under the previously described conditions. The fractionate solution containing DKP was placed on a Bond Elut C8 cartridge to desalt the phosphate buffer. After the cartridge was washed with 3 ml water, it was eluted with 2 ml of 20 % acetonitrile. The eluate was then evaporated to dryness under reduced pressure for MS analysis. The identity of DKP in soft drinks was confirmed by MS.

RESULTS AND DISCUSSION

Chromatographic Conditions

In general, Caffeine (Caf) and sodium benzoate (BA) are added to the APM-sweetened soft drinks. Since these additives might interfere with the determination of DKP on HPLC chromatograms, the operating conditions of the HPLC were examined. Nucleosil 5-C_{18}, a typical reverse phase column, was employed in this study with the mixed phosphate buffer and acetonitrile solution as the mobile phase.

Figures 2 and 3 show the effect of acetonitrile concentration and pH of the mobile phase on the capacity factor (k'). Each k'value of all four compounds (DKP, Caf, APM, and BA) decreased with an increase in the concentration of acetonitrile. The k' value of BA showed a pH dependene with the mobile phase much stronger than that of the others. When the pH was less than 3, DKP could not be well separated from Caf, but when its value was greater than 6, BA interfered with DKP. It was observed that a good baseline separation of these compounds was achieved when weakly acidic solvent (pH 4.0) containing 15 % acetonitrile was used.

On the other hand, the effect of potassium dihydrogenphosphate concentration was studied over the range of 10 to 50 mM. It was observed that a 10 mM phosphate buffer gave a sufficient separation.

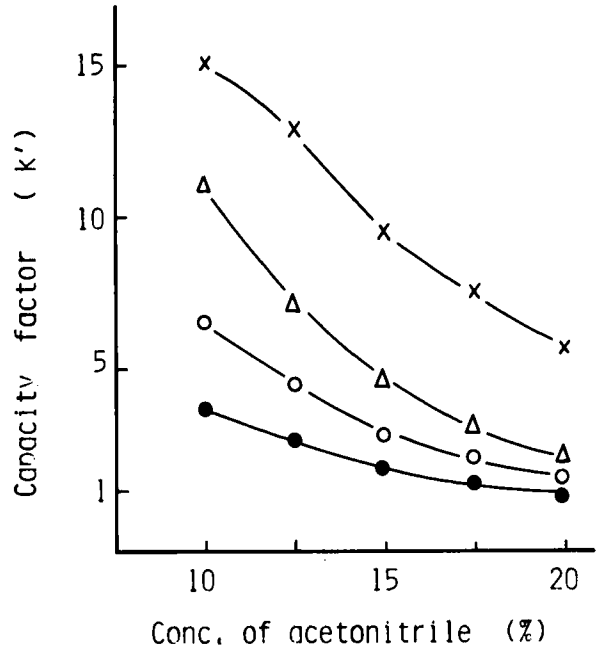

FIGURE 2. Effect of acetonitrile concentration of the mobile phase on capacity factor of DKP(●—●), Caf(○—○), APM(Δ—Δ), and BA(x—x).

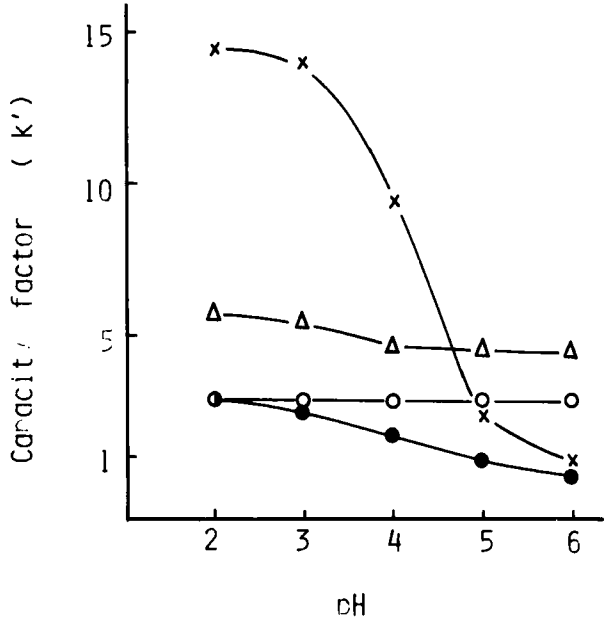

FIGURE 3. Effect of pH of the mobile phase on capacity factor of DKP(●—●), Caf(○—○), APM(Δ—Δ), and BA(x—x).

