Waters
Lab Highlights

An Internal Communication
of Applications and Techniques

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AN/LS/ED/DR/OT

1985 PITTSBURGH CONFERENCE NEW ORLEANS, LA

NO. 786 USE OF ABSORBANCE RATIOS IN LIQUID CHROMATOGRAPHY

Attached is documentation of a presentation given at the 1985 PITTSBURGH CONFERENCE held in New Orleans from February 25 to March 1, 1985. The published abstract is included below.

J. B. LI, R. L. COTTER, E. J. HILLIER and M. W. ANDREWS
Waters Chromatography Division, Millipore, Milford, MA 01757

The isolation of a substance from a complex biological mixture is a tedious procedure. To minimize this, simultaneous use of on-line monitoring of absorbance and ratios of absorbance at two different wavelengths increases the amount of information obtained during a single chromatographic run. This isolation of Cephalosporin C (Ceph C) from fermentation broth is an example.

The liquid chromatography instrumentation used is available from Waters (Milford, MA). It consisted of a U6K Sample Injector, modified with two 2-ml sample loops in series; M6000A Solvent Delivery System; a 490 Programmable Multiwavelength Detector; a Model 401 Refractometer; and the 840 Data and Chromatography Control System or a 730 Data Module. The columns used were the Waters Radial-PAK™ μBONDAPAK™ C_{18} cartridge (8 mm x 10 cm) in a Z-Module™ radial compression system and a semi-preparative μBONDAPAK™ C_{18} column (19 mm x 15 cm). The buffer was 20 mM ammonium acetate, pH 6.2, with 1% acetonitrile added. Flow rates were 2 and 4 ml/min with the Radial-PAK cartridge and 9.9 ml/min with the semi-preparative column.

Standard Ceph C eluted from the Radial-PAK cartridge in 16 min at 2 ml/min. A stop-flow spectral scan using the 490 Detector revealed absorbance maxima at 205 and 260 nm. Absorbance ratio of 280/260 nm was 0.35 across the entire absorbance peak, indicating homogeneity of the Ceph C. When 10 μl of fermentation broth were injected onto the column, without preliminary sample clean-up, Ceph C was chromatographically well separated from the other components. The absorbance ratio was useful in identifying the Ceph C peak and assessing its relative purity.

A direct scale-up procedure from the analytical column to a semi-preparative column containing the same resin was done. Because of the large quantity of material, UV absorbance was no longer useful and refractive index of the effluent was monitored instead. Rechromatography of fractions from the Ceph C peak on the analytical column indicated the fractions contained one component with the characteristic retention time and absorbance ratio of 0.35 at 280/260 nm for Ceph C.

Another feature of the Waters 490 Detector is Windowplot. When the absorbance ratio of a compound is known, a ratio window can be set, e.g. 0.33-0.37 for Ceph C. A Windowplot is observed only when compounds which have that absorbance ratio pass through the detector. Windowplot can be used to trigger external events like a fraction collector to permit collection of only the compound in the peak of interest.

Thus, in this application the Waters 490 Detector with its four channels of output proved to be useful for simultaneous monitoring of absorbance, absorbance ratios and Windowplot. This can provide quantitative, as well as qualitative information about the compounds of interest.

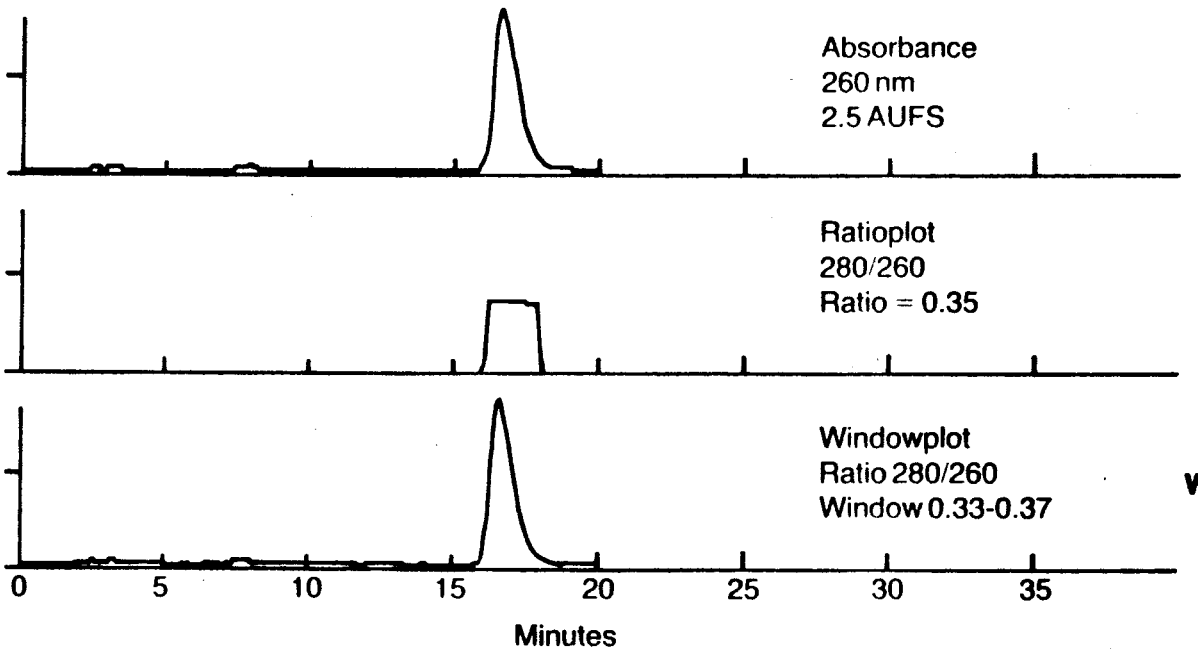
Any questions regarding the paper should be directed to the author listed at the bottom of this page. Slides are available through Marketing Communications in Milford (X2303) or directly from the photographer, Kevin Monaghan (617/528-8579).

Waters
Division of MILLIPORE 34 Maple St. / Milford, MA 01757 / 617-478-2000

Jeanne Li

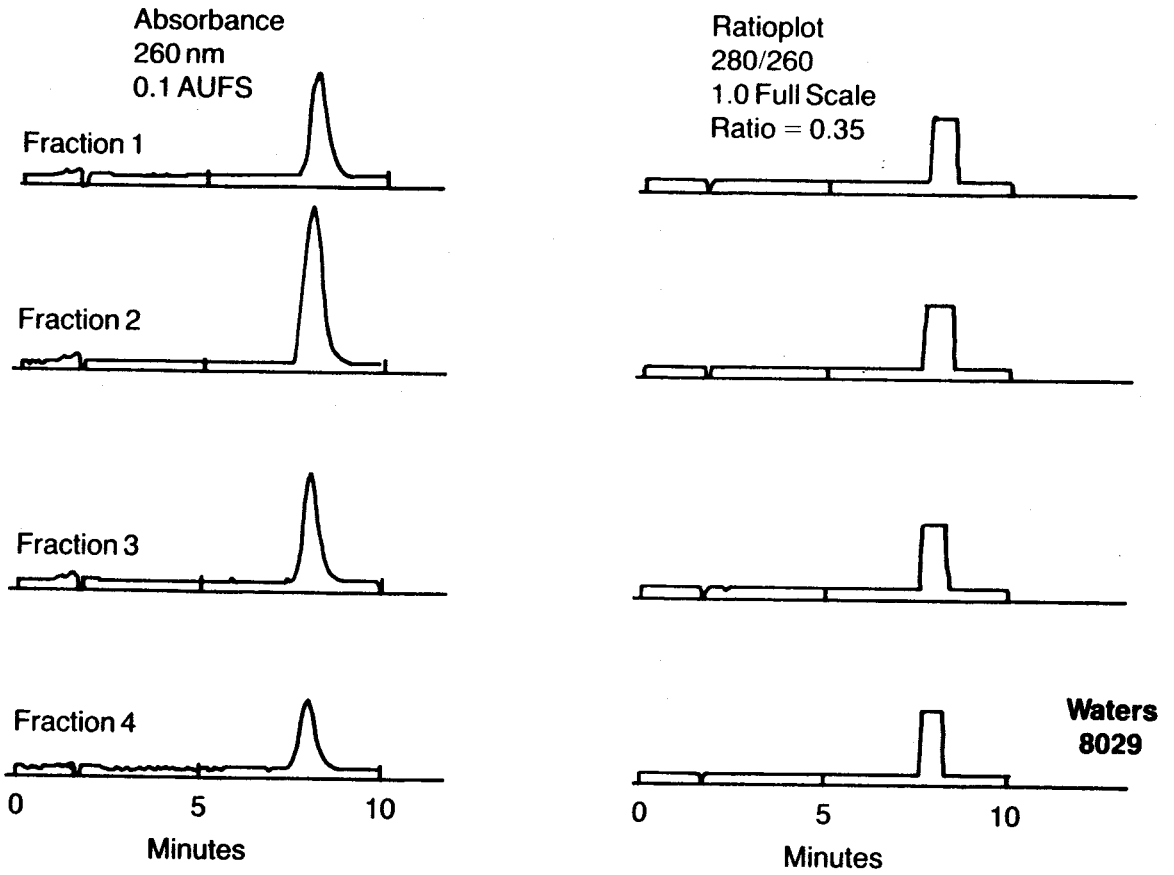
Windowplot
Cephalosporin C Standard

COLUMN: Radial-PAK C_{18}, 8 mm × 10 cm
SOLVENT: 20 mM Ammonium Acetate +
1% Acetonitrile (2 ml/min)
DETECTOR: 490



