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ATTACHMENT 2



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Analytical Method for the Determination of Gentamicin in Pears

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I. INTRODUCTION

This analytical method describes a procedure for the determination of residues of gentamicin in pears. Gentamicin is an antibiotic containing three major components, and is normally produced and used as the sulfate salt (Appendix A).

The method begins with extraction of a sample with a methanol/buffer mixture. The extract is isolated by centrifugation and filtration, and passed through a CM-Sephadex column for cleanup and concentration. The eluate from the cleanup column is derivatized with *o*-phthalaldehyde (OPA) reagent. After derivatization the solvent is exchanged *via* a solid-phase extraction (SPE) column to methanol. The methanol solution is analyzed *versus* derivatized standards on a high-performance liquid chromatography (HPLC) system using a reversed phase column and a fluorescence detector. The limit of quantitation for the method is 0.03 ppm total gentamicin in pears.

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II. MATERIALS AND REAGENTS

The following list of materials and reagents is provided to assist the analyst in successfully performing this method. Other materials and reagents may be used if they are functionally equivalent.

A. Materials

Top loading balance

Analytical balance

Polypropylene centrifuge bottles

Centrifuge capable of 3000 rpm

Graduated cylinders, 100-mL

Mechanical Shaker

Homogenizer, Virtishear catalog number 225318

Whatman GF/A filter paper (12.5 cm) Fisher catalog number 09-874-160

MSI G15 filter paper (12.4 cm) Fisher catalog number G15WP124245

Büchner funnel (12.5 cm)

Vacuum adapter

Flat-bottom vacuum flask, 500-mL

pH Meter, calibrated

Disposable filtration column, 6-mL with 20- μ m frit, JT Baker Cat. No. 7121-06

C₁₈ SPE column 6-mL, JT Baker Cat. No. 7020-07

Reservoirs, capacity 15 and 70 mL

Solid phase vacuum extraction manifold, Supelco Cat. No. 5-7030M

Test tubes: capacity 10 and 15 mL

Disposable pasteur pipets

Syringe filters (2 μ m)

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Disposable syringe (3-mL)

Shimadzu Model LC-6A HPLC pumps

Shimadzu Model SIL-6B auto injector

Autosampler vials

HPLC C₈ column, Zorbax C8, 25 cm x 4.6 mm

HPLC C₈ column, Supelco LC-8-DB 25 cm x 4.6 mm (optional)

Column heater (optional)

Shimadzu RF-551 spectrofluorometer

B. Reagents

All reagents are reagent grade, ACS-certified or better. All solvents are pesticide-grade or better.

Water, Milli-Q or equivalent, 18 megaohm.

Methanol

Celite, analytical filter aid, Fisher Cat. No. 211-500

CM-Sephadex (C-25), Pharmacia Cat. No. 17-0210-01

Sodium sulfate, anhydrous

Sodium phosphate, monobasic

Sodium heptane sulfonate

Ammonium acetate

Acetic Acid

Sodium hydroxide solution, 50%

Phosphoric acid, concentrated

Sulfuric acid, concentrated

Fluoraldehyde, Pierce Cat. No. 26025

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Gentamicin sulfate, USP or BP grade, analytical standard

C. Preparation of reagents

Extraction buffer. 0.1 M Na₂SO₄, 0.1 M NaH₂PO₄, pH 2. Dissolve 28.4 g Na₂SO₄ and 27.6 g NaH₂PO₄ in 2000 mL water. Adjust the pH to 2 with concentrated H₂SO₄.

0.2 M Na₂SO₄. Dissolve 28.4 g Na₂SO₄ in 1000 mL water.

2 M Phosphoric acid. Dissolve 34 mL of concentrated phosphoric acid in 200 mL water. Adjust the volume to 250 mL.

2 M NaOH. Dissolve 26.3 mL 50% NaOH in 200 mL water. Adjust the volume to 250 mL.

Alkaline buffer. Dissolve 28.4 g Na₂SO₄ in 500 mL water, add 5 mL 2 M NaOH, dilute to 1000 mL with water.

CM-Sephadex (C-25) ion exchange resin. Add 150 g of resin to 1400 mL of 0.2 M Na₂SO₄. Let equilibrate for 24 hours before using.

Ion pair reagent. Dissolve 0.5 g sodium heptane sulfonate in 500 mL methanol.

Gradient mobile phase A. 5 g sodium heptane sulfonate, 50 mL glacial acetic acid, 450 mL Milli-Q water, adjust the volume to 1000 mL with methanol. Filter through 0.45- μ m membrane.

Gradient mobile phase B. Methanol

Isocratic Mobile Phase (optional). Buffer: 0.02 M ammonium acetate, 0.005 M sodium heptane sulfonate, pH 4 with acetic acid. Dissolve 1.54 g ammonium acetate and 1 g sodium heptane sulfonate in 1000 mL water. Adjust the pH to 4 with acetic acid (~5 mL). Combine with methanol to give 25/75 buffer/MeOH. Filter through 0.45- μ m membrane.

D. Stock solutions

The following description is for the preparation of gentamicin stock solutions. The concentrations given are for total gentamicin. The concentration of any given component of gentamicin is determined by the relative amount of each component to the total.

Primary Stock Solution

A primary stock solution is prepared by weighing out (to the nearest 0.1 mg) gentamicin sulfate equivalent to 0.100 g gentamicin into a 100-mL volumetric flask. The flask is filled to the mark with 20% methanol in water. This solution contains 1000 μ g/mL gentamicin.

It is necessary to consider both the potency of the gentamicin sulfate reference standard (Appendix B) and the amount of water present in a lot of gentamicin standard that is not dry. The optimal approach is to determine water content by the USP or an alternate method, then apply a mathematical correction to amounts weighed out to prepare analytical calibration solutions. Alternatively, if the potency is provided on a wet-weight basis, that value may be used for freshly-opened standard.

The equation used to calculate the amount of gentamicin sulfate to contain 0.100 g of gentamicin is:

$$\text{g gentamicin sulfate} = 0.100 / (\text{potency} / 1000)$$

For example, a typical wet-weight basis potency of gentamicin sulfate is 625 $\mu\text{g}/\text{mg}$. The requisite amount of gentamicin sulfate needed to prepare the stock solution described above is:

$$\text{g gentamicin sulfate} = 0.100 / (625 / 1000) = 0.160 \text{ g}$$

Secondary Stock Solutions

Pipet 5.00 mL of the 1000 $\mu\text{g}/\text{mL}$ solution into a 50-mL volumetric flask and fill to the mark with 20% methanol in water. This solution contains 100 $\mu\text{g}/\text{mL}$ gentamicin.

Pipet 15.0 mL of the 100 $\mu\text{g}/\text{mL}$ solution into a 100-mL volumetric flask and fill to the mark with 20% methanol in water. This solution contains 15.0 $\mu\text{g}/\text{mL}$ gentamicin.

Pipet 25.0 mL of the 15.0 $\mu\text{g}/\text{mL}$ solution into a 50-mL volumetric flask and fill to the mark with 20% methanol in water. This solution contains 7.50 $\mu\text{g}/\text{mL}$ gentamicin.

Pipet 10.0 mL of the 15.0 $\mu\text{g}/\text{mL}$ solution into a 50-mL volumetric flask and fill to the mark with 20% methanol in water. This solution contains 3.00 $\mu\text{g}/\text{mL}$ gentamicin.

Pipet 5.00 mL of the 15.0 $\mu\text{g}/\text{mL}$ solution into a 50-mL volumetric flask and fill to the mark with 20% methanol in water. This solution contains 1.50 $\mu\text{g}/\text{mL}$ gentamicin.

Pipet 10.0 mL of the 3.00 $\mu\text{g}/\text{mL}$ solution into a container and add 50 mL of 20% methanol in water. This solution contains 0.500 $\mu\text{g}/\text{mL}$ gentamicin.

E. Derivatized Standard Solutions

Derivatized standard solutions for HPLC calibration are prepared fresh with each set of samples. Using the following table as a guide, pipette 1.00 mL of the secondary stock solution into a clean 10-mL test tube and add 1 mL of Fluoraldehyde (*o*-phthalaldehyde reagent solution). Let stand 7 minutes. The concentrations given in the table are for total gentamicin. See Section F, below, for calculation of individual component concentrations.

Concentration of Stock Solution ($\mu\text{g/mL}$)	Volume of Stock Solution (mL)	Final Standard Volume (mL)	Final Standard Concentration ($\mu\text{g/mL}$)
15.0	1.00	5.00	3.00
7.50	1.00	5.00	1.50
3.00	1.00	5.00	0.600
1.50	1.00	5.00	0.300
0.500	1.00	5.00	0.100

Once the 7 minutes are up, use a vacuum manifold to load each standard onto a separate C_{18} SPE column that has been conditioned with 4 mL of methanol followed by 4 mL of water. Use the reservoir and control the vacuum to facilitate loading. Do not allow the column to go dry during conditioning or during loading. Rinse the test tube with 4 mL of water and transfer this washing to the column. After the wash is loaded, draw the fluid through the column and allow the column to go dry for approximately 1 minute. Add to the column 3.5 mL of methanol and collect the eluate in a 10-mL test tube calibrated at 5.00 mL. Add ion pair reagent (1.0 mL) to the test tube and then bring the volume to 5.00 mL with methanol. The resulting solution is a standard solution used for instrument calibration.

F. Calculation of Individual Component Concentrations

The content of the gentamicin reference standard is specified by potency (assay) and by the percent of each component (Appendix B). To calculate the concentration of each component in the HPLC standard solutions, multiply the total gentamicin concentration by the percent component divided by 100. The concentration of the individual component in the standard solutions is used in calculation of the concentrations of individual components in injected sample extracts. The table below shows calculated component concentrations for a hypothetical reference standard that contains 30% C_1 , 20% C_{1a} , and 50% C_{2a}, C_2 .

Standard, $\mu\text{g/mL}$		0.10	0.30	0.60	1.50	3.00
Component	%	Concentration of Component, $\mu\text{g/mL}$				
C_1	30	0.03	0.09	0.18	0.45	0.90
C_{1a}	20	0.02	0.06	0.12	0.30	0.60
C_{2a}, C_2	50	0.05	0.15	0.30	0.75	1.50

III. Analytical Procedure

Samples are received and stored frozen. The samples are prepared for analysis by mixing with dry ice and grinding in a cutter mixer. After the dry ice has sublimed, subsamples can be taken for analysis.

A. Extraction

Weigh subsamples (50.0 ± 0.1 g) into 250-mL polypropylene centrifuge bottles. (It is most convenient to weigh the subsamples while they are still frozen.) Make method validation fortifications at this time by pipetting 1.00 mL of the appropriate secondary stock solution directly onto an untreated control subsample. For example, to fortify at 0.03 ppm, 1.00 mL of 1.50 $\mu\text{g}/\text{mL}$ stock solution is pipetted onto a 50-g untreated control subsample. Add approximately, 3-4 g of Celite to each sample. Add 50 mL extraction buffer plus 50 mL methanol to each sample. Macerate the samples with a Virtishear mixer at medium speed for 3 minutes, followed by centrifugation at 3000 rpm for 3 minutes. Decant the supernatant into a 500-mL flat-bottom vacuum flask through a Büchner funnel attached to a vacuum source and fitted with two filter papers, Whatman GF/A on top and MSI G15 on bottom.

Add 50 mL extraction buffer plus 25 mL methanol to the centrifuge bottle containing the solids from the initial extraction. Cap the bottle and shake it on a mechanical shaker for 10 minutes. After shaking, centrifuge the bottle for 3 minutes at 3000 rpm. Again decant the supernatant through the Büchner funnel into the 500-mL flat-bottom flask that contains the initial extract solution.

Extract the material in the bottle a third time with 50 mL extraction buffer plus 25 mL methanol by shaking on the mechanical shaker for 10 minutes, followed by centrifugation at 3000 rpm for 3 minutes, and decanting through the Büchner funnel into the 500-mL flat bottom flask.