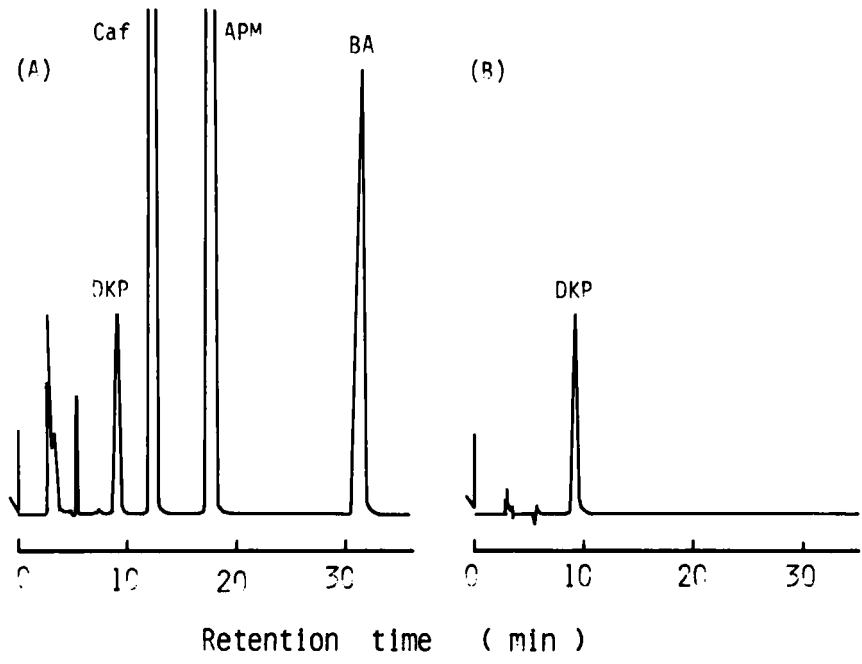

FIGURE 4. Liquid chromatograms of DKP obtained from untreatment(A) or treatment(B) with the clean up column.

On the basis of these experiments, 10 mM potassium dihydrogen phosphate - acetonitrile (85:15, v/v), acidified pH to 4.0 with phosphoric acid, was used as the mobile phase.

The calibration curve, using the peak height versus DKP content, showed a linear relationship in the range of 0.5 to 10 μg/ml for DKP. The minimum detectable amount was 2.5 ng for DKP at a sensitivity of 0.08 AUFS.

Clean up procedure of DKP

Figure 4(A) shows that the peaks of Caf, APM, and BA appear over scale on the chromatograms because of their much larger

quantities than DKP in soft drinks, when they were injected directly into the HPLC. Besides these compounds, coloring substances, such as caramel or acids, are usually added to soft drinks. Therefore, direct injection may lead to a loss in the ability of the HPLC column to separate compounds. In addition, since the retention time of BA is about 3 times longer than that of DKP, it is very time consuming to analyse many samples by HPLC. Therefore, it should be desirable to eliminate as much as possible these additives from samples in order to determine DKP with rapidity and high sensitivity.

Attempts were made to clean up samples by use of several types of Bond Elut cartridges. It was observed that DKP, Caf, APM, and BA were all retained on the C8 cartridge, and Caf and APM were retained on the SCX cartridge while DKP and BA passed through the cartridge. Based on these experiments, the combination of SCX and C8 cartridges were studied, i.e., the SCX was connected to the C8. DKP and BA could then be separated from Caf and APM. That is, DKP and BA were retained on the C8, while Caf and APM were retained on the SCX.

The elution pattern of DKP with 20 % acetonitrile from the C8 cartridge was studied by using 20 μg DKP and 200 μg BA. No DKP was found in the 0.5 ml of the first eluate, where other contaminants, such as caramel color when present in soft drinks were found. All the DKP was found in the followed 1.5 ml of 20 % acetonitrile, whereas no BA was found in this fraction. Therefore, the 0.5 ml was discarded with the subsequent 1.5 ml eluates taken as the DKP. Consequently, Caf, APM, and BA could be totally removed from the samples using this procedure. A typical chromatogram is presented in Figure 4 (B) for a APM-sweetened soft drink after removed of impurity.

Recovery Study and Analysis of Commercial Samples

Commercial soft drinks fortified at a level of 10 μg/ml and 2.5 μg/ml of DKP were used for the recovery study. Table 1

TABLE 1.

Recoveries of Diketopiperazine from Soft Drink

Added(μg/ml)	Recovery(%) *	C.V.(%)
10.0	99.8	3.7
.2.5	96.0	2.4

C.V.: Coefficient of Variation

* : Average of 5 determinations

TABLE 2.

