NAA

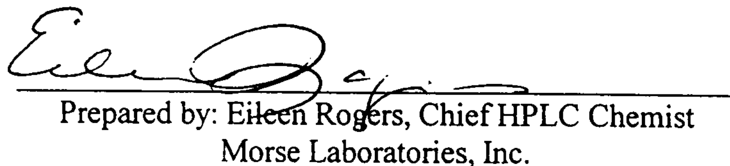
Rec'd 11/6/98
Method now required by
registrant (AMVAC)
[illegible]

Recommended Analytical Residue Method for 1-Naphthaleneacetic acid, 1-Napthalacetamide, and/or 1-Naphthaleneacetic acid, ethyl ester, in Grape (red and white), Grapefruit, Tomato, and Green Bell Pepper Matrices

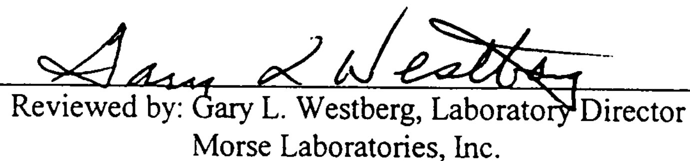
Analytical Method: NAA-AM-003

Date of Issue: June 5, 1998

Morse Laboratories, Inc.
1525 Fulton Avenue
Sacramento, California 95825



Prepared by: Eileen Rogers, Chief HPLC Chemist
Morse Laboratories, Inc.



Reviewed by: Gary L. Westberg, Laboratory Director
Morse Laboratories, Inc.

A. Principle

This method was developed based on AMVAC Chemical Corporation Analytical Method: NAA-AM-001 titled "Recommended Analytical Residue Method for 1-Naphthaleneacetic acid, 1-Naphthalacetamide, and/or 1-Naphthaleneacetic acid, ethyl ester in Apple and Pear Matrices" dated September 19, 1996. The method is capable of consistently meeting a target LOQ of 0.01 ppm for each analyte in combination (expressed as NAA equivalents), or individually.

Residues of 1-Naphthaleneacetic acid, 1-Naphthalacetamide, and 1-Naphthaleneacetic acid, ethyl ester are extracted from Grape (red and white), Grapefruit, Tomato, and Green Bell Pepper matrices by blending with deionized water then converted to the parent 1-Naphthaleneacetic acid through base hydrolysis. Extracts are filtered then partitioned into dichloromethane. An SPE cleanup using silica gel is performed prior to the determination of analyte concentration by High Performance Liquid Chromatography (HPLC) using fluorescence detection. Results are calculated using linear regression from external standards as NAA then a conversion factor may be applied to express results in terms of the desired analyte.

B. Apparatus Required (Equivalent items may be used provided they are functionally equivalent)

1. HPLC equipped with an autosampler and a fluorescence detector
2. HPLC Analytical Column
3. Mason jars, pint
4. Flat bottom evaporation flasks, 500 mL
5. Separatory funnels, 1000 mL
6. Rotary evaporator with cold finger condenser and temperature controlled water bath, Buchler or equivalent
7. Omni-mixer with variable speed adjustment equipped with blades appropriate for pint Mason jars.
8. Büchner filter funnel (9 cm diameter)
9. Teflon sleeves, 24/40
10. 500 mL side arm flasks (for Büchner filtration)
11. Whatman GF/A glass fiber filter media, 9 cm diameter
12. Reflux condensers, 300 mm
13. Heating mantles to fit 500 mL evaporation flasks
14. Culture tubes, screw cap (13 mm x 100 mm and 16 mm x 125 mm)
15. Centrifuge tubes, 15 mL conical, with screw caps.
16. Antifoam 1430 emulsion (Dow Corning), or equivalent

17. Vac Elut SPS 24 Solid Phase Extraction (SPE) Vacuum manifold, or equivalent
18. Varian Mega Bond Elut SI SPE columns (12 cc/2g)

    Note: Use of equivalent columns may require modification to the cleanup elution scheme. Any substitution should be rigorously tested for suitability.

19. 2.5 mL Luer-Lok glass syringes and 0.45 μm PTFE filter discs (Gelman Acrodisc 13 CR PTFE 0.45 μ or equivalent)
20. pH indicator strips, pH 1-12 range
21. Typical laboratory equipment (eg. pH meter, vortex mixer, nitrogen manifold, etc.)
22. Typical laboratory glassware (eg. graduated cylinders, funnels, Class A pipets, beakers, volumetric flasks, etc.)
23. Glass wool
24. N-Evap Analytical Evaporator with temperature controlled water bath and nitrogen manifold, or VWR Heat Block equipped with suitable test tube insert and Pierce Reacti-Vap manifold evaporating unit. Equivalent apparatus may be used to evaporate samples under a gentle stream of nitrogen at a controlled temperature.
25. Vacuum filtration apparatus equipped with 47 mm, 0.45μ nylon 66 filtering membrane

C. <u>Reagents Required</u>

1. Dichloromethane, Omni Solve, glass distilled
2. Hexane, Omni Solve, glass distilled
3. Acetonitrile, Burdick and Jackson, HPLC grade
4. Water, Burdick and Jackson, HPLC grade
5. Glacial acetic acid, reagent grade
6. Hydrochloric acid, concentrated, reagent grade
7. Deionized water
8. Sodium sulfate, reagent grade
9. Sodium hydroxide, reagent grade
10. Potassium Phosphate, monobasic (KH_2PO_4), reagent grade

D. **Standards Required:** (Lot numbers and purities may vary)

1. 1-Naphthaleneacetic acid, technical MW: 186.2
2. 1-Naphthalacetamide, technical MW: 185.24
3. 1-Naphthaleneacetic acid, ethyl ester, technical MW: 214.26

Known purity and lot numbers supplied by:

AMVAC Chemical Corporation
4100 E. Washington Blvd.
Los Angeles, CA 90023-4406

E. **Preparation of Solutions and Reagents**

1. Liquid Chromatographic Standard Solutions

   Prepare a solution of approximately 1 mg/mL NAA analytical standard in acetonitrile. Mix thoroughly and dilute with 1:1 acetonitrile/water to concentrations appropriate for HPLC analysis (typically 50 ng/mL, 25 ng/mL, 10 ng/mL, and 3 ng/mL -- lowest standard is equivalent to 60% of LOQ for NAA and NAA Amide, 70% NAA Acetate).

2. Fortification Solutions

   Prepare separate solutions of approximately 1 mg/mL of each analytical standard in acetonitrile. Mix thoroughly and serially dilute the stock to prepare necessary fortification concentrations. Amide and ester recoveries may be calculated by applying an appropriate conversion factor to the residue result expressed as NAA.

3. 50% Sodium Hydroxide Solution

   Dissolve 500 grams of sodium hydroxide pellets into a volume of approximately 800 mL deionized water in a precalibrated one liter polypropylene container. Mix thoroughly until pellets dissolve. Fill to the mark when solution cools. Mix thoroughly.

4. <u>49.5% Dichloromethane 49.5% Hexane with 1% Acetic Acid</u>

For each 200 mL of solution, combine 99.0 mL dichloromethane to 99.0 mL hexane. Add 2 mL of acetic acid and mix thoroughly.

5. <u>99% Dichloromethane with 1% Acetic Acid</u>

For each 200 mL of solution, combine 2 mL acetic acid to 198 mL dichloromethane and mix thoroughly.

6. <u>1:1 Acetonitrile/Water Dilution Solution</u>

For each liter of solution, combine 500 mL acetonitrile and 500 mL HPLC grade water and mix thoroughly.

7. <u>0.025m KH_2PO_4 pH 5 Buffer</u>

For each liter of buffer weigh 3.40 g KH_2PO_4 into a 1 liter volumetric flask. Dilute to volume with HPLC grade water. Using pH meter adjust to pH 5 with 1N NaOH. Filter through Nylon 66, 0.45μ membrane to remove particulates.

8. <u>HPLC Mobile Phase Solution</u>

For each liter of solution combine a volume of 0.025m KH_2PO_4 with an appropriate volume of acetonitrile. For example: to make a buffer of 25% acetonitrile 75% 0.025m KH_2PO_4 add 250 mL of acetonitrile to 750 mL 0.025m KH_2PO_4, pH 5. Mix thoroughly and degas continuously with helium.

F. Typical HPLC Chromatographic Conditions

HPLC Chromatograph:	Thermo Separation Products Sp8800 Ternary HPLC pump with Sp 8880 Autosampler equipped with LabNet System control
Detector:	Thermo Separation Products Fluor LC 304
Excitation Wavelength:	220 nm
Emission Wavelength:	340 nm

Photomultiplier setting:	600
Column:	DuPont Zorbax SAX 4.6 mm × 25 cm, 5μ, or equivalent
Column Temperature:	40 °C
Mobile Phase:	25% acetonitrile 75% 0.025m KH_2PO_4, pH 5 (isocratic)
Flow Rate:	0.8 mL/min.
Injection Volume:	20 μL
Run Time:	20 minutes

Note: The previous are typical operating conditions and parameters. Conditions may be modified to optimize chromatography. In ion exchange chromatography the analyst may choose to modify both the ionic strength (molarity of the buffer) and the concentration of the organic modifier. A decrease in either the ionic strength or the organic modifier should increase retention of the analyte, though the effect on interfering compounds may differ depending on the variable. Also, in ion exchange chromatography it is common to make frequent small adjustments in the mobile phase to compensate for changes in the column condition. The exact parameters must be documented with each chromatographic set.

G. Linearity Check

Inject 10 μL (or other suitable volume to achieve an acceptable response of at least 5X signal/noise ratio for low standard) of the 1-Naphthaleneacetic acid calibration standards (liquid chromatographic standard solutions) and plot the peak height for each versus its concentration (μg/mL or ng/mL) to demonstrate linearity of response. Significant departure from linearity and retention time may indicate instrumental or operational problems which must be corrected before proceeding. Monitor system suitability on a daily basis.

H. Extraction Procedures

1. Weigh 50 grams of homogenous sample into pint Mason jars. Fortify samples for quality control purposes with appropriate standards.

2. Add 100 mL deionized water and blend with Omni mixer for 1 minute. Adjust speed to blend thoroughly, yet minimize splashing up into blade gasket. Rinse Omni blade with additional DI water into sample container.

3. Transfer sample (without filtration) by pouring through a glass funnel into a 500 mL evaporation flask. Thoroughly rinse sample container and funnel with additional portions of deionized water.

4. Add 65 mL of 50% sodium hydroxide to each sample (the resulting mixture should be at least 10% sodium hydroxide). Use the funnel from the previous step for the addition of sodium hydroxide avoiding contact with the ground glass surfaces. This caustic solution may cause glass joints to fuse to one another.

5. Add 10 drops antifoam (up to 20 drops total if necessary) to each flask to prevent excessive bumping during hydroloysis. Rinse reflux condensers with DI water and discard. Attach each flask to a cooled reflux condenser. (Reflux condensers are cooled via water recirculating through a dry ice/water bath. Bath must be monitored and dry ice added as necessary). Set each flask in a heating mantle (controlled via a Variac). It is advisable to use teflon joint sleeves to prevent glass joints from fusing. Set Variac to maintain a moderate boil and reflux for 3 hours.

6. After samples have cooled, rinse condenser with small amount of DI water into flask, then vacuum filter into a 500 mL side arm flask using a 9 cm Buchner funnel equipped with 9 cm Whatman GF/A glass fiber filter media. Rinse flask and filter disc with additional small portions of DI water while rinsing the walls of the Büchner funnel. Transfer contents of side arm flask into a 1000 mL separatory funnel with additional deionized water rinses.

   NOTE: Use extreme caution due to the caustic nature of these extracts. The use of gloves and a protective face shield is advisable during this step.

7. Partition the basic extract one time with 100 mL dichloromethane by shaking gently for 1 minute. Allow layers to separate and discard the lower organic layer to waste.

   NOTE: Vent separatory funnels frequently during extraction steps.

8. Acidify the extract to <pH 2 by adding approximately 60-65 mL concentrated hydrochloric acid in small increments while swirling separatory funnel. Check the pH with pH indicator strips and adjust if necessary. Allow extracts to cool before proceeding. Extracts may continue to boil and evolve fumes for sometime.

   NOTE: A color change will occur at or near the desired pH end point.

   CAUTION: This step causes significant evolution of vapors and generates a significant amount of heat. Use approriate protective clothing and eye wear and always work in a fume hood! Handle concentrated hydrochloric acid carefully, pouring from a small secondary container into the separatory funnel as necessary. Do not stopper separatory funnels until they have cooled.

9. Partition the acidic extract with 100 mL dichloromethane for 1 minute. Care should be exercised to not agitate extract too vigorously to prevent the formation of an emulsion. (Use a gentle shake, or a swirling motion with an end over end tumble to agitate separatory funnels.) If emulsion forms, a glass rod may be used to help break an emulsion. After layers separate, drain lower phase through approximately 150 grams pre-rinsed sodium sulfate into a 500 mL evaporation flask.

   To pre-rinse sodium sulfate: Pour approximately 150 g sodium sulfate into a glass funnel plugged with a small amount of glass wool. Rinse with two 25 mL portions of dichloromethane. Discard dichloromethane.

10. Repeat step 9. Allow emulsion, if present, to pass onto the sodium sulfate.

   NOTE: Minimize emulsion if possible. The emulsion contains water which may be carried through to the SI SPE cleanup. Since water deactivates the silica gel this can adversely effect analyte recoveries.

11. Rinse sodium sulfate with two 25 mL portions of dichloromethane.

12. Evaporate combined extracts to approximately 2-3 mL by rotary evaporation at 35°C.

    Transfer extract to a test tube calibrated at 10.0 mL. Rinse flask with several small portions of dichloromethane and add to test tube. Adjust test tube volume to calibration mark with dichloromethane. Mix thoroughly.

I. Solid Phase Extraction/Silica Gel Cleanup

Silica gel cleanup is necessary to help eliminate matrix interferences.

1. Condition SPE-Si column by rinsing with two 5 mL portions of hexane. It is important to keep hexane layer approximately 0.5 cm above the sorbent bed at all times. Allow the column to elute approximately 2 drops per second, to avoid channeling. Discard eluent to waste.

2. Prepare sample extract from Step #12 above for SPE column:

   Plug a glass test tube funnel with a small piece of glass wool. Add 2 g sodium sulfate to funnel. Filter sample extract from Step 12 through sodium sulfate. Discard approximately the first 0.5 mL. Collect at least 1.5 to 2.0 mL additional filtrate.

   NOTE: This step was added to ensure all residual water is removed from extract prior to cleanup. As discussed above, residual water in the sample extract introduced to the SPE cartridge may adversely effect analyte recovery.

3. Pipet 1.0 mL aliquot of the sample extract prepared above to the SPE-Si column. Discard eluent to waste.

4. Wash the SPE-Si column with 5 mL 49.5% dichloromethane 49.5% hexane with 1% acetic acid. Discard eluent to waste.

5. Elute residues from SPE-Si cartridge into a 15 mL conical centrifuge tube (precalibrated at 15.0 mL) with three 5 mL portions of 99% dichloromethane 1% acetic acid. Dilute to calibration mark with 99% dichloromethane 1% acetic acid. Mix thoroughly.

J. Solvent Exchange

The following procedure is used to exchange the dichloromethane solution with 1:1 acetonitrile/water so that the extract is suitable for HPLC analysis. Do not allow sample extracts to go to dryness during the solvent exchange process or analyte loss may occur.

1. Pipette 6.0 mL sample extract from step #5 above into a 13 mm x 100 mm culture tube precalibrated at 2.0 mL and 4.0 mL.

2. Place tubes into a water bath at 35°C and evaporate extract to approximately 0.5 mL using a gentle stream of nitrogen gas. Alternatively, evaporate extracts on a heating block at 35 °C using a gentle stream of nitrogen supplied by a Reactivap.