B. Cleanup and Concentration (CM-Sephadex Column)

Adjust the pH of the combined extract to 6.4-6.5 using 50% NaOH solution. If a pH greater than 6.5 is reached, use 2 M phosphoric acid to lower the pH to within the correct range.

Prepare a CM-Sephadex column by pipetting 4 mL of prepared Sephadex resin into a disposable 6-mL filtration column and marking the liquid level. Once the resin has settled, add additional resin to bring the bed level to 4 mL. Attach a 70-mL reservoir to the top of the column using an adapter. Rinse the column with 10 mL 0.2 M Na_2SO_4 , allowing the liquid level to just reach the top of the resin bed. Load the entire extract onto the Sephadex column using a slight vacuum, maintaining a flow of 5-10 mL/min. Loss in recovery will occur if the Sephadex column goes dry at any point during the washing or loading phases. After the extract has been loaded, rinse the 500-mL flat-bottom flask with 10 mL 0.2 M Na_2SO_4 . Transfer the rinse to the column. Wash the column with 2 mL 10 mM NaOH in 0.2 M Na_2SO_4 . Elute the analytes from the

Sephadex column with 10 mL 10 mM NaOH in 0.2 M Na₂SO₄, collecting the eluate in a 15-mL test tube. The columns may be drained dry at this point. Other elution profiles from the Sephadex column may be obtained with different lots. It is important to determine the correct collection fraction for each lot of resin.

C. Derivatization and Solvent Exchange

Derivatize the eluate by adding 3 mL Fluoraldehyde to the test tube and letting stand for 7 minutes. Once the 7 minutes are up, use a vacuum manifold to load the Sephadex column eluate onto a C₁₈ Solid Phase Extraction (SPE) column that has been conditioned with 4 mL of methanol followed by 4 mL of water. Use a 5-mL reservoir and control the vacuum to facilitate the loading. Do not allow the column to go dry during conditioning or during loading. Rinse the test tube with 4 mL water and transfer this washing to the column. After the wash is loaded, draw the fluid through the column and allow the column to go dry for approximately 1 minute. Add 3.5 mL methanol to the column and collect the eluate in a 10-mL test tube calibrated at 5.00 mL. Add 1.0 mL ion pair reagent to the test tube and then bring the volume to 5.00 mL with methanol. The concentration of sample in the extract solution is 10.0 g/mL.

IV. INSTRUMENT PARAMETERS

The isolated residues are quantitated by reverse-phase ion-pair HPLC using fluorescence detection.

A. Gradient Elution

The following operating conditions are given as a guide. Other conditions may be used to separate interfering compounds from the compounds of interest. The elution order of the three major components of gentamicin (Appendix A) is based on previously reported results (1,2). The elution order is C₁, C_{1a}, C_{2a}, C₂. Example chromatograms are provided in Appendix C.

Mobile Phase:

Solvent A: 5 g Sodium Heptane Sulfonate, 50 mL Glacial Acetic Acid, 450 mL Milli-Q water, adjust volume to 1000 mL with Methanol.

Solvent B: Methanol

Gradient: 25% Solvent B and 75% Solvent A from time 0 to time 5 minutes, linear gradient from 25% Solvent B to 75% Solvent B at time 15 minutes.

Flow Rate: 1.5 mL/min

Column: Zorbax C8, 25 cm x 4.6 mm

Temperature: Ambient

Detection: Fluorescence
Excitation: 345 nm
Emission: 445 nm

B. Isocratic Elution

Isocratic elution can be used to quantitate components C_{1a} and C_{2a}, C_{2b} , but may not adequately resolve C_1 from coextractives to allow quantitation. It is unlikely that residue levels of C_1 will differ greatly from levels of C_{1a} and C_{2a}, C_{2b} , however, so isocratic elution could be useful to scan quickly for detectable levels of gentamicin, with quantitation done by gradient elution if necessary. In a magnitude of the residue study on gentamicin residues in pears, in which 12 samples were analyzed using gradient elution, no gentamicin residues were found above the 0.03 ppm limit of quantitation for total gentamicin. Examination of analytical results for individual components indicated that no component predominated when apparent residues below the quantitation limit were observed. These results support the observation that C_1 residue levels do not appreciably exceed levels of the other two major components.

The following operating conditions are given as a guide. Other conditions may be used to separate interfering compounds from the compounds of interest. The elution order of the three major components of gentamicin (Appendix A) is based on previously reported results (1,2). The elution order is $C_1, C_{1a}, C_{2a}, C_{2b}$. Example chromatograms are provided in Appendix D.

Mobile Phase: 25:75, Solvent A:Solvent B
Solvent A: 0.02 M Ammonium Acetate: 0.005 M Sodium Heptane Sulfonate, pH 4 with Acetic Acid.
Solvent B: Methanol.

Flow Rate: 1.5 mL/min

Column: Supelco LC-8-DB, 25 cm x 4.6 mm

Temperature: 50 °C

Detection: Fluorescence
Excitation: 345 nm
Emission: 445 nm

V. METHOD OF CALCULATION

The epimers C_{2a} and C_{2b} are resolved in both the isocratic and the gradient elution system, so that two peaks appear in the elution region of C_{2a}, C_{2b} (Appendices D and E). The integrated areas of the two peaks are summed, to give a single response for C_{2a}, C_{2b} . This procedure is required because the ratio of the components in the analytical standard is typically given for only the three major components, *i.e.*, C_1, C_{1a} , and C_{2a}, C_{2b} . Thus,

individual component concentrations for the epimers of C₂ cannot be assigned. Refer to Appendices A and B for more information.

A. Gentamicin Component Concentration in the Injected Solution.

The concentration of each of the three major components of gentamicin in the sample solution is determined by external calibration. A nonweighted linear least-squares fit of the responses and concentrations of the injection standards is used to calculate the concentration of each component in the sample solution. The concentrations of the individual components are summed to give the total gentamicin concentration in the sample solution.

If the peak area of any component in a sample solution is outside the range of responses for the corresponding component in the standard solutions, the component concentration in the sample solution is outside the range at which the least-squares calibration is valid. If the sample response exceeds the response of the most concentrated standard, the sample must be diluted and reinjected. Alternatively, a higher standard may be injected with the sample set if linearity is still demonstrated.

If the response of any component in a sample solution is less than the response of the least concentrated standard, the apparent analyte concentration in the extract may be calculated *versus* the response factor of the least concentrated standard. Typically, concentrations below the calibration range would only be calculated for control samples, to allow background correction of fortification recoveries.

1. *Gentamicin Concentration within the Calibration Range.*

Calibration:

Peak areas are determined for each component at several concentrations of calibration solutions. For each component, linear regression is used to give an equation of the form

$$y = m \cdot x + b \qquad \text{Eq. (1)}$$

where y = the component peak area,
 m = the slope;
 x = the component concentration, µg/mL; and
 b = the intercept.

The correlation coefficient of the regression curve must be 0.995 or above.

Concentration of individual component in the solution injected:

Rearrangement of Eq. 1 gives Eq. 2, by which the concentration of the component in the extract solution is calculated

$$x = \frac{(y - b)}{m} \quad \text{Eq. (2)}$$

where the symbols have the same meanings as in Eq. 1.

2. *Gentamicin Concentration below the Calibration Range.*

A response factor is calculated from the concentration and response of the least concentrated standard solution:

$$RF = \frac{y}{x} \quad \text{Eq (3)}$$

where RF = the component response factor;
 y = the component peak area, and
 x = the component concentration, $\mu\text{g/mL}$.

Rearrangement of Eq. 3 gives Eq. 4, by which the concentration of the component in the injected extract solution is calculated.

$$x = y \cdot RF \quad \text{Eq. (4)}$$

where the symbols have the same meanings as in Eq. 3.

B. Gentamicin Residue Levels in the Sample

1. *Calculation of total gentamicin concentration in the sample extract solutions.*

The **total** concentration of gentamicin is the sum of the individual concentrations:

$$x_t = x_1 + x_{1a} + x_{2,2a} \quad \text{Eq. (5)}$$

where x_t = the total concentration of gentamicin in the injected extract, $\mu\text{g/mL}$;

x_1 = the concentration of C_1 in the injected extract, $\mu\text{g/mL}$;

x_{1a} = the concentration of C_{1a} in the injected extract, $\mu\text{g/mL}$; and

$x_{2,2a}$ = the concentration of C_{2a}, C_2 in the injected extract, $\mu\text{g/mL}$.

2. *Calculation of gentamicin residue level in sample.*

The residue level of total gentamicin in the sample is calculated using the following equation:

$$\text{ppm gentamicin} = \frac{x_t}{C} \quad \text{Eq. (6)}$$

where C = concentration of sample in injected extract solution, g/mL.

In the method validation test reported herein, $C = 50.0$ g per 5.00 mL, or 10.0 g/mL.

If desired, the concentration of any individual component can be calculated, by using Eq. 6 and the concentration of the individual component in the injected extract rather than the total concentration of gentamicin in the injected extract.

VI. RESULTS AND DISCUSSION

Table I shows recoveries for all three components, using gradient elution. The recovery for total gentamicin was 99.5% at the lower limit of quantitation, 0.03 ppm and 83.9% at the upper limit of quantitation of quantitation, 0.15 ppm. Individual recoveries are tabulated for reference although such recoveries would not typically be determined or reported for sample analyses.

Recoveries for total gentamicin residues using isocratic elution, based on the fortified amounts of components C_{1a} and C_{2a}, C_2 are given in Table II. Again, recoveries of individual components are tabulated, to allow calculation of individual standard deviations, and to allow comparison with individual recoveries determined using gradient elution. Recovery for total C_{1a} and C_{2a}, C_2 gentamicin ranged from 75.8% to 89.3%, with a mean of 81.1% and a coefficient of variation of 4.8%. Recoveries for individual components C_{1a} and C_{2a}, C_2 ranged from 73.0% to 96.7, with mean of 82.6% for C_{1a} and 81.1% for C_{2a}, C_2 , and coefficients of variation of 8.1% for C_{1a} and 3.9% for C_{2a}, C_2 . These results show adequate sensitivity, accuracy, and precision of the analytical method, including the extraction, clean-up, and derivatization steps

Using gradient elution, individual recoveries for C_{1a} and C_{2a}, C_2 are comparable to or better than those determined by isocratic elution. These results, together with the excellent total recoveries of total gentamicin from fortifications at the high and low ends of the quantitation range, demonstrate the validity of the gradient HPLC method for quantitation of total gentamicin residues, including all three major gentamicin components, C_1 , C_{1a} , and C_{2a}, C_2 .

VII. CONCLUSIONS

The method described herein has been shown to be valid for determination of total gentamicin residues in pears. The method is sufficiently sensitive to determine residue levels at 0.03 ppm. The total gentamicin concentration range in which the method has been shown to be valid is 0.03 ppm to 0.15 ppm. Analysis of untreated pear control samples demonstrated that interferences from coextractives are well below the 0.03 ppm limit of quantitation. Recoveries from fortifications were between 70% and 120% in all instances, which demonstrates adequate accuracy for quantitation of gentamicin residues in the tested range. The coefficients of variation for 12 separate recovery

determinations at three levels were below 10%, for both individual components and total gentamicin, which demonstrates excellent precision.

VIII. METHOD NOTES

It is worth noting that the Sephadex column step requires special attention. If the column gets any air on it, a loss in recovery will result. The reasoning behind the volume of column chosen also deserves mention. A smaller column gave poorer recoveries, and larger columns gave increased resistance (slower flow rates). A 4-mL column was chosen because it gave good recovery while maintaining high flow rates.

Ion pair reagent is added to the final extract to improve chromatography. A buffer at low pH is not used because the derivatized gentamicin appears to be unstable under acidic conditions.

The HPLC elution pattern is affected by the pH of the mobile phase as well as organic content and buffer strength. Some adjustment to the mobile phase may be necessary to optimize the separation.

To avoid carry-over from high-concentration calibration solutions, the HPLC standards should be injected in order from lowest to highest concentration. The control extract solution should be injected immediately after the lowest-concentration calibration solution.