Contents of Diketopiperazine (DKP) in Carbonated Soft Drinks Containing Aspartame

Sample	Formulation of vessel	DKP (μg/ml)	Storing period after manufactured (day)
A	Aluminum can	16.9	42
B	Aluminum can	19.7	71
C	Aluminum can	26.0	76
D	Glass bottle	9.5	-
E	Glass bottle	18.4	-
F	Glass bottle	23.2	-

- : No indication

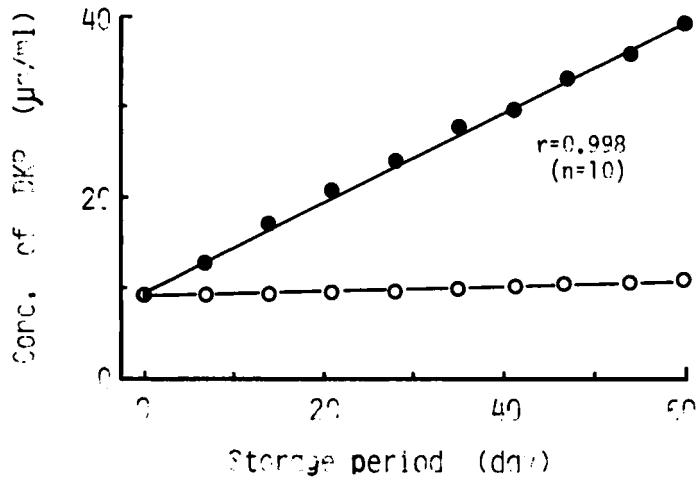

FIGURE 5. Correration between DKP concentration and storage periods of APM-sweetened soft drink stored at room temperature (●—●), and at 4 ℃ (o—o).

demonstrates greater than 95 % overall mean recoveries and 4 % coefficient of variations. The detection limit of the method was 0.5 μg/ml for DKP.

The formation of DKP in APM sweetened soft drinks with three aluminum can and three glass bottles of the same brand were investigated. DKP was found in all samples, and the content of DKP was varied among them as shown in Table 2. It was considered that the variation arose from both the difference in storage temperature and storage period in a store.

Therefore, the variation of DKP content with time was investigated so as to prove this hypothesis. Sample "D" (shown in Table 2) was divided into two portions. One of them was stored at room temperature (25 - 27 ℃) and the other at 4 - 5 ℃ in a refrigerator for 2 months, respectively. Figure 5 shows that the DKP content at room temperature increased with the increase in storage time, and finally reached a concentration of 39.1 μg/ml.

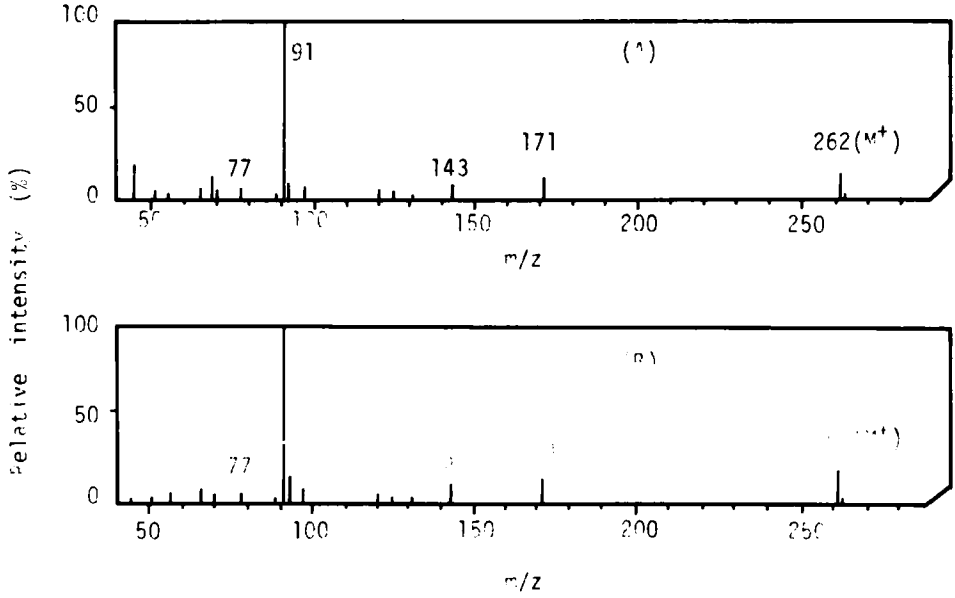

FIGURE 6. Mass spectra of an authentic standard of DKP (A) and of a DKP sample (B).

The increase in concentration of the DKP was about 4 times that of its initial concentrations. On the other hand, the DKP content in sample stored at 4 - 5 ℃ did not show any noticeable variation.

Consequently, this result suggests the fear that APM-sweetened soft drinks may lose their quality because of the rapid degradation of APM, if they were stored at room temperature after manufacture.

Therefore, much attention may need to look at the storage conditions of APM-sweetened soft drinks.

Confirmation of DKP

Identification by MS was carried out to confirm the presence of DKP, in fractions obtained by preparative HPLC in commercial

samples. Figure 6 represents the mass spectra of authentic standard DKP ($C_{13}H_{14}N_2O_4$, Mw. 262) and a sample. The positions and the intensity of sample peaks coincided with those of the standard DKP. The fraction was thus identified as DKP.

Consequently, these results indicate that the quantitative analysis of DKP can be evaluated in terms of quality assessment of APM-sweetened soft drinks.

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