3. When extract volume nears a 0.5 mL volume, add approximately 1 mL of acetonitrile. Continue to evaporate extract to approximately 0.5 mL, and add another 1 mL portion of acetonitrile to the tubes. Allow extract to evaporate to approximately 0.5 mL and remove from the water bath.

4. After tubes have cooled to room temperature, add acetonitrile to the 2.0 mL calibration mark.

5. Add HPLC grade water to each tube to the 4.0 mL mark. Mix tube thoroughly with a vortex mixer. A light precipitate may form during this step.

6. Filter sample extracts using a 2.5 mL glass syringe equipped with a 0.45 μm Gelman PTFE filter disc. Extracts are ready for HPLC analysis.

K. Automated Liquid Chromatography Analyses

Begin automated HPLC analysis with a minimum of a four point standard curve. Follow the standard curve with the unknown (study) samples alternating with standard injection (curve check standards). Arrange samples in the autosampler tray so that no more than four samples are injected between curve check standards. If a sample peak height exceeds the peak height of the most concentrated curve standard the sample must be diluted with 1:1 acetonitrile/water such that its peak height will be bracketed by the standard curve. Document the dilution required and incorporate into the calculation as described in section L below. Always end each chromatographic run with at least one standard injection. Follow established guidelines for determining identity of peaks based

on retention time. Example chromatograms for 1-naphthaleneacetic acid from red grapes, white grapes, grapefruit, tomatoes, and green bell peppers are found in Appendix I.

L. Calculation

Calculations for instrumental analysis are conducted using a validated software application to create a standard curve based on linear regression. These regression functions are used to calculate a best fit line (from a set of standard concentrations in ng/mL versus peak height response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line.

The example calculations for ppm found and percent recovery (for fortified samples) are:

1. The amount of analyte (in ppm) found in the sample is calculated according to the following equation:

$$ppm\ found = ng/mL \times \frac{mL\ Solv}{sample\ wt.\ (g)} \times \frac{Adj.\ Vol.\ (mL)}{mL\ aliquot} \times \frac{SPE\ Elut.\ Solv\ (mL)}{SPE\ Aliquot\ (mL)} \times$$

$$\frac{FV\ (mL)}{mL\ Aliquot\ Fr.\ SPE\ Elution} \times \frac{1\ \mu g}{1000\ ng} \times HPLC\ Dil.\ Fact. \times MWCF$$

where,

- ng/mL = Analyte concentration reported by integrator
- mL Solvent =Extraction solvent volume (100 mL)
- Sample Weight (g) = Amount of sample extracted (50 g)
- Adj. Vol. (mL) = Volume of extract adjusted after rotary evaporation (10 mL)
- mL Aliquot = Aliquot of original extract taken to reflux step (100 mL)
- SPE Elut. (mL) = Volume of solvent required to elute analyte from SPE column (15 mL)
- SPE Aliquot (mL) = Volume of extract loaded on to the SPE (1.0 mL)
- FV (mL) = Final volume of extract (4 mL)
- mL Aliquot Fr. Elut. = Portion of SPE elution evaporated (6 mL) for final concentration

HPLC Dilution Factor = the magnitude of dilution required to bracket the response of the sample within the standard curve responses. When the sample requires no dilution, the HPLC dilution factor = 1.

MWCF = Molecular Weight Conversion Factor:

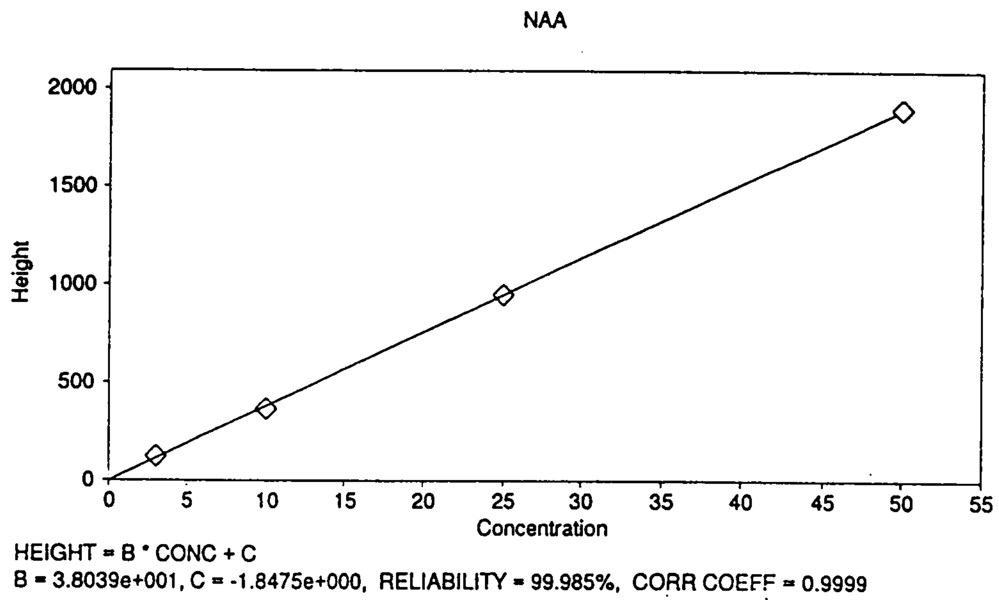
NAA =	1.00
NAA Amide =	0.995
NAA Acetate =	1.15

2. The percent recovery for fortified control samples is calculated as follows:

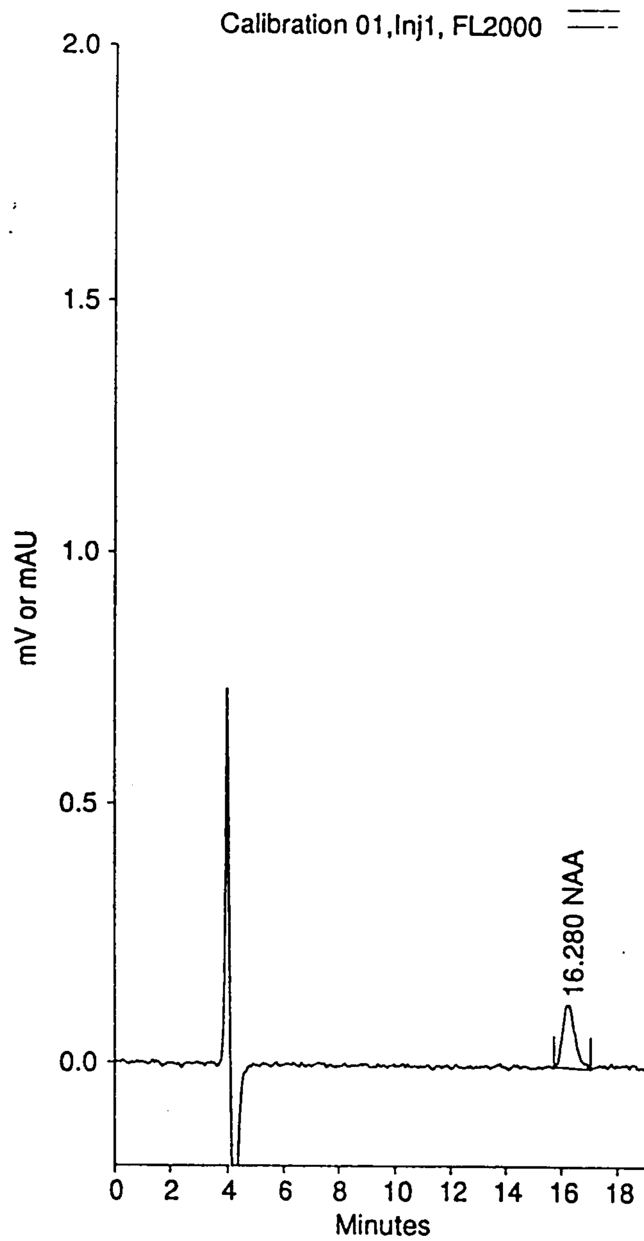
$$\% \ Recovery = \frac{ppm \ analyte - ppm \ control}{Fort. \ level \ of \ analyte} \times 100$$