Rechromatographed Fractions
Purified Cephalosporin C

COLUMN: Radial-PAK C_{18}, 8 mm × 10 cm
SOLVENT: 20 mM Ammonium Acetate +
1% Acetonitrile (4 ml/min)
DETECTOR: 490



By Jeanne Li, Ernie Hillier, and Richard Cotter

Computerized HPLC detection in pharmaceutical research

IN THE pharmaceutical industry, researchers producing active compounds by chemical synthesis or biological processes require a fast, easy way to confirm the identity of the compound of interest in a crude mixture and isolate that compound with maximum purity. This is especially important to researchers developing procedures to scale-up to production levels.

The application of high performance liquid chromatography (HPLC) to the separation and purification of many compounds from difficult matrices has rapidly expanded in recent years. With the recent growth of computerization in HPLC instrumentation, researchers have many new tools with advanced capabilities to facilitate compound isolation and synthesis.

In particular, computerized HPLC detection capabilities have greatly enhanced compound identification and purification. These capabilities are available in the Waters model 490 programmable multiwavelength detector (*Figure 1*). Using the instrument's four output channels and microprocessor control, researchers can employ many different preprogammed routines using user-selectable wavelength and ratio values in a single analysis to help identify compounds and verify purity.

Dr. Li and Mr. Hillier are Applications Chemists, and Mr. Cotter is Manager, Product Evaluation Laboratory, Waters Chromatography Division, Millipore Corp.

Some of the routines of particular importance to pharmaceutical research will be demonstrated in the following isolation example.

Isolating and identifying biologically active compounds in pure form from fermentation broths is typically very difficult because of the many impurities and contaminants that are present. The initial chromatographic step to identify the compound of interest is to find the ultraviolet (UV) absorbance maximum of that compound. Typically, researchers will use a benchtop spectrophotometer to run a spectral UV scan to find the wavelength of maximum absorbance for that compound and then use HPLC to perform the separation and purification. With programmable multiwavelength detection, finding the UV maximum absorbance can be readily accomplished by continually monitoring up to 12 wavelengths while analyzing a pure standard of the compound. This affords the added advantage of using a single instrument to find the maximum absorbance as well as perform the separation.

To verify purity of compounds separated from the crude mixtures, researchers typically go back to a spectrophotometric scan, or use TLC, bio-assay or other off-line techniques. With programmable multiwavelength detection, elution characteristics and peak purity can be inferred in a single analysis by using routines such as RATIOPLOT and WINDOWPLOT, which allow actual visualization of peak purity or contamination (see *Figure 2*).

Figure 1 *Model 490 programmable multiwavelength detector.*

RATIOPLOT divides absorbances at two selected wavelengths and plots the resulting ratiogram on any of four channels to help detect hidden components that might be present under individual peaks. As shown in Figure 2, the flat portion at the top of the RATIOPLOT of the protein standard indicates a relatively pure standard. The RATIOPLOT reveals the contaminant in the spike sample by indicating a large disturbance in the plot. To further verify purity, one channel can be used to plot a ratio while the other three channels can monitor additional wavelengths.

WINDOWPLOT will only plot the absorbance at a selected wavelength if the absorbance ratio of a peak is found to fall within a particular ratio window. A normal symmetrical peak will be plotted

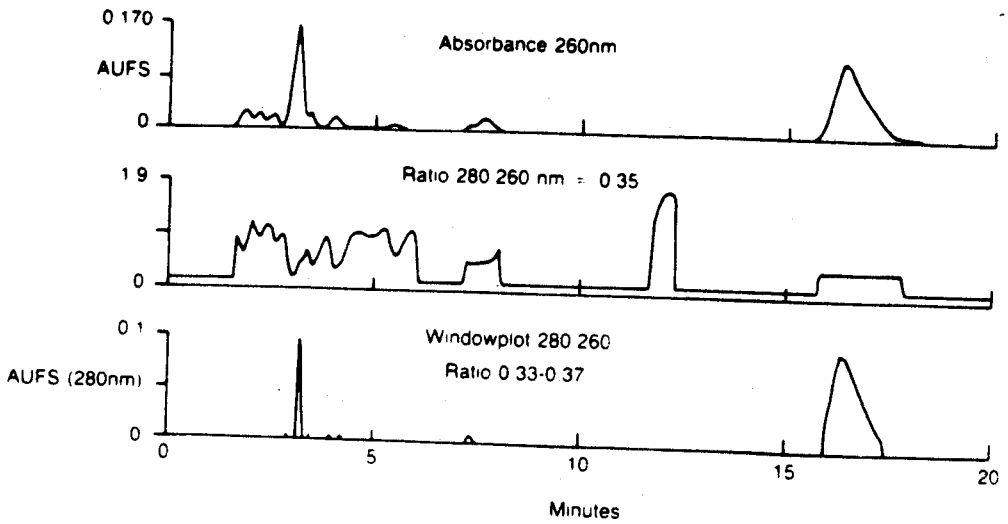


Figure 5 *Analysis of Cephalosporin C in crude fermentation broth*

SAMPLE 10 µl Fermentation Broth after Millex-SR Filtration and C18 SEP-PAK
CARTRIDGE Radial-PAK µBONDAPAK C18. 8 mm x 10 cm
ELUENT 20 mM NH_4OAc. pH 6.2. in 1% CH_3CN H_2O
FLOW RATE 2.0 ml min
DETECTOR Model 490 Programmable Multiwavelength Detector

was then used for comparison of the crude sample.

A 10-µL sample of the crude fermentation broth containing about 30 µg of Cephalosporin C was injected into the chromatograph and showed good separation of the antibiotic from the extraneous components (*Figure 5*). RATIOPLOT on channel 2 indicated the material in the Cephalosporin C peak was fairly homogeneous. The WINDOWPLOT routine on channel 3 suggested that the peak contains contaminants as indicated by the cut-off portion of the peak. Here the value

