

APPENDIX II

NUTRASWEET R&D ANALYTICAL DEPARTMENT

PAGE 1 of 12

SPECIFICATIONS AND METHODS DOCUMENT

JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

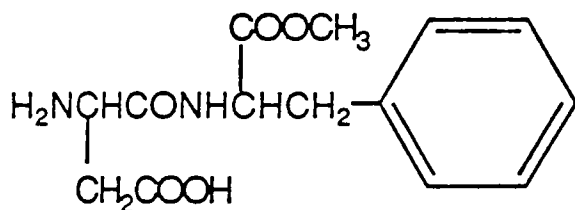
AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

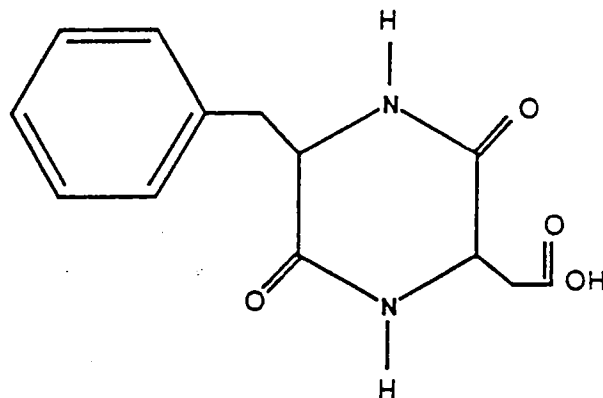
SUPERSEDES: NEW

METHOD SUMMARY:

This ion pairing isocratic HPLC method is provided for the analysis of low levels (parts per million range) of APM and DKP in ultralight beer. The method requires a placebo to check for, and subtract if necessary, any placebo interference with DKP. APM is fully resolved from all placebo peaks.

STRUCTURES:

(L,L-α-Aspartame)



(Diketopiperazine)

EXPERIMENTAL:Reagents and Chemicals:

1. HPLC grade water
2. HPLC grade acetonitrile
3. 85% o-Phosphoric acid, (reagent grade)
4. Pentane sulfonic acid sodium salt, monohydrate (Kodak)
5. 10 N sodium hydroxide (reagent grade)
6. Triethylamine (reagent grade)

NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 2 of 12
JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

Solution Preparation:

PIC-B5 reagent:

1. In a 600-mL beaker, add 57.5 g of o-phosphoric acid to 300 mL of water.
2. Add 48 g of pentane sulfonic acid and stir to dissolve.
3. Adjust the pH to 2.5 with triethylamine.
4. Transfer the solution to a 500-mL volumetric flask and dilute to volume with water.

Mobile Phase:

1. Mix 1820 mL of water with 20 mL of the PIC-B5 reagent and 160 mL of acetonitrile.
2. Adjust the pH to 2.5 with o-phosphoric acid.
3. Filter using 0.45 micron nylon filter and degas for 5 minutes using vacuum.

Sample Preparation:

1. Open the beer bottle and pour contents into a wide mouth flask.
2. Stir and sonicate, skimming off the foam occasionally, until the foaming subsides and the beer is thoroughly degassed.
3. Transfer aliquots into WISP vials (in duplicate).

NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 3 of 12
JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

Standard Preparation:

1. In duplicate, weigh 10 mg of APM standard into separate 100-mL volumetric flasks. Dissolve and dilute to volume with mobile phase.
2. Pipet 5 mL of the solution into a 50-mL volumetric flask and dilute to volume with mobile phase. These are the APM std and std check solutions.
3. In duplicate, weigh 10 mg of DKP standard into separate 100-mL volumetric flasks. Dissolve and dilute to volume with mobile phase.
4. Pipet 5 mL of the solution into a 50-mL volumetric flask and dilute to volume with mobile phase. These are the DKP std and std check solutions.
5. Prepare a 1:10 dilution of the DKP std solution to set the integration parameters.

Apparatus:

HPLC system equipped with a UV detector and autosampler.

