

**Analytical Method for the Determination of
Aspartame and Diketopiperazine in
Baked Goods and Baking Mixes**

October 8, 1992

**The NutraSweet Company
1751 Lake Cook Road, Deerfield IL**

METHOD OUTLINE

- I. Summary and Overview of the Method**
- II. Method for the Determination of Aspartame and Diketopiperazine in Baked Goods and Baking Mixes**
- III. Analytical Considerations for the Use of the Method**

I. Summary and Overview of the Method

A simple reverse-phase isocratic high performance liquid chromatography method was developed to quantitate the amount of aspartame (APM) and diketopiperazine (DKP) in baked goods and baking mixes and in finished formulations prior to baking. The method involves a two-phase liquid extraction system in which hexane is used to remove lipid material, and APM and DKP remain in the aqueous phase. Standard curves prepared in buffer were linear for the determination of both APM and DKP. The precision, accuracy, specificity, and sensitivity of the method were established. Recoveries of APM and DKP from various products were very high for both analytes.

The method is intended to ensure compliance with the use limit of aspartame in baked goods set in the regulation (21 CFR 172.804(c)(23)). As such, the method should be used as follows:

- 1) To measure aspartame in refrigerated or frozen ready-to-bake products and dry mixes prepared for baking according to manufacturers' directions.
- 2) To measure aspartame in finished formulations prior to baking.
- 3) To measure aspartame and DKP in ready-to-eat baked goods. The measured levels of aspartame and DKP are converted to the level of aspartame in the finished formulations prior to baking.

II. Method for the Determination of APM and DKP in Baked Goods and Baking Mixes

Purpose:

This method is applicable to the separation and quantitation of aspartame (APM) and diketopiperazine (DKP) in baked goods, dry mixes, refrigerated or frozen ready-to-bake products, and finished formulations prior to baking. Only APM need be measured in refrigerated or frozen ready-to-bake products and finished formulations prepared from commercial dry mixes or from individual ingredients. Both APM and DKP concentrations must be determined in ready-to-eat baked goods. APM and DKP concentrations in baked goods are used to derive APM use levels in finished formulations prior to baking as described in Part III below.

Apparatus:

1. HPLC system consisting of suitable pump, injector, variable UV detector, recorder and integrator
2. Laboratory centrifuge
3. Mechanical shaker
4. Nalgene centrifuge cup, 250 mL
5. Ultrasonic bath and a vacuum source of degassing
6. pH meter

Reagents:

1. Hexane, HPLC grade
2. o-phosphoric acid, 85%, ACS grade
3. Sodium phosphate monobasic, monohydrate, ACS grade

4. Acetonitrile, HPLC grade
5. Water, HPLC grade
6. APM & DKP Reference Standards (available from the United States Pharmacopeia)

Preparation of 0.0125 M Sodium Phosphate Buffer:

1. Weigh 9.3 grams of sodium phosphate monobasic and transfer to a 6 L flask. Add 5400 mL water and stir to dissolve the salt.
2. Transfer 1800 mL of the buffer to a 2 L flask. Adjust the pH to 2.5 with phosphoric acid.
3. Adjust the pH of the remaining 3600 mL of the buffer to 3.5 with phosphoric acid.

Preparation of 65:35 Acetonitrile:Phosphate Buffer Solution:

1. Add together in a 1 L flask, 325 mL acetonitrile and 175 mL sodium phosphate buffer, pH 2.5.
2. Stir until well mixed.

Preparation of Mobile Phase:

1. Add 400 mL acetonitrile to the 3600 mL of pH 3.5 sodium phosphate buffer. Stir until well mixed. In some instances, the pH may be varied slightly to improve the resolution of APM and DKP from closely eluting peaks.
2. Filter through a 0.45 micron membrane (such as Millipore HAWP-025-00). Degas by sonification under vacuum or by helium sparge.