APPENDIX I

Example Chromatograms

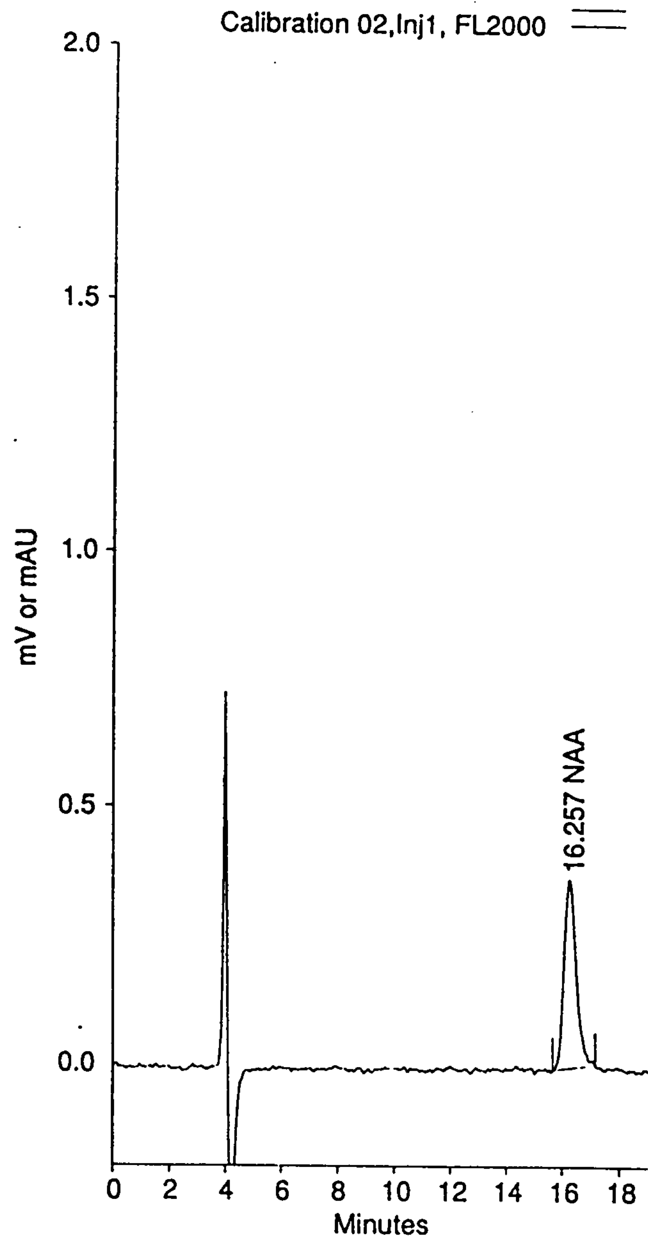


Typical calibration curve for NAA in Red Grapes



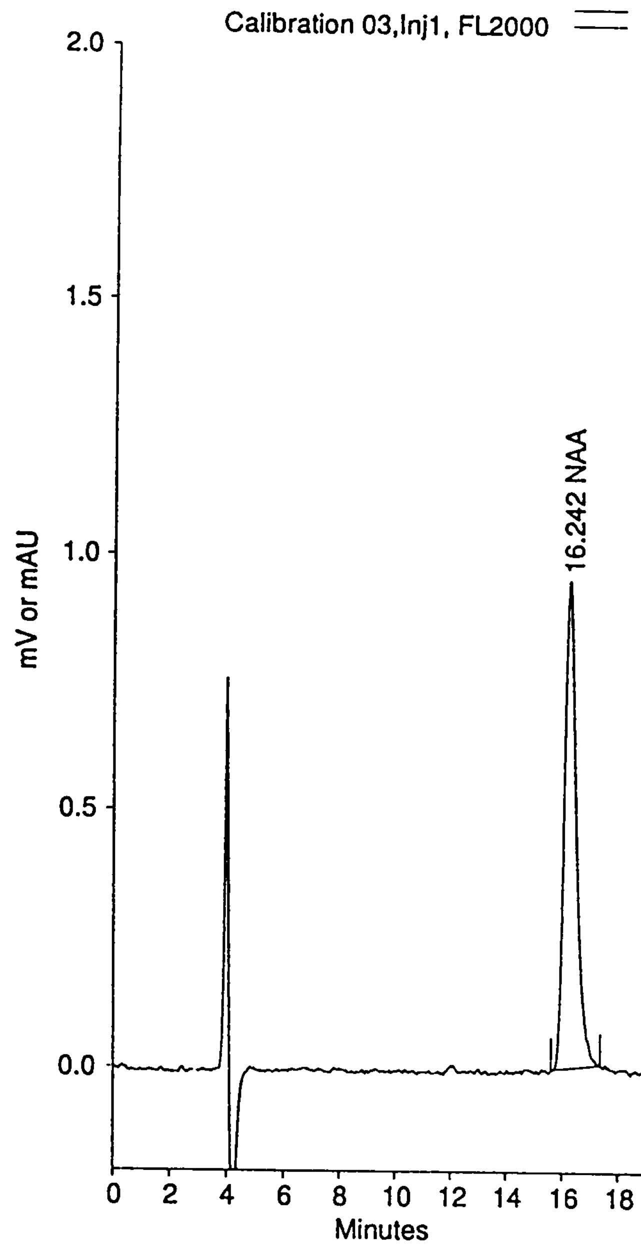
Red Grape matrix

NAA calibration standard (3 ng/mL) 20 μL injected



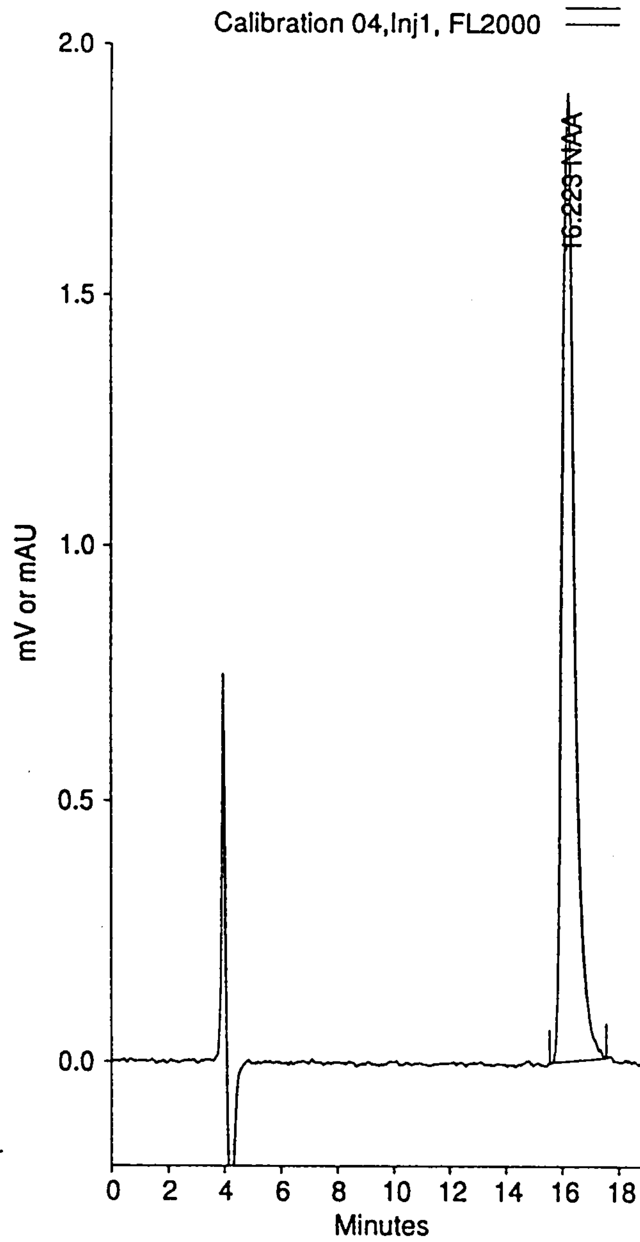
Red Grape matrix

NAA calibration standard (10 ng/mL) 20 μL injected



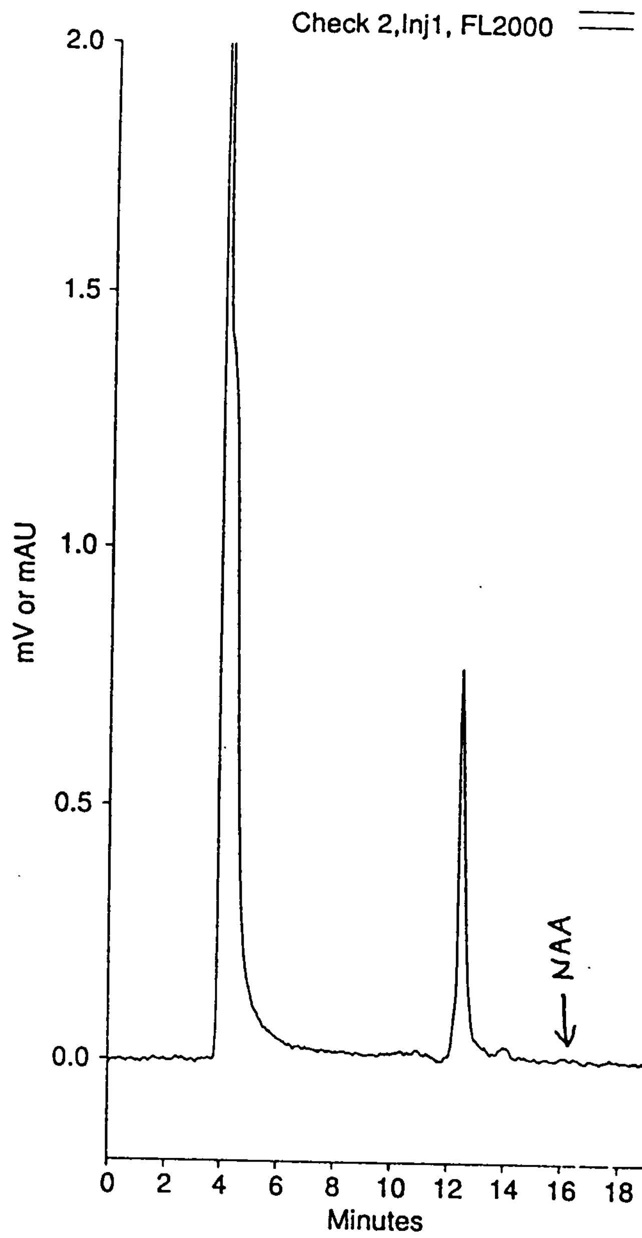
Red Grape matrix

NAA calibration standard (25 ng/mL) 20 μL injected

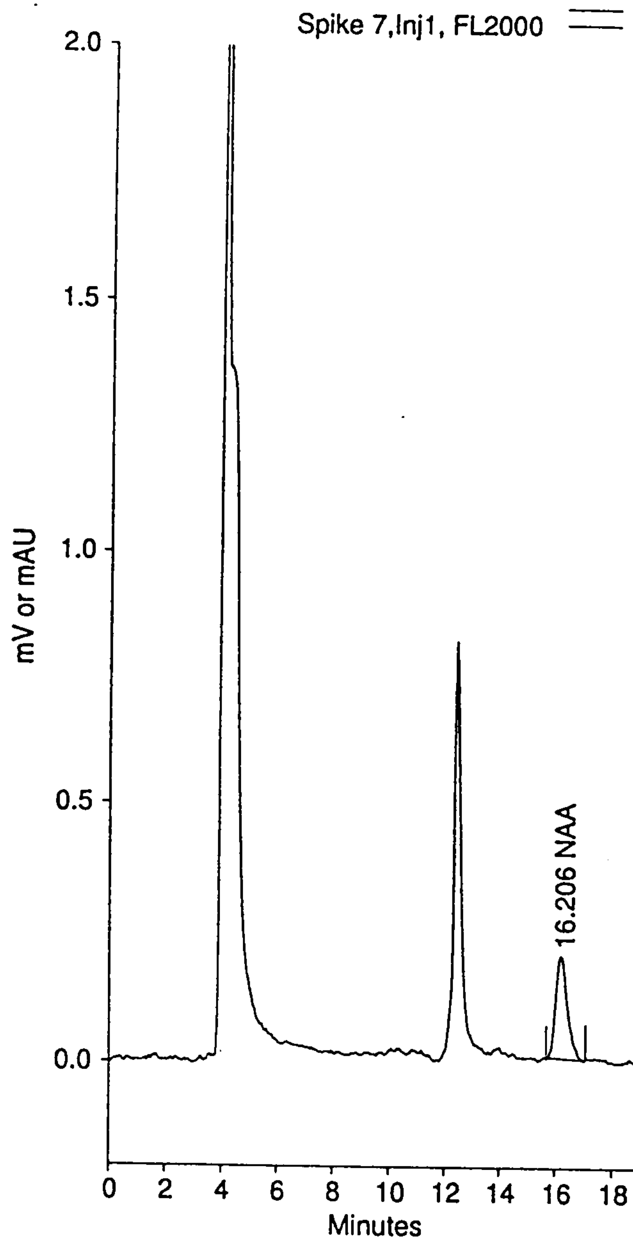


Red Grape matrix

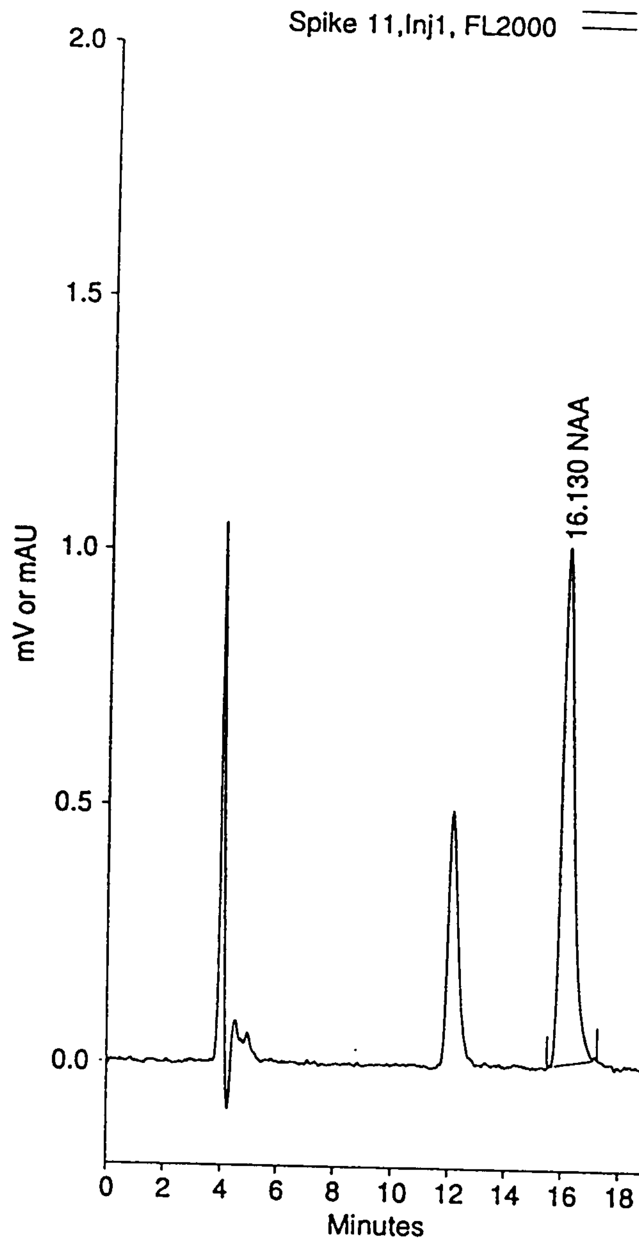
NAA calibration standard (50 ng/mL) 20 μL injected



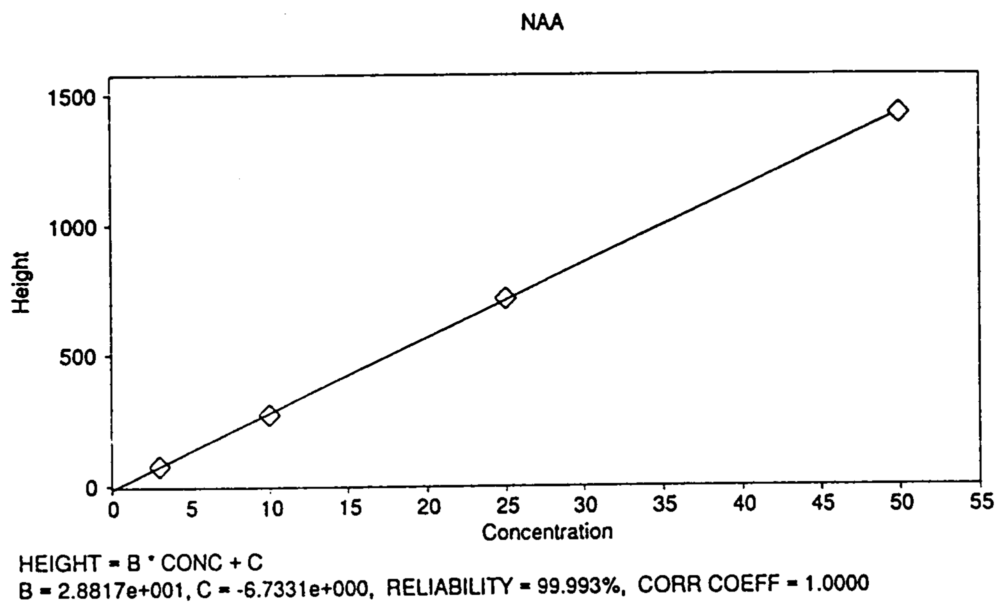
Red Grape Control (0.5 g/mL) 20 μL injected
None detected (Less than 0.01 ppm)



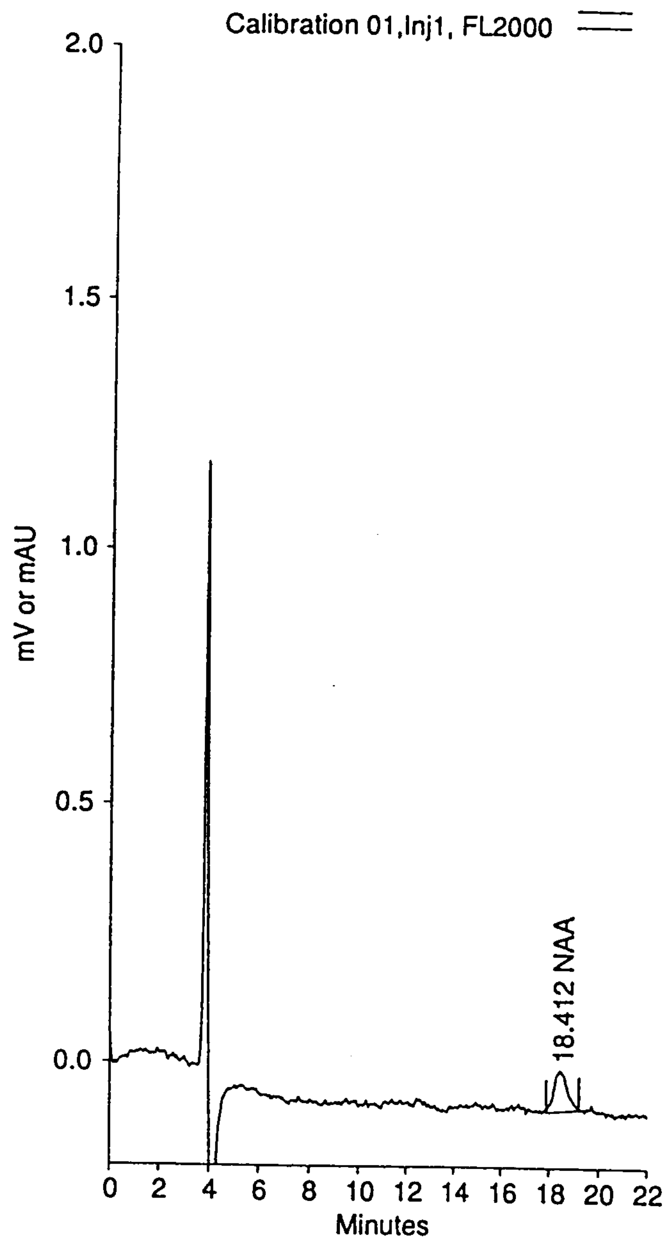
Red Grape spike @ 0.01 ppm with NAA (0.5 g/mL)
20 μL injected 104% Recovery



Red Grape spike @ 0.5 ppm with NAA (1 to 10 dilution of 0.5 g/mL)
20 μL injected 106% Recovery

