

**ANALYTICAL METHODS  
FOR**

- 1) GELLED**
- 2) NOUGAT**
- 3) CARAMEL**
- 4) COMPOUND COATING**

**ASPARTAME IN  
SOFT CANDY**

**APPENDIX II  
PAGE 2**

METHOD# NAM87-030

**DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
IN GELLED CANDIES**

ISSUED: DECEMBER 1987

SUPERSEDES: NEW

*Mary Della Costa (sk)*  
AUTHOR

*Henry Anderson (sk)*  
APPROVED BY

SCOPE:

This method is provided for the determination of aspartame, A-AP and diketopiperazine in gelled candies.

APPARATUS:

1. HPLC system consisting of suitable pump, injector, variable UV detector, strip chart recorder and, or A/D converter and integrator. The system used for the method validation was a Waters 840 system.
2. Laboratory centrifuge.
3. Blender (osterizer type).
4. Mini-blend containers, 4 oz.
5. Nalgene centrifuge cups, 250-ml.
6. Ultrasonic bath and a vacuum source for degassing.

REAGENTS:

1. Methanol, HPLC Grade
2. Sodium Phosphate Monobasic, ACS Grade
3. Phosphoric Acid, ACS Grade
4. Acetonitrile, HPLC Grade
5. Milli-Q water or equivalent

PREPARATION OF 0.0125 M SODIUM PHOSPHATE BUFFER:

1. Weigh 3.4 grams of sodium phosphate monobasic and transfer to a 2 liter flask. Add 2000 ml of Milli-Q water and stir to dissolve the salt.
2. Adjust the pH to 3.5 with phosphoric acid.

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PREPARATION OF SAMPLE SOLVENT, 40:60 METHANOL:MILLI-Q WATER

1. Add together in a 1 liter flask, 400 ml methanol and 600 ml Milli-Q water.
2. Stir until well mixed.
3. Adjust pH to 3.5 with phosphoric acid.

PREPARATION OF MOBILE PHASE:

1. In a 4 liter flask, mix together, 1800 ml sodium phosphate buffer and 200 ml acetonitrile. Stir until well mixed.
2. Filter through a 0.45 micron membrane (such as Millipore HAWP-025-00). Degas by sonification under vacuum or by helium purge.

PREPARATION OF APM STANDARDS:

1. Accurately weigh, to the nearest 0.01 mg, 6 mg, 8 mg, and 10 mg amounts of Aspartame Working Standard into separate 100-ml volumetric flasks. Label the flasks APMSTD-1, APMSTD-2 and APMSTD-3, respectively.
2. Dissolve the aspartame in about 70 ml of mobile phase. Q.S. each flask with mobile phase and mix well.
3. Filter each standard solution through a 0.45 micron membrane (such as a Millex-HA filter) into WISP vials.

PREPARATION OF DKP/AP STANDARDS:

1. Accurately weigh, to the nearest 0.01 mg, 10 mg DKP and AP reference standard into 100 ml volumetric flask.
2. Dissolve the DKP and AP in about 70 ml of mobile phase. Label these flasks AP/DKP-1, AP/DKP-2 and AP/DKP-3, respectively. Q.S. the flask with mobile phase and mix well. This is the Stock Solution.
3. Pipet 5-, 10-, and 30-ml aliquotes of the Stock Solution into separate 50 ml volumetric flasks. Q.S. each flask with mobile phase and mix well.
4. Filter each standard solution through a 0.45 micron membrane into WISP vials.

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**SAMPLE PREPARATION:**

1. Cut samples into small pieces.
2. Accurately weigh, in duplicate, to the nearest 1.0 mg an amount of the gelled candy equivalent to 7.5 mg APM into a 4 oz blender-cup.
3. Accurately pipet, to each sample, 100 mg of the 40:60 Methanol: Water.
4. Blend each sample with a blender for 1-2 minutes, or until samples are reasonably chopped.
5. Sonicate samples in a warm water bath for 15 minutes. Blend at high speed for 30-60 seconds. Repeat sonication. Blend. Sample should be completely dissolved. If not repeat steps.  
NOTE: SAMPLE PREPARATION SHOULD BE DONE QUICKLY. SAMPLES SOLIDIFY WHEN LEFT STANDING.
6. Filter the supernatant through a 0.45 micron membrane into WISP vials.

**CHROMATOGRAPHIC CONDITIONS:**

**Column:**

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

**Mobile Phase:**

10% Acetonitrile/90% 0.0125 M NaH<sub>2</sub>PO<sub>4</sub> adjusted to pH 3.5 with phosphoric acid.

**Flow Rate:**

2.0 ml/min

**Injection Volume:**

50 uL (microliters)

**Detection:**

UV, 210 nm, 0.1 AUFS

**Pressure:**

2000 psig

**Temperature:**

Ambient

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Approximate Run Time:

17 minutes

SAMPLE ANALYSIS:

1. Make several injections of the APMSTD-2 and AP/DKPSTD-2. Check the chromatograms to verify reproducible retention times and acceptable peak shapes.
2. Make duplicate injections of each APMSTD, AP/DKPSTD and sample.

CALCULATIONS:

PART I. APM

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

$$\text{CONC. APM, mg/ml} = \frac{W \times F}{100}$$

Where, W = Weight of Aspartame Working Standard in each APM standard, mg.

F = Correction for Aspartame Standard Purity

2. Record the peak area for each APM Standard injection and calculate the average peak area for each set of duplicate injections.
3. Plot the average peak area versus the corresponding APM standard concentration. Using least squares or regression analysis, calculate the slope and y-intercept of the APM standard curve.
4. Record the APM peak area for each sample injection and calculate the average peak area for each set of duplicate injections. Subtract the area of any placebo interference.
5. Determine the APM concentration (Cx) for each sample using the slope and y-intercept of the standard curve.