Preparation of APM Standards:

1. Accurately weigh, to the nearest 0.01 mg, 10 mg, 15 mg, and 25 mg of APM standard into separate 100 mL volumetric flasks. Label the flasks APMSTD-1, APMSTD-2, and APMSTD-3, respectively.
2. Dissolve the APM in about 70 mL of mobile phase. Dilute to volume with mobile phase and mix well.
3. Filter each standard solution through a 0.45 micron membrane (such as a Millex-HA filter) into HPLC vials.

Preparation of DKP Standards (Baked Goods Only):

1. Accurately weigh, to the nearest 0.01 mg, 20 mg DKP Reference Standard into a 100 mL volumetric flask.
2. Dissolve the DKP in about 70 mL of mobile phase. Dilute the flask to volume with mobile phase and mix well. This is the stock solution.
3. Pipet 5, 10, and 20 mL aliquots of the stock solution into separate 50 mL volumetric flasks. Dilute each flask to volume with mobile phase and mix well.
4. Filter each standard solution through a 0.45 micron membrane into HPLC vials.

Sample Preparation:

1. For finished formulations prior to baking and dry mixes, use the product directly. For baked products, cut and chop the sample using a blender.
2. Accurately weigh, in duplicate, 10.0 g (see Notes of Interest 1) of each sample. Transfer each sample into a 250 mL centrifuge cup.

3. If the method of quantitation is by standard addition, weigh four additional 10.0 g samples into each of four 250 mL centrifuge cups. Add the DKP stock solution with baked good samples only.
 - a. Into each of two cups containing preweighed samples, accurately weigh 5 mg of APM Reference Standard and add 10 mL of DKP stock solution.
 - b. To each of the remaining cups, accurately weigh 10 mg of APM Reference Standard and 20 mL of DKP stock solution.
4. To each of the six cups, add 50 mL of hexane and 25 mL of 65:35 Acetonitrile:Phosphate Buffer. (Note: hexane is used to reduce the quantity of lipid material in the aqueous fraction and to liberate APM from the matrix.)
5. Shake each sample on a mechanical shaker for 30 minutes at its maximum arm swing setting.
6. To the first two sample weighings, (Step 2), add 75 mL of pH 2.5 phosphate buffer to each sample. To samples initially prepared in 3a, add 65 mL of pH 2.5 phosphate buffer. To samples initially prepared in 3b, add 55 mL of pH 2.5 phosphate buffer.
7. Shake each cup on a shaker for 45 minutes and then centrifuge for 10 minutes at 300 rpm. (Note: extraction times can be changed to maximize recovery or to reduce processing time).
8. Remove the hexane layer by suction and discard. Using a syringe, carefully withdraw a portion of the supernatant layer and filter through a 0.45 micron filter unit into an HPLC vial for analysis.

Chromatographic Conditions:

Column: uBondapak C-18, 30 cm x 3.9 mm i.d.
equipped with a uBondapak C-18 Guard-Pak

Mobile Phase: 10% Acetonitrile/90% 0.0125 M NaH₂PO₄
adjusted to pH 3.5 with phosphoric acid

Flow Rate: 2.0 ml/minute

Injection Volume: 20 µL

Detection: UV, 210 nm, 0.1 AUFS

Pressure: 2000 psig

Temperature: ambient

Approximate Run Time: 17 minutes

Typical chromatograms are shown in Figures 1 - 4.

Sample Analysis:

1. Inject each APM standard, DKP standard (baked goods only), and sample. Inject standard solutions at the end of the run.
2. If the response of any unspiked sample is greater than the response of the highest standard, dilute the sample solution with mobile phase accordingly.

Calculations Using Standard Curves:

1. APM

- a. Calculate the concentration of APM in each APM standard solution using the following formula:

$$\text{Conc. APM, mg/ml} = \frac{W}{100}$$

Where, W = Weight of APM standard in
each APM standard solution,
mg

- b. Record the peak area for each APM standard injection.