IX Tables

Table I. Recoveries of Individual Components and Total Gentamicin, Gradient HPLC

Table II: Recoveries of Individual Components and Total C₁, and C₂, C₃, Isocratic HPLC

Table I. Recoveries of Individual Components and Total Gentamicin, Gradient HPLC

Sample Name	Percent Recovery			
	C ₁	C _{1a}	C _{2a} , C ₂	Total
F 40831-044 WAPE-01-A C + 0.03 ppm ¹	107	103	91.6	99.5
F 40831-044 WAPE-01-A C + 0.15 ppm	74.9	89.1	87.1	83.9

¹ Fortification level

Table II. Recoveries of Individual Components and Total C₁ and C₂, C₂, C₂, Isocratic HPLC

Sample Name		Percent Recovery		
		C ₁	C ₂ , C ₂	Total ¹
F 40861-003	C + 0.03 ppm ²	82.8	85.2	84.2
F 40861-004	C + 0.03 ppm	96.7	78.4	86.2
F 40861-005	C + 0.06 ppm	77.2	81.5	79.6
F 40861-006	C + 0.06 ppm	83.5	79.6	81.2
F 40861-007	C + 0.15 ppm	75.2	78.6	77.2
F 40861-008	C + 0.15 ppm	78.1	80.3	79.4
F 40862-011	C + 0.03 ppm	73.0	77.9	75.8
F 40862-012	C + 0.03 ppm	90.6	88.3	89.3
F 40862-013	C + 0.06 ppm	84.9	79.8	82.0
F 40862-014	C + 0.06 ppm	84.9	84.1	84.5
F 40862-015	C + 0.15 ppm	78.8	78.8	78.8
F 40862-016	C + 0.15 ppm	85.1	81.1	82.8
	Mean	82.6	81.1	81.8
	CV ³	8.1%	3.9%	4.8%

¹ Based on total fortification level of C₁ and C₂, C₂

² Fortification level.

³ CV = Coefficient of Variation = (100 x Standard Deviation)/Mean

X. REFERENCES

1. Claes, P.; Vanderhaeghe, H. *Pharmacoepial Forum*. 1987, Sept.-Oct., 2977-2985.
2. Albracht, J.H.; DeWit, M.S. *J. Chrom.* 1987, 389, 306-311.

Appendix A

Structure and Composition of Gentamicin

Gentamicin, sometimes referred to as "Gentamicin C complex" is a mixture of three polyfunctional bases produced during fermentative growth of a common and wide-spread microorganism, *Micromonospora purpurea*. Gentamicin is easily converted to gentamicin sulfate, a salt, which is the sole gentamicin-containing antibiotic available in commerce.

Each of the three components in gentamicin contains two amino sugars, garosamine and purpurosamine, linked to a central aminocyclitol known as 2-deoxystreptamine. Figure 1, depicting the structure of gentamicin, also shows the amino sugars and 2-deoxystreptamine.

The components of gentamicin are designated C_1 , C_{1a} , and C_2 . These components differ only in the substituents R and R_1 on the purpurosamine sugar at Carbon 6 (refer to Figure 1). One component, C_2 , is epimeric at Carbon 6. The epimers are designated as C_2 and C_{2a} and correspond to the R and S configurations, respectively. A distinction between C_2 and C_{2a} is not usually made, and the two epimers are instead listed together as C_{2a}, C_2 or often simply as C_2 . For historical reasons the symbol C_2 can represent both epimers, or the single epimer with R configuration. Generally no ambiguity is introduced, because in most cases the epimers are not differentiated. In this method the sum of the C_2 epimers will be symbolized by C_{2a}, C_2 .

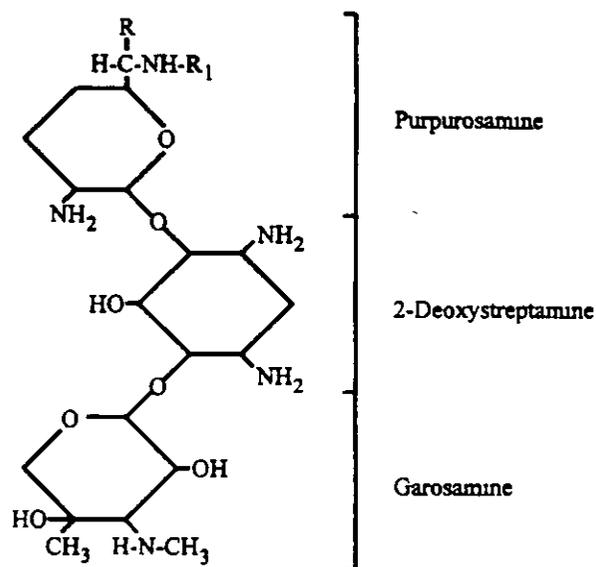


Figure 3

USP/BP gentamicin sulfate, CAS No. 1403-66-3, contains the three components (as the corresponding sulfate salts) within specified proportions. The proportions are indicated below, along with the substituents at Carbon 6 (refer also to Figure 1, above).

<u>Isomer</u>	<u>R</u>	<u>R₁</u>	<u>Proportion</u>
C_1	CH_3	CH_3	25 - 50 %
C_{1a}	H	H	15 - 40 %
C_2	CH_3	H	20 - 50 % (sum of R and S epimers)

Appendix B

Gentamicin Analytical Standard

Gentamicin reference standard is supplied as the sulfate salt. Gentamicin itself is a mixture of three principal aminoglycosides, each containing 5 amino groups of approximately equal basic strength. Commercial USP/BP grade gentamicin sulfate contains all three main components, at varying proportions. A mole of one of the component aminoglycosides contains 5 equivalents of base. When neutralized with sulfuric acid, one mole of aminoglycoside contains 2.5 moles of sulfate ion.

The content of gentamicin in the reference standard is specified in terms of biological activity measured *versus* a primary standard maintained by the USP. The activity of the primary standard is based on a level of 1000 $\mu\text{g}/\text{mg}$ (dry basis) originally assigned to a master standard that contained only gentamicin in the free (basic) form. One milligram of gentamicin with a potency of 690 $\mu\text{g}/\text{mg}$ is equivalent to 690 micrograms of the original master standard.

Practically, the assay of a given reference standard of gentamicin is equivalent to its potency. That is, gentamicin sulfate with a potency of 690 $\mu\text{g}/\text{mg}$ has a gentamicin content that is close to 690 micrograms gentamicin per mg, equivalent to an assay of 69 %. Therefore 100 mg of this reference standard contains $0.69 \times 100 = 69$ mg gentamicin, expressed as the free base. The balance, 31 mg, is present as sulfate.

USP-grade gentamicin, including any lot used as a reference standard, contains three components within proportions set by the USP. The three components (C_1 , C_{1a} , and C_2, C_2) differ slightly in molecular weight and biological activity, so the potency of a lot of gentamicin depends to an extent on the exact ratio of components it contains. This dependency is relatively small, however, and for practical analyses no appreciable error is introduced by apportioning the potency among the components according to their ratio.

Appendix C
Example Chromatograms
Gradient Elution

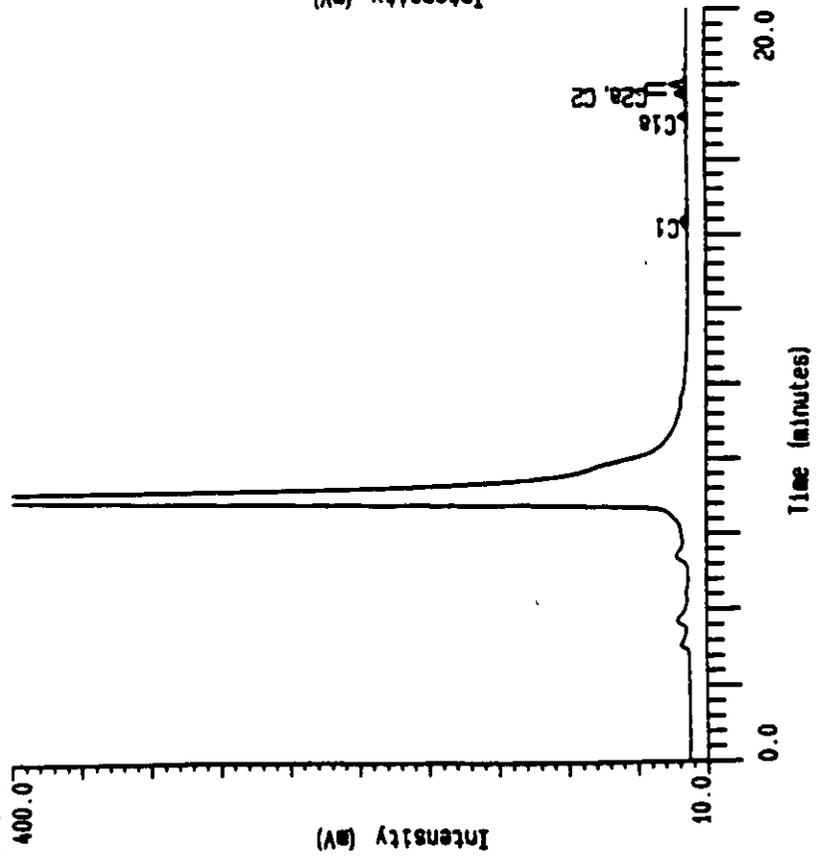
ABC Laboratories, Inc.

MCPD ver 2.3
Date of Report: 10 DEC 93 at 11:30:44

MCD0 (40831) 4 MAPE, 1.1
Std 0.1 ug/mL

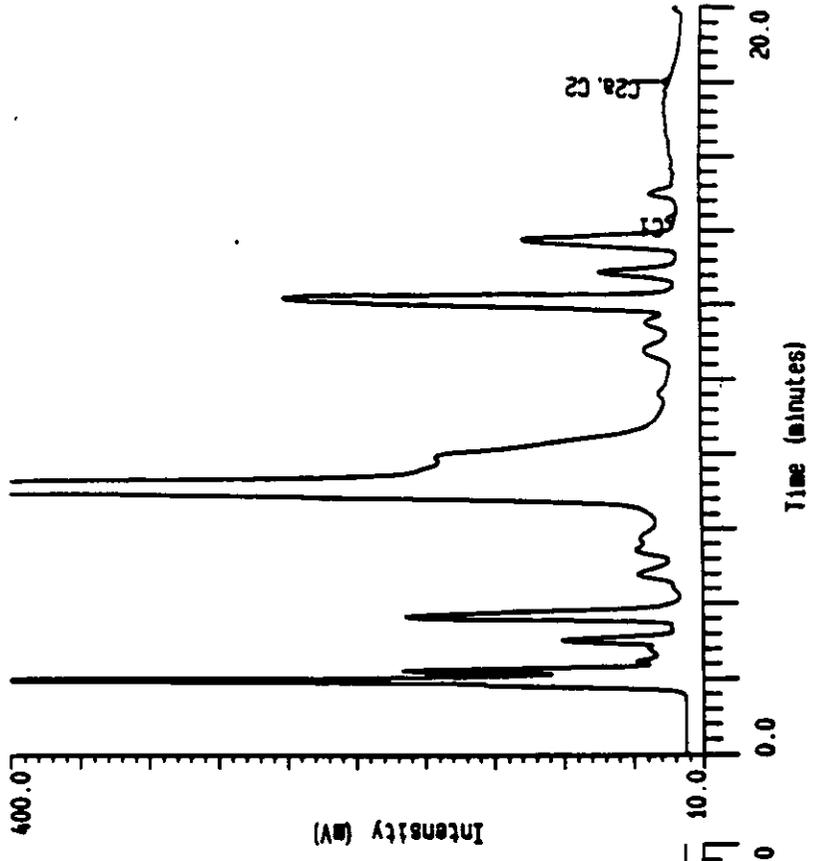
Acquired on 8-Dec-1993 at 19:46

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.35	48952	0.03386
C1a	17.14	36538	0.03505
C2a, C2	17.87	124801	0.04857