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6. Calculate the percent APM found in each sample.

$$\text{Percent APM} = \frac{(Cx) \times 100 \text{ ml} \times 100}{Ws}$$

Where: Cx = Concentration of APM in the sample, mg/ml

Ws = Weight of sample, mg

PART II. AP/DKP

1. Calculate the concentration of AP or DKP in each AP/DKP std.
2. Record the peak areas of AP/DKP in each AP/DKP Std injection and calculate the average peak areas for each set of duplicate injections.
3. Plot the average peak areas of the AP/DKP standards versus the corresponding standard concentrations. Using least squares or regression analysis, calculate the slope and y-intercept of the AP/DKP standard curve.
4. Record the AP/DKP peak areass for each sample injection and calculate the average peak areas for each set of duplicate injections.
5. Determine the AP/DKP concentration (Cy) in mg/ml for each sample using the slope and y-intercept of the standard curves.
6. Calculate the percent AP/DKP for each sample.

$$\text{Percent DKP} = \frac{(Cy) \times 100 \text{ ml} \times 100}{Ws}$$

Where: Cy = Concentration of AP/DKP in the sample, mg/ml

Ws = Weight of sample, mg

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Figure 1  
Chromatogram of APM Standard

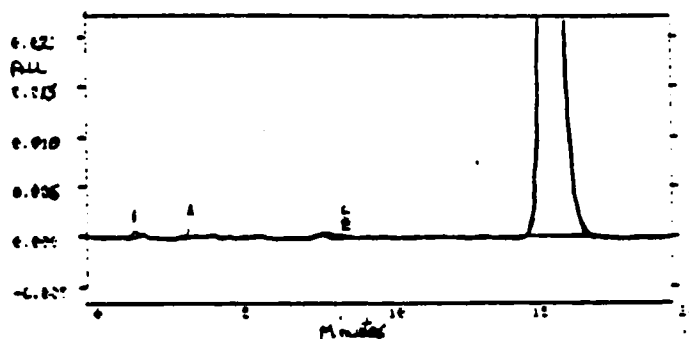
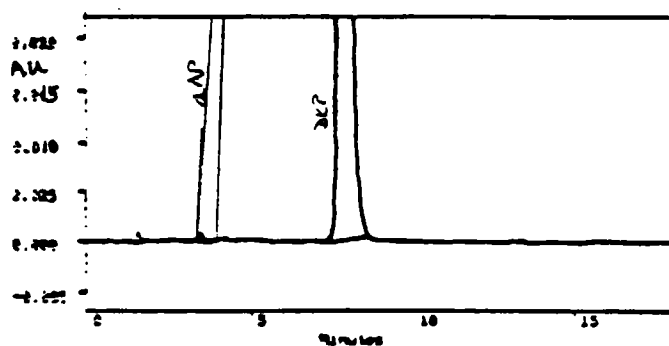


Figure 2  
Chromatogram of AP/DKP Standard



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Figure 3  
Chromatogram of Gelled Candy Sample

Chromatogram of PLACES





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Method Validation Summary

APM

Precision:

The between run precision of the method using the spiked placebos was 2.4%. The within run RDS is 1.9%. The 95% upper confidence limit for the within run RDS is 3.1%. The 95% upper confidence limit for the total RDS was 12.7%. (N=12, 2 days/2 analysts).

Accuracy

The average percent recovery of the method determined from the spiked placebo recoveries was 97.6% (N=12). (2days/2 analysts)

Specificity

There is no interference between APM and placebo peaks in orange gelled candy.

Sensitivity

The minimum detectable quantity is 1 ppm. The sensitivity is considered satisfactory for this method.

References

N.B. Ref. 256, p 211-215, M. L. Dalla Costa  
N.B. Ref. 259, p. 247-249, B. Joanino

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DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
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Method Validation Summary

DKP

Precision:

The between run precision of the method using the spiked placebos was 2.3%. The within run RDS is 1.2%. (N=6 1 Day/1 Analyst). The 95% upper confidence limit for within run RDS is 3.6%. The 95% upper confidence limit for the total RDS was 8.7%

Accuracy

The average percent recovery of the method determined from the spiked placebo recoveries was 93.7% (N=6)

Specificity

There is no interference between DKP and placebo peaks in orange gelled candy.

Sensitivity

The minimum detectable quantity is 1 ppm. The sensitivity is considered satisfactory for this method.

References

N.B. Ref. 256, p. 226 to 229, M. L. Dalla Costa

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DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
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Method Validation Summary

a-AP

Precision:

The between run precision of the method using the spiked placebos was 2.9%. The within run RDS is 1.6%. (N=6 1 Day/1 Analyst). The 95% upper confidence limit for within run RDS is 4.1%. The 95% upper confidence limit for the total RDS was 9.8%.

Accuracy

The average percent recovery of the method determined from the spiked placebo recoveries was 94.8% (N=6)

Specificity

There is no interference between AP and placebo peaks in orange gelled candy.

Sensitivity

The minimum detectable quantity is 1 ppm. The sensitivity is considered satisfactory for this method.

References

N.B. Ref. 256, p. 226 to 229, M. L. Dalla Costa

DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
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METHOD# NAM88-008

ISSUED: JUNE 1988

SUPERSEDES: NEW

Mary Delle Cote (A.A.)  
AUTHOR

Jerry Hansen (A.A.)  
APPROVED BY

SCOPE:

This method is provided for the determination of aspartame, alpha aspartylphenylalanine, and diketopiperazine in nougat candies. The method has been validated for a lycasin formula of nougat candy. The method should be revalidated if any other formulations are used.

APPARATUS:

1. HPLC system consisting of suitable pump, injector, variable UV detector, strip chart recorder and/or A/D converter and integrator. The system used for the method validation was a Waters 840 HPLC System.
2. Laboratory centrifuge.
3. Mallet.
4. Nalgene centrifuge cups, 250-ml.
5. Ultrasonic bath and a vacuum source for degassing.

REAGENTS:

1. Dry ice.
2. Phosphoric Acid, ACS Grade.
3. Sodium Phosphate Monobasic, ACS Grade.
4. Acetonitrile, HPLC Grade.
5. Milli-Q water or equivalent.

PREPARATION OF 0.0125 M SODIUM PHOSPHATE BUFFER:

1. Weigh 6.2 grams of sodium phosphate monobasic and transfer to a 4 liter flask. Add 3600 ml Milli-Q water and stir to dissolve the salt.
2. Adjust the pH to 3.5 with phosphoric acid.

PREPARATION OF 65:35 ACETONITRILE: PHOSPHATE BUFFER SOLUTION:

1. Measure 650 ml of acetonitrile in a 1 liter flask. Add 350 ml of 0.0125 M sodium phosphate buffer, pH 3.5.
2. Stir until well mixed.

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**PREPARATION OF MOBILE PHASE:**

1. In a 2 liter flask, mix together, 1800 ml sodium phosphate buffer and 200 ml acetonitrile. Stir until well mixed.
2. Filter through a 0.45 micron membrane (such as Millipore HAWP-025-00). Degas by sonification under vacuum or by helium purge.