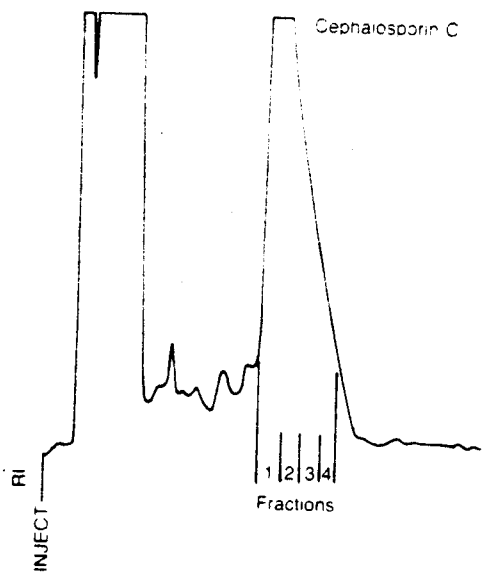


Figure 6 *Preparative isolation of Cephalosporin C.*

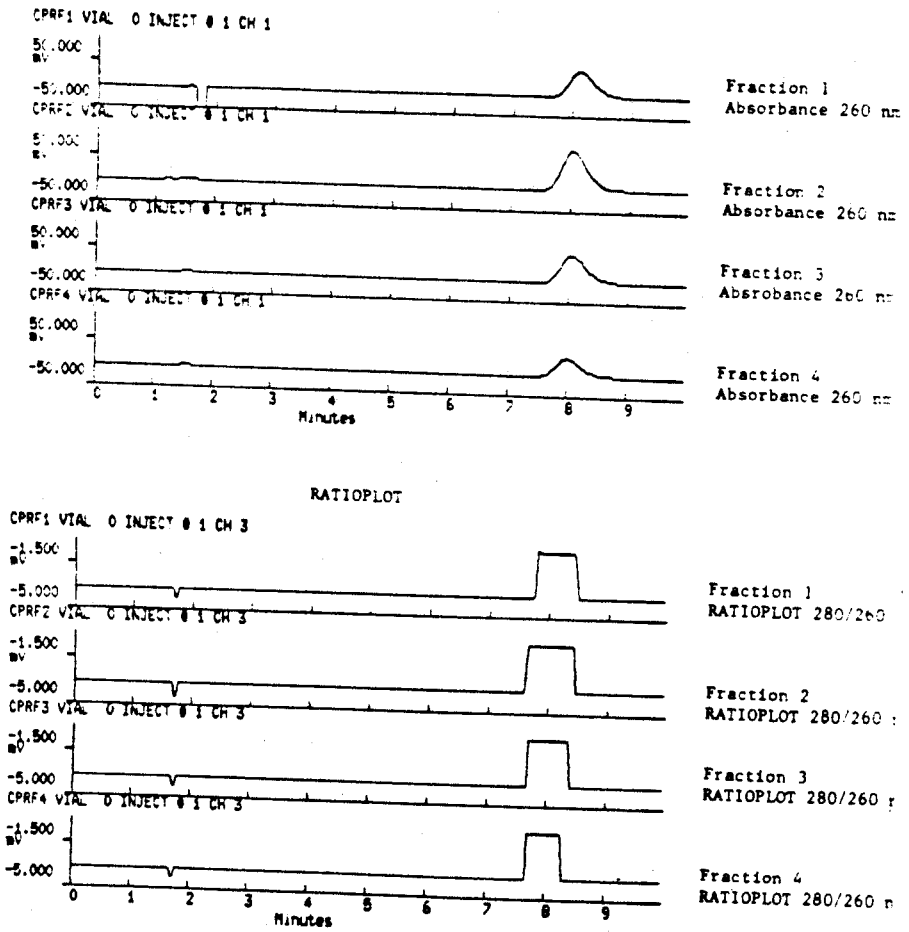


Figure 7 *Analysis of Cephalosporin C fractions.*

of the new detection capabilities become apparent. In just a single analysis, the separation was recorded, the Cephalosporin C peak was tentatively identified, and possible contaminants, shown by the tailing portion of the peak, were discovered quickly and simply. This information also illustrated where fractions could be collected for preparative chromatography purifications.

To isolate the Cephalosporin C on a preparative scale, a 4-mL sample of the crude broth was injected onto a 19-mm C_{18} column and detected by a refractive index detector. Even with the larger amount of crude material, Cephalosporin C was well separated on the larger column (*Figure 6*).

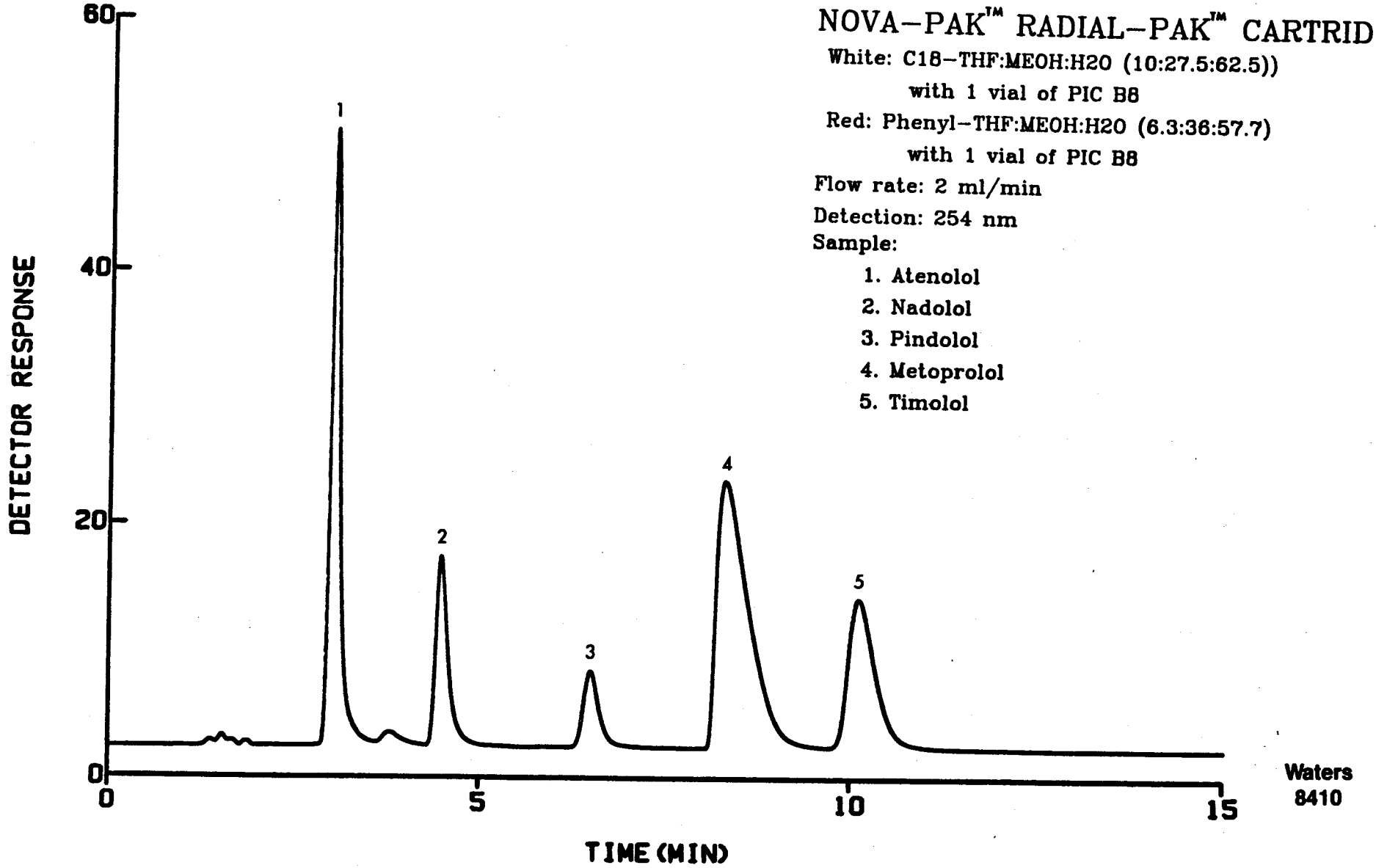
To confirm the purity of the Cephalosporin C separated on the preparative scale, four collected fractions of the Cephalosporin C peak were analyzed using an absorbance plot at 260 nm and RATIOPLOT at 280/260 nm, the same as in the initial analysis. Results indicated single peaks with retention times and ratios the same as the standard, confirming purity of Cephalosporin C (*Figure 7*) and demonstrating that pure fractions were collected at the proper time.

Conclusion

Programmable multiwavelength HPLC detection provides a fast, easy way to help confirm the identity of compounds of interest in complex mixtures as well as aid in the verification of purity. As demonstrated in the isolation and purification of Cephalosporin C, use of preprogrammed detection routines plus multichannel capabilities greatly simplifies the researcher's chemical synthesis and biological processes by providing more information about a compound in a single analysis.