- c. Plot the peak area versus the corresponding APM standard concentration. Calculate the slope and y-intercept of the APM standard curve analysis using linear regression analysis.
- d. Record the APM peak area for each sample.
- e. Determine the APM concentration (Cx) in mg/mL for each sample using the slope and y-intercept of the standard curve.
- f. Calculate the APM concentration in mg/g for each sample.

$$\text{Conc. APM, mg/g} = \frac{(C_x) \times 100}{W_s}$$

Where, Cx = Concentration of APM in the sample, mg/ml
Ws = Weight of sample, g

2. DKP (Baked Goods Only)

- a. Calculate the concentration of DKP in each DKP standard.
- b. Record the peak area of DKP in each DKP standard injection.
- c. Plot the peak area of the DKP standards versus the corresponding standard concentration in mg/mL. Using linear regression analysis, calculate the slope and y-intercept of the DKP standard curve.
- d. Record the DKP peak area for each sample injection.
- e. Determine the DKP concentration (Cy) in mg/mL for each sample using the slope and y-intercept of the standard curve.
- f. Calculate the DKP concentration in mg/g for each sample.

$$\text{Conc. DKP, mg/g} = \frac{(C_y) \times 100}{W_s}$$

Where, Cy = Concentration of DKP in the sample, mg/mL
Ws = Weight of sample, g

- Note: 1) The standard curves for APM and DKP must have a correlation coefficient of not less than 0.98. If not, repeat the standard preparation.
- 2) An appropriate computer program may be used for the calculations.

Calculations Using Standard Addition Method:

1. APM

- a. Record the APM peak area for each sample, unspiked and spiked with APM and DKP standards.
- b. Plot the peak area versus the corresponding APM standard concentration in mg/mL. Using linear regression analysis, calculate the slope and y-intercept.
- c. Using the intercept, determine the concentration of APM in mg/mL (Cx) in the unspiked sample by extrapolation.
- d. Calculate the APM concentration in mg/g for the unspiked sample:

$$\text{Conc. APM, mg/g} = \frac{(C_x) \times 100}{W_s}$$

Where, Cx = Concentration of APM in the sample, mg/mL
Ws = Weight of sample, g

2. DKP (Baked Goods Only)

- a. Record the DKP peak area for each sample, unspiked and spiked with APM and DKP standards.

- b. Plot the peak area versus the corresponding spiked DKP standard concentration, mg/mL. Using linear regression analysis, calculate the slope and y-intercept.
- c. Determine the concentration of DKP in mg/mL, (Cx), in the unspiked sample by extrapolation.
- d. Calculate the DKP concentration in mg/g for the unspiked sample.

$$\text{Conc. DKP, mg/g} = \frac{(C_y) \times 100}{W_s}$$

Where, C_y = Concentration of DKP in the sample, mg/mL
 W_s = Weight of sample, g

System Clean-Up:

Wash the system and column with 150 mL of HPLC water followed by 75 mL of HPLC grade acetonitrile.

Notes of Interest:

1. Sample amount taken for analysis may be varied, depending upon the expected level of APM.
2. As in any HPLC method, a system suitability check must be performed before starting the analysis.
3. If the slope for the standard addition method of response versus the concentration of the analyte added is significantly less (greater than 20%) than that for the standard curve, then values obtained from the standard addition method should be used.

FIGURES

Figure 1
Chromatogram of APH Standard

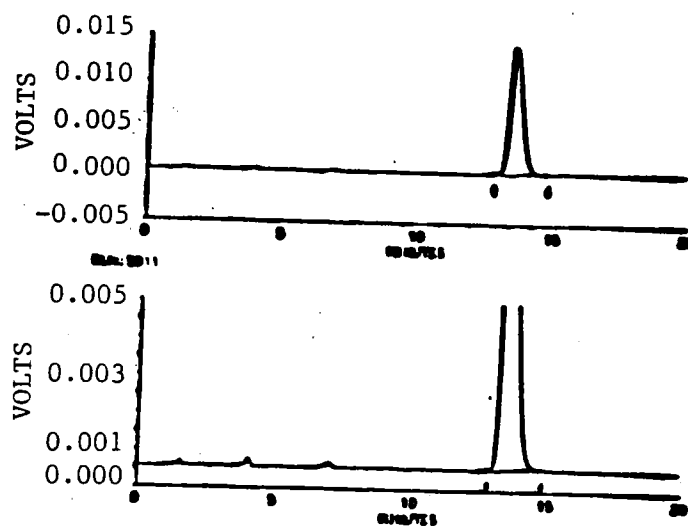
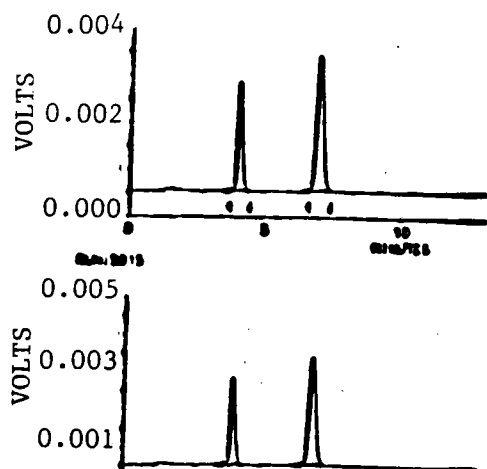