**PREPARATION OF APM STANDARDS:**

1. Accurately weigh, to the nearest 0.01 mg, 8 mg, 12 mg, and 14 mg amounts of Aspartame Working Standard into separate 100-ml volumetric flasks. Label the flasks APMSTD-1, APMSTD-2 and APMSTD-3, respectively.
2. Dissolve the aspartame in about 70 ml of mobile phase. Q.S. each flask with mobile phase and mix well.
3. Filter each standard solution through a 0.45 micron membrane (such as a Millex-HA filter) into WISP vials.

**PREPARATION OF DKP/AP STANDARDS:**

1. Accurately weigh, to the nearest 0.01 mg, 25 mg DKP and 20 mg of AP Reference Standards into a 100-ml volumetric flask.
2. Dissolve the DKP/AP standards in about 15 ml of 65:35 CH<sub>3</sub>CN:buffer. Q.S. the flask with mobile phase and mix well. This is the Stock Solution.
3. Pipet 5-, 10-, and 15-ml aliquots of the Stock Solution into separate 100-ml volumetric flasks. Q.S. each flask with mobile phase and mix well.
4. Filter each standard solution through a 0.45 micron membrane into WISP vials.

**SAMPLE PREPARATION:**

1. Wrap samples in foil and freeze until hard. With a mallet, break samples into pieces. For the lycasin samples, weigh samples as you break them. Due to the composition of lycasin, the samples melt as soon as they are removed from the ice.

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SAMPLE PREPARATION:

2. Accurately weigh approximately 5 grams of samples into a nalgene cup.
3. Accurately pipet, to each sample, 25 ml of 65:35 CH<sub>3</sub>CN:buffer solution and 25 ml of hexane.
4. Shake each sample on a wrist shaker for one half hour or until samples are dissolved.
5. Remove samples from shaker. Pipet 75 ml of phosphate buffer to each sample. Shake for an additional half hour.
5. Centrifuge samples for ten minutes.
6. Remove the fat layer from the aqueous layer by suction.
7. Filter the supernatant through a 0.45 micron CR membrane into WISP vials.

CHROMATOGRAPHIC CONDITIONS:

|                          |   |
|--------------------------|---|
| Column:                  | Supelcosil LC-18, 25 cm. x 4.6 mm i.d.<br>Supelco C-18 Guard column |
| Mobile Phase:            | 10% Acetonitrile/90% 0.0125 M NaH <sub>2</sub> PO <sub>4</sub>      |
| Flow Rate:               | 2.0 ml/minute.  |
| Injection Volume:        | 50 uL (microliters).  |
| Detection:               | UV, 210 nm, 0.1 AUFS.   |
| Pressure:                | 2000 psig.  |
| Temperature:             | ambient.  |
| Approximate<br>Run Time: | 30 minutes.   |

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SAMPLE ANALYSIS:

1. Make duplicate injections of the APM STD-2 and the DKP-AP STD-2. Check the chromatograms to verify reproducible retention times and acceptable peak shapes.
2. Make duplicate injections of each APM std, DKP-AP std, and sample.

CALCULATIONS:

PART I. APM

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

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

Where, W = Weight of Aspartame Working Standard in each APM standard, mg.

F = Correction for Aspartame Standard Purity.

2. Record the peak area for each APM Standard injection and calculate the average peak area for set of duplicate injections.
3. Plot the average peak area versus the corresponding APM standard concentration. Using least squares or regression analysis, calculate the slope and y-intercept of the APM standard curve.
4. Record the APM peak area for each sample injection and calculate the average peak area for each set of duplicate injections. Subtract the area of any placebo interference.
5. Determine the APM concentration (Cx) for each sample using the slope and y-intercept of the standard curve.
6. Calculate the percent APM found in each sample.

$$\text{Percent APM,} = \frac{(Cx) \times 100 \text{ ml}}{Ws} \times 100$$

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

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PART II. DKP/AP

1. Calculate the concentration of DKP and AP in each DKP/AP Std.
2. Record the peak areas of DKP and AP in each DKP/AP Std injection and calculate the average peak areas for each set of duplicate injections.
3. Plot the average peak areas of the DKP and AP standards versus the corresponding standard concentrations. Using least squares or regression analysis, calculate the slope and y-intercept of the DKP standard curve and the AP standard curve.
4. Record the DKP and AP peak areas for each sample injection and calculate the average peak areas for each set of duplicate injections.
5. Determine the DKP concentration (Cy) in mg/ml and the AP concentration (Cz) in mg/ml for each sample using the slope and y-intercept of the standard curves.
6. Calculate the percent DKP and the percent AP for each sample:

$$\text{Percent DKP} = \frac{(\text{Cy}) \times 100}{\text{Ws}} \times 100$$

(AP)

Cy = Concentration of DKP or AP in the sample, mg/ml  
Ws = Weight of sample, mg



DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
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Figure 1  
Chromatogram of APM Standard

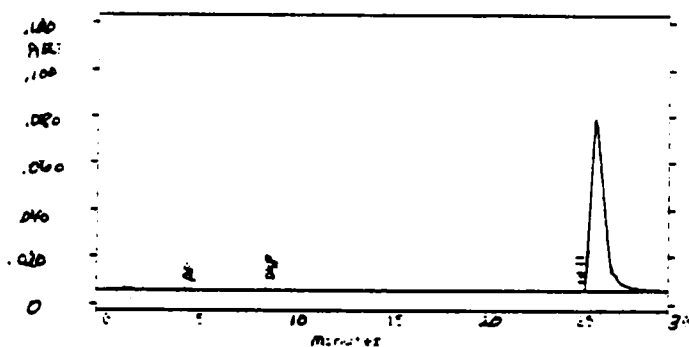
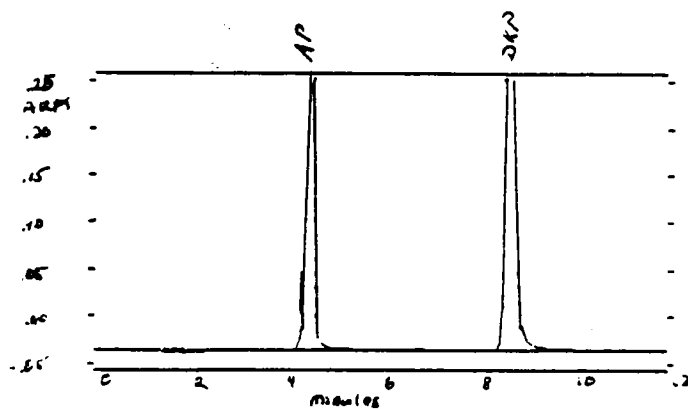