NOVA-PAK C18 VERSUS NOVA-PAK PHENYL

BETA-ADRENERGIC BLOCKERS



SUMMARY

NOVA–PAK™ FAMILY

BROAD RANGE OF POLARITY

BROAD SELECTIVITY

HIGH EFFICIENCY

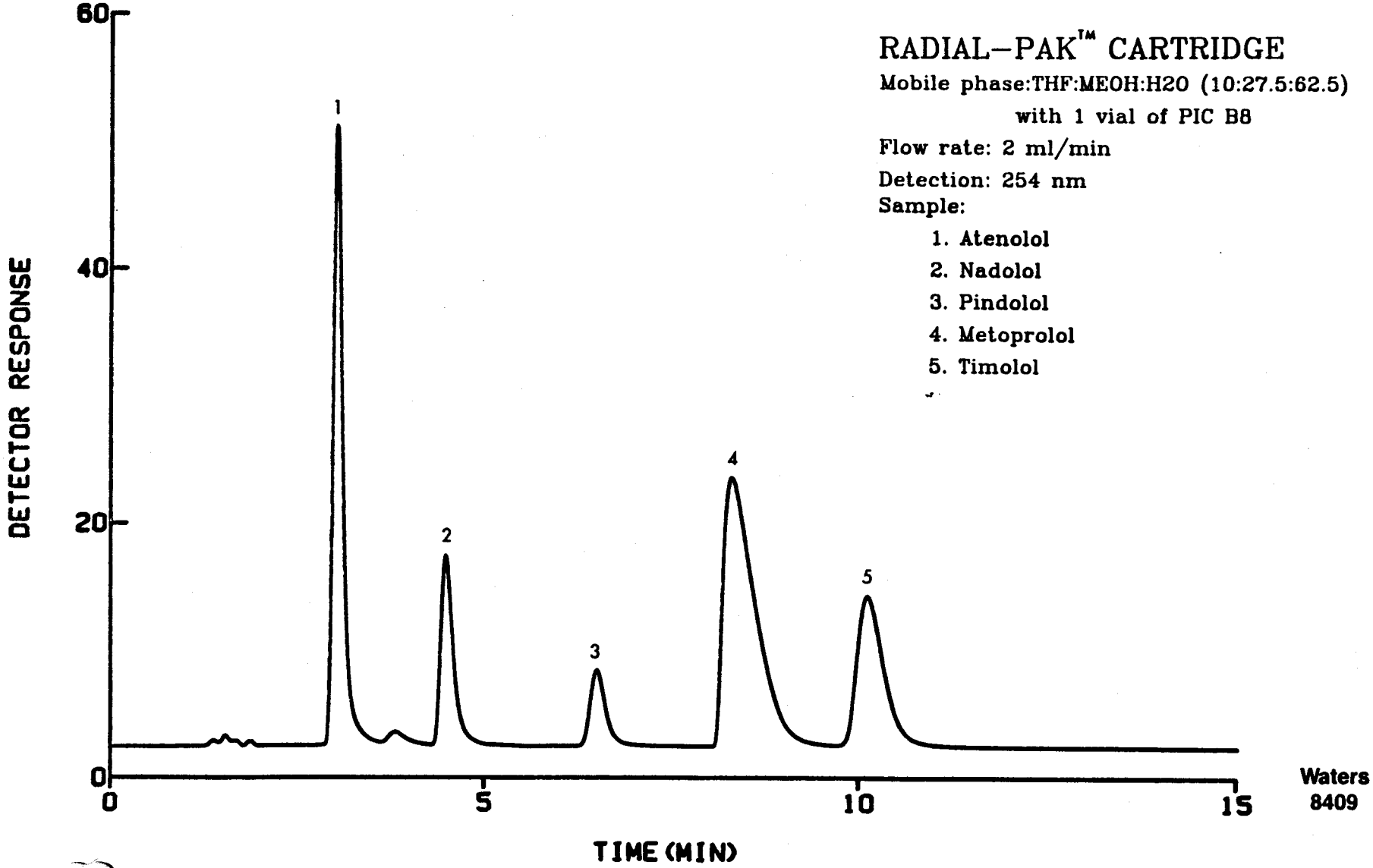
REPRODUCIBILITY

COST EFFECTIVE

Waters
8412

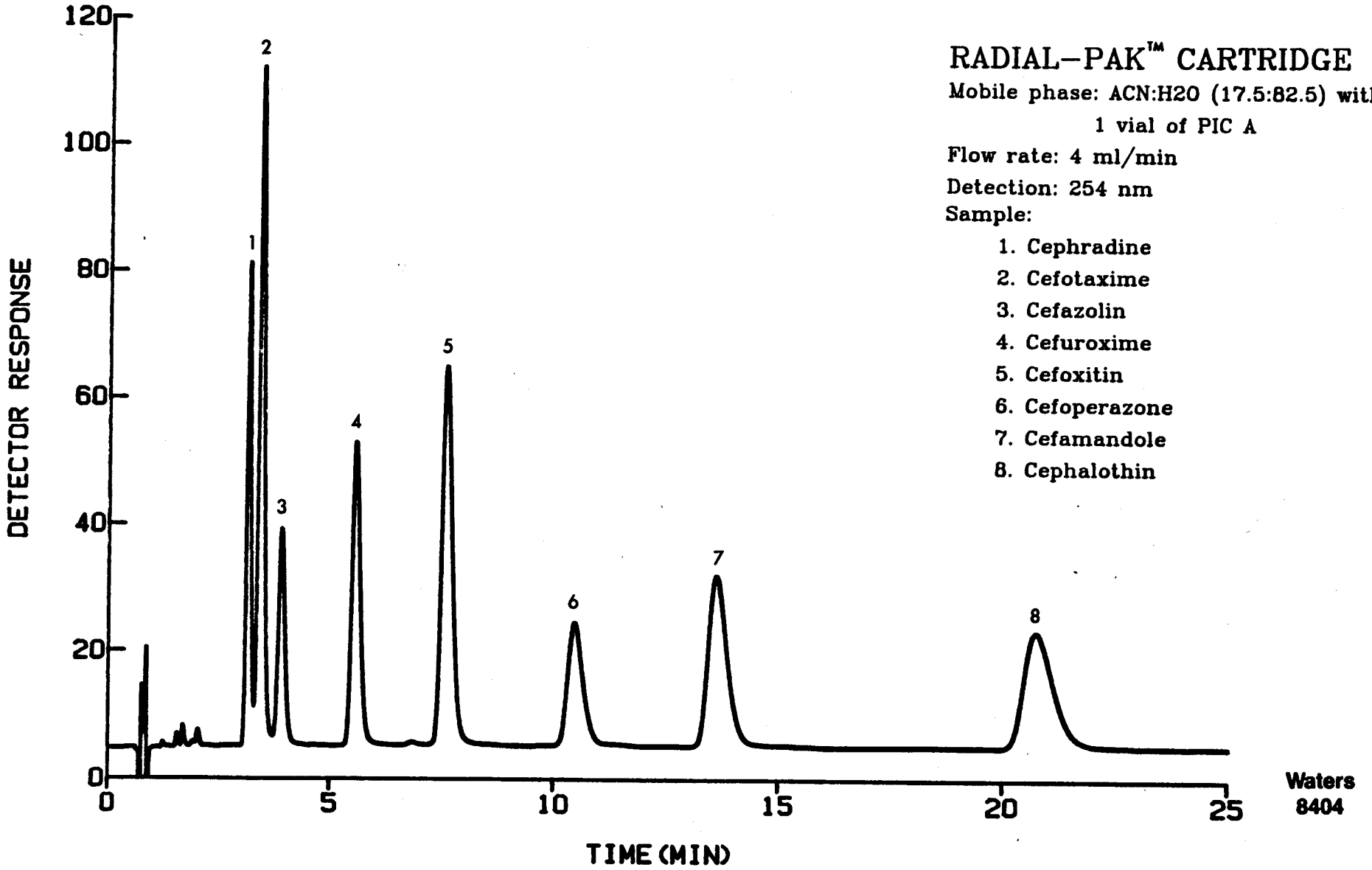
NOVA–PAK™ C18

BETA-ADRENERGIC BLOCKERS



NOVA–PAK™ C18

CEPHALOSPORIN ANTIBIOTICS



RADIAL–PAK™ CARTRIDGE

Mobile phase: ACN:H2O (17.5:82.5) with 1 vial of PIC A

Flow rate: 4 ml/min

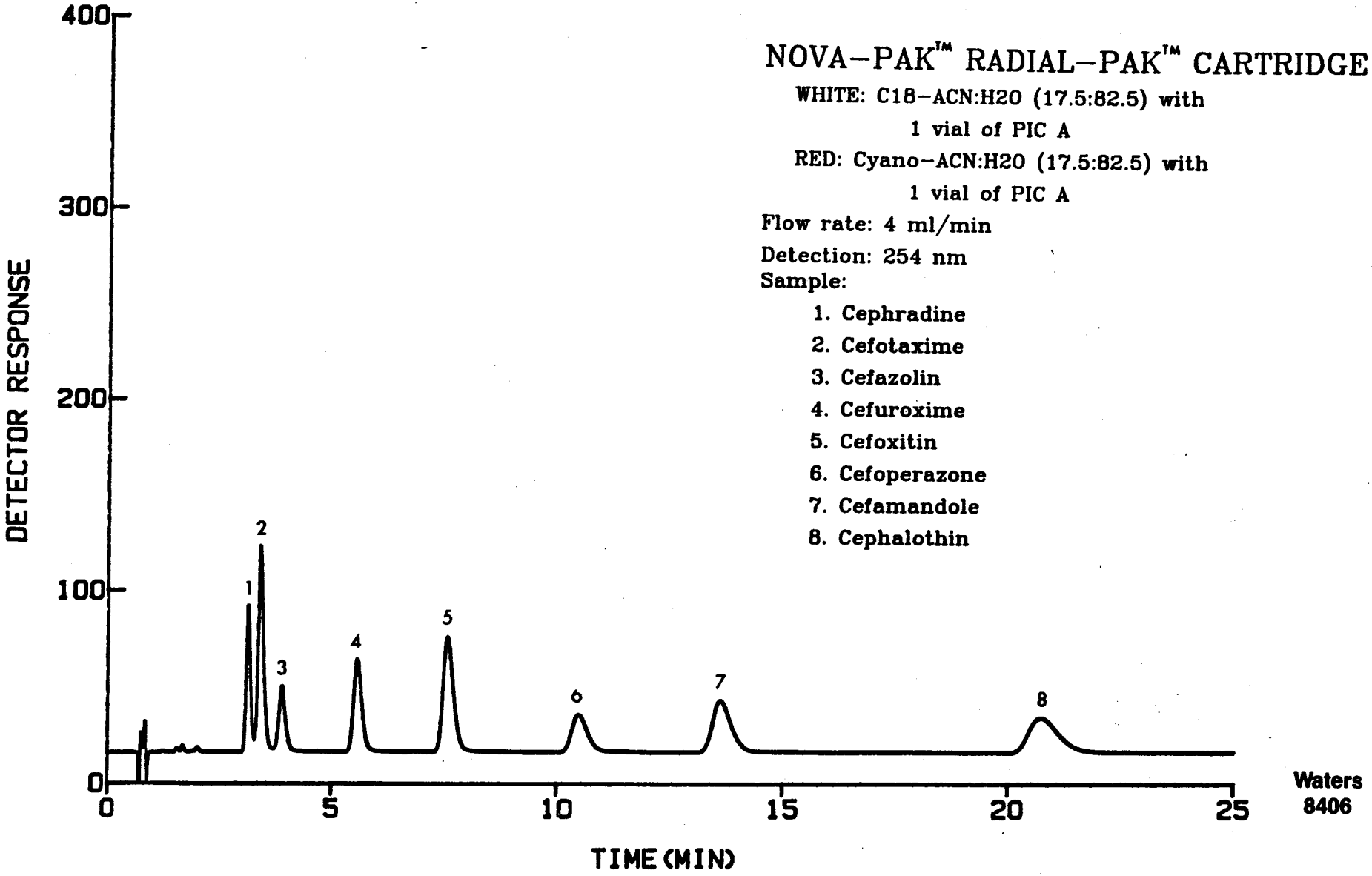