MCD0 (40831) 4 MAPE, 2.1
C 40831-043 93-MAPE-01-A Control
Acquired on 8-Dec-1993 at 20:08

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.30	45359	0.00323
C1a	0.00	0	0
C2a, C2	18.00	27541	0.00283



40831

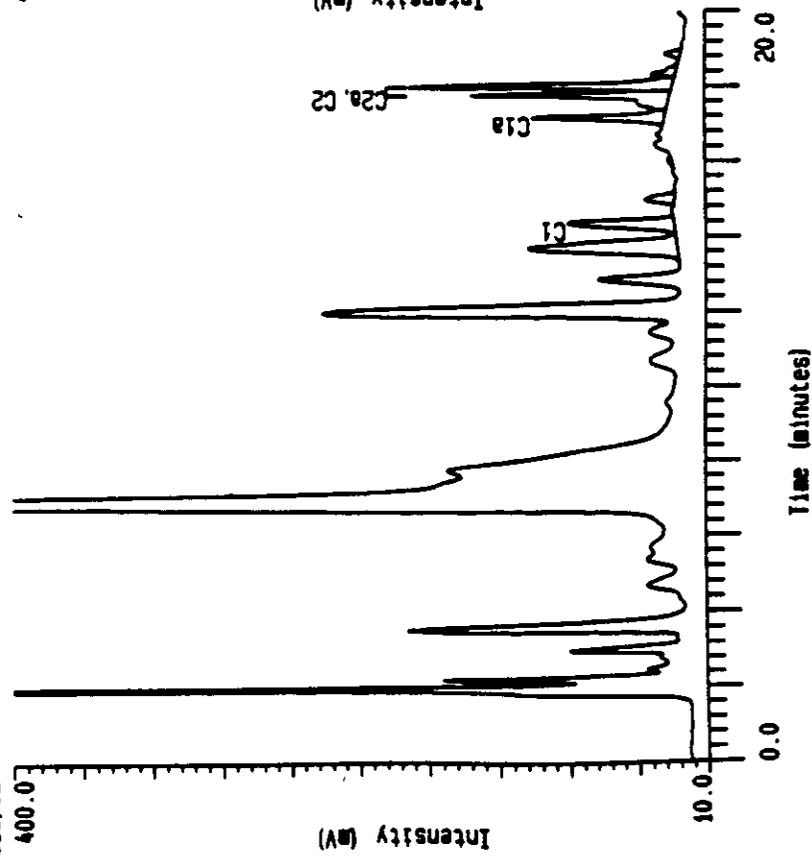
96008

ABC Laboratories, Inc.

Date of Report: 10 DEC 93 at 11:30:44
 MCPP ver 2.3

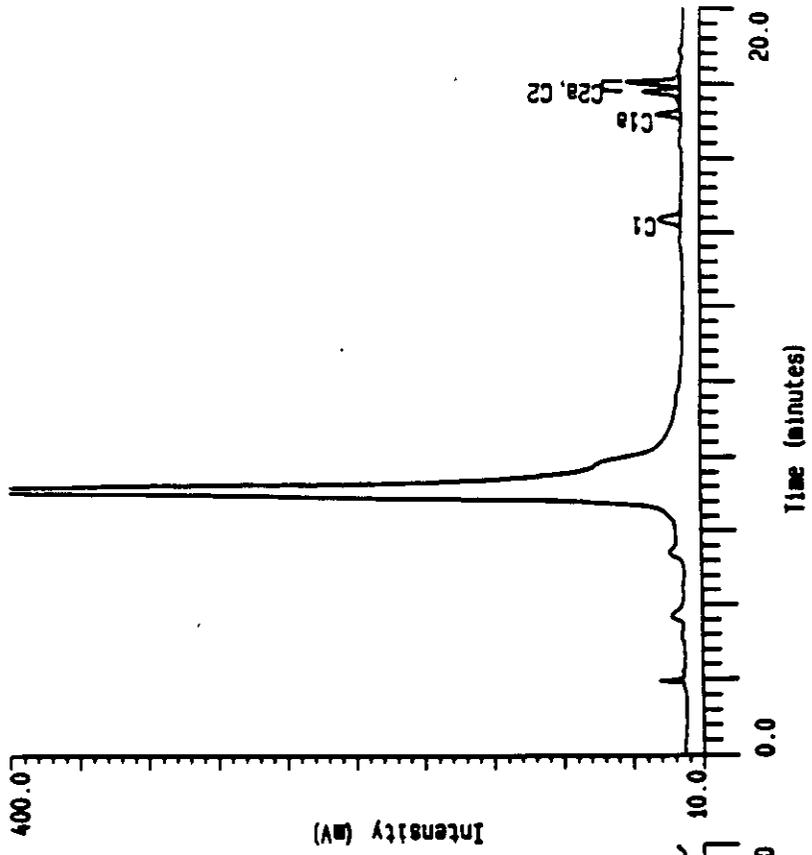
MC00 [40831] 4 WAPE, 3.1
 F 40831-044 93-WAPE-01-A C + 0.15 ppm
 Acquired on 8-Dec-1993 at 20:30

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.33	842737	0.03762
C1a	17.13	706247	0.03961
C2a, C2	17.86	2410437	0.05237



MC00 [40831] 4 WAPE, 4.1
 Std 0.3 ug/mL
 Acquired on 8-Dec-1993 at 20:52

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.36	172990	0.08736
C1a	17.14	123656	0.08285
C2a, C2	17.87	432956	0.11264

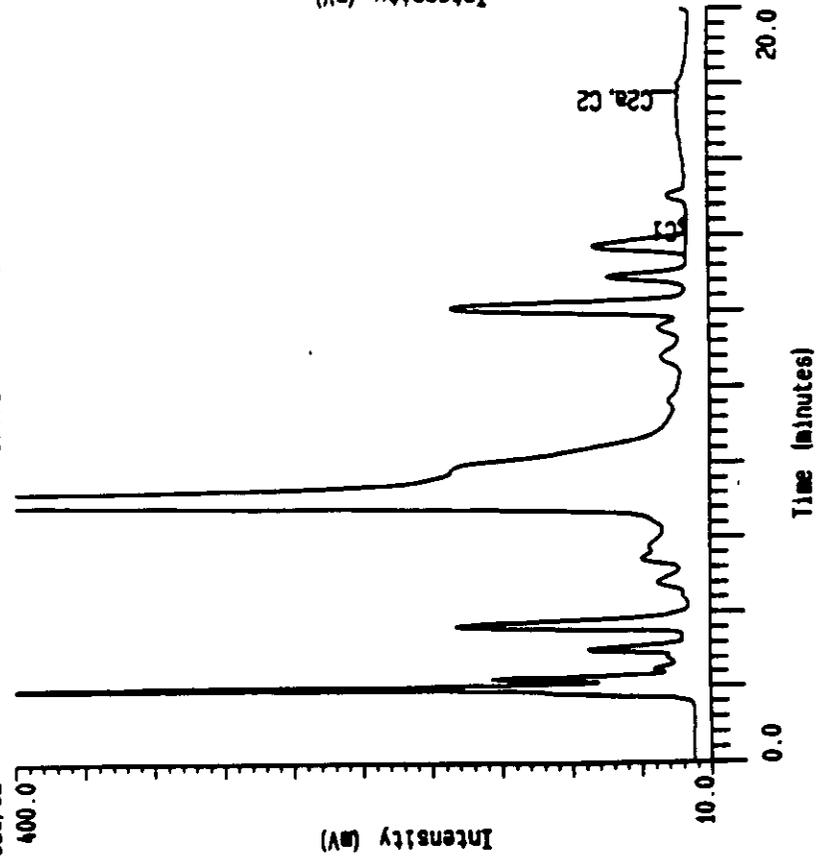


ABC Laboratories, Inc.

Date of Report: 10 DEC 93 at 11:30:44
 MCPP ver 2.3

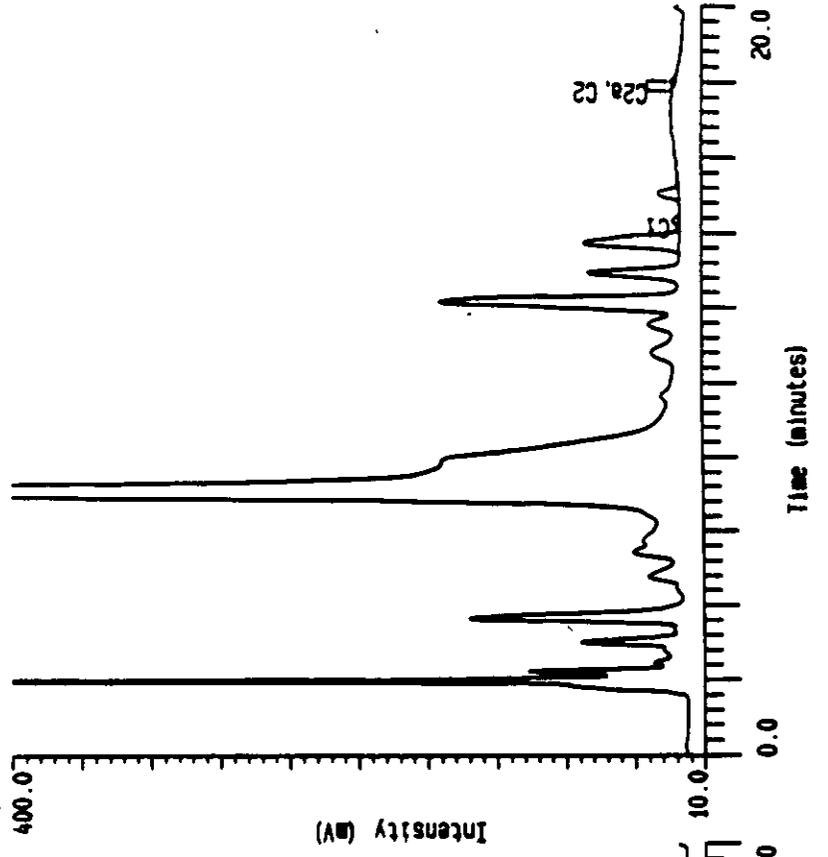
MC00 (40831) 4 WAPE, 5, 1
 T 40831-045 93-WAPE-02-A
 Acquired on 8-Dec-1993 at 21:15

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.29	49327	0.00340
C1a	0.00	0	0
C2a, C2	17.76	0	0.00226



MC00 (40831) 4 WAPE, 6, 1
 T 40831-046 93-WAPE-02-B
 Acquired on 8-Dec-1993 at 21:37

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.32	53235	0.00357
C1a	0.00	0	0
C2a, C2	17.88	23046	0.00274



40831

890080

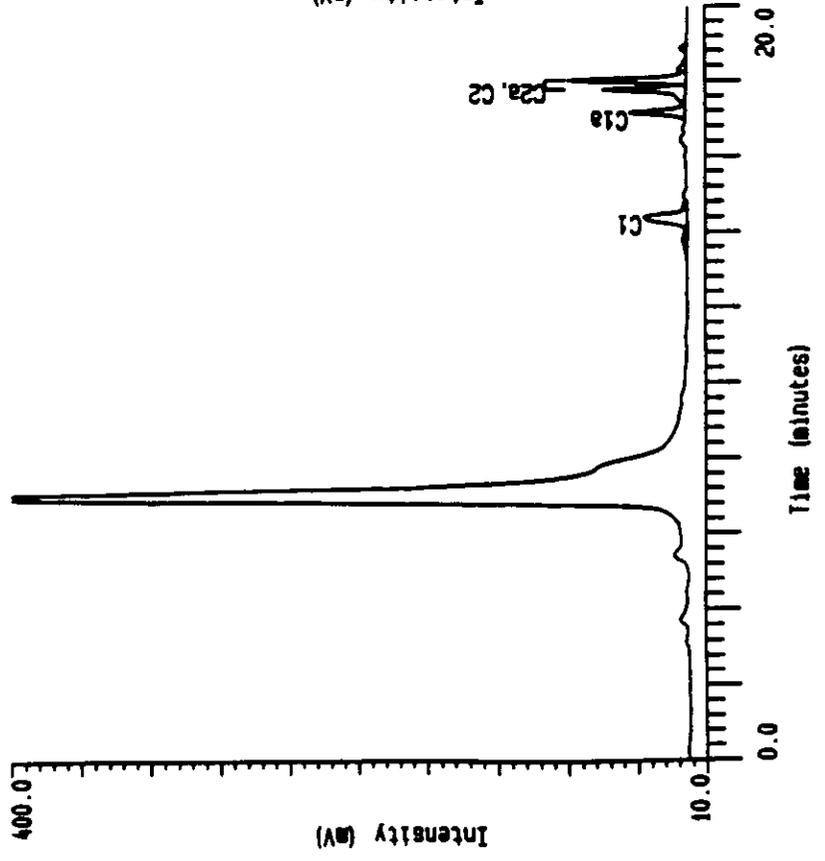
ABC Laboratories, Inc.