Figure 2  
Chromatogram of DKP/AP Standard



DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
IN NOUGAT CANDIES

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Method Validation Summary

APM

Precision:

The within run RSD of the method using the spiked placebos was 0.98%. (N=12, 2 days/2 analysts)  
The between run precision of the method using spiked placebos was 0.39%. (N=12, 2days/2analysts)  
The total precision of the method was 1.05% with a 95% upper confidence limit for the total RSD of 1.83%. (N=12)

Accuracy

The average percent recovery of the method determined from the spiked placebo recoveries was 100.55%, (N=12, 2 days/2 analysts). The 95% confidence limits were 95.50% to 105.60%.

The method is linear in the range of 0.08 to 0.14 mg/ml.

Specificity

There is no interference between APM and placebo peaks.

Sensitivity

The minimum detectable quantity is 1 ppm. The sensitivity is considered satisfactory for this method.

References

N.B. References: 326, p.295-300, Bernie Joanino  
336, p.67-70, 105-108 M. Dalla Costa

**DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
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**METHOD# NAM88-008**

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Method Validation Summary

DKP

Precision:

The within run RSD of the method using the spiked placebos was 1.47%. (N=12, 2 days/2 analysts).

The between run precision of the method using spiked placebos was 2.60%. (N=12, 2 days/2 analysts)

The total precision of the method was 2.99% with a 95% upper confidence limit for within run RSD of 18.82%.

Accuracy

The average percent recovery of the method determined from the spiked placebo recoveries was 98.15%, (N=12, 2 days/2 analysts). The 95% confidence limits were 74.58% to 121.75%.

The method is linear in the range of 0.02 to 0.10 mg/ml.

Specificity

There is interference between DKP and placebo peaks, however, it is less than four percent.

Sensitivity

The minimum detectable quantity is 1 ppm. The sensitivity is considered satisfactory for this method.

References

N.B. References: 336, p. 67-70, 105-108, M. Dalla Costa  
326, p. 295-300, B. Joanino

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METHOD# NAM88-008

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Method Validation Summary

alpha-AP

Precision:

The within run RSD of the method using the spiked placebos was 1.60%. (N=12, 2 days/2 analysts)  
The between run precision of the method using spiked placebos was 0. (2 days/2 analysts)  
The total precision of the method was 1.60% with a 95% upper confidence limit of the total RSD of 2.55%. (N=12)

Accuracy

The average percent recovery of the method determined from the spiked placebo recoveries was 101.5%, (N=12, 2 days/2 analysts). The 95% confidence limits were 98.62% to 100.68%.

The method is linear in the range of 0.02 to 0.08 mg/ml.

Specificity

There is interference between AP and placebo peaks, however, it is less than four percent.

Sensitivity

The minimum detectable quantity is 1 ppm. The sensitivity is considered satisfactory for this method.

References

N.B. References: 336, p. 67-70, 105-108, M. L. Dalla Costa  
326, p. 295-300, B. Joanino

DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
IN CARAMEL CANDIES

METHOD# NAM88-003

ISSUED: March 1988

SUPERSEDES: NEW

Mary Della Costa  
AUTHOR

James Harrison  
APPROVED BY

SCOPE:

This method is provided for the determination of aspartame, alpha aspartylphenylalanine, and diketopiperazine in caramel candies. The method has been validated for a lycasin formula of caramel candy. The method should be revalidated if any other formulations are used.

APPARATUS:

1. HPLC system consisting of suitable pump, injector, variable UV detector, strip chart recorder and/or A/D converter and integrator. The system used for the method validation was a Waters 840 HPLC System.
2. Laboratory centrifuge.
3. Blender (osterizer type).
4. Mini-blend containers, 4 oz..
5. Nalgene centrifuge cups, 250-ml.
6. Ultrasonic bath and a vacuum source for degassing.

REAGENTS:

1. Methanol, HPLC Grade.
2. Phosphoric Acid, ACS Grade.
3. Sodium Phosphate Monobasic, ACS Grade.
4. Acetonitrile, HPLC Grade.
5. Milli-Q water or equivalent.
6. Sodium sulfate.

PREPARATION OF 0.0125 M SODIUM PHOSPHATE BUFFER:

1. Weigh 3.4 grams of sodium phosphate monobasic and transfer to a 2 liter flask. Add 2000 ml Milli-Q water and stir to dissolve the salt.
2. Adjust the pH to 3.0 with phosphoric acid.

PREPARATION OF SAMPLE SOLVENT: SODIUM SULFATE IN METHANOL AND WATER

1. Weigh 10 grams of sodium sulfate. Place in a 2 liter flask. Add 1 liter of Milli-Q water, mix well. Add 1.5 liters of methanol.

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IN CARAMEL CANDIES**

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2. Stir until well mixed.

**PREPARATION OF MOBILE PHASE:**

1. In a 4 liter flask, mix together, 1800 ml sodium phosphate buffer and 200 ml acetonitrile. Stir until well mixed.
2. Filter through a 0.45 micron membrane (such as Millipore HAWP-025-00). Degas by sonification under vacuum or by helium purge.

**PREPARATION OF APM STANDARDS:**

1. To the nearest 0.01 mg, 10 mg, 15 mg, and 25 mg amounts of Aspartame Working Standard into separate 100-ml volumetric flasks. Label the flasks APMSTD-1, APMSTD-2 and APMSTD-3, respectively.
2. Dissolve the aspartame in about 70 ml of mobile phase. Q.S. each flask with mobile phase and mix well.
3. Filter each standard solution through a 0.45 micron membrane (such as a Millex-HA filter) into WISP vials.

**PREPARATION OF DKP/AP STANDARDS:**

1. Accurately weigh, to the nearest 0.01 mg, 20 mg DKP and 15 mg of AP Reference Standards into a 100-ml volumetric flask.
2. Dissolve the DKP/AP standards in about 70 ml of mobile phase Q.S. the flask with mobile phase and mix well. This is the Stock Solution.
3. Pipe 5-, 10-, and 20-ml aliquots of the Stock Solution into separate 50-ml volumetric flasks. Q.S. each flask with mobile phase and mix well.
4. Filter each standard solution through a 0.45 micron membrane into WISP vials.