Detection: 254 nm

Sample:

1. Cephradine
2. Cefotaxime
3. Cefazolin
4. Cefuroxime
5. Cefoxitin
6. Cefoperazone
7. Cefamandole
8. Cephalothin

NOVA–PAK™

CEPHALOSPORIN ANTIBIOTICS



NOVA–PAK™ RADIAL–PAK™ CARTRIDGE

WHITE: C18–ACN:H20 (17.5:82.5) with
1 vial of PIC A

RED: Cyano–ACN:H20 (17.5:82.5) with
1 vial of PIC A

Flow rate: 4 ml/min

Detection: 254 nm

Sample:

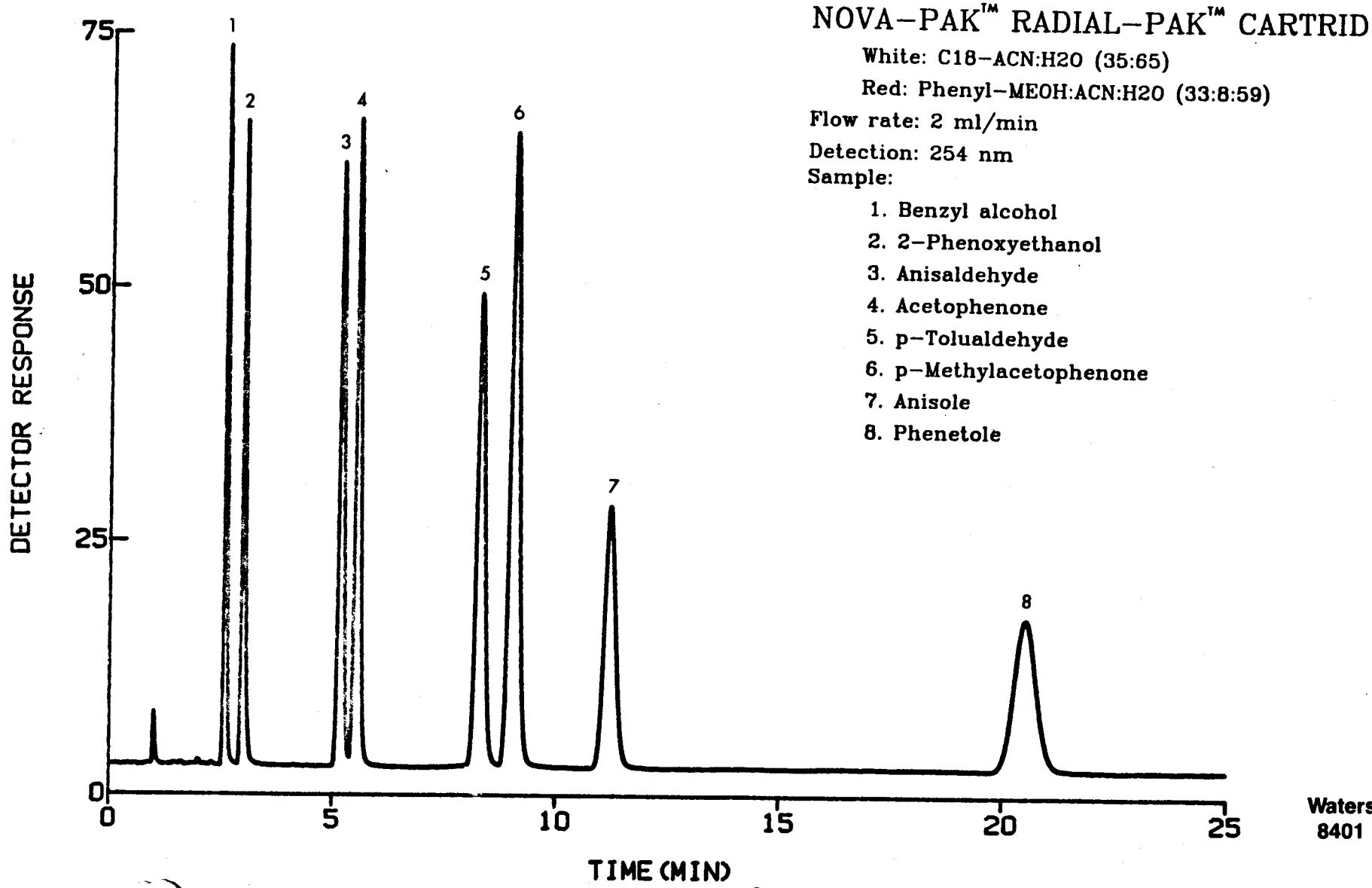
1. Cephradine
2. Cefotaxime
3. Cefazolin
4. Cefuroxime
5. Cefoxitin
6. Cefoperazone
7. Cefamandole
8. Cephalothin