Figure 2
Chromatogram of DFP-AP Standard



FIGURES

Figure 3
Chromatogram of Placebo Cake

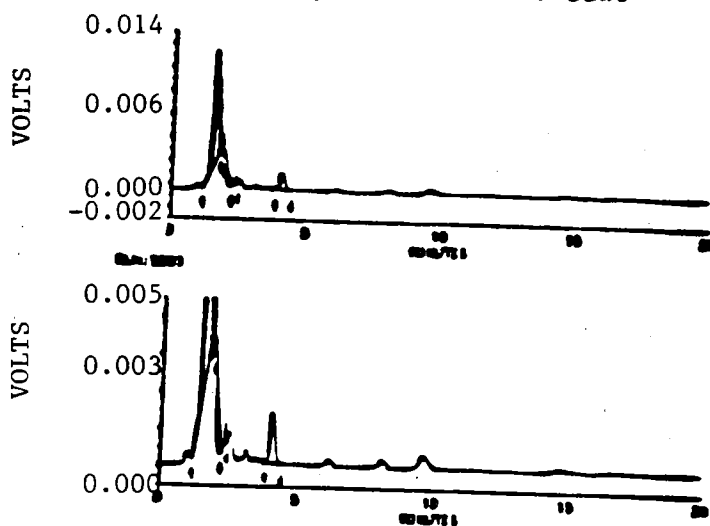
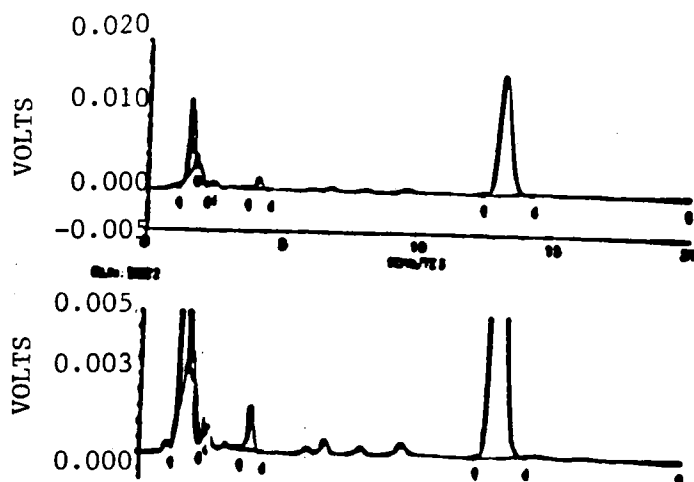


Figure 4
Chromatogram of Cake Containing Encapsulated APH



III. Analytical Considerations for the Use of the Method

This section describes the derivation of APM use levels in finished formulations prior to baking using APM and DKP levels measured in ready-to-eat baked goods.