Date of Report: 10 DEC 93 at 11:30:44
 MCPP ver 2.3

MCD0 [40831] 4 WAPE, 7.1
 Std 0.6 ug/mL

Acquired on 8-Dec-1993 at 21:59

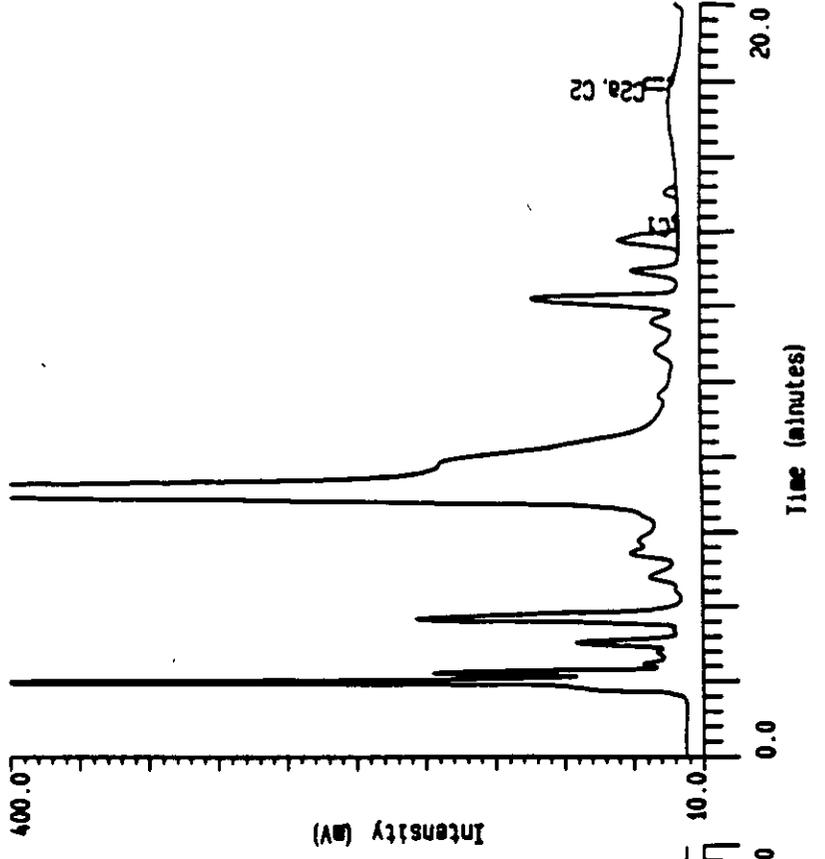
Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.37	421870	0.19473
C1a	17.15	279130	0.16647
C2a, C2	17.87	994321	0.22936



MCD0 [40831] 4 WAPE, 8.1
 T 40831-047 93-WAPE-03-A

Acquired on 8-Dec-1993 at 22:21

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.34	37292	0.00288
C1a	0.00	0	0
C2a, C2	17.91	23950	0.00276



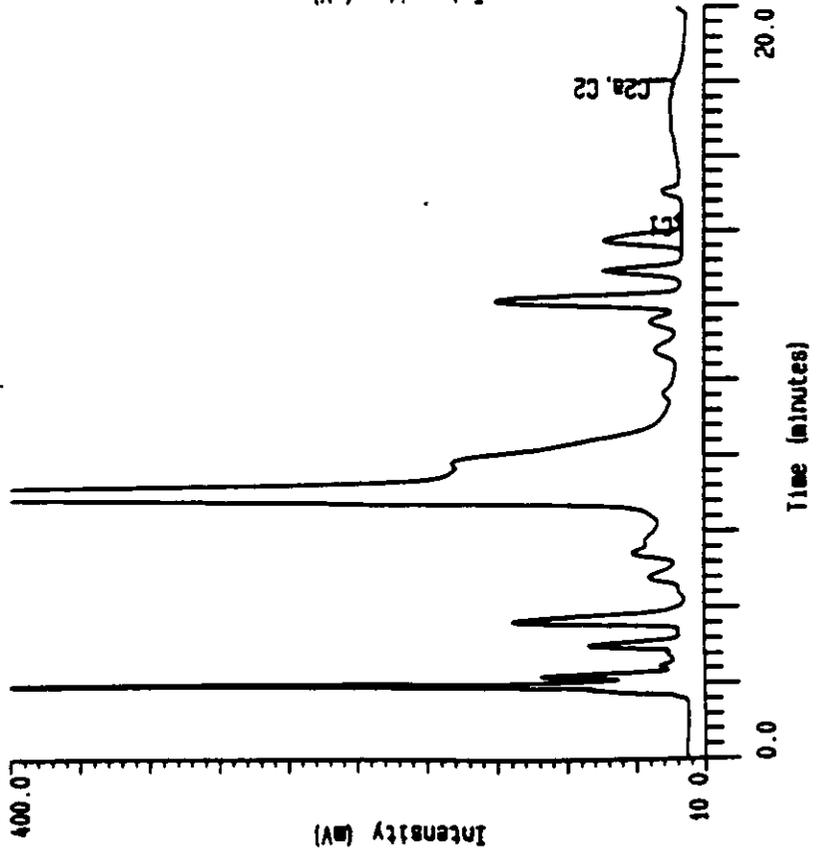
ABC Laboratories, Inc.

MCPD ver 2.3

Date of Report: 10 DEC 93 at 11:30:44

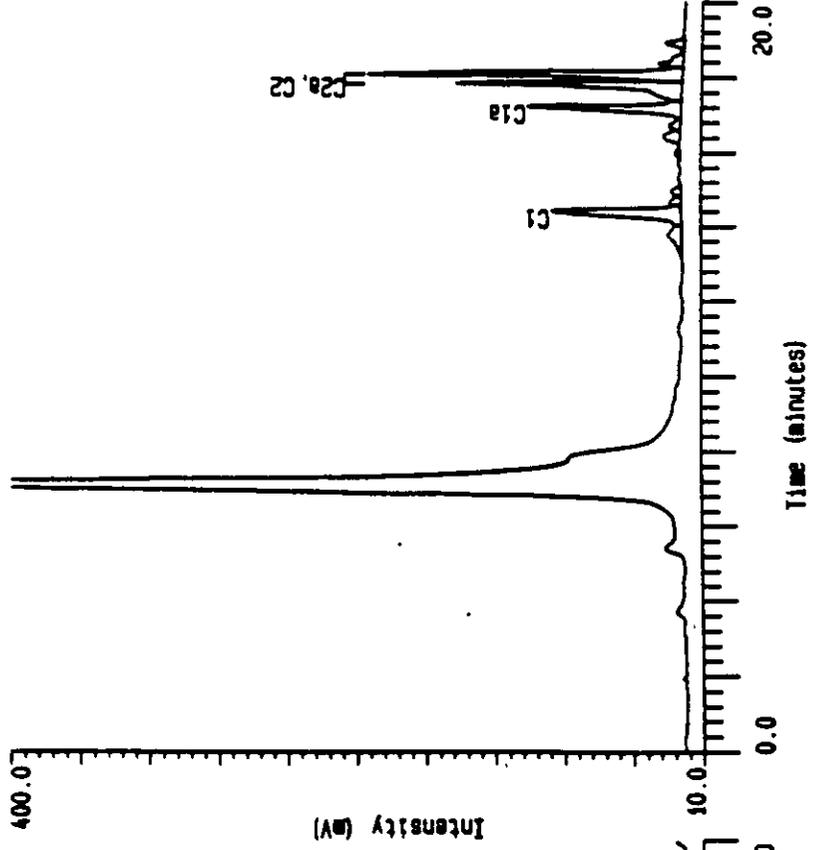
MCD0 (40831) 4 WAPE, 9.1
 T 40831-048 93-WAPE-03-B
 Acquired on 8-Dec-1993 at 22:43

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.31	45409	0.00323
C1a	0.00	0	0
C2a, C2	18.02	0	0.00226



MCD0 (40831) 4 WAPE, 10.1
 Std 1.5 ug/mL
 Acquired on 8-Dec-1993 at 23:06

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.36	1023146	0.45411
C1a	17.16	829022	0.46224
C2a, C2	17.88	2745878	0.59354



1804-03-40831

00100

ABC Laboratories, Inc.