Instrumental Conditions:

Chromatographic Conditions:

Column:	Whatman Partisil 5 ODS-3 RAC II, 10 cm x 4.6 mm
Column Temperature:	Ambient
Mobile Phase:	8% acetonitrile, 1% PIC-B5, 91% HPLC grade water, pH adjusted to 2.5 with phosphoric acid.
Flow Rate:	2 mL/min.
Injection Volume:	100 microliters
Detection:	UV at 210 nm at 0.01 AUFS
Run Time:	Approximately 20 minutes
Integration parameters:	Set to detect 1 ppm solution of DKP

NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 4 of 12
JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

Chromatographic Profiles:

1. Approximate Retention:

DKP: 3.7 min., at $k' = 6.4$

APM: 10.1 min., at $k' = 19.2$

2. Typical chromatograms are shown in figures 1 and 2.

ANALYSIS PROCEDURE

System Suitability:

Before actual run, make sure the system is suitable by injecting 3 replicates of standards. The RSD of the areas and retention times should be within +/- 2%.

Assay Sequence:

1. Perform a standard check by injecting the standard check solution between standards. The standard check should be within +/- 2% of the theory. If not, repeat standard preparation.
2. Bracket injections of the samples by the injections of the APM and DKP standards.

NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 5 of 12
JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

Calculations:

1. Standard Check:

$$\% \text{ Standard} = \frac{R \text{ std} \times \text{mg STD check}}{R \text{ std check} \times \text{mg STD}} \times 100$$

Where: R std = average peak response of component
in standard injections.

R std check = average peak response of component
in standard check injection.

2. Analysis:

- a) Accurately determine the peak responses of interest (preferably peak area) in all standard and sample injections.
- b) Calculate the concentration, X, in mcg/mL, for each component in the sample according to:

$$X = \frac{R_u}{R_s} \times C_s$$

Where: X = concentration for each component (mcg/mL or ppm)
Ru = peak response for each component in the sample.
Rs = peak response for each component in the standard
Cs = concentration for each component in the standard.
mcg/mL..

c) Report the individual values.

d) Average the individual values and report along with SD.

NOTE: Appropriate computer programs may be used for these calculations.

NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 6 of 12
JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

VALIDATION SUMMARY

The HPLC assay for α -APM and DKP in beer was validated by two analysts performing one run on each of two days on different instrumentations. On each run, two sample results per targeted concentration were collected. The performance characteristics of the method are as follows:

Precision:

	<u>Within Run RSD</u>	<u>Between Run RSD</u>	<u>Total RSD</u>
α -APM Level			
20 ppm	1.23	0.84	1.18
10 ppm	1.06	5.07	5.18
5 ppm	1.33	10.62	10.71

DKP Level

12 ppm	1.27	2.11	2.46
6 ppm	1.21	3.62	3.82
3 ppm	3.78	4.37	5.78

Accuracy:

% Mean Recovery

α -APM Level

20 ppm	100.2
10 ppm	100.2
5 ppm	97.9

DKP Level

12 ppm	97.5
6 ppm	92.2
3 ppm	89.5

NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 7 of 12
JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

Linearity:

The response for α -APM is linear from 2 ppm to 20 ppm.

Slope	~ 90867
Intercept	16197
Correlation Coefficient	0.99990

The response for DKP is linear from 1 ppm to 20 ppm.

Slope	96874
Intercept	45902
Correlation Coefficient	0.99980

Sensitivity:

The minimum quantifiable quantities are approximately 5 ppm for α -APM and 3 ppm for DKP. Values between 5 to 2 ppm α -APM and 3 to 1 ppm DKP are detected but the effect of baseline at those levels makes measurements semi-quantitative.

Selectivity:

The method is selective for α -APM. No interference was observed from the placebo beer. There is placebo interference in the DKP peak observed by one analyst in one system. A correction for this peak is necessary for an accurate quantitation.

NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 8 of 12
JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

COMMENTS:

Column/Systems Clean-up:

1. Flush the entire system with 300 mL of filtered, degassed, distilled water or HPLC grade bottled water.
2. At the end of the water washing, wash the autoinjector as follows:
 - a) for the WISP, run the purge cycle;
 - b) for the H.P. autoinjector, run the wash cycle for 10 minutes.
3. Flush the system with 150 mL of HPLC grade, degassed acetonitrile

REFERENCE:

NS 945, pp 129-134 E. Victorino

NS 928, pp 168-190 T. Albano

NUTRASWEET R&D ANALYTICAL DEPARTMENT

PAGE 9 of 12

SPECIFICATIONS AND METHODS DOCUMENT

JANUARY, 1992

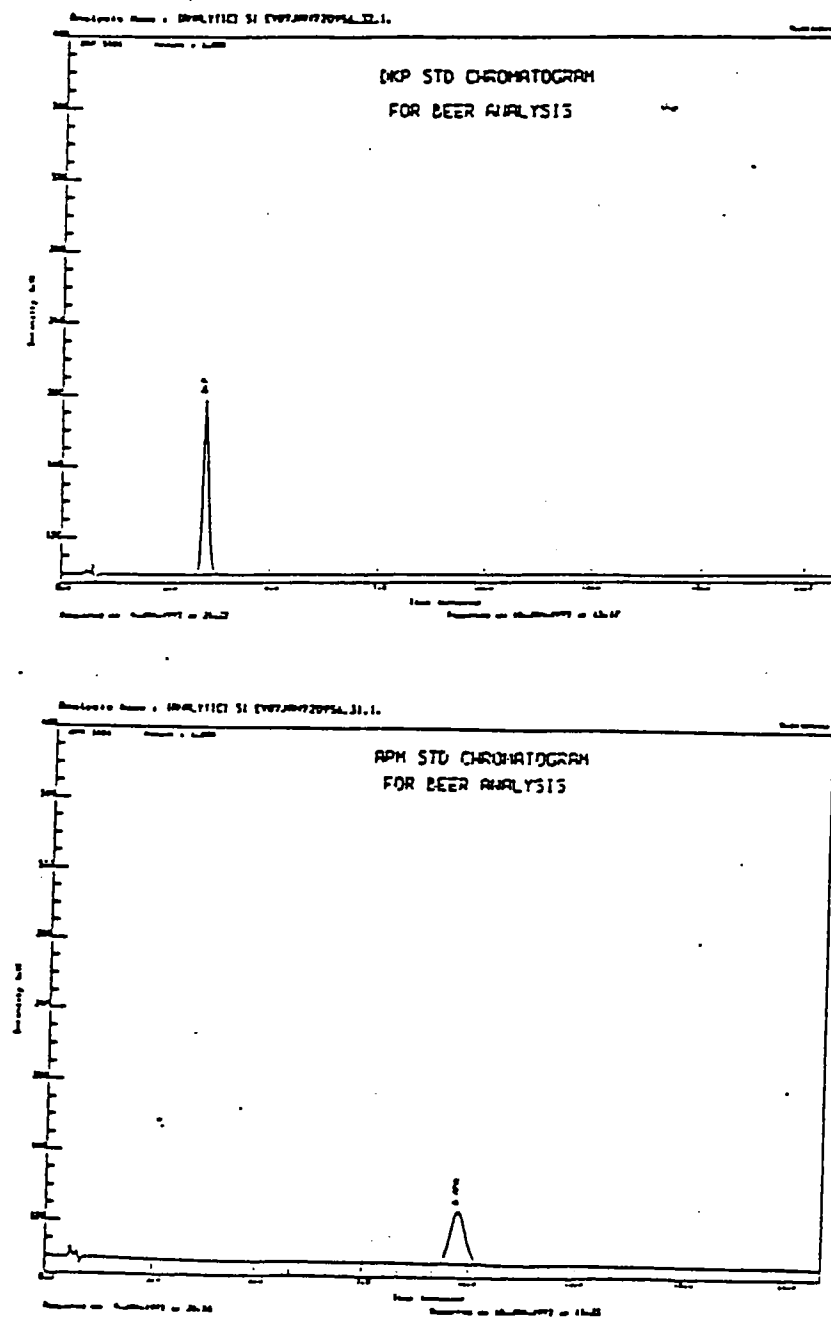
TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