The concentration in the finished formulation prior to baking can be calculated as follows:

$$\text{APM Conc. in Finished Formulation (mg/g)} = \left[\text{APM Conc. in Baked Good (mg/g)} + \left[\text{DKP Conc. in Baked Good (mg/g)} \times \frac{\text{Molecular Wt.}}{\text{Correction Factor}} \right] \right] \times \frac{\text{Moisture Loss Correction Factor}}{1}$$

The molecular weight correction factor is the ratio of the molecular weights of APM (M.W. 294) and DKP (M.W. 262), i.e. 1.12, and serves to convert DKP concentration to APM concentration. A correction factor for moisture loss during baking is also introduced to avoid an overestimate of the APM concentration in the finished formulation prior to baking. Moisture loss during baking usually varies between 5% and 15% depending on time, temperature, product surface area, and moisture content in the finished formulation and, ideally, should be determined for each baked product to be analyzed. However, because the regulation (21 CFR 172.804(c)(23)) imposes a limit on APM use level not because APM is unsafe but to ensure that manufacturers follow GMP (Good Manufacturing Practice) a correction factor may be used that is typical for most baked goods. This value is 0.9 and corresponds to 10% moisture loss, which is intermediate between 5% and 15% loss usually observed during baking. A correction factor different from 0.9 may be used if moisture loss has been accurately determined.



DEPARTMENT OF HEALTH & HUMAN SERVICES

Robert C. Peterson
Public Health Service

Food and Drug Administration
Washington DC 20204

10-7-93

Robert C. Peterson
Director, Regulatory Compliance
The NutraSweet Company
Box 730
1751 Lake Cook Road
Deerfield, IL 60015-5239
Facsimile 708-940-1447

Dear Mr. Peterson:

This is in response to your telephone inquiry of October 7, 1993. No objections to the Final Rule for use of aspartame in baked goods and baking mixes (published in the U.S. Federal Register of April 19, 1993) have been received.

We hope this response is adequate. Please feel free to contact us if you have questions regarding any of the above.

Sincerely yours,

F. Owen Fields
F. Owen Fields, Ph.D.
Novel Ingredients Branch, HFS-207
Division of Product Policy
Center for Food Safety
and Applied Nutrition



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

September 4, 1992

ROBERT C. PETERSON

SEP 10 1992

Robert C. Peterson
Director
The Nutrasweet Co.
1751 Lake Cook Rd.
Deerfield, IL 60015-5239

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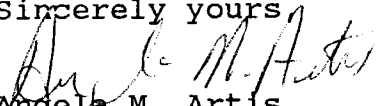
Re: Food Additive Petition No. 7A4044

Dear Mr. Peterson:

This will acknowledge receipt of your amendment dated
September 1, 1992, transmitting additional information in
support of the above referenced food additive petition.

Because of the nature of the information submitted in support
of your petition, we will need an extended review period.

Sincerely yours


Angela M. Artis
Division of Food & Color Additives
Center for Food Safety
and Applied Nutrition

The NutraSweet Company

Box 730, 1751 Lake Cook Road, Deerfield, Illinois 60015-5239
Telephone: 708/940-9800



September 1, 1992

Owen Fields, Ph.D.
Center for Food Safety
and Applied Nutrition
Food and Drug Administration
1110 Vermont
Washington, DC 20204

Re: Clarification of the Amendment of the Aspartame Regulation to
Allow Use in Baked Goods and Baking Mixes

Dear Dr. Fields:

It is apparent from recent conversations with Food and Drug Administration scientists that a clarification in the requested amendment of 21 CFR 172.804 allowing use of aspartame in baked goods and baking mixes is necessary. Our proposed amendment detailed in a letter to you from me dated ~~July~~ 16, 1992, requested approval in "Baked goods and baking mixes in combination with safe and suitable ingredients at levels not to exceed 0.5% aspartame." Our intention was to set the limit of 0.5% aspartame on the finished formulation prior to baking and not on aspartame concentration in dry mixes or finished baked goods. Therefore, for purposes of clarification, we respectfully request that the amendment read "Baked goods and baking mixes in combination with safe and suitable ingredients at levels not to exceed 0.5% aspartame in the finished formulation prior to baking."

This requested amendment is the most practical manner to regulate aspartame use in baking. It is easily understood by the food industry and compliance can be readily monitored. The reference method can be used to quantitate aspartame in finished formulations, baking mixes and baked goods.

If you have any questions, please contact me.

Sincerely,

Robert Peterson
Director, Regulatory Compliance