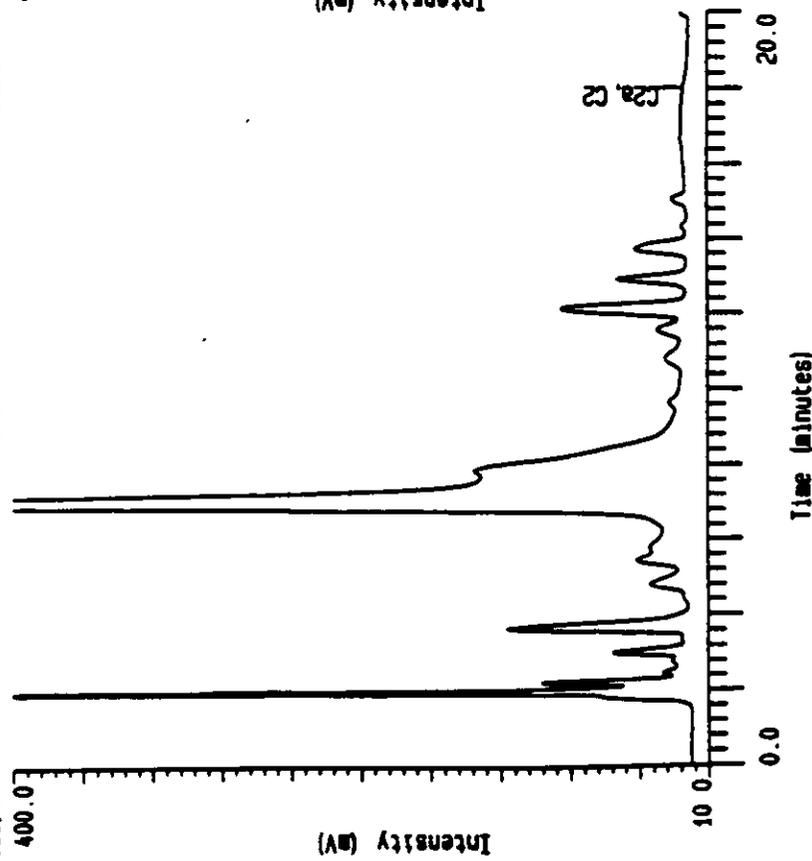
MCPD ver 2.3

Date of Report: 10 DEC 93 at 11:30:44

MC00 [40831] 4 WAPE, 11.1
T 40831-049 93-WAPE-04-A

Acquired on 8-Dec-1993 at 23:28

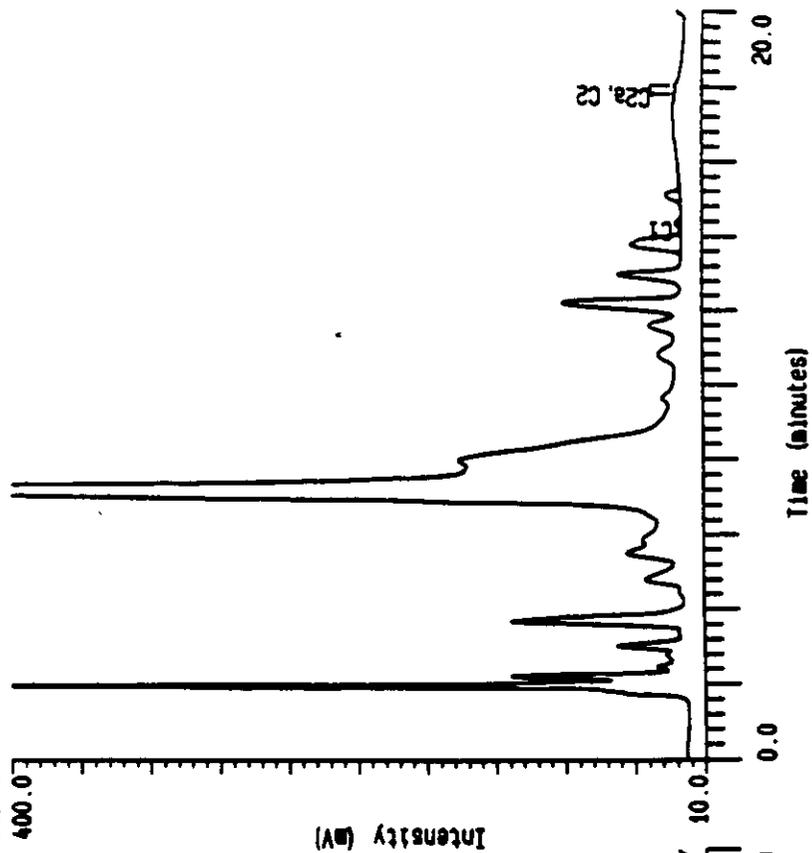
Peak Name	RT (mins)	Area	ug/mL or ppm
C1	0.00	0	0
C1a	0.00	0	0
C2a, C2	18.01	0	0.00226



MC00 [40831] 4 WAPE, 12.1
T 40831-050 93-WAPE-04-B

Acquired on 8-Dec-1993 at 23:50

Peak Name	RT (mins)	Area	ug/mL or ppm
C1	14.34	41142	0.00305
C1a	0.00	0	0
C2a, C2	17.90	0	0.00226



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101080

ABC Laboratories, Inc.

MCPD ver 2.3

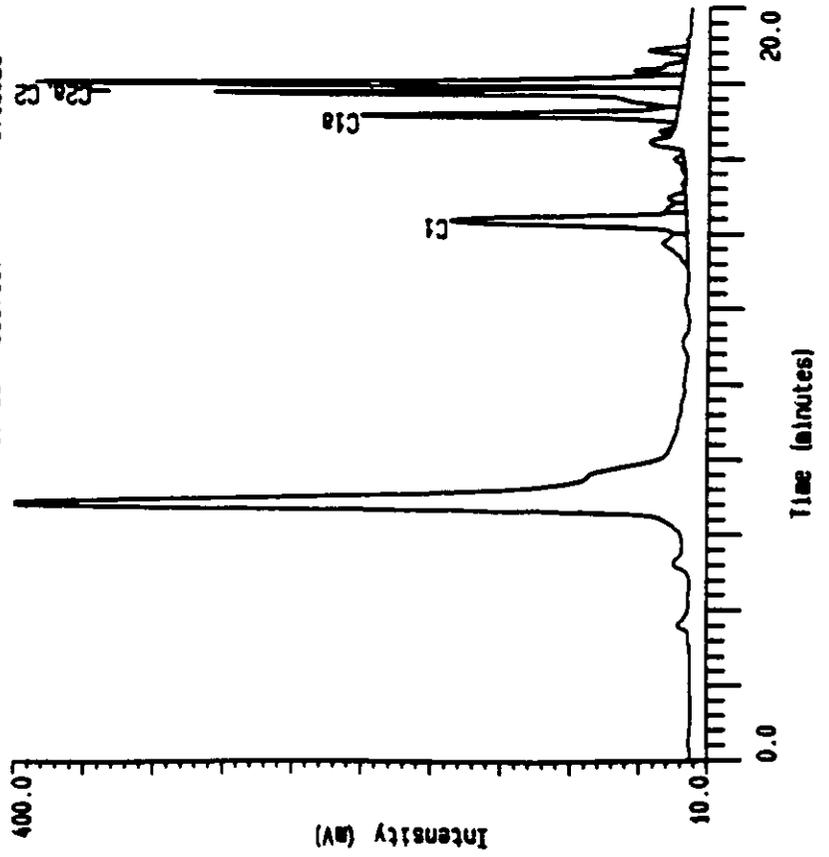
Date of Report: 10 DEC 93 at 11:30:44

MCD0 (40831) 4 MAPE, 13.1

Std 3.0 ug/mL

Acquired on 9-Dec-1993 at 00:12

Peak Name	RT (mins)	Area	ug/mL or ppb
C1	14.35	2130112	0.93154
C1a	17.16	1513323	0.88409
C2a, C2	17.88	5567937	1.18029



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20102

Appendix D
Example Chromatograms
Isocratic Elution

ABC Laboratories, Inc.

MCPD ver 2.3

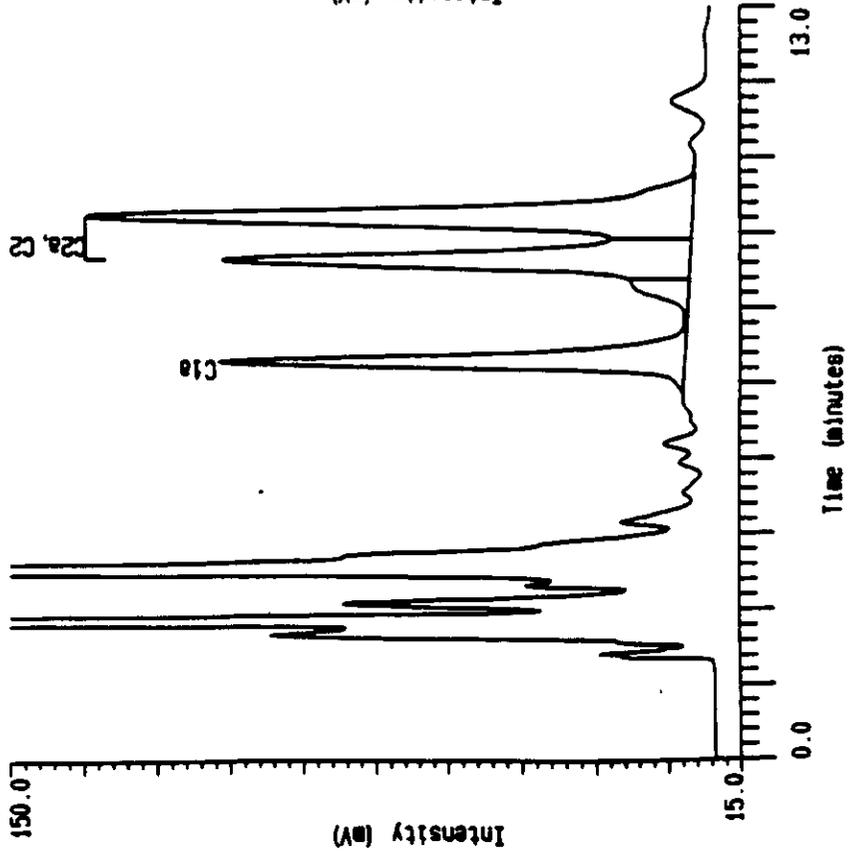
Date of Report: 21 DEC 93 at 14:53:32

MCD0 [40862] 11 PearMV2, 1.1

Std 3.0 ug/mL

Acquired on 21-Sep-1993 at 14:34

Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.88	1417825	0.90538
C2a, C2	8.99	4267455	1.21005

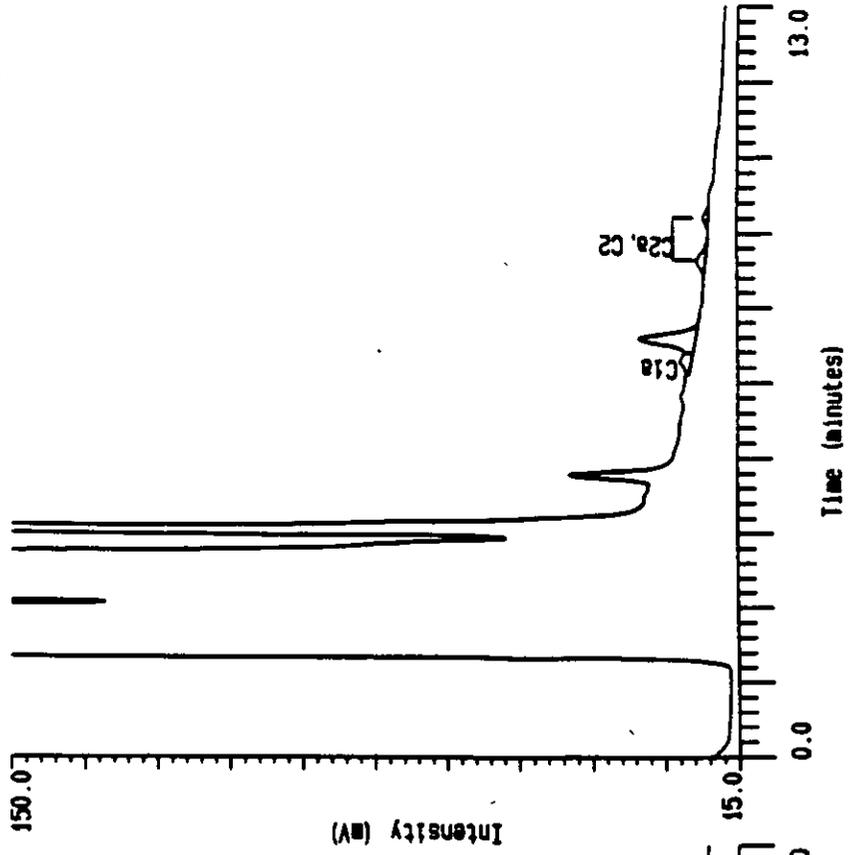


MCD0 [40862] 11 PearMV2, 2.1

C 40862-009 Control Pear

Acquired on 21-Sep-1993 at 14:48

Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.87	34327	0.00257
C2a, C2	8.99	46435	0.00270



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40104

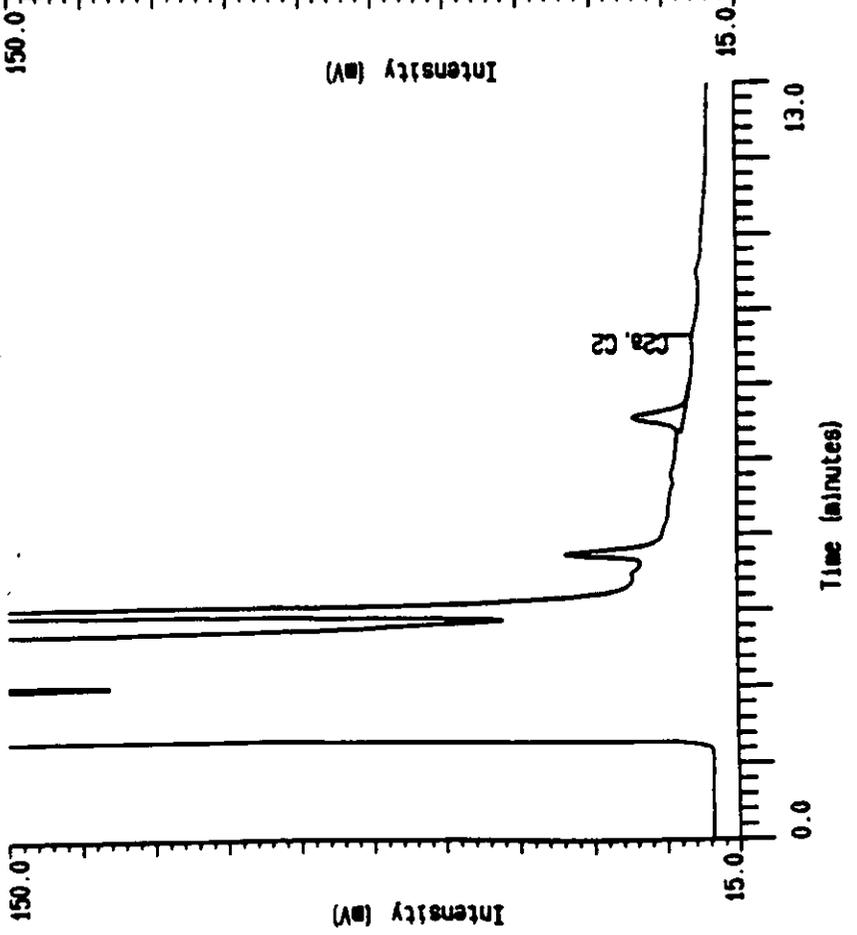
ABC Laboratories, Inc.