**SAMPLE PREPARATION:**

1. If samples are elastic type, wrap samples in foil and freeze until hard. If samples are submitted hard, wrap in foil and continue as follows. Add the hard samples to a blender cup and grind with a blender. Transfer to a suitable sample container.

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IN CARAMEL CANDIES**

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**SAMPLE PREPARATION:**

2. Accurately weigh approximately 10 grams into a nalgene cup.
3. Accurately pipet, to each sample, 100 ml of sample solvent and 25 ml of hexane.
4. Shake each sample on a wrist shaker for one half hour or until samples are dissolved.
5. Centrifuge samples for ten minutes.
6. Extract the fat layer from the aqueous layer by suction.
7. Filter the supernatant through a 0.45 micron CR membrane into WISP vials.

**CHROMATOGRAPHIC CONDITIONS:**

|                       |   |
|-----------------------|---|
| Column:               | - Supelco C-18, 25 cm. x 4.6 mm i.d.  |
| Mobile Phase:         | 10% Acetonitrile/90% 0.0125 M NaH <sub>2</sub> PO <sub>4</sub> adjusted to pH 3.0 with phosphoric acid. |
| Flow Rate:            | 2.0 ml/minute.  |
| Injection Volume:     | 50 uL (microliters).  |
| Detection:            | UV, 210 nm, 0.1 AUFS.   |
| Pressure:             | 2000 psig.  |
| Temperature:          | ambient.  |
| Approximate Run Time: | 30 minutes.   |

DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
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SAMPLE ANALYSIS:

1. Make duplicate injections of the APM STD-2 and the DKP-AP STD-2. Check the chromatograms to verify reproducible retention times and acceptable peak shapes.
2. Make duplicate injections of each APM std, DKP-AP std, and sample.

CALCULATIONS:

PART I. APM

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

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

Where, W = Weight of Aspartame Working Standard in each APM standard, mg.

F = Correction for Aspartame Standard Purity.

2. Record the peak area for each APM Standard injection and calculate the average peak area for set of duplicate injections.
3. Plot the average peak area versus the corresponding APM standard concentration. Using least squares or regression analysis, calculate the slope and y-intercept of the APM standard curve.
4. Record the APM peak area for each sample injection and calculate the average peak area for each set of duplicate injections. Subtract the area of any placebo interference.
5. Determine the APM concentration (Cx) for each sample using the slope and y-intercept of the standard curve.
6. Calculate the percent APM found in each sample.

$$\text{Percent APM,} = \frac{(Cx) \times 100 \text{ ml}}{Ws} \times 100$$

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



DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
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PART II. DKP/AP

1. Calculate the concentration of DKP and AP in each DKP/AP Std.
2. Record the peak areas of DKP and AP in each DKP/AP Std injection and calculate the average peak areas for each set of duplicate injections.
3. Plot the average peak areas of the DKP and AP standards versus the corresponding standard concentrations. Using least squares or regression analysis, calculate the slope and y-intercept of the DKP standard curve and the AP standard curve.
4. Record the DKP and AP peak areas for each sample injection and calculate the average peak areas for each set of duplicate injections.
5. Determine the DKP concentration (Cy) in mg/ml and the AP concentration (Cz) in mg/ml for each sample using the slope and y-intercept of the standard curves.
6. Calculate the percent DKP and the percent AP for each sample:

$$\text{Percent DKP (AP)} = \frac{(C_y) \times 100}{W_s} \times 100$$

Cy = Concentration of DKP or AP in the sample, mg/ml  
Ws = Weight of sample, mg

DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
IN CARAMEL CANDIES

METHOD# NAM88-003

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Figure 1  
Chromatogram of APM Standard

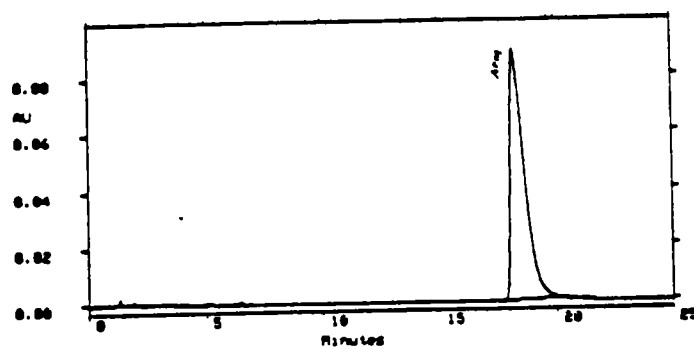
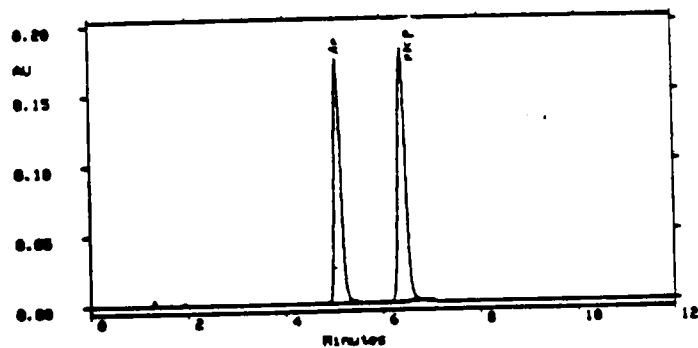


Figure 2  
Chromatogram of DKP/AP Standard



DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
IN CARAMEL CANDIES

METHOD# NAM88-003

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Method Validation Summary

APM

Precision:

The within run RSD of the method using the spiked placebos was 0.51% (N=6, 1day/1analyst).  
The 95% upper confidence limit for within run RSD is 1.21%.

Accuracy

The average percent recovery of the method determined from the spiked placebo recoveries was 94.04%, (N=6).

Specificity

There is no interference between APM and placebo peaks.

Sensitivity

The minimum detectable quantity is 1 ppm. The sensitivity is considered satisfactory for this method.

References

N.B. Ref. 326, p.148-154, Bernie Joanino.

DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
IN CARAMEL CANDIES

METHOD# NAM88-003

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Method Validation Summary

DKP

Precision:

The within run RSD of the method using the spiked placebos was 3.65% (N=6, 1day/1analyst).