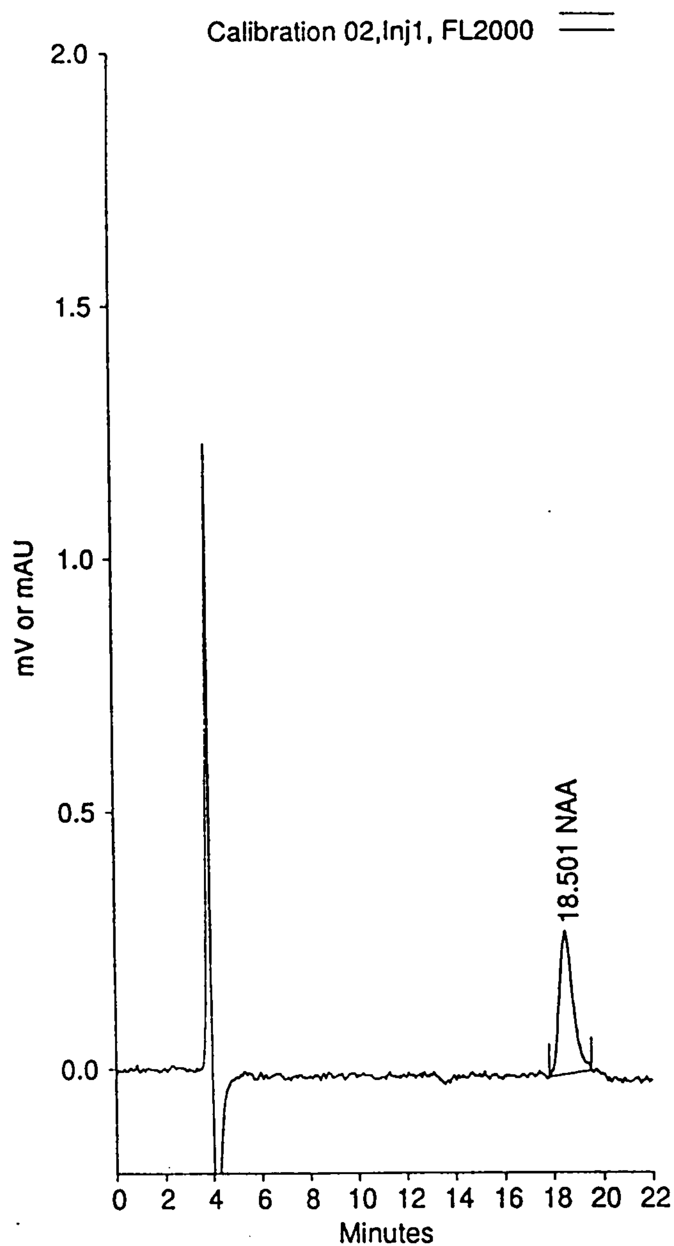


Typical calibration curve for NAA in White Grapes



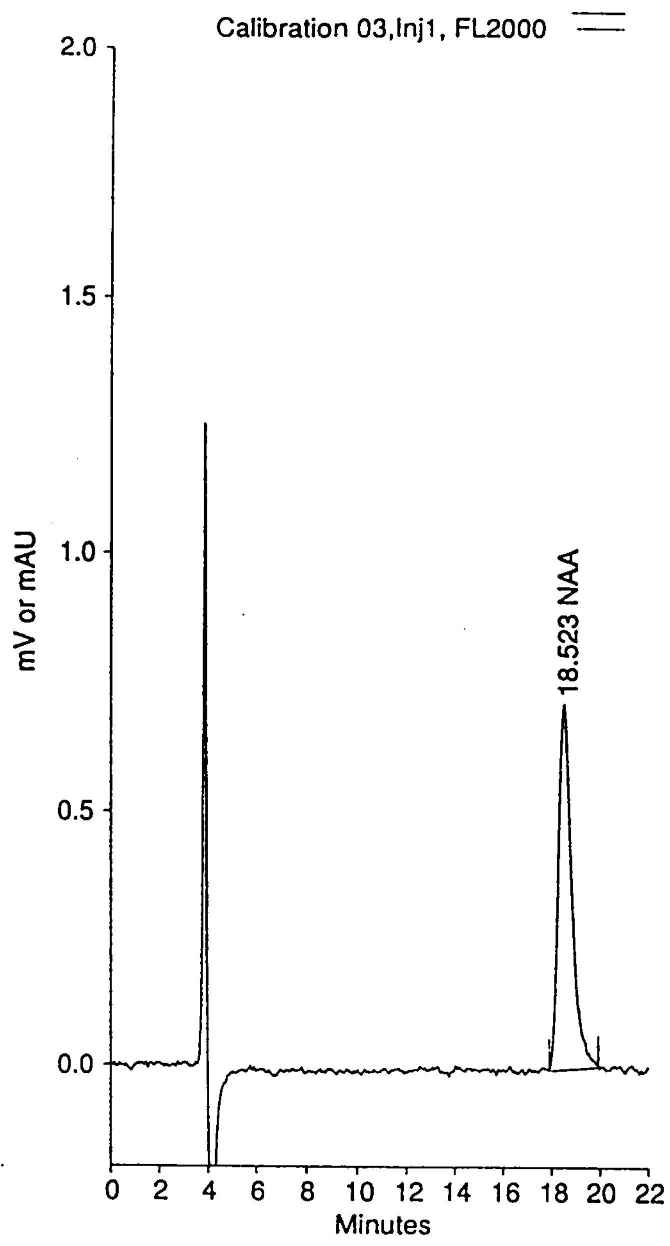
White Grape matrix

NAA calibration standard (3 ng/mL) 20 μL injected



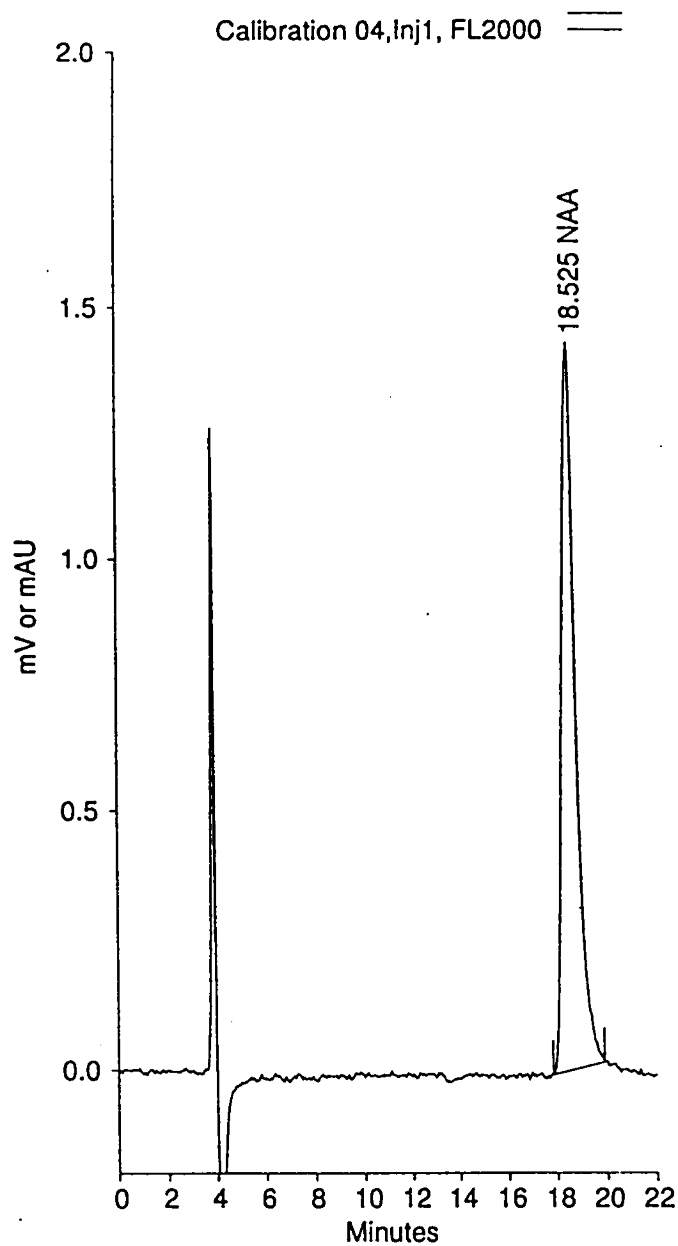
White Grape matrix

NAA calibration standard (10 ng/mL) 20 μL injected



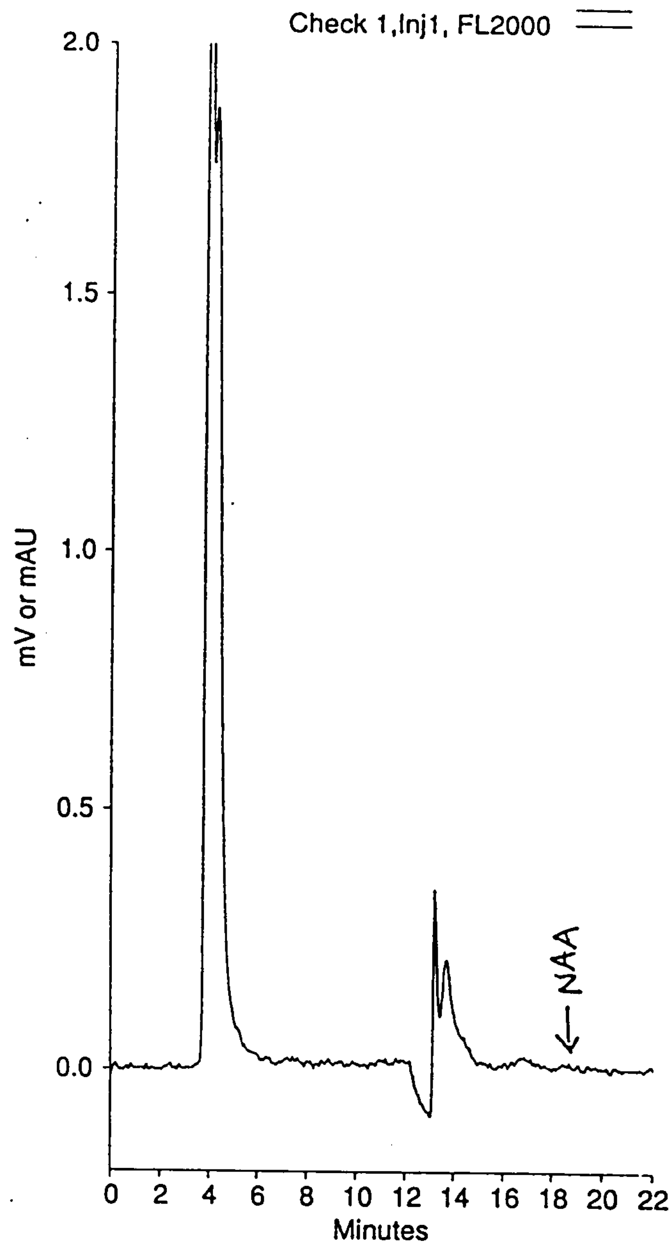
White Grape matrix

NAA calibration standard (25 ng/mL) 20 μL injected

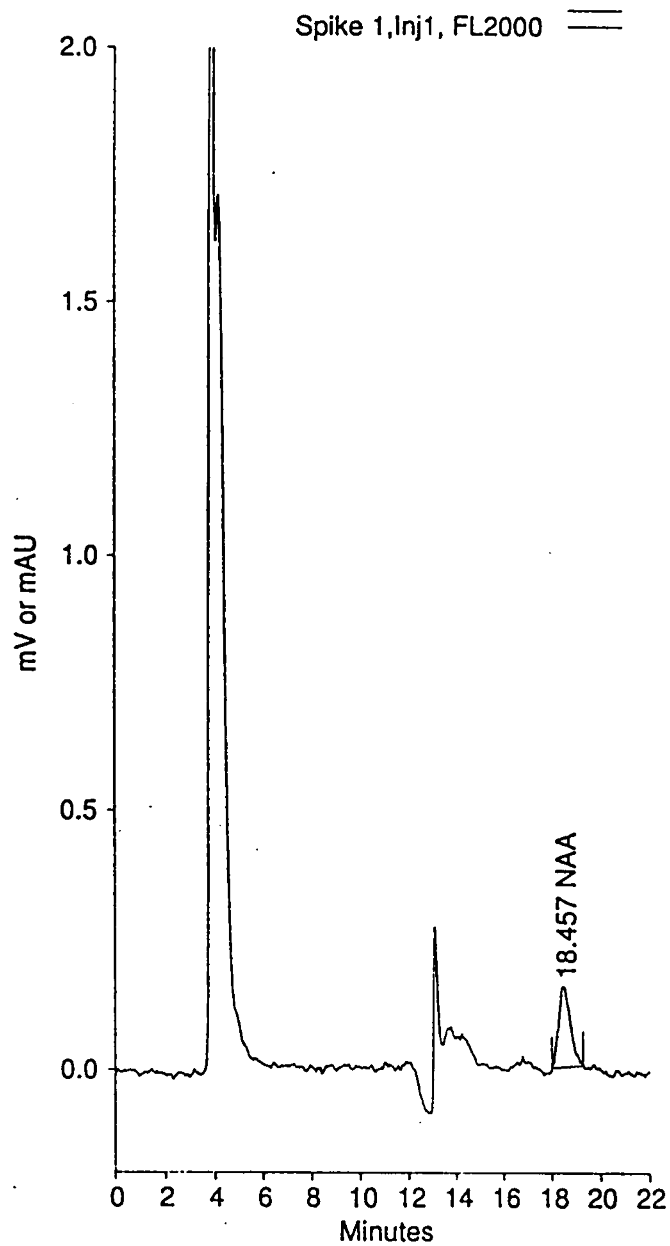


White Grape matrix

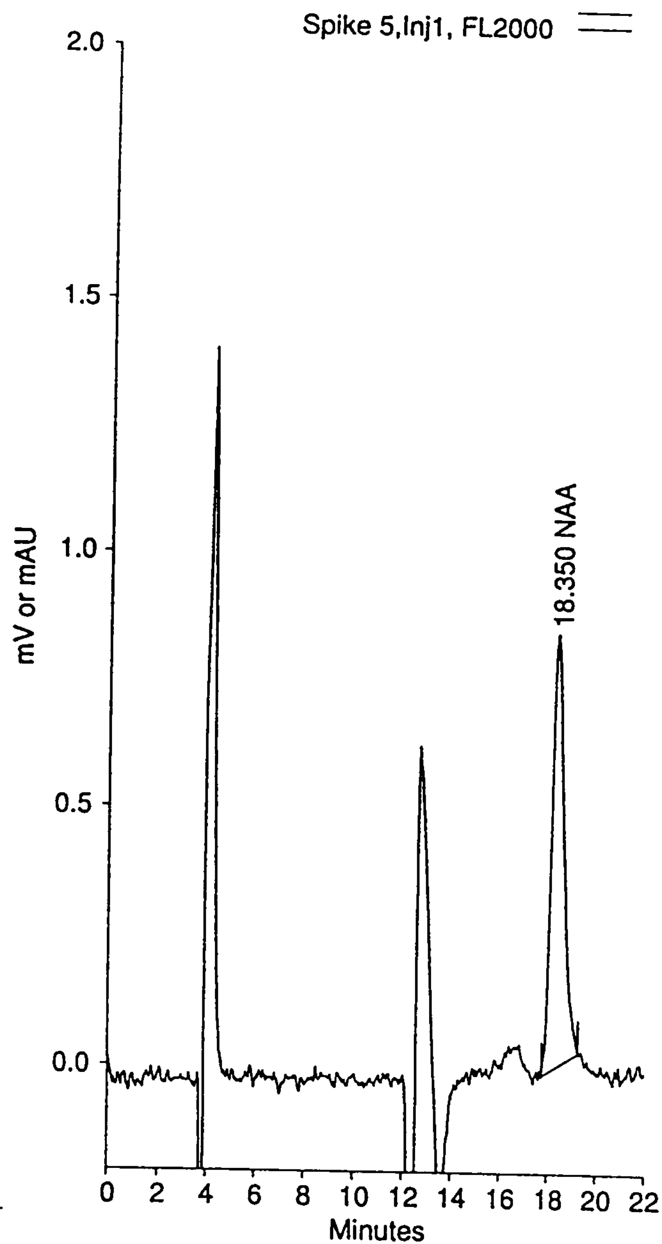
NAA calibration standard (50 ng/mL) 20 μL injected



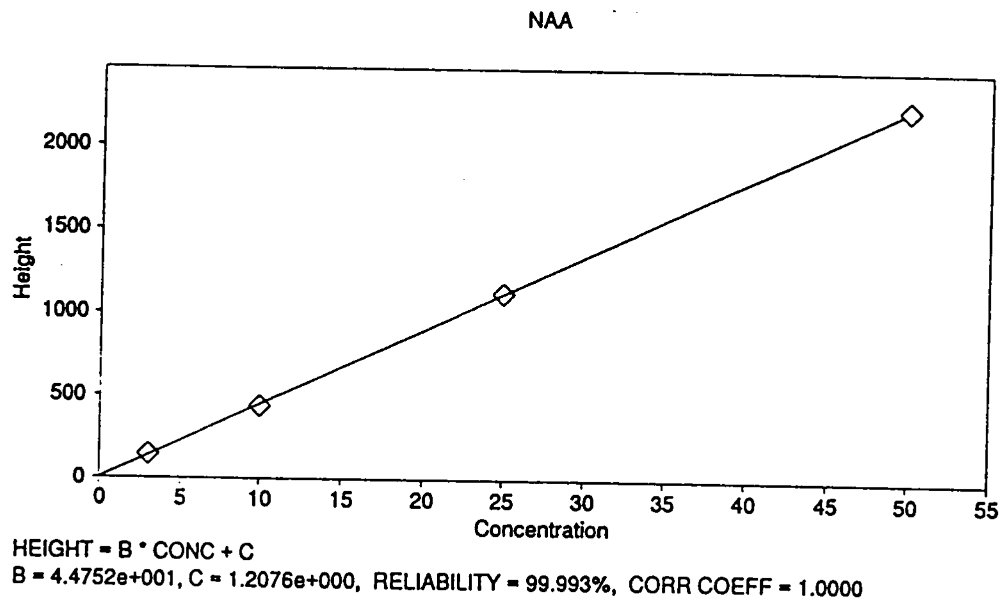
White Grape Control (0.5 g/mL) 20 μL injected
None detected (Less than 0.01 ppm)