MCPD ver 2.3

Date of Report: 21 DEC 93 at 14:53:32

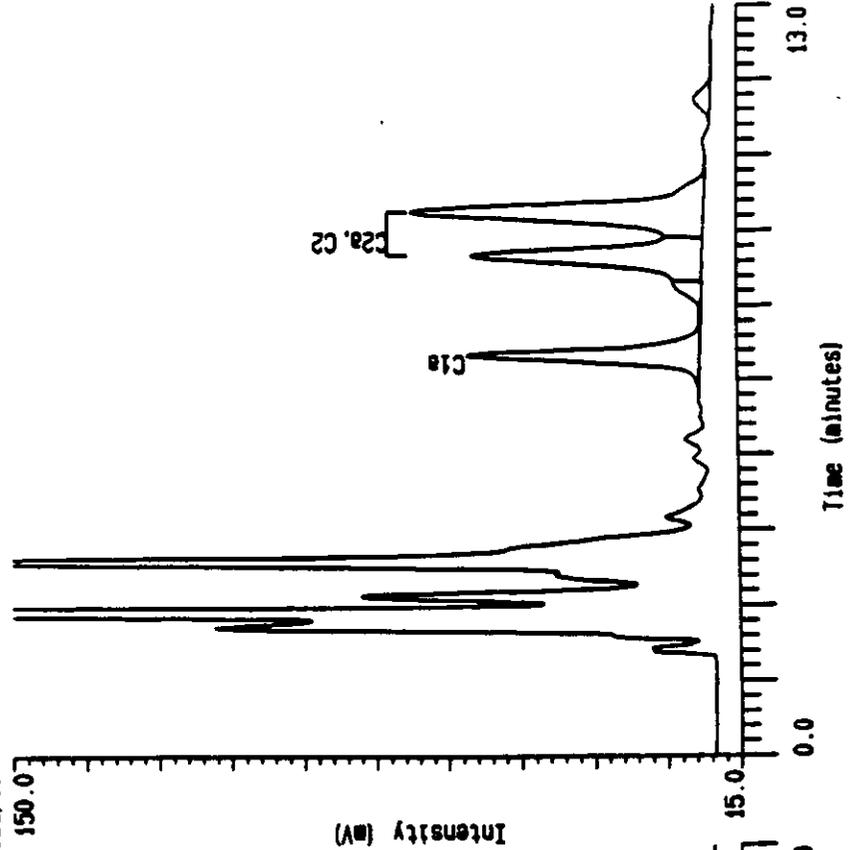
MCD0 (40862) 11 PearMV2, 3, 1
 C 40862-010 Control Pear
 Acquired on 21-Sep-1993 at 15:02

Peak Name	RT (mins)	Area	ug/mL or ppa
C1a	0.00	0	0
C2a, C2	8.64	0	0.00140



MCD0 (40862) 11 PearMV2, 4, 1
 Std 1.5 ug/mL
 Acquired on 21-Sep-1993 at 15:16

Peak Name	RT (mins)	Area	ug/mL or ppa
C1a	6.86	716007	0.45917
C2a, C2	8.98	2114807	0.60672



ABC Laboratories, Inc.

MCPD ver 2.3

Date of Report: 21 DEC 93 at 14:53:32

MCD0 [40862] 11 PearMV2, 5, 1
F 40862-011 C + 0.03 ppm

Acquired on 21-Sep-1993 at 15:29

Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.87	97974	0.00662
C2a, C2	8.97	265336	0.00940

150.0

Intensity (mV)

15.0

0.0

Time (minutes)

MCD0 [40862] 11 PearMV2, 6, 1
F 40862-012 C + 0.03 ppm

Acquired on 21-Sep-1993 at 15:43

Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.85	123068	0.00822
C2a, C2	8.96	330077	0.01065

150.0

Intensity (mV)

15.0

0.0

Time (minutes)

15801087 40831

90106

ABC Laboratories, Inc.

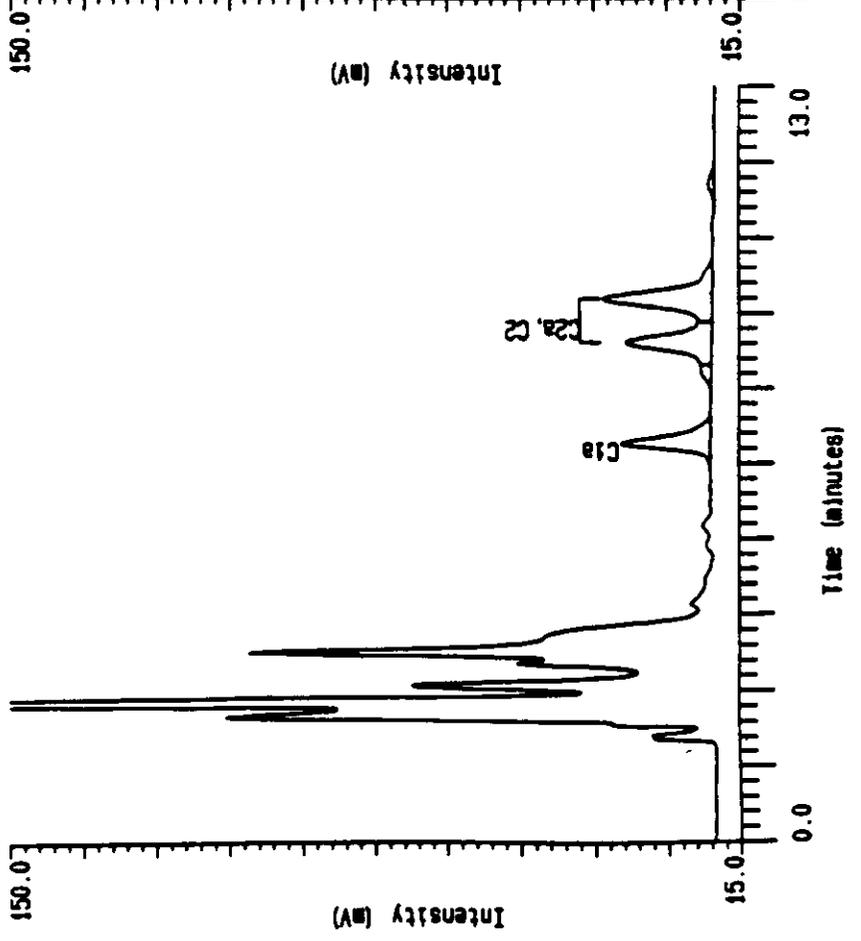
MCPD ver 2.3

Date of Report: 21 DEC 93 at 14:53:32

MCD0 (40862) 11 PearHV2, 7.1
Std 0.6 ug/mL

Acquired on 21-Sep-1993 at 15:57

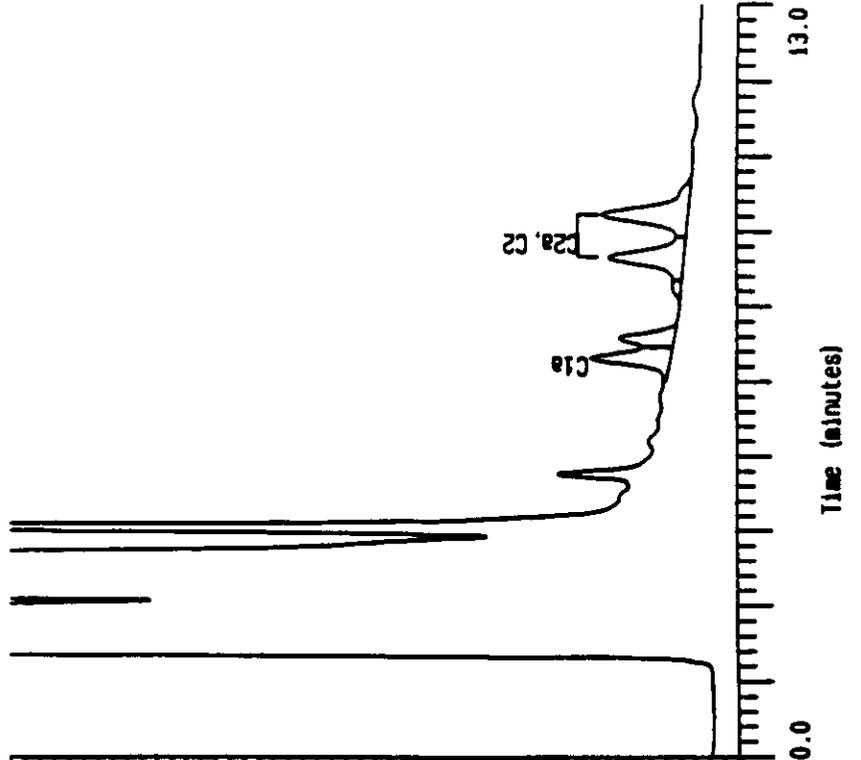
Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.86	271923	0.17681
C2a, C2	8.97	773877	0.23090



MCD0 (40862) 11 PearHV2, 8.1
F 40862-013 C + 0.06 ppm

Acquired on 21-Sep-1993 at 16:11

Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.88	235999	0.01540
C2a, C2	8.99	636444	0.01924



138048 040831

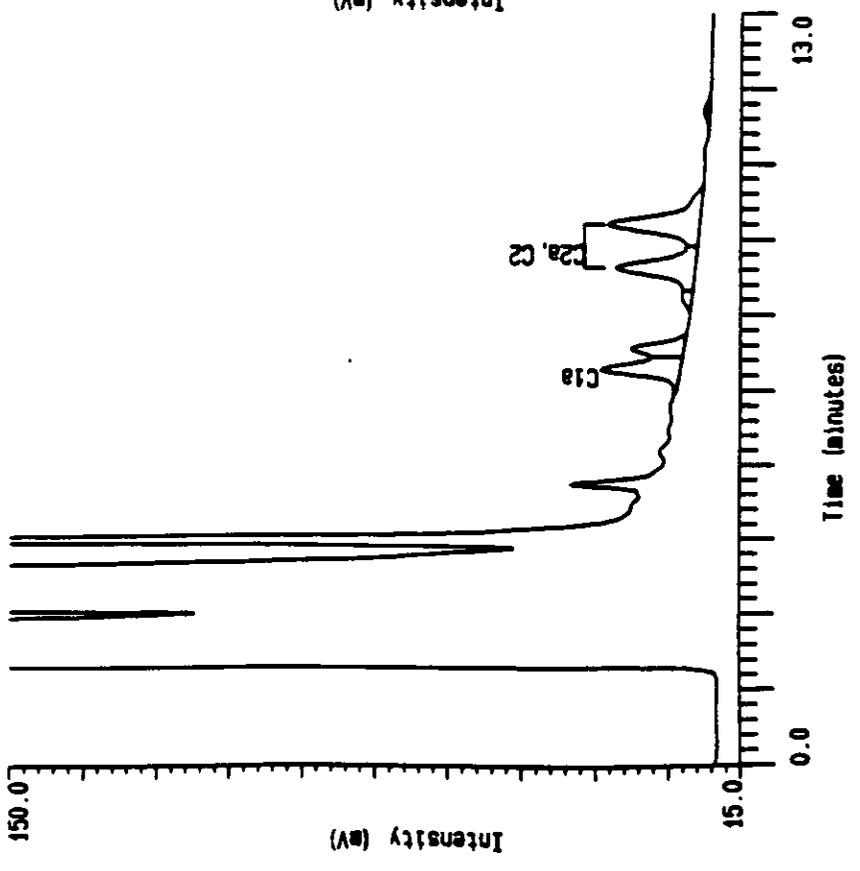
2010107

ABC Laboratories, Inc.