The 95% upper confidence limit for within run RSD is 8.65%.

Accuracy

The average percent recovery of the method determined from the spiked placebo recoveries was 96.18%, (N=6).

Specificity

There is interference between DKP and placebo peaks, however, it is less than four percent.

Sensitivity

The minimum detectable quantity is 1 ppm. The sensitivity is considered satisfactory for this method.

References

N.B. Ref. 326, p.148-149, Bernie Joanino.

**DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS  
IN CARAMEL CANDIES**

**METHOD# NAM88-003**

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Method Validation Summary

alpha-AP

Precision:

The within run RSD of the method using the spiked placebos was 2.81% (N=6, 1day/1analyst).

The 95% upper confidence limit for within run RSD is 6.68%.

Accuracy

The average percent recovery of the method determined from the spiked placebo recoveries was 101.5%, (N=6).

Specificity

There is interference between AP and placebo peaks, however, it is less than four percent.

Sensitivity

The minimum detectable quantity is 1 ppm. The sensitivity is considered satisfactory for this method.

References

N.B. Ref. 336, p.22-26, M. L. Dalla Costa.

**ASPARTAME IN  
SOFT CANDY**

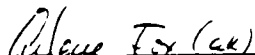
**APPENDIX II  
PAGE 30**

METHOD# NAM87-031

TITLE: DETERMINATION OF ASPARTAME AND DEGRADATION PRODUCTS IN  
COMPOUND COATINGS

DATE OF ISSUE: DECEMBER, 1987

SUPERSEDES: NEW

  
AUTHOR

  
APPROVED BY

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SCOPE:

This method is provided for the determination of aspartame (APM), diketopiperazine (DKP) and  $\alpha$ -Aspartyl-L-phenylalanine ( $\alpha$ -AP) in milk compound coatings.

APPARATUS:

- 1) HPLC system consisting of suitable pump, injector, variable UV detector and integrator.
  - a.  $\alpha$ -AP and DKP analyses require HP 1090M with QuickRes Curve Resolution Software.
- 2) Wrist action shaker.
- 3) Nalgene centrifuge cups, 250-ml.
- 4) Mouli-grater.
- 5) Ultrasonic bath and a vacuum source for degassing.
- 6) Laboratory centrifuge.
- 7) pH meter.

REAGENTS:

- 1) Acetonitrile, HPLC grade.
- 2) Milli Q water or equivalent.
- 3) Sodium Phosphate Monobasic, ACS grade.
- 4) Phosphoric acid, 85%, ACS grade.
- 5) Hexane, HPLC grade.
- 6) Reference standards for APM,  $\alpha$ -AP and DKP.

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**PREPARATION OF MOBILE PHASE:**

- 1) DMOA/H<sub>3</sub>PO<sub>4</sub> stock solution preparation: 0.05 M DMOA in 3.3 M H<sub>3</sub>PO<sub>4</sub>.
  - a. Weigh 0.77 g of DMOA into 100 ml volumetric flask on a top loading balance.
  - b. Add approximately 50 ml Milli-Q water.
  - c. Add 23.0 ml of concentrated H<sub>3</sub>PO<sub>4</sub> (85%).
  - d. Dilute to volume with Milli-Q water and mix.
  - e. Refrigerate for long term storage (up to 3 months).
- 2) Pipet 20.0 ml of the DMOA/H<sub>3</sub>PO<sub>4</sub> stock solution into an Erlenmeyer flask.
- 3) Add 3980 ml of Milli-Q water and mix well.
- 4) Adjust pH to 3.45 with 50% NaOH.
- 5) Add 348 ml of acetonitrile and mix well.
- 6) Filter through a 0.45 micron membrane (such as Millipore HAWP-025-00). Degas by sonication under vacuum or by helium purge.

**PREPARATION OF 0.0125 M SODIUM PHOSPHATE BUFFER:**

- 1) Weigh 3.44 g of sodium phosphate monobasic and transfer to a 2 liter flask. Add 2000 ml. Milli-Q water and stir to dissolve salt.
- 2) Adjust the pH to 3.00 with phosphoric acid.

**PREPARATION OF 30:70 ACETONITRILE: PHOSPHATE BUFFER SOLUTION:**

- 1) Add together in a 1 liter flask, 300 ml acetonitrile and 700 ml sodium phosphate buffer.
- 2) Stir until well mixed.

**PREPARATION OF APM STANDARDS:**

- 1) Accurately weigh to the nearest 0.01 mg, 10 mg, 15 mg, and 25 mg amounts of Aspartame Reference Standard into separate 100-ml volumetric flasks. Label the flasks APM STD 1, APM STD 2 and APM STD 3.
- 2) Dissolve the APM in 50 ml of 30:70 Acetonitrile: Phosphate Buffer. Dilute to volume with Phosphate Buffer and mix.
- 3) Filter through a 0.45 micron membrane (such as Gelman #4217).

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**PREPARATION OF  $\alpha$ -AP/DKP STANDARDS:**

- 1) Accurately weigh, to the nearest 0.01 mg, 12 mg of  $\alpha$ -AP Reference Standard plus 12 mg DKP Reference Standard into a 100 ml volumetric flask.
- 2) Dissolve  $\alpha$ -AP and DKP in 50 ml of 30:70 Acetonitrile: sample solvent. Dilute to volume with phosphate buffer and mix well. This is the  $\alpha$ -AP/DKP Stock Solution.
- 3)  $\alpha$ -AP/DKP Standard 1: Pipet 15.0 ml Stock Solution into a 100 ml volumetric flask. Dilute to volume with phosphate buffer and mix well. Filter through a 0.45 micron membrane.
- 4)  $\alpha$ -AP/DKP Standard 2: Pipet 15.0 ml Stock Solution into a 50 ml volumetric flask. Dilute to volume with sample solvent and mix well. Filter through a 0.45 micron membrane.
- 5)  $\alpha$ -AP/DKP Standard 3: Pipet 10.0 ml Stock Solution into a 25 ml volumetric flask. Dilute to volume with sample solvent and mix well. Filter through a 0.45 micron membrane.