ACIDIC COMPOUNDS

Waters
8403

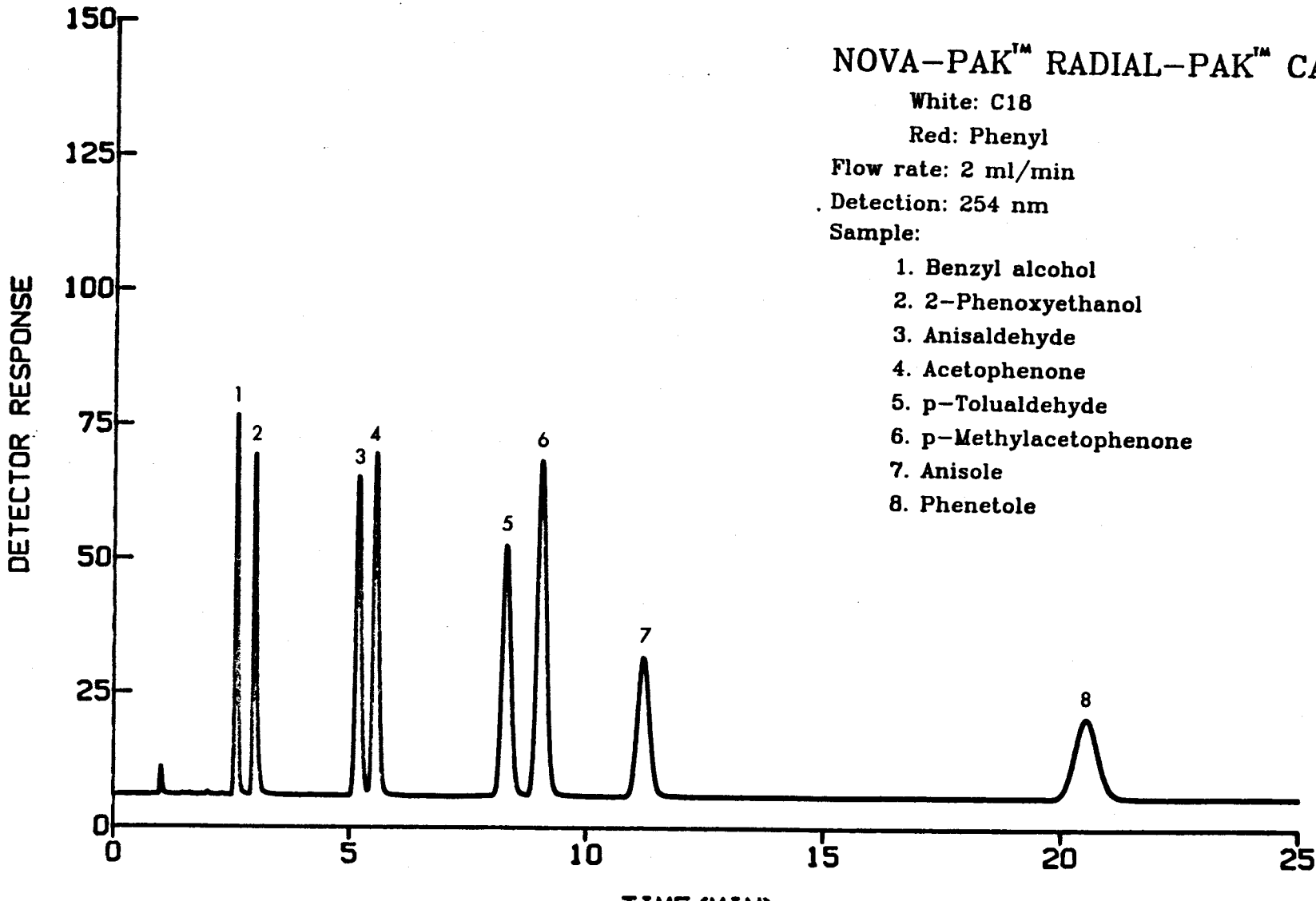
NOVA-PAK C18 VERSUS NOVA-PAK PHENYL

OPTIMIZED NOVA-PAK PHENYL MOBILE PHASE



NOVA-PAK™ C18 VERSUS NOVA-PAK™ PHENYL

ACN: H20 (35: 65)



NOVA-PAK™ RADIAL-PAK™ CARTRIDGE

White: C18

Red: Phenyl

Flow rate: 2 ml/min

Detection: 254 nm

Sample:

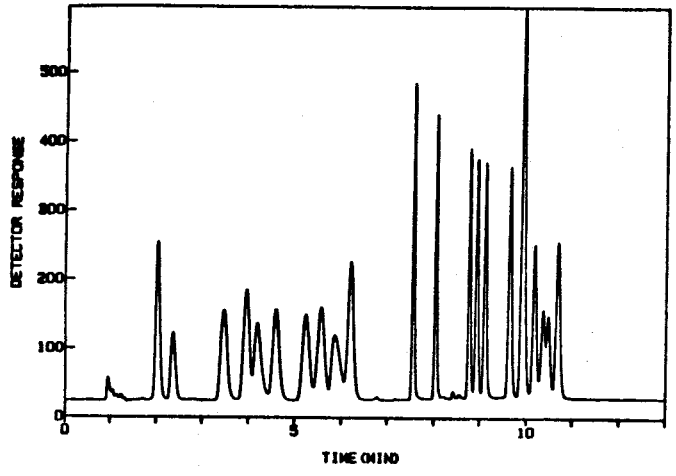
1. Benzyl alcohol
2. 2-Phenoxyethanol
3. Anisaldehyde
4. Acetophenone
5. p-Tolualdehyde
6. p-Methylacetophenone
7. Anisole
8. Phenetole

NEUTRAL COMPOUNDS

Waters
8397

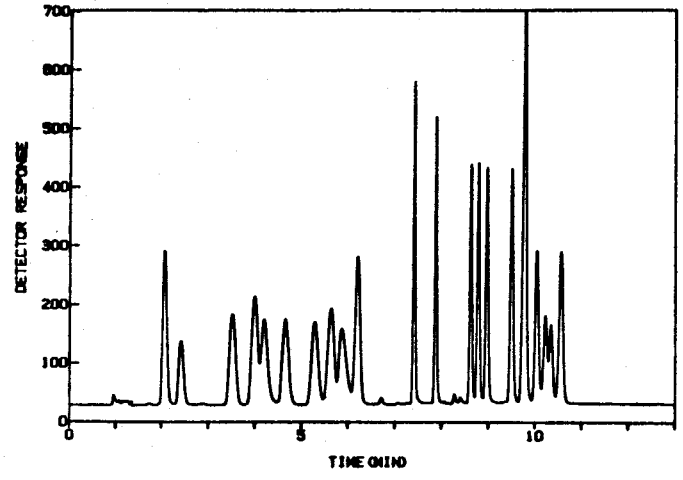
PTH AMINO ACIDS ON NOVA-PAK C18

BATCH # 1017



PTH AMINO ACIDS ON NOVA-PAK C18

BATCH # 1022

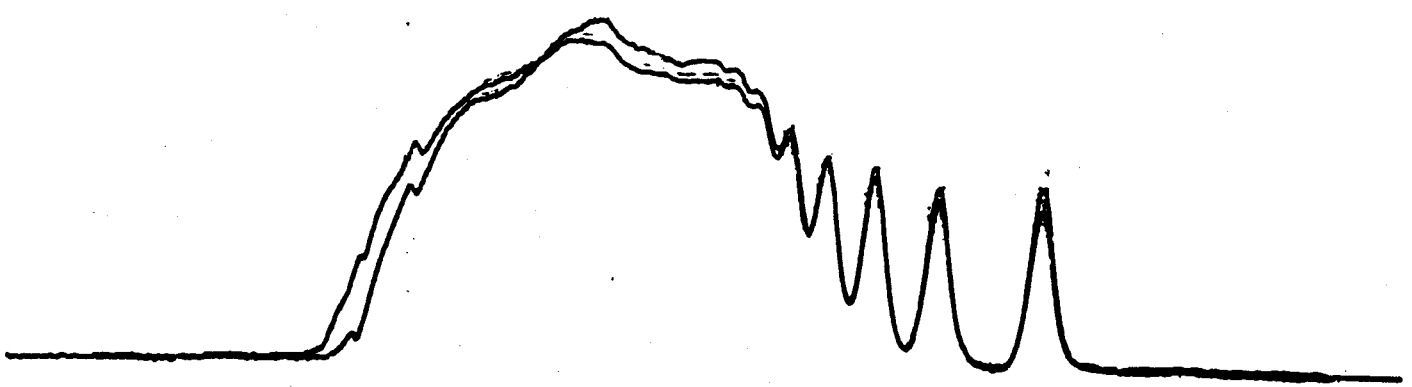


Waters
8395

Fro-Dex® 15 vs. Corn Syrup Solids 16 DE

Experimental 100-500-10^3 Å GPC Column St

SAMPLE CONCENTRATION: 2%



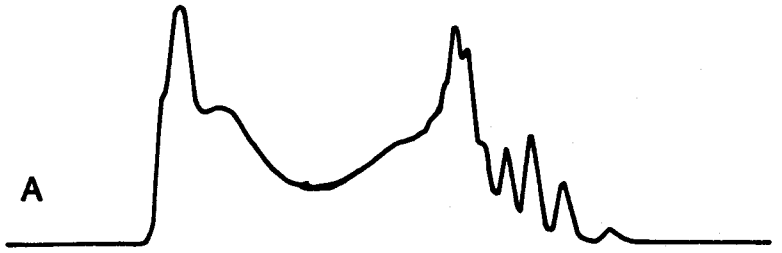
Waters
8074

Four Starch Hydrolyzates

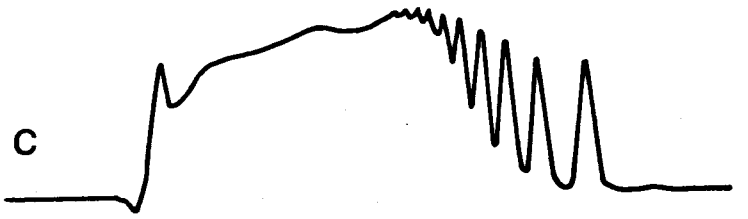
500-500-10^3 Å Column Set

SAMPLE CONCENTRATION: 2%
INJECTION VOLUME: A,B: 45µl
C,D: 60 µl
COLUMNS: Experimental GPC Columns