MCPD ver 2.3
 Date of Report: 21 DEC 93 at 14:53:32

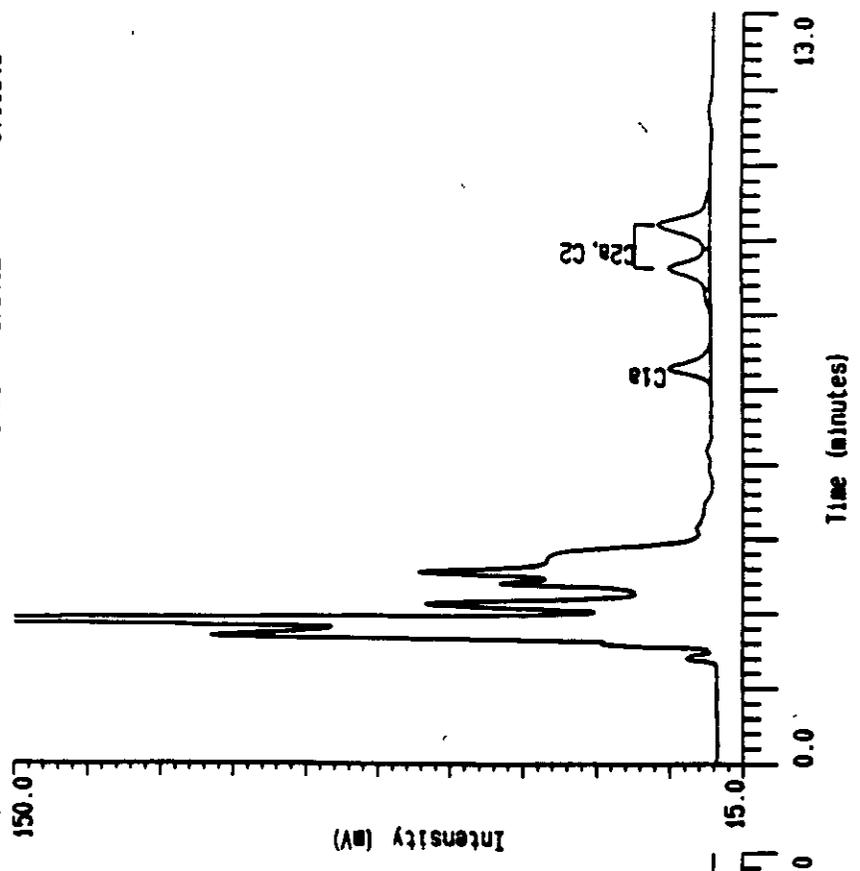
MCD0 [40862] 11 PearNV2, 9, 1
 F 40862-014 C + 0.06 ppm
 Acquired on 21-Sep-1993 at 16:25

Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.85	235274	0.01541
C2a, C2	8.96	673960	0.02029



MCD0 [40862] 11 PearNV2, 10, 1
 Std 0.3 ug/mL
 Acquired on 21-Sep-1993 at 16:39

Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.85	132799	0.08836
C2a, C2	8.96	376402	0.11949



15804 0181 708

80108

ABC Laboratories, Inc.

MCPD ver 2.3

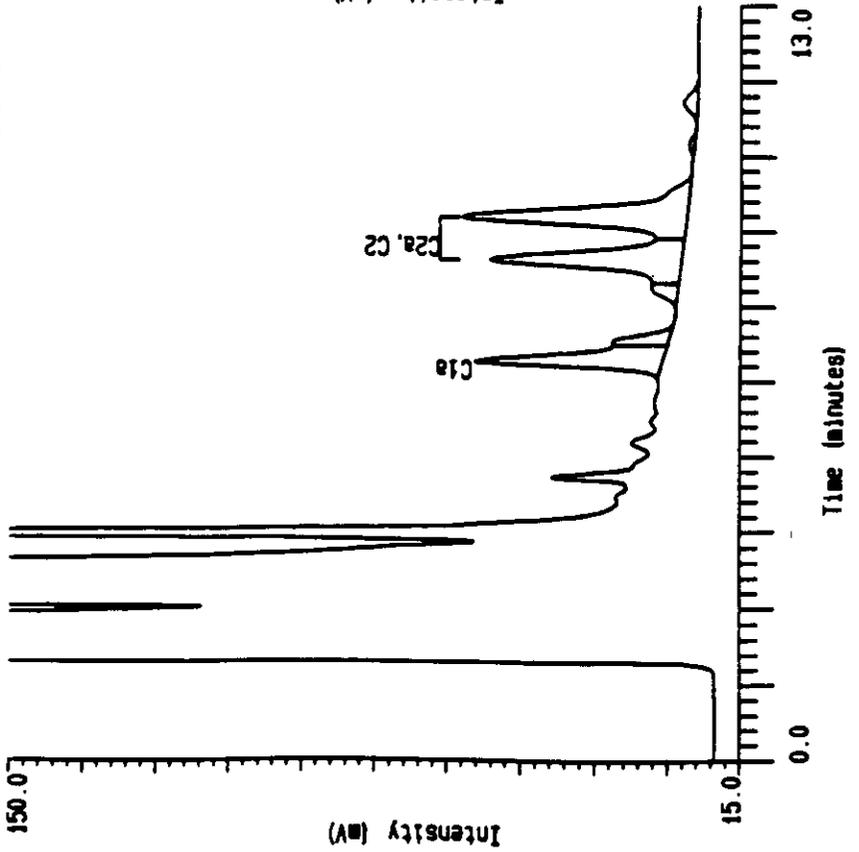
Date of Report: 21 DEC 93 at 14:53:32

MCD0 [40862] 11 PearHV2, 11.1

F 40862-015 C + 0.15 ppm

Acquired on 21-Sep-1993 at 16:53

Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.87	555723	0.03572
C2a, C2	8.98	1646286	0.04754

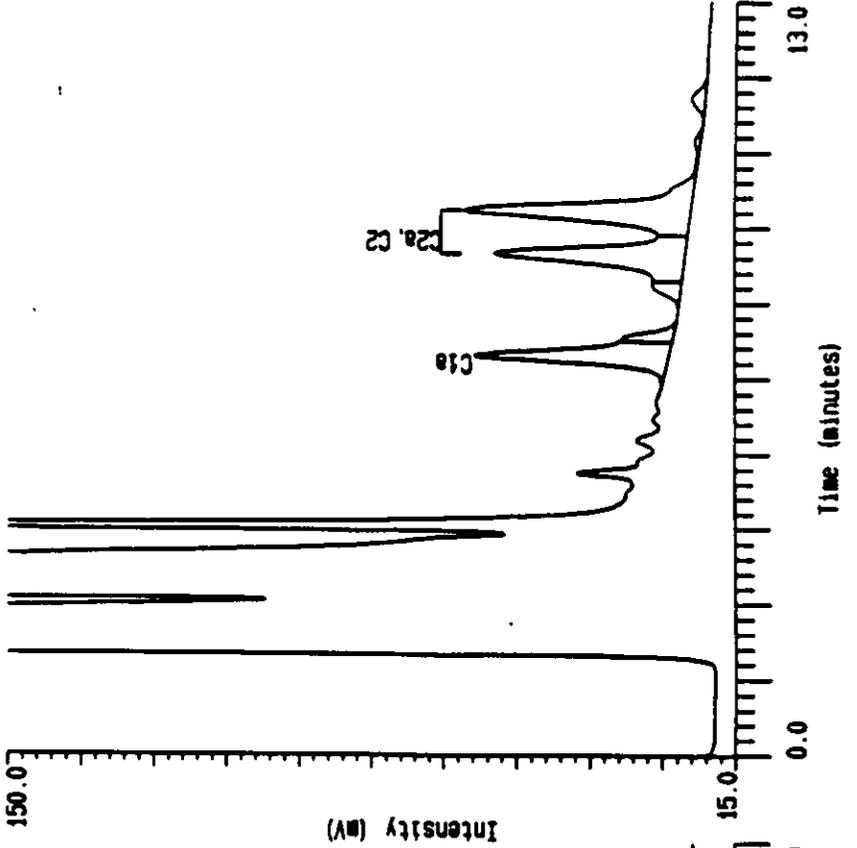


MCD0 [40862] 11 PearHV2, 12.1

F 40862-016 C + 0.15 ppm

Acquired on 21-Sep-1993 at 17:06

Peak Name	RT (mins)	Area	ug/mL or ppm
C1a	6.86	600891	0.03860
C2a, C2	8.96	1694505	0.04889



ABC Laboratories, Inc.

MCPD ver 2.3

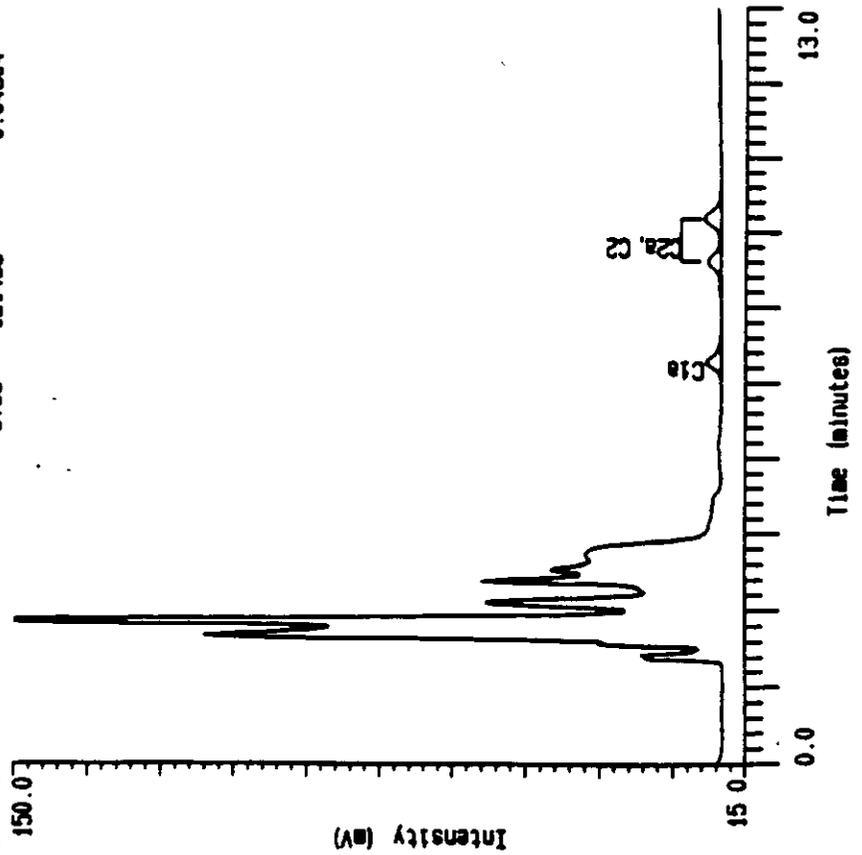
Date of Report: 21 DEC 93 at 14:53:32

MCD0 (40862) 11 PearNV2.13.1

Std 0.1 ug/mL

Acquired on 21-Sep-1993 at 17:20

Peak Name	RT (mins)	Area	ug/mL or ppb
C1a	5.86	46010	0.03318
C2a, C2	8.96	121465	0.04804



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