**SAMPLE PREPARATION:**

- 1) Grate chocolate with mouli grater.
- 2) Accurately weigh, in duplicate, 5.00 g of each compound coating. Transfer each sample to a 250 ml Nalgene centrifuge cup.
- 3) Add to each sample 50 ml. hexane and 25.0 ml of 30:70 Acetonitrile: Phosphate Buffer.
- 4) Shake each sample on a mechanical shaker for 30 minutes.
- 5) Add 25.0 ml of Phosphate Buffer to each sample. Shake on a mechanical shaker for 30 minutes and then centrifuge for 10 minutes at 3000 rpm.
- 6) Remove the hexane layer by suction. Filter a portion of the supernatant through a 0.45 micron Autovial nylon membrane.
- 7) This procedure (steps 1-6) should be followed in preparing a placebo and an  $\alpha$ -AP/DKP spiked placebo to check for the possible presence of other materials eluting at the same retention time as  $\alpha$ -AP, APM or DKP.



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CHROMATOGRAPHIC CONDITIONS:

Column: Supelcosil C-18DB, 25 cm. x 4.6 mm. i.d. with 2 cm. LC-18 Supelcoguard column.

Mobile Phase: 8.0% Acetonitrile/92.0% 0.26 mM DMOA in 0.017 M Phosphate Buffer, pH adjusted to 3.45 with NaOH.

Flow Rate: 2.0 ml./min.

Injection Vol.: 20 uL (microliters).

Detection: UV, 210 nm  
Store All Spectra  
Range 201 to 301 nm

Pressure: 2500 psig.

Temperature: Ambient

Approximate  
Run Time: 30 minutes

SYSTEM SUITABILITY CHECK:

- 1) Make duplicate injections of each APM standard and each  $\alpha$ -AP/DKP standard. APM standard 1 is reinjected in the middle and at the end of a long run of samples. The relative standard deviation of the area count for the APM Standard 1 should be less than 2.0%.
- 2) Check the placebo and the standard chromatograms to determine if there are any placebo interferences for  $\alpha$ -AP, APM and DKP. If necessary, small adjustments (0.5%) in the concentration of acetonitrile can be made to optimize the resolution. The placebo changes analytically over time so it may be necessary to use curve resolution for both  $\alpha$ -AP and DKP. The recovery determined by the  $\alpha$ -AP/DKP spiked placebo is used to determine whether curve resolution is being performed correctly.

SAMPLE ANALYSIS:

- 1) Make two injections of each sample preparation. Record the area counts of the analytes.

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CALCULATIONS:

PART 1: APM

- 1) Record the peak area for each APM Standard injection and calculate the average peak area for the set of duplicate injections.
- 2) Plot the average peak area versus the corresponding APM standard concentration (ppm). Using least squares or regression analysis, calculate the slope and y-intercept of the APM standard curve. From this information calculate the APM concentration (ppm) for each sample preparation (Cs1).
- 3) Determine the APM in each sample using the following equation:

$$\text{APM (ppm)} = \frac{\text{Cs1} \times 50}{\text{Ws}}$$

Where, Ws = Weight in grams of the sample.

PART 2: α-AP

- 1) Record the peak area for each α-AP Standard injection and calculate the average peak area for the set of duplicate injections.  
  
If QuickRes is necessary, peak area must be determined by creating a Total Wavelength Chromatogram (TWC) for the standard using QuickRes.
- 2) Plot the average peak area versus the corresponding α-AP standard concentration (ppm). Using least squares or regression analysis, calculate the slope and y-intercept of the α-AP standard curve. From this information, calculate the α-AP concentration in each sample preparation (Cs2).
- 3) Determine the α-AP in each sample by using the following equation:

$$\alpha\text{-AP (ppm)} = \frac{\text{Cs2} \times 50}{\text{Ws}}$$

**METHOD# NAM87-031**

**PART 3: DKP**

- 1) Record the peak area for each DKP Standard injection and calculate the average peak area for the set of duplicate injections.

If QuickRes is necessary, peak area must be determined by creating a TWC for the standard using QuickRes.

- 2) Plot the average peak area versus the corresponding DKP standard concentration (ppm). Using least squares or regression analysis, calculate the slope and y-intercept of the DKP standard curve. From this information calculate the DKP concentration for each sample preparation (Cs3).

$$\text{DKP (ppm)} = \frac{\text{Cs3} \times 50}{\text{Ws}}$$

Ws

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Figure 1  
Chromatogram of APM Standard

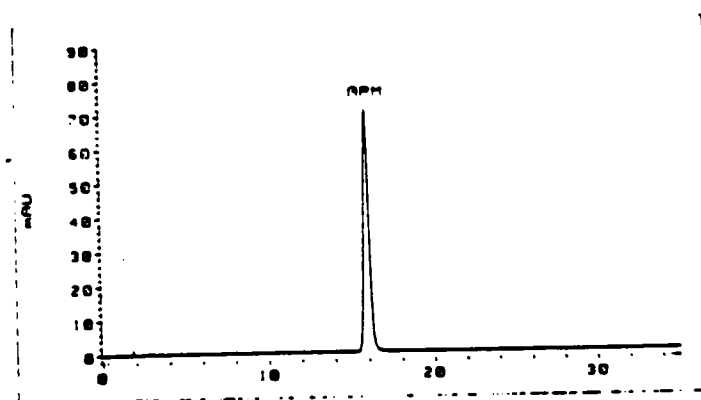
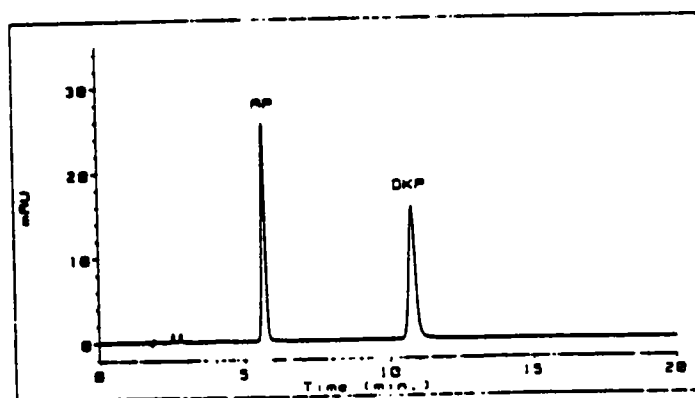