FIGURE 1



NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 10 of 12
JANUARY, 1992

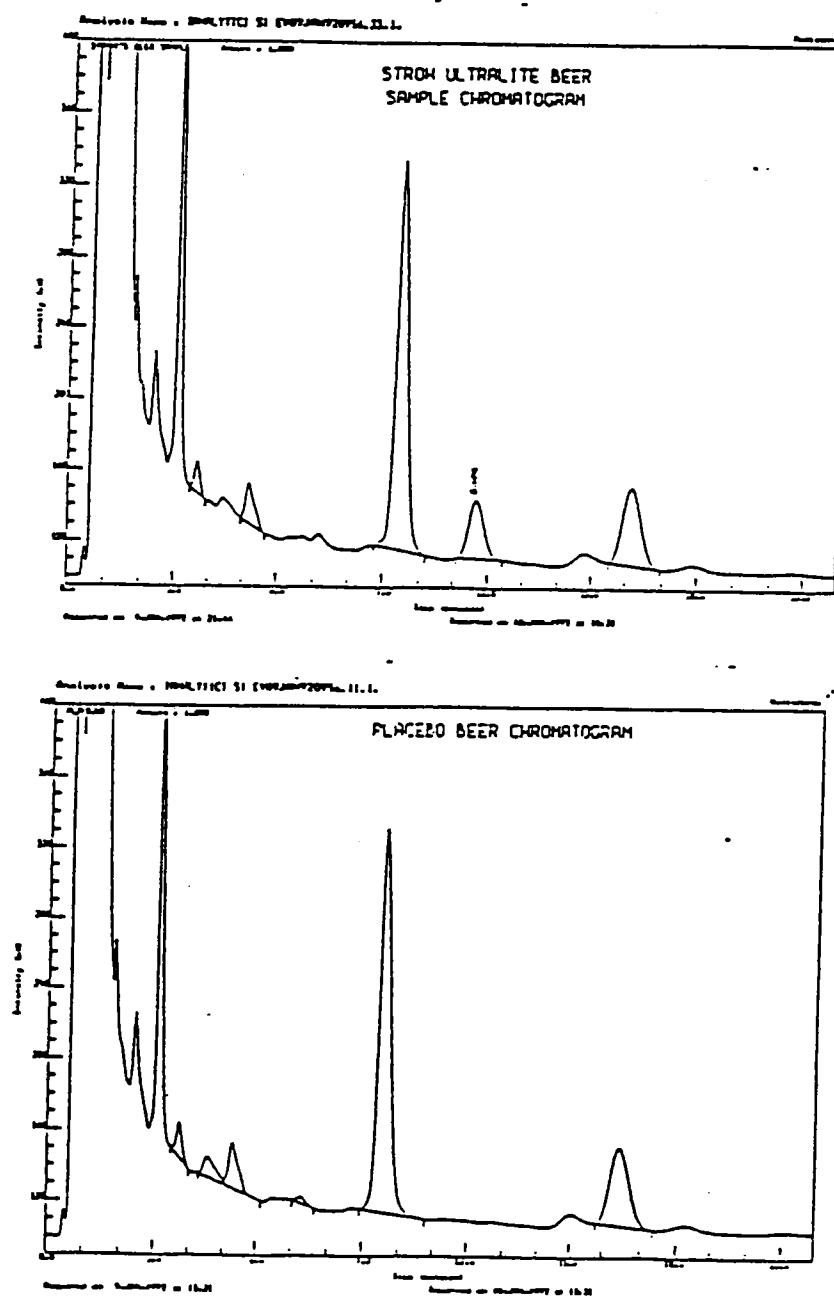
TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

FIGURE 2



NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 11 of 12
JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

APPENDIX 1

I N T E R O F F I C E M E M O R A N D U M

Date: 13-Jan-1992 04:36pm CST
From: Tom Carr
CARRT
Dept: Statistical Services
Tel No: 506-2304

TO: Ted Albano

(ALBANO)

CC: Zenaida Tagamolila

(TAGAMOLILA)

Subject: APM and DKP in Beer -- Method Validation

I have reviewed the precision, accuracy and linearity portions of the method validation document you provided me with today. The protocols and analyses are statistically appropriate and correct. The results summarized in the document are accurate when compared to the corresponding VALSUM computer output.

NUTRASWEET R&D ANALYTICAL DEPARTMENT
SPECIFICATIONS AND METHODS DOCUMENT

PAGE 12 of 12
JANUARY, 1992

TITLE: HPLC ASSAY FOR DKP AND APM IN BEER

AUTHOR: T. ALBANO

DOCUMENT NO.: NS-M92-005-A

SUPERSEDES: NEW

Approval Signatures:

Teddy P. Albano 1-15-92
Author Date

Estelle R. Wilkins 1-16-92
Author Date

J. P. Tagamoh 1/16/92
Supervisor Date

Pollan Shi 1/17/92
Referee, NutraSweet R&D Analytical Sciences Date

Michael F. Cleary 1/20/92
Manager, NutraSweet R&D Analytical Sciences Date

Project Code: 20001