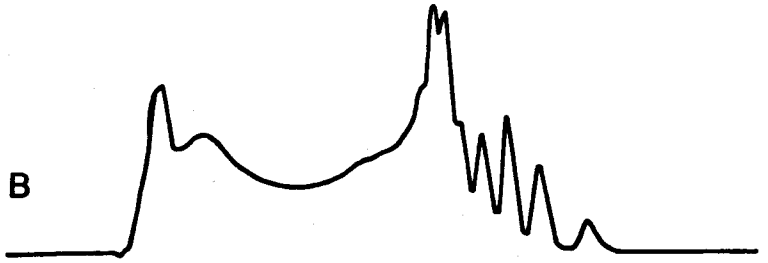
FLOW RATE: 0.5 ml/min
MOBILE PHASE: Water with 50 mg/L $CaNa_2$ EDTA
TEMPERATURE: 80°C
DETECTOR: RI 8X



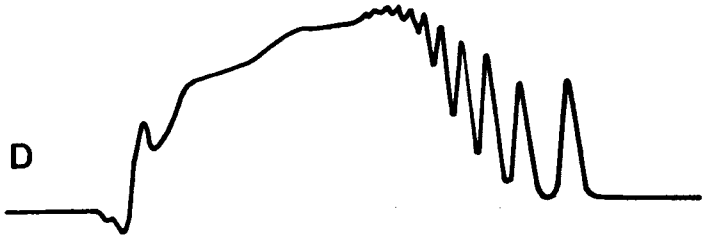
Maltodextrine 12 DE



Fro-Dex® 15



Maltrin® 15

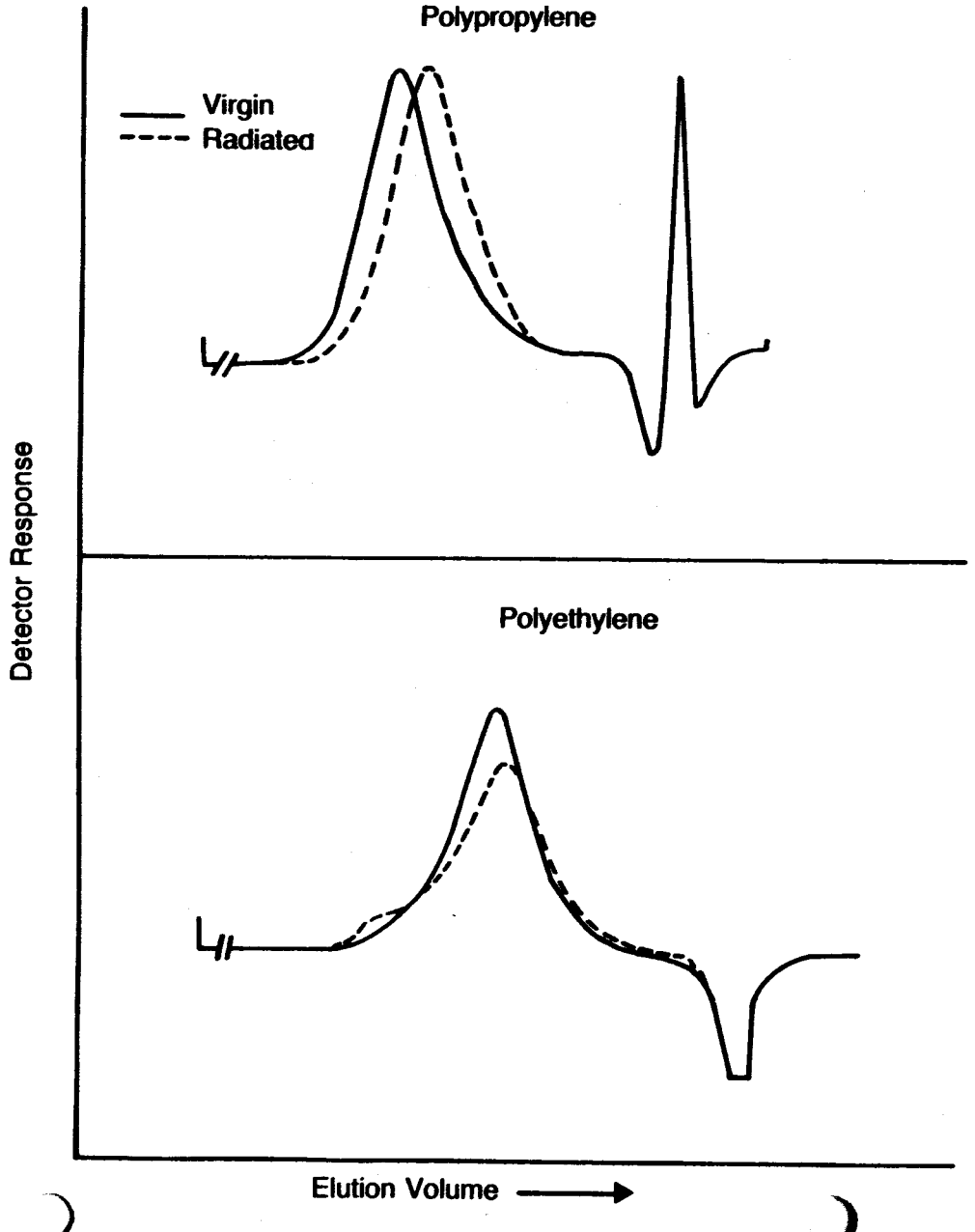


Corn Syrup Solids 16 DE

Waters
8081

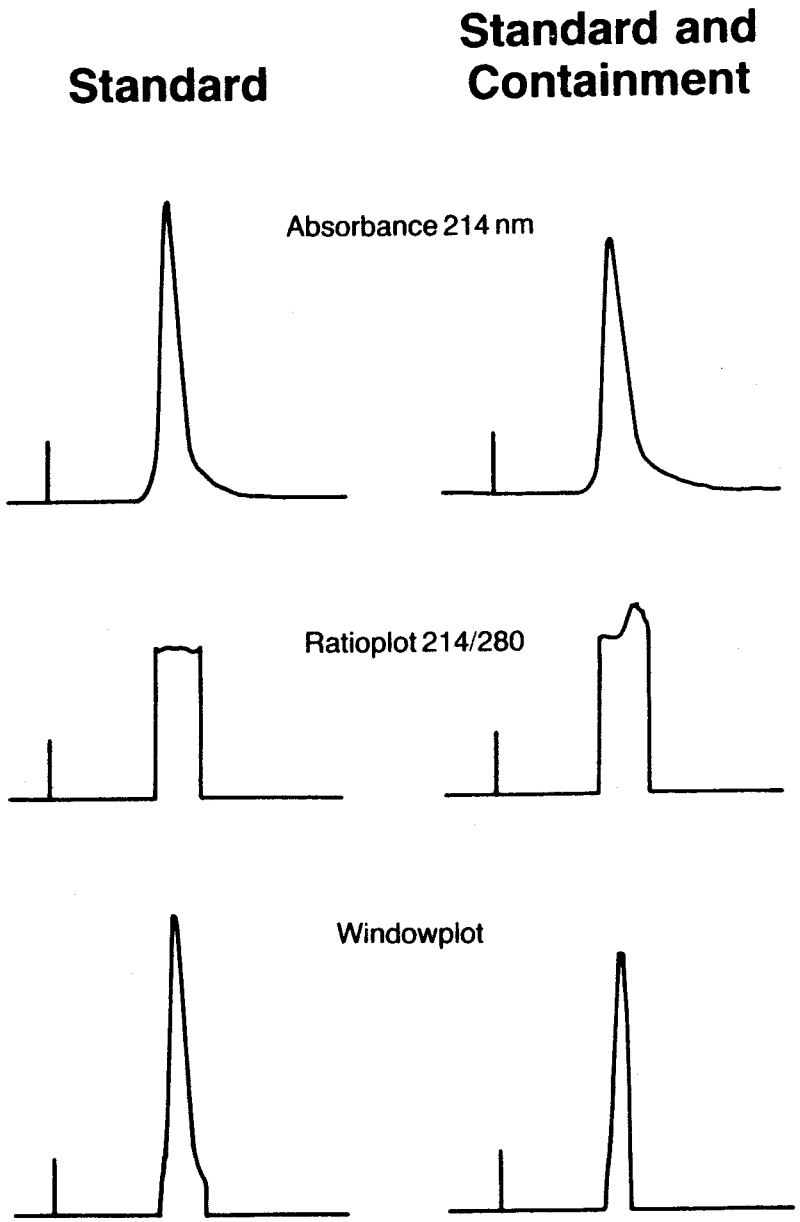
Medical Device Grade Polyolefin Resins

Effect of Radiation Sterilization



SAMPLE: PE and PP
COLUMN: ULTRASTYRAGEL™
SOLVENT: TCB (140°C)
FLOW RATE: 1 ml/min
DETECTOR: RI

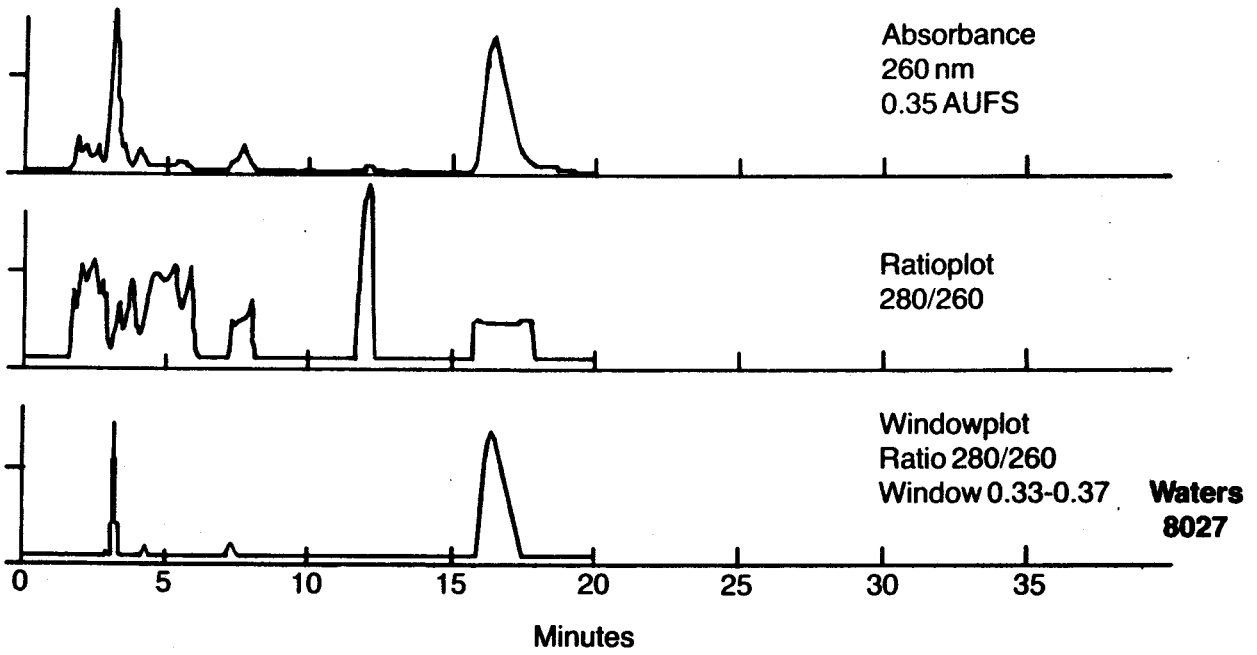
Waters
8416


Standard
Standard and Containment
Absorbance 214 nm
Ratioplot 214/280
Windowplot

Waters
8026

Fermentation Broth, 10 µl
Cephalosporin C Isolation

COLUMN: Radial-PAK C_{18}, 8 mm × 10 cm
SOLVENT: 20 mM Ammonium Acetate +
1% Acetonitrile (2 ml/min)
DETECTOR: 490



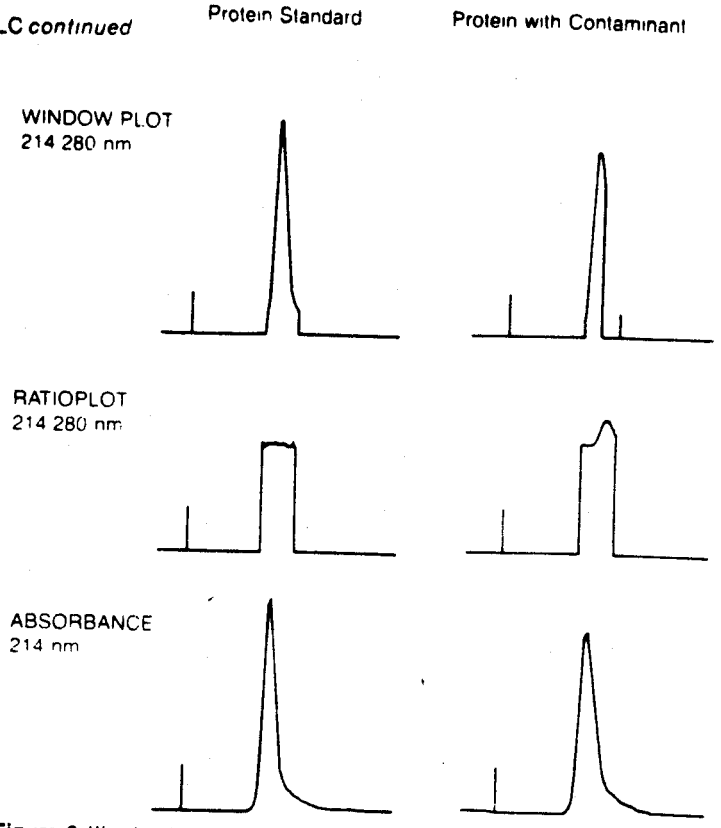


Figure 2 *Illustration of protein standard and protein with contaminant.*

if the absorbance ratio stays within an operator-specified range (window). If a hidden impurity causes the ratio to fall outside of that window at any point in the peak, it will be detected by a sharply attenuated response as shown in Figure 2. In addition to its utility as a purity check routine, WINDOWPLOT is also useful in determining where on the peak researchers should begin collecting fractions for preparative purification.