Figure 2  
Chromatogram of  $\alpha$ -AP and DKP Standard



METHOD# NAM87-031

Figure 3

Chromatogram of Compound Coating Placebo

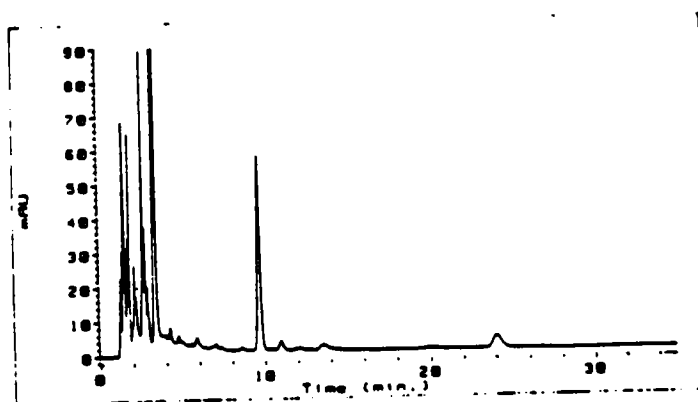
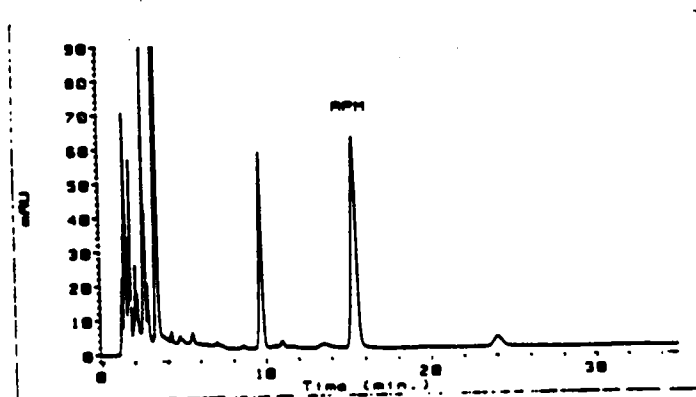


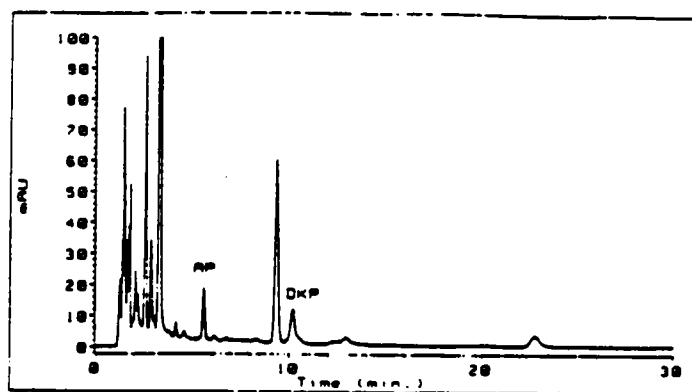
Figure 4

Chromatogram of Compound Coating Placebo Spiked with APM



METHOD# NAM87-031

Figure 5  
Chromatogram of Compound Coating Placebo Spiked  
with  $\alpha$ -AP and DKP



**METHOD# NAM87-031  
METHOD VALIDATION SUMMARY**

**APM**

**COMPOUND COATING**

**PRECISION:**

The within run precision of the method determined using placebo compound coating spiked with APM standard in the range of 0.75 to 1.88 mg/g of compound coating was 1.58% (N = 12, 2 days/2 analysts).

The between run precision of the method using the spiked placebos above was 2.17%.

The total precision of the method determined using the spiked placebos was 269% (N = 12).

**ACCURACY:**

The average percent recovery of the method determined from the spiked placebo recoveries was 96.70%.

The method is linear in the range of 0.05 mg/ml to 0.25 mg/ml.

**SPECIFICITY:**

The following seven degradation products will not interfere with the determination of APM: DKP, PA, PM,  $\alpha$ -AP, B-AP, PHE and B-APM.

There is no placebo interference for APM.

**SENSITIVITY:**

The minimum detectable quantity (MDQ) is 0.025 mg/g compound coating.

**REFERENCES:**

N.B. 326, pp. 30-32, B. Joanino  
N.B. 310, pp. 28-29 & 34-36, A. Fox

**METHOD# NAM87-031  
METHOD VALIDATION SUMMARY**

**α-AP**

**COMPOUND COATING**

**PRECISION:**

The within run precision of the method determined using placebo compound coating spiked with α-AP standard in the range of 0.12 to 0.40 mg/g of compound coating was 4.13% (N = 7, 2 days/1 analyst).

The between run precision of the method using the spiked placebos above was 1.48%.

**ACCURACY:**

The average percent recovery of the method determined from the spiked placebo recoveries was 99.65%.

The method is linear in the range of 0.017 mg/ml to 0.050 mg/ml.

**SPECIFICITY:**

APM and the following six degradation products will not interfere with the determination of α-AP: DKP, PA, PM, B-AP, PHE and B-APM.

Curve Resolution is used to eliminate problems with placebo interference.

**SENSITIVITY:**

The minimum detectable quantity (MDQ) is 0.010 mg/g compound coating.

**REFERENCES:**

N.B. 310, pp. 34-36 & 85-86, A. Fox.



**METHOD# NAM87-031  
METHOD VALIDATION SUMMARY**

**DKP**

**COMPOUND COATING**

**PRECISION:**

The within run precision of the method determined using placebo compound coating spiked with DKP standard in the range of 0.12 to 0.40 mg/g compound coating was 2.56% (N = 4, 1 day/1 analyst) when placebo interference did not require curve resolution. The within run precision for the spiked placebo runs where curve resolution had to be used was 2.56% (N = 7, 2 days/1 analyst).

The between run precision of the method with curve resolution was 0.83%.

The total precision of the method determined using the spiked placebos was 2.90% with curve resolution.

**ACCURACY:**

The average percent recovery of the method determined from the spiked placebo recoveries was 98.41% for the runs without curve resolution and 96.72% for the runs with curve resolution.

The method is linear in the range of 0.018 mg/ml to 0.050 mg/ml.

**SPECIFICITY:**

APM and the following six degradation products will not interfere with the determination of DKP: PA, PM,  $\alpha$ -AP, B-AP, PHE and B-APM.

The placebo for compound coating changes with time. Sometimes these changes result in problems with placebo interference that can be solved by curve resolution.

**SENSITIVITY:**

The minimum detectable quantity (MDQ) is 0.010 mg/g compound coating.

**REFERENCES**

N.B. 310, pp. 25-26, 81-82, & 85-86.