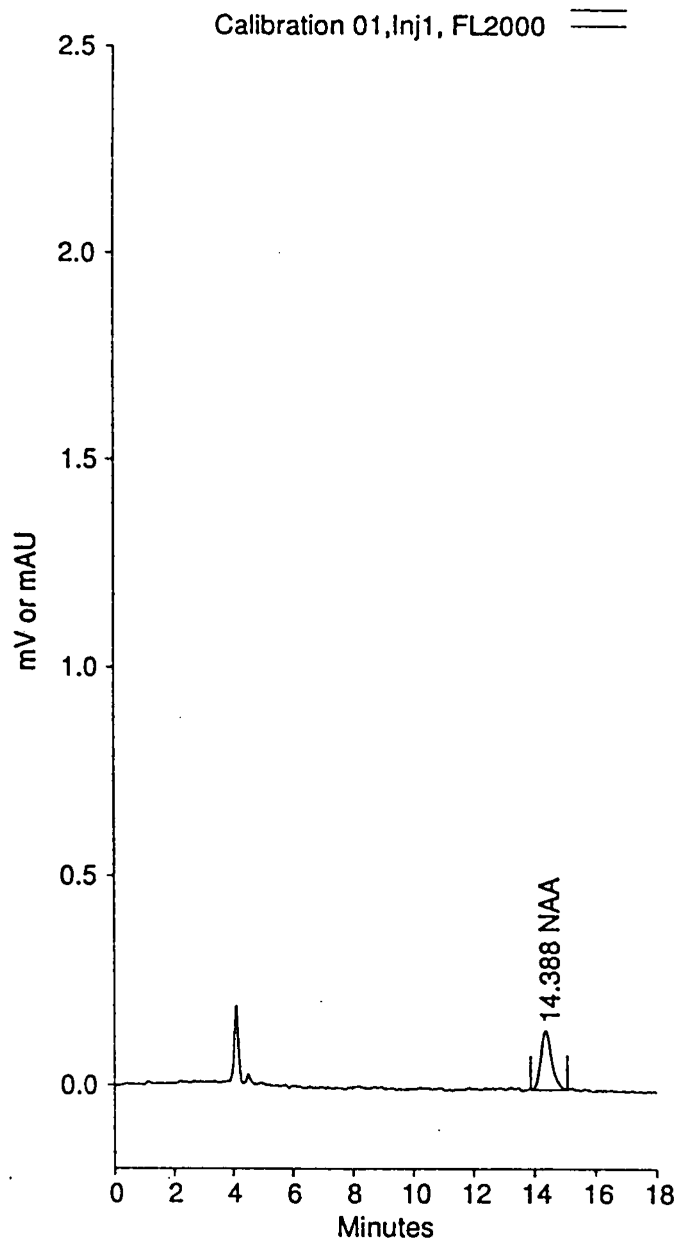
White Grape spike @ 0.01 ppm with NAA (0.5 g/mL)
20 μL injected 113% Recovery



White Grape spike @ 0.5 ppm with NAA (1 to 10 dilution of 0.5 g/mL)
20 μL injected 118% Recovery

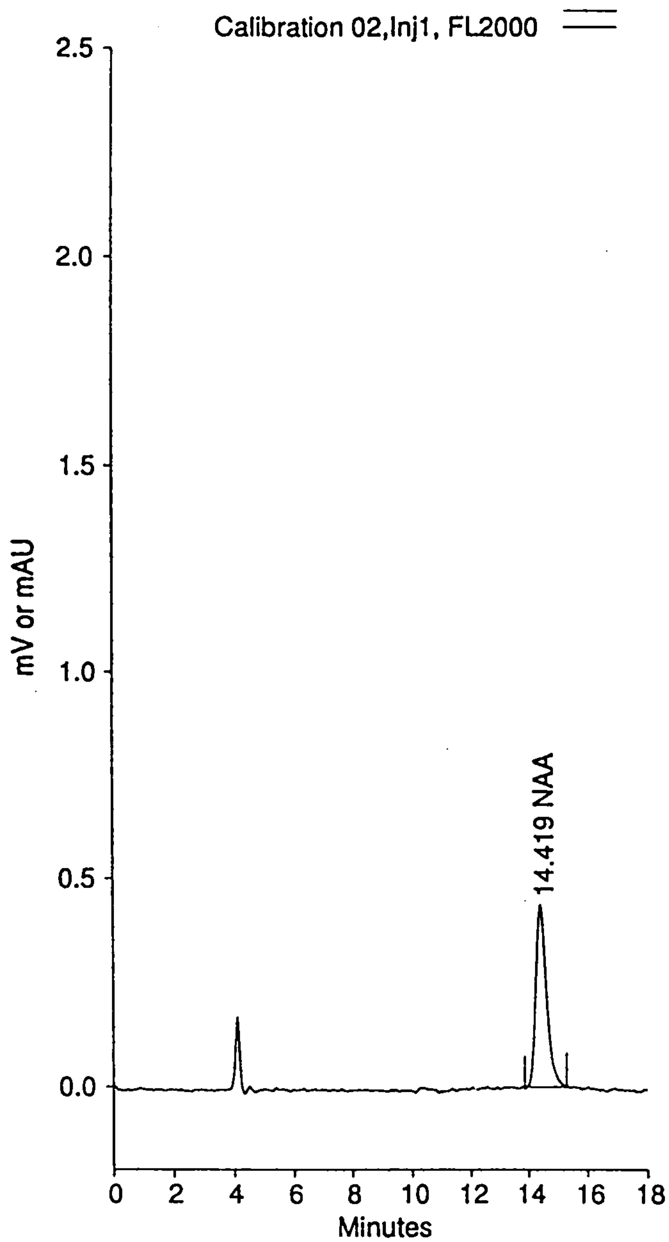


Typical calibration curve for NAA in Grapefruit



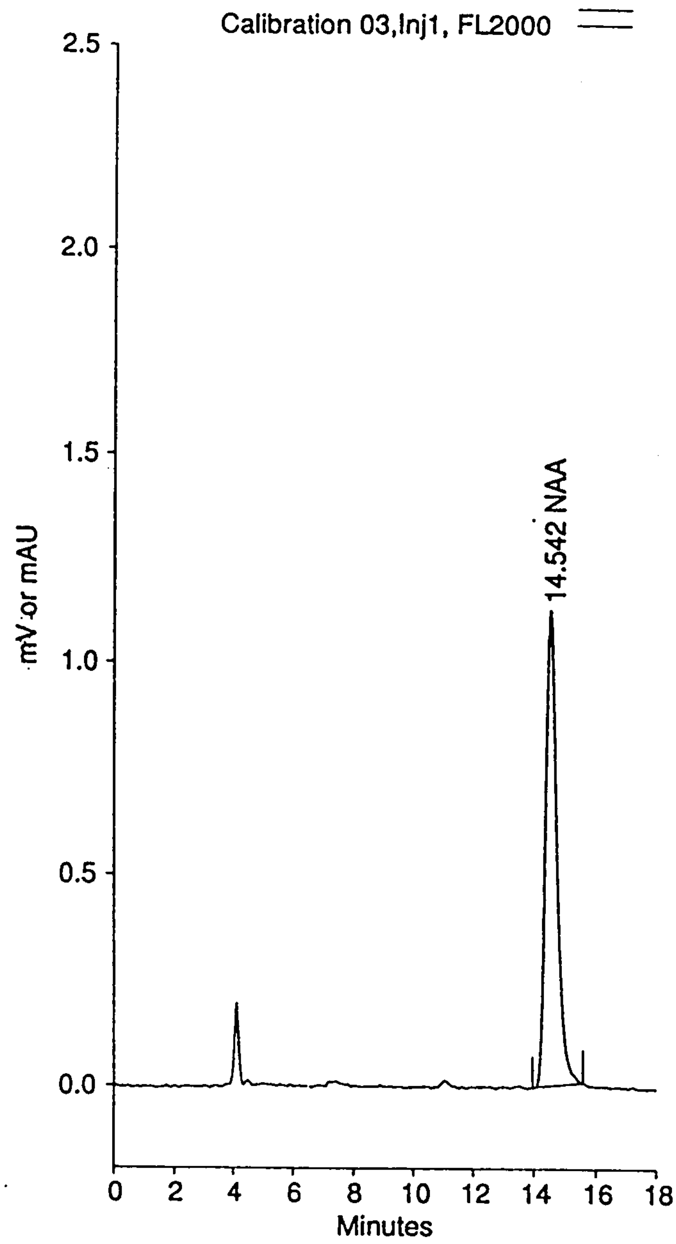
Grapefruit matrix

NAA calibration standard (3 ng/mL) 20 μL injected



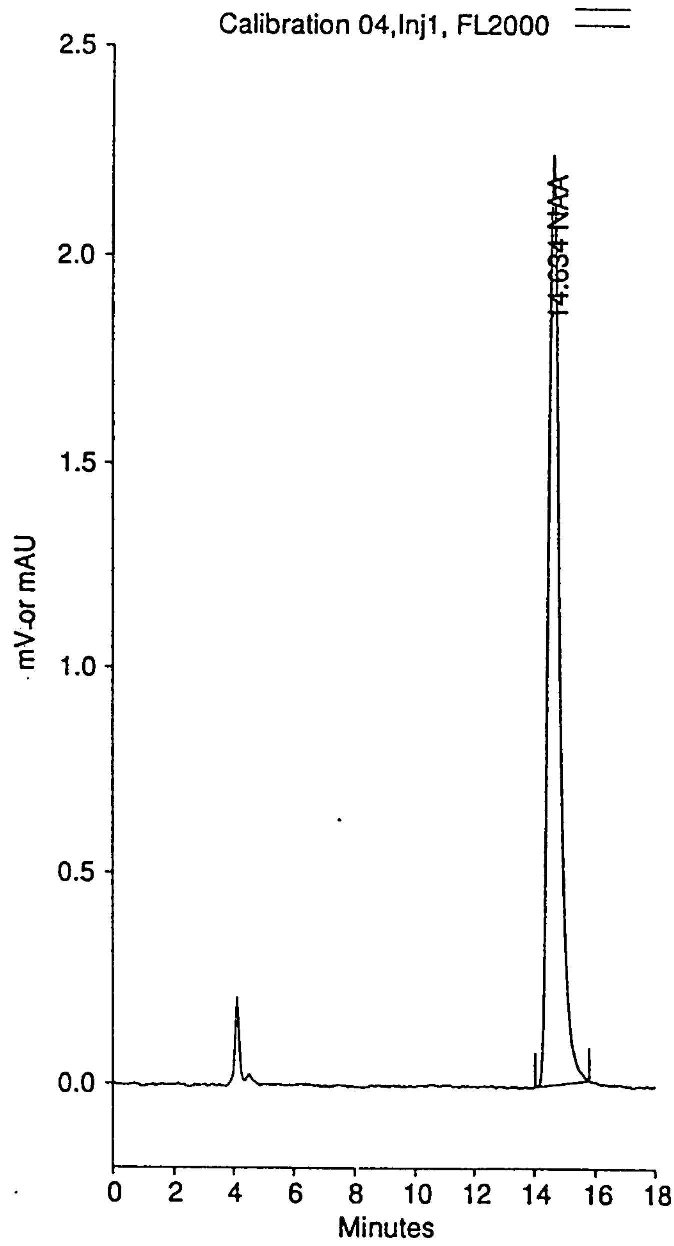
Grapefruit matrix

NAA calibration standard (10 ng/mL) 20 μL injected



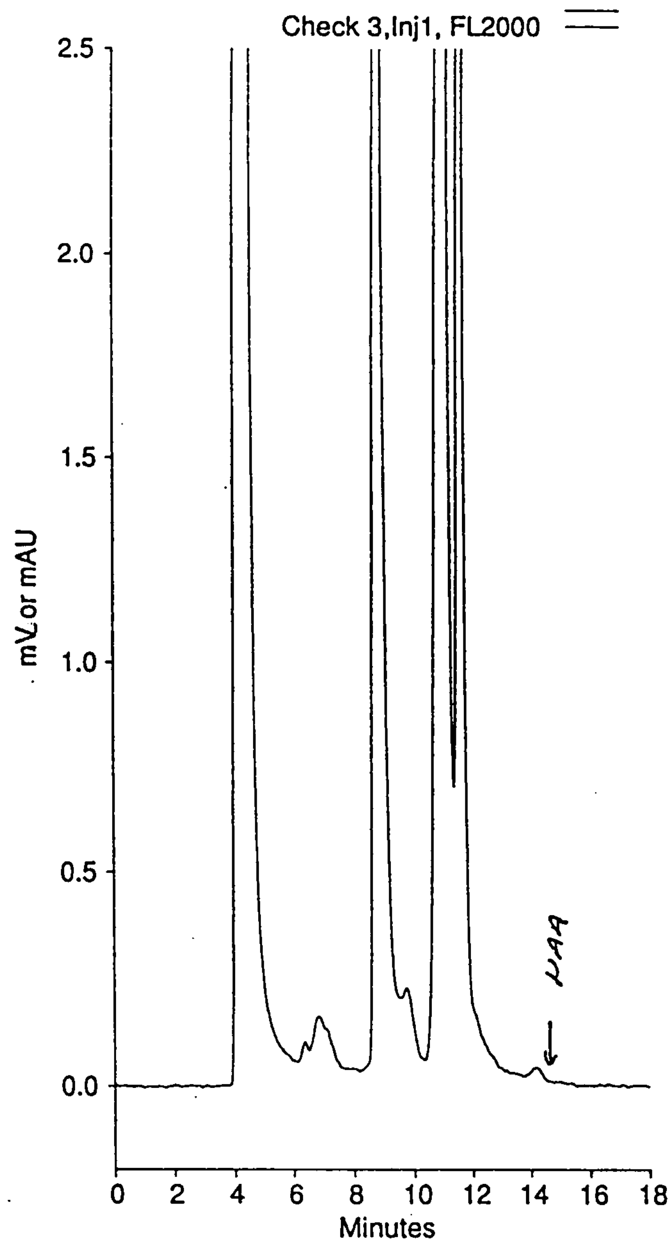
Grapefruit matrix

NAA calibration standard (25 ng/mL) 20 μL injected

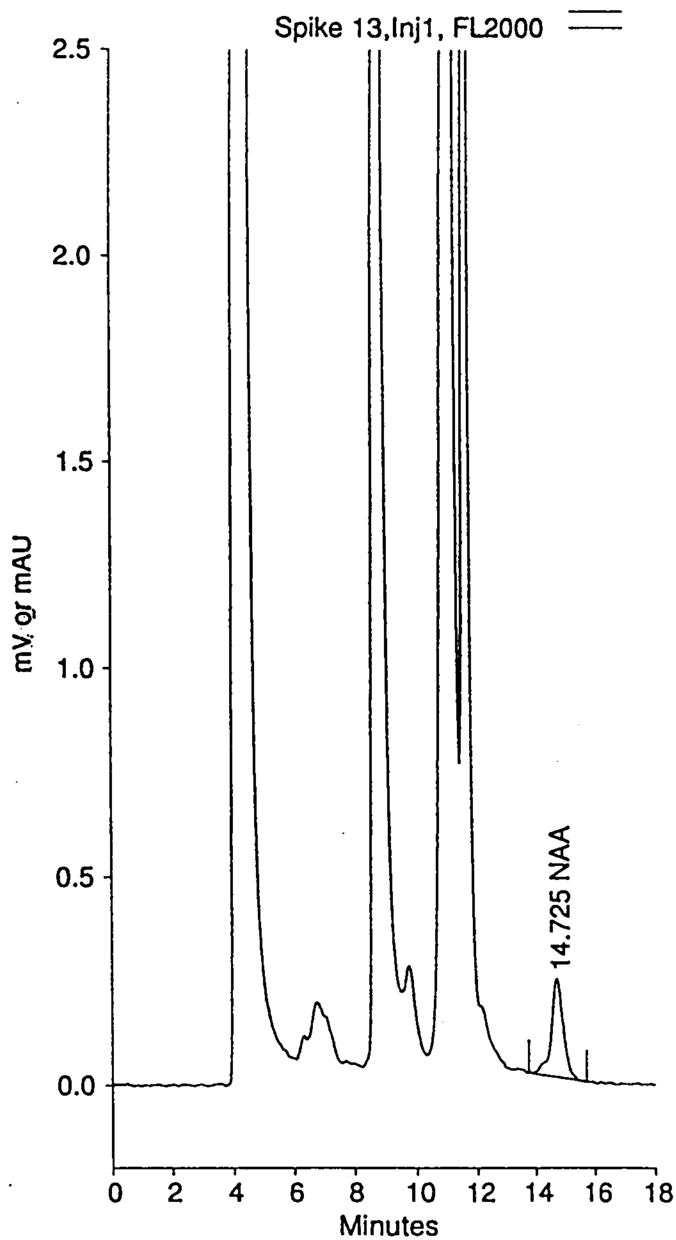


Grapefruit matrix

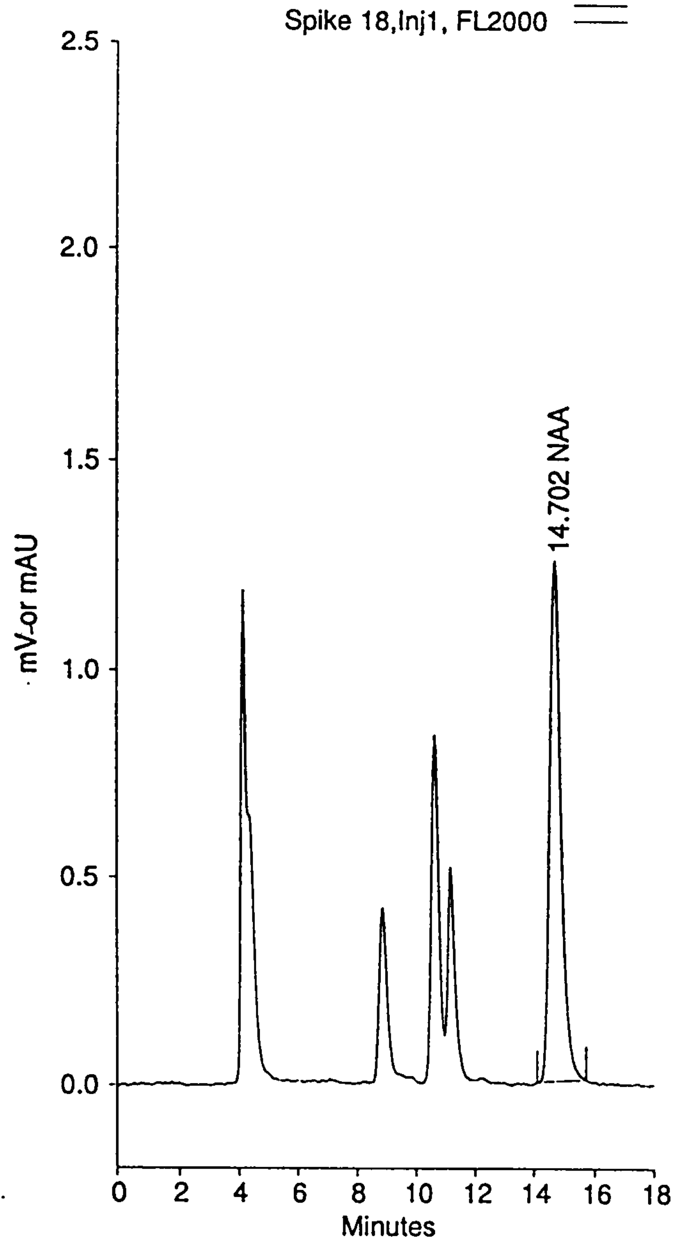
NAA calibration standard (50 ng/mL) 20 μL injected



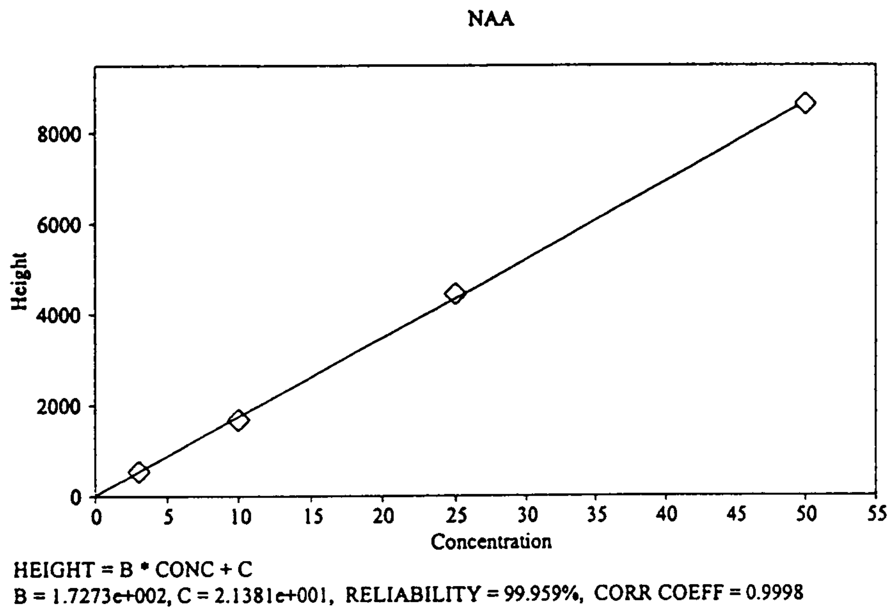
Grapefruit Control (0.5 g/mL) 20 μL injected
None detected (Less than 0.01 ppm)



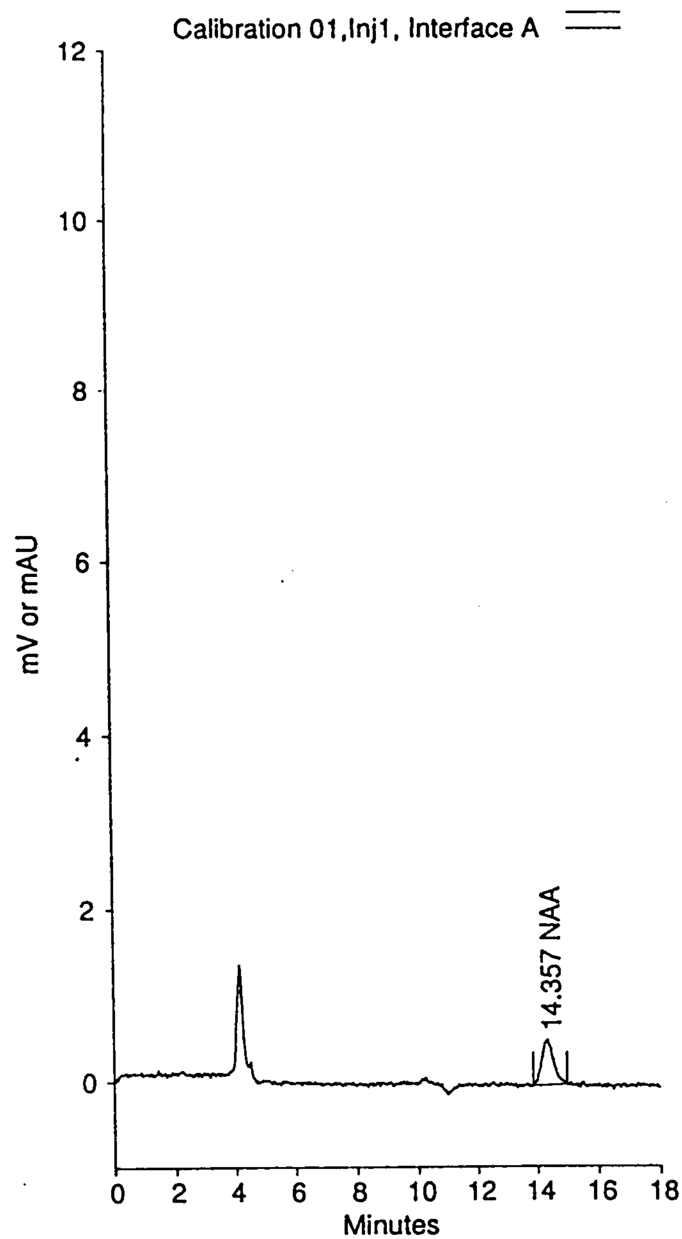
Grapefruit spike @ 0.01 ppm with NAA (0.5 g/mL)
20 μL injected   105% Recovery



Grapefruit spike @ 0.5 ppm with NAA (1 to 10 dilution of 0.5 g/mL)
20 μL injected 112% Recovery

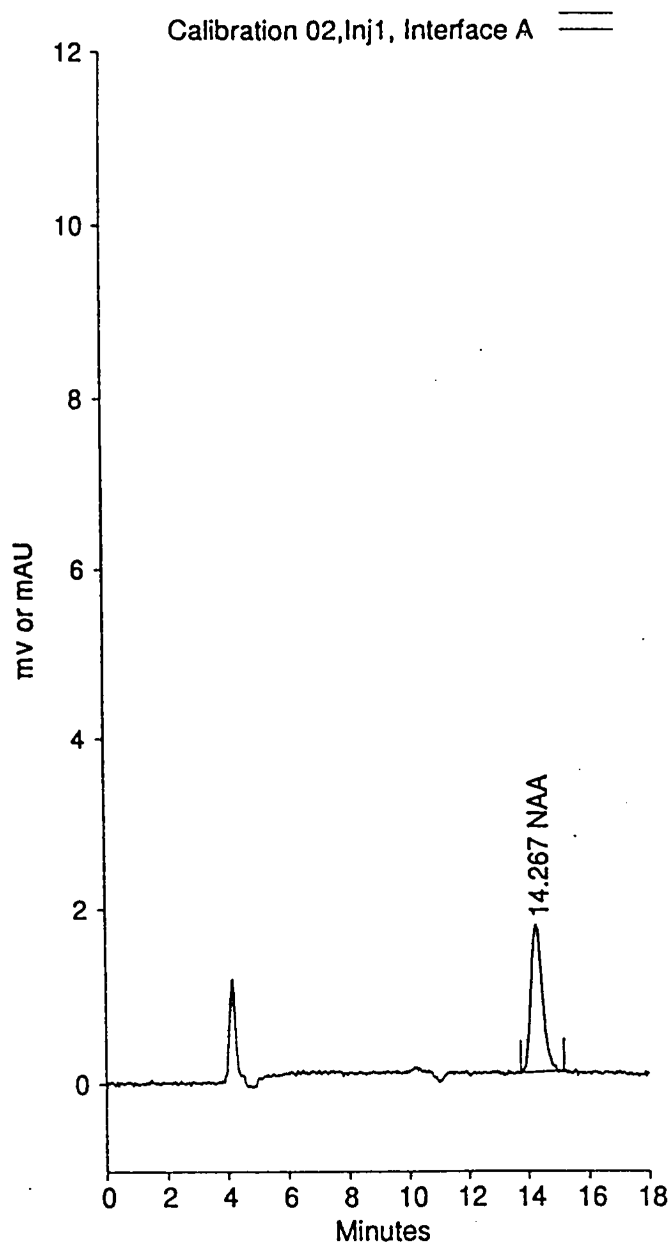


Typical calibration curve for NAA in Tomato



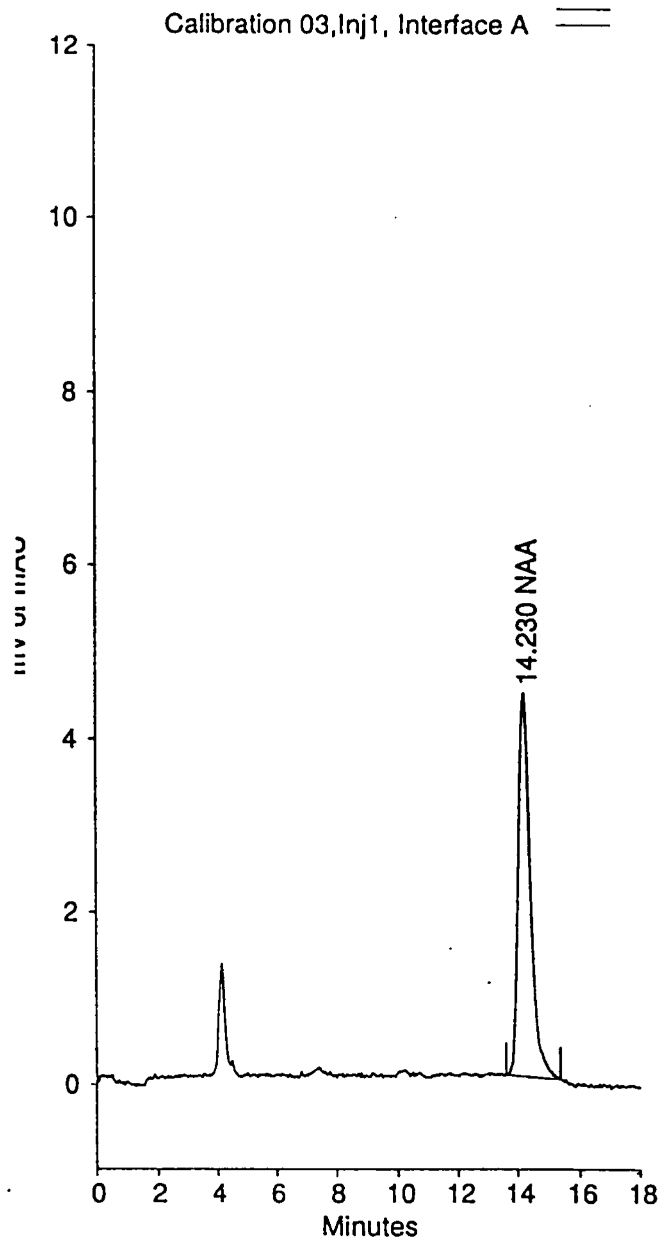
Tomato matrix

NAA calibration standard (3 ng/mL) 20 μL injected



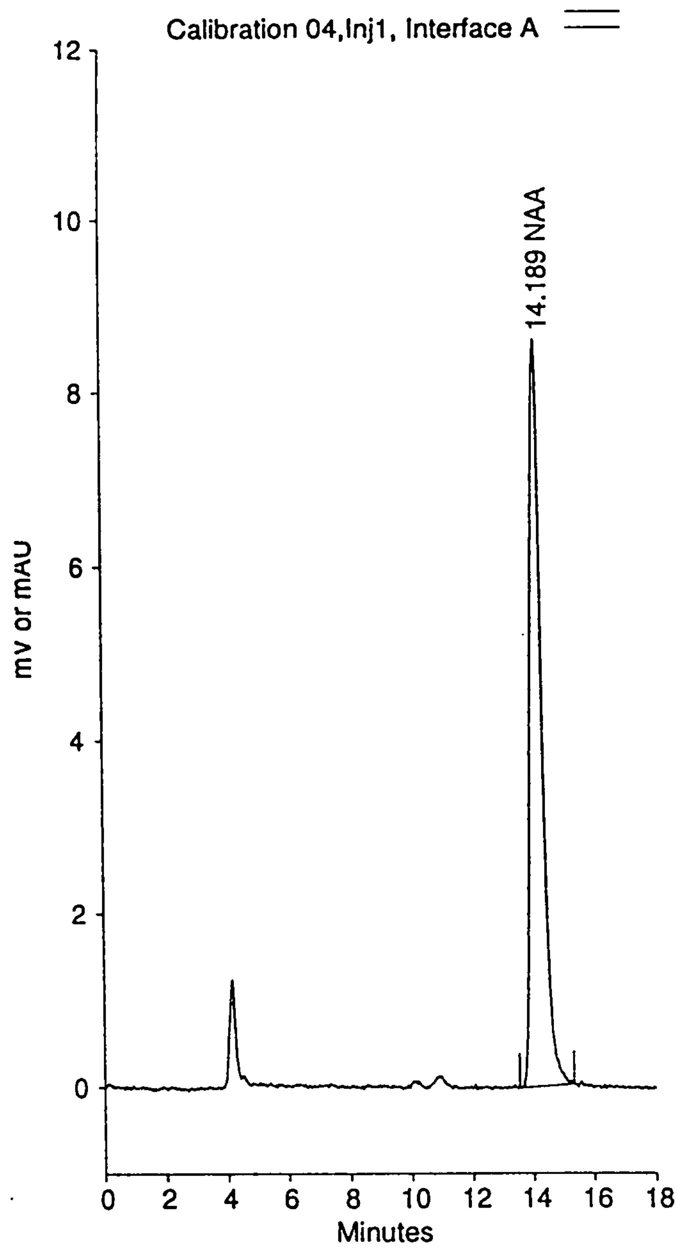
Tomato matrix

NAA calibration standard (10 ng/mL) 20 μL injected



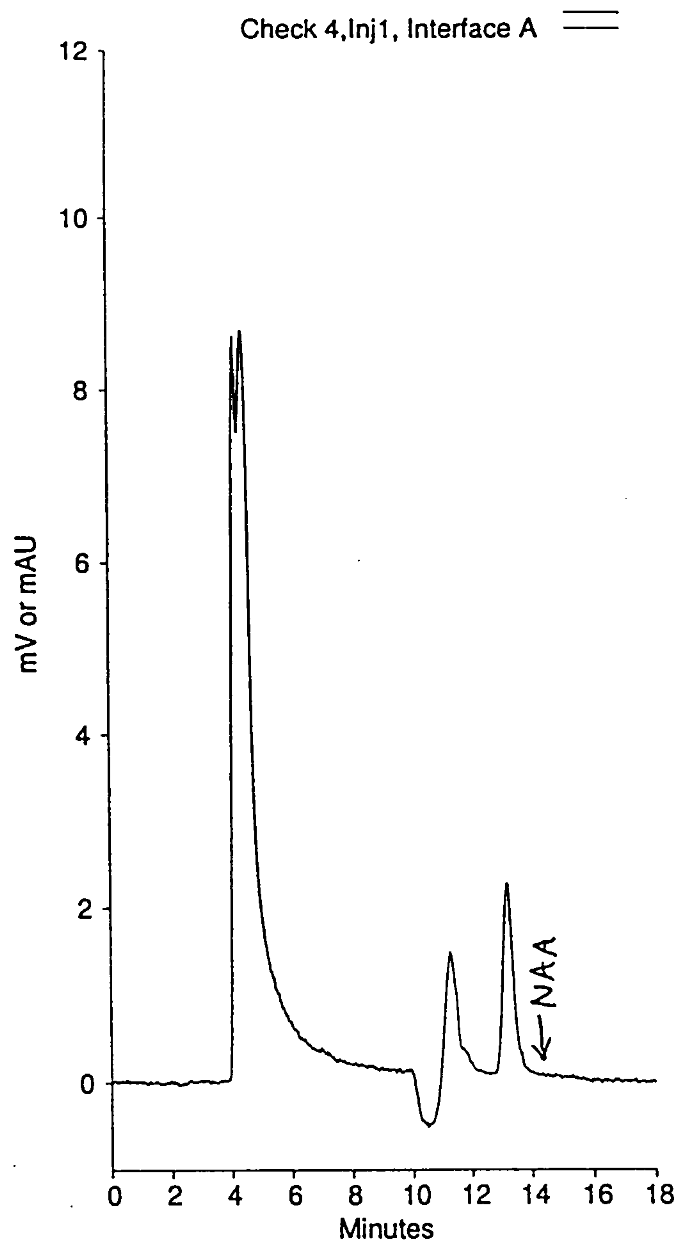
Tomato matrix

NAA calibration standard (25 ng/mL) 20 μL injected

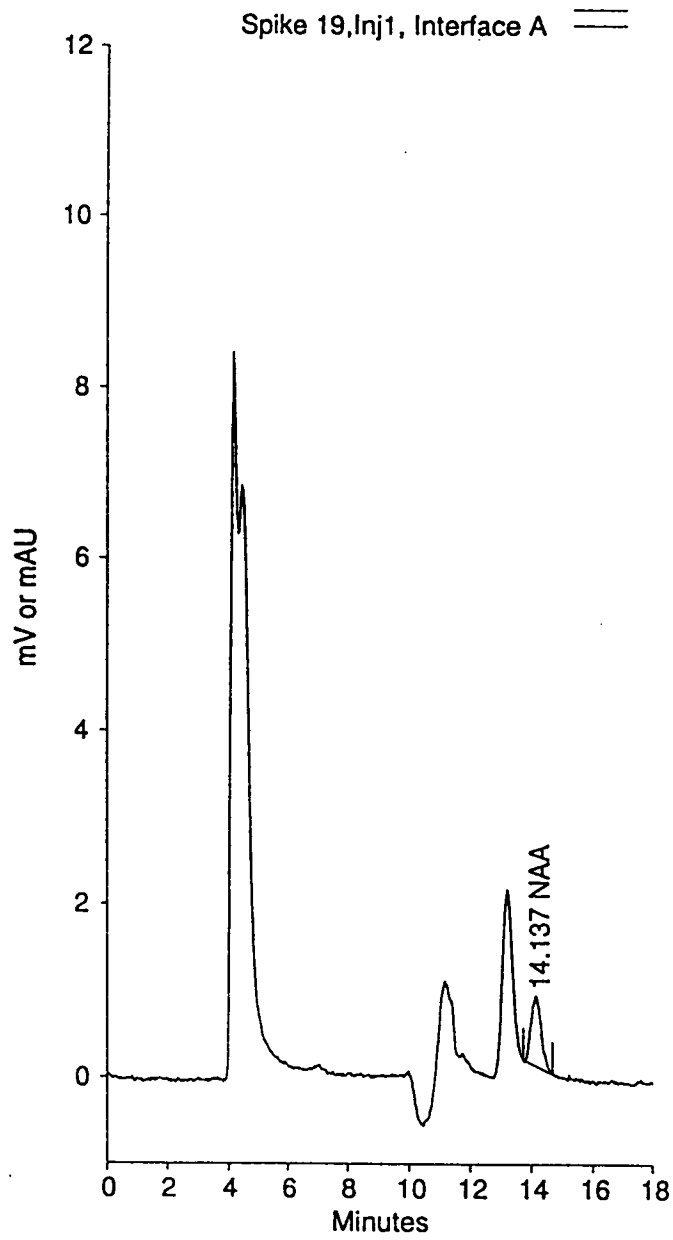


Tomato matrix

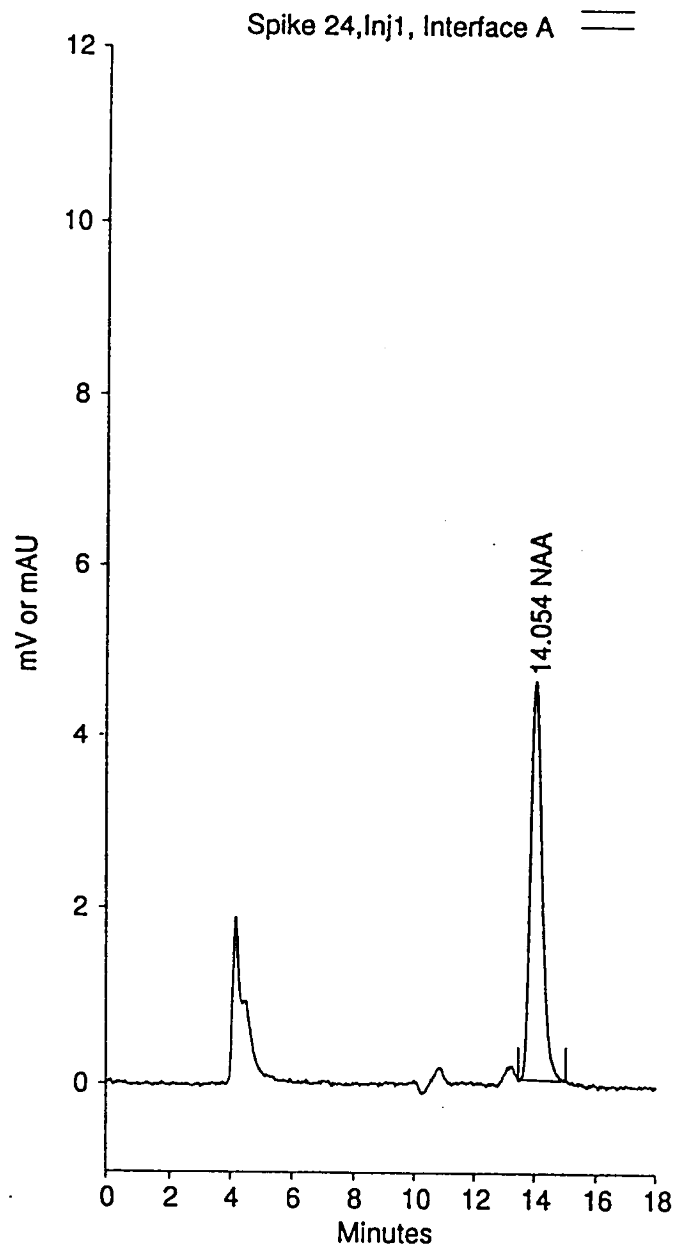
NAA calibration standard (50 ng/mL) 20 μL injected



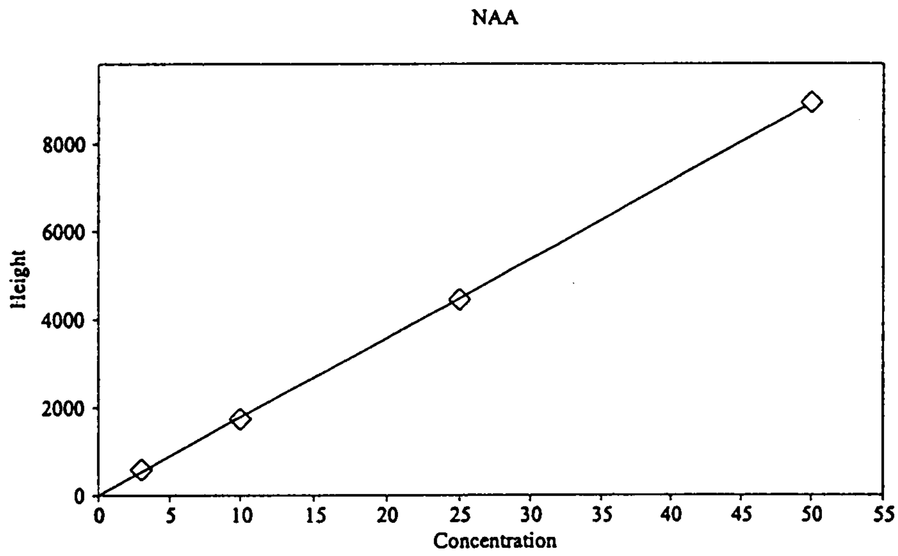
Tomato Control (0.5 g/mL) 20 µL injected
None detected (Less than 0.01 ppm)



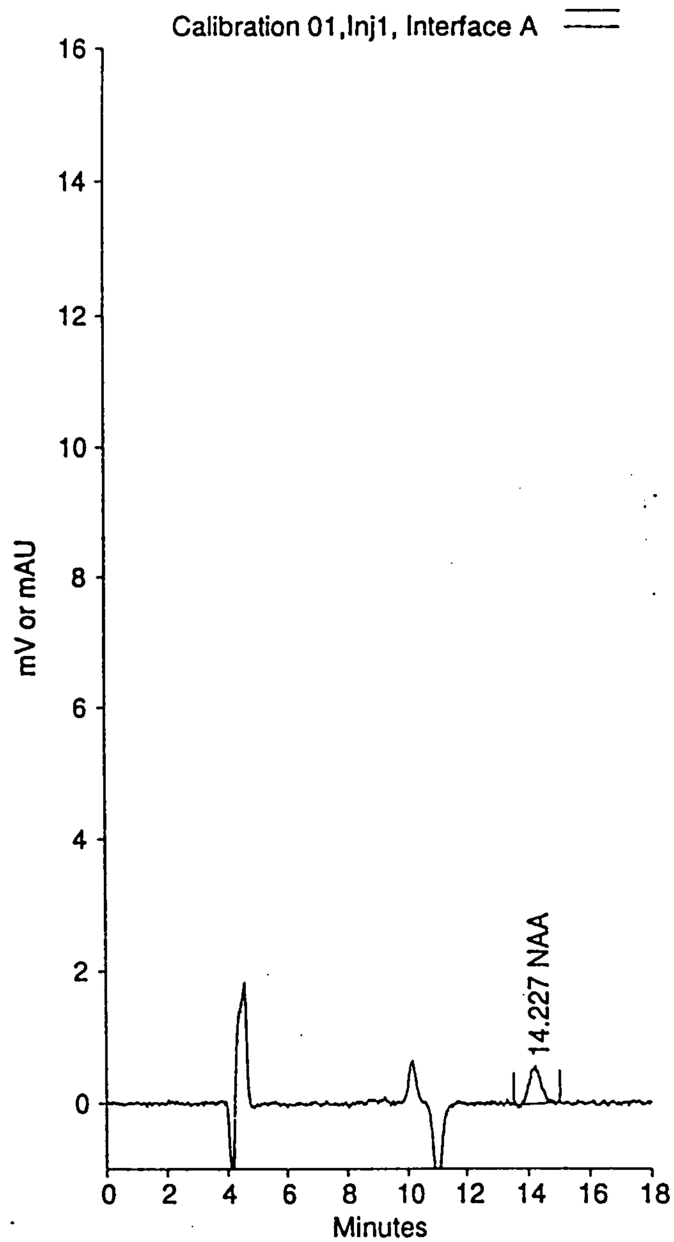
Tomato spike @ 0.01 ppm with NAA (0.5 g/mL)
20 μL injected 91% Recovery



Tomato spike @ 0.5 ppm with NAA (1 to 10 dilution of 0.5 g/mL)
20 μL injected 106% Recovery

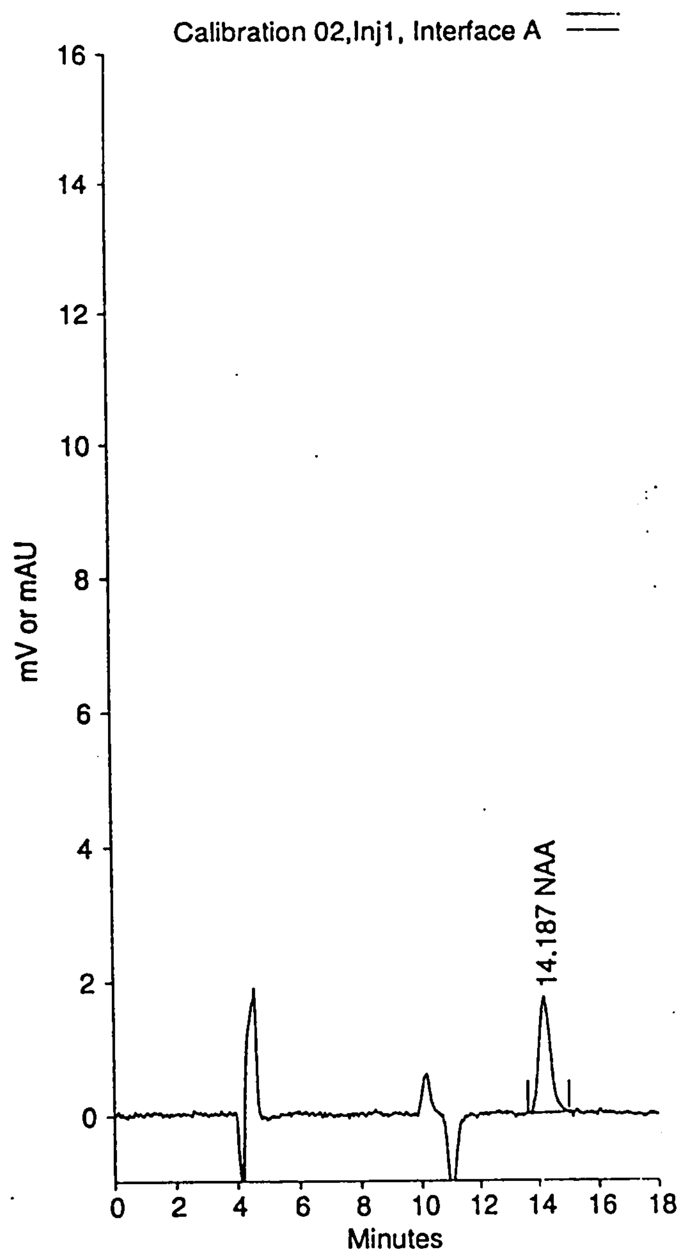


Typical calibration curve for NAA in Green Bell Pepper



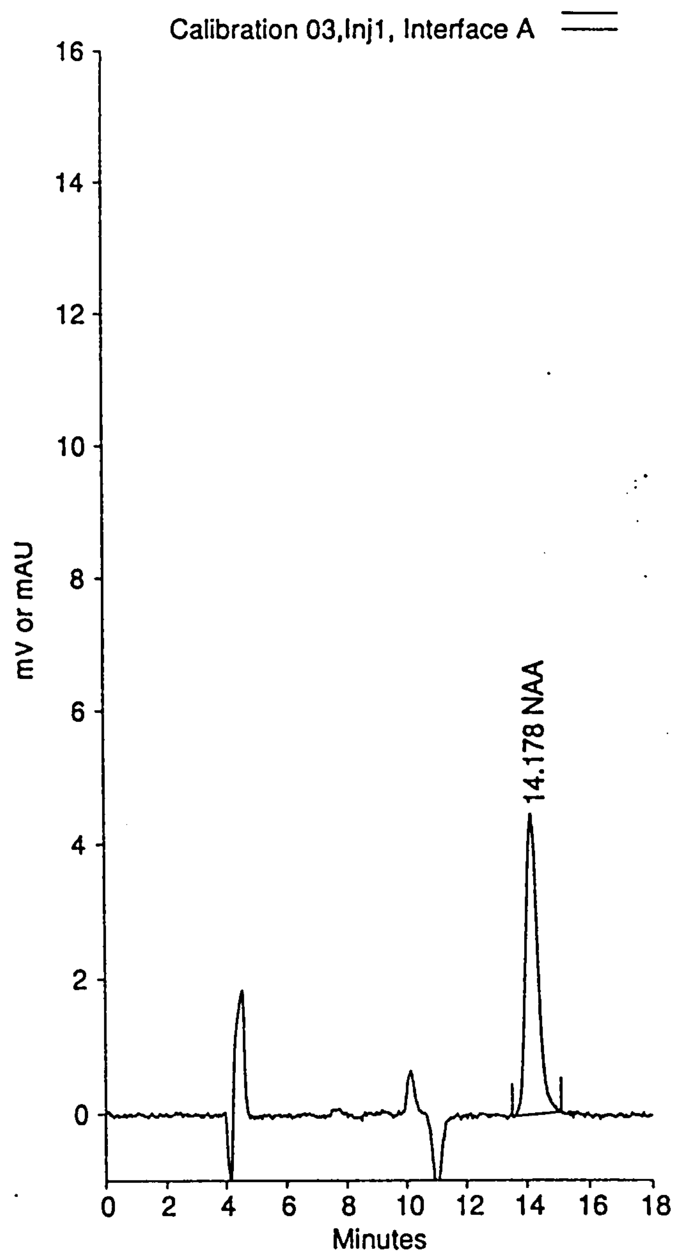
Green Bell Pepper Matrix

NAA calibration standard (3 ng/mL) 20 μL injected



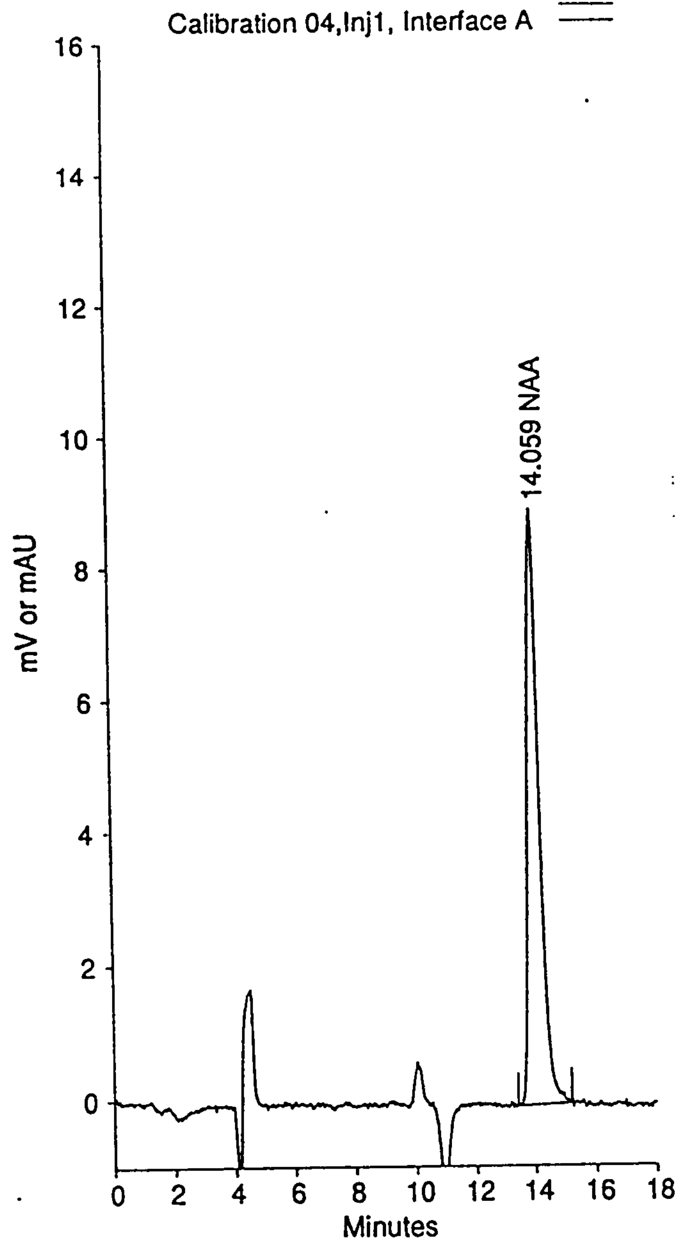
Green Bell Pepper Matrix

NAA calibration standard (10 ng/mL) 20 μL injected



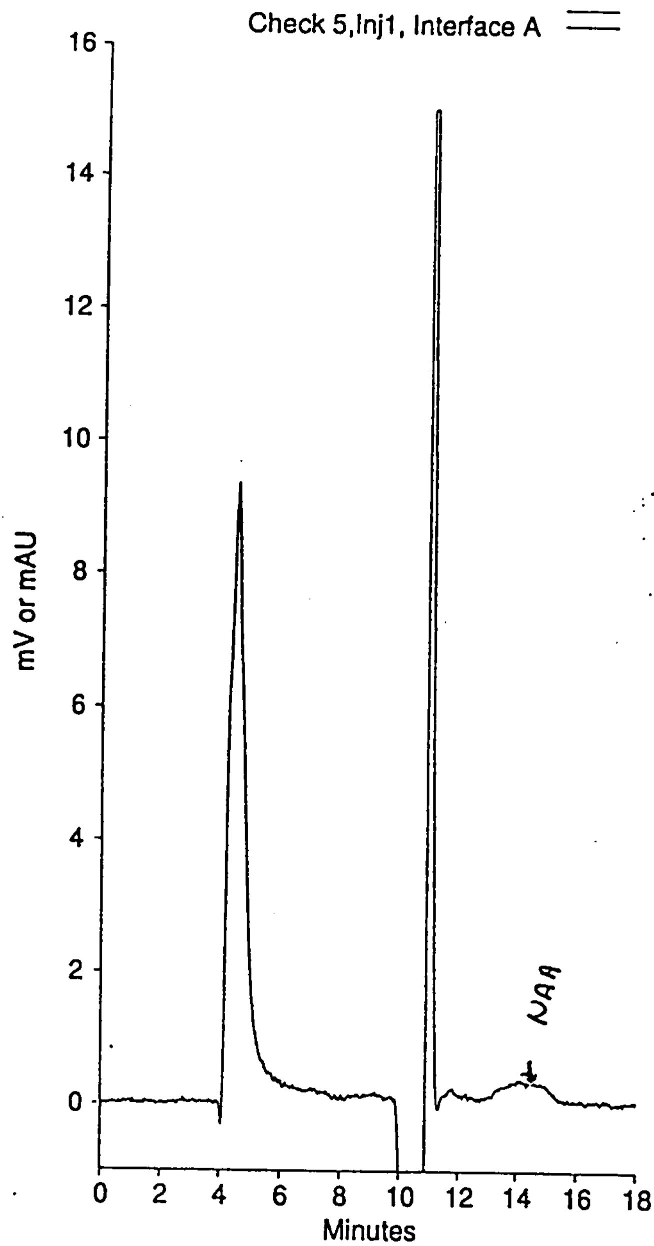
Green Bell Pepper matrix

NAA calibration standard (25 ng/mL) 20 μL injected

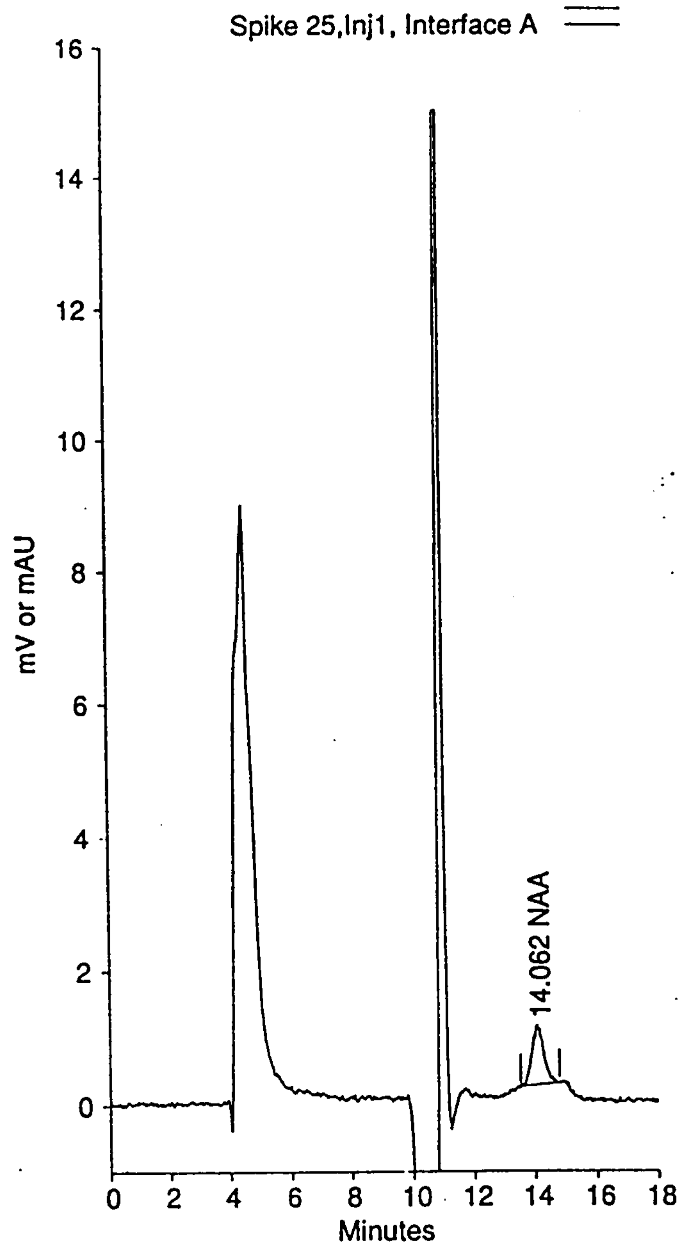


Green Bell Pepper matrix

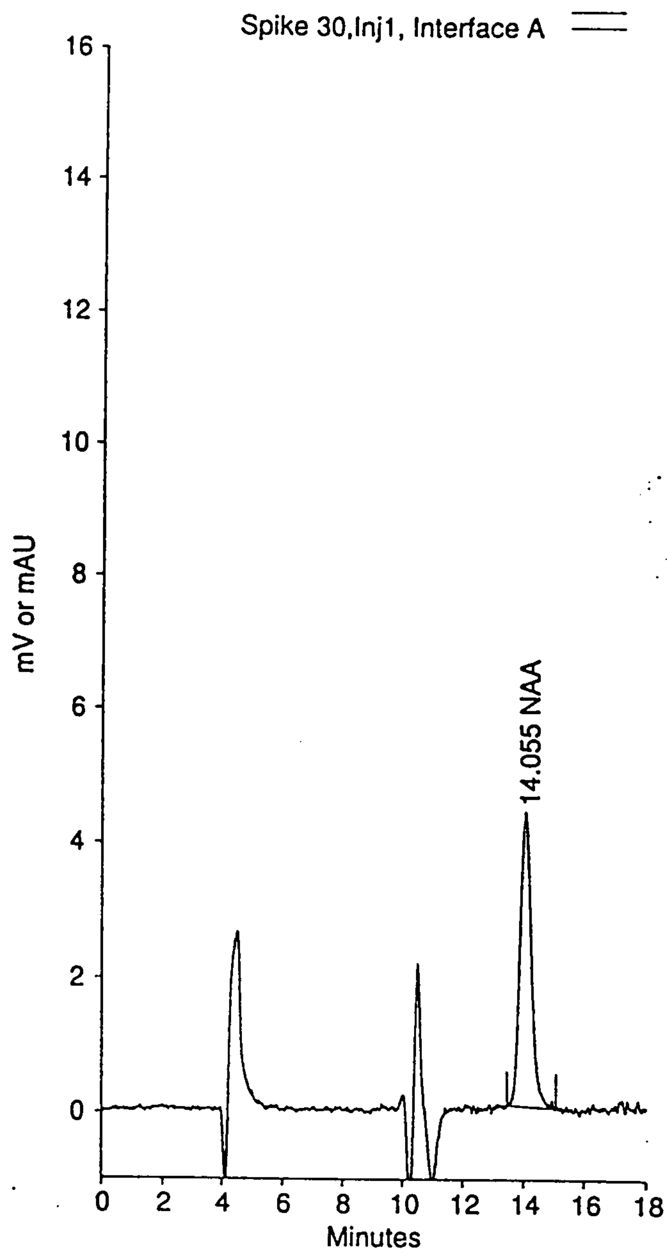
NAA calibration standard (50 ng/mL) 20 μL injected



Green Bell Pepper Control (0.5 g/mL) 20 μL injected
None detected (Less than 0.01 ppm)



Green Bell Pepper spike @ 0.01 ppm with NAA (0.5 g/mL)
20 μL injected   98% Recovery



Green Bell Pepper spike @ 0.5 ppm with NAA (1 to 10 dilution of 0.5 g/mL)
20 μL injected 97% Recovery