The advantage of detection modes such as RATIOPLOT and WINDOWPLOT is the ability to continuously monitor peak purity while analyses are being run, thereby eliminating time-consuming post-run calculations or computer manipulations.

Isolation and purification of Cephalosporin C from a crude fermentation broth

The usefulness of the routines afforded by programmable multiwavelength detection is quite apparent when applied to pharmaceutical research. In the following example, an HPLC separation was performed to isolate and purify Cephalosporin C, an antibiotic formed in a complex fermentation broth.

The first step was to find the maximum absorbance of Cephalosporin C. Using the automatic absorbance monitoring

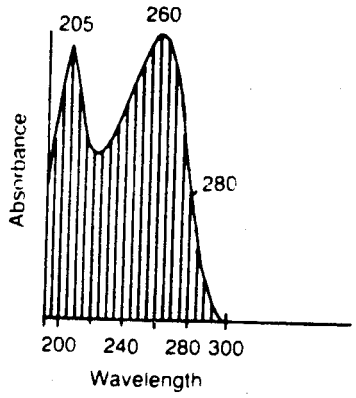
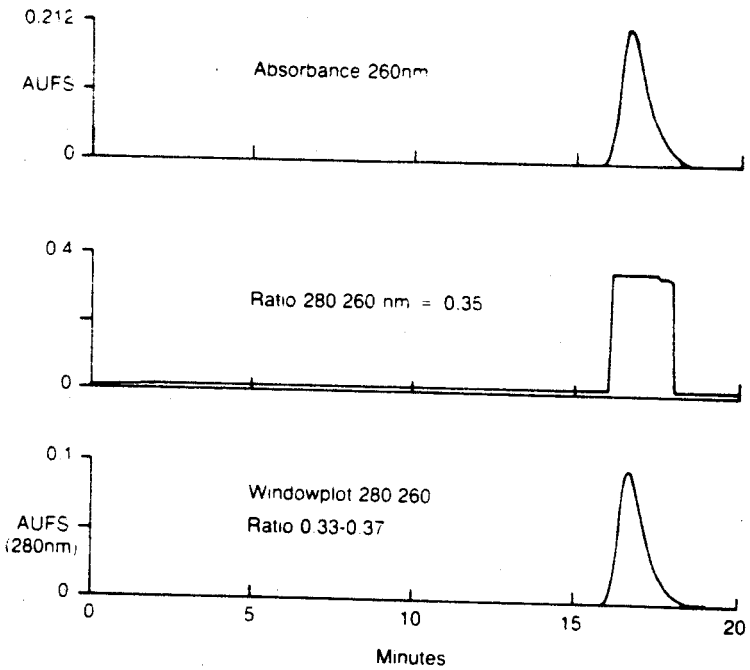


Figure 3 *Absorbance spectrum of Cephalosporin C.*

routine, a spectral scan of the Cephalosporin C was performed from 190 to 600 nm in 1-nm increments. The spectrum indicated two maximum absorbances at 205 and 260 nm (*Figure 3*).

Next, Cephalosporin C standards were analyzed simultaneously using three different modes of detection. Output was recorded and stored on a four-channel computer station, Waters 840 data and chromatography control station. The 490 detector simultaneously monitored absorbance at 260 nm while plotting RATIOPLOT at 280/260 nm and WINDOWPLOT at 280/260 nm (*Figure 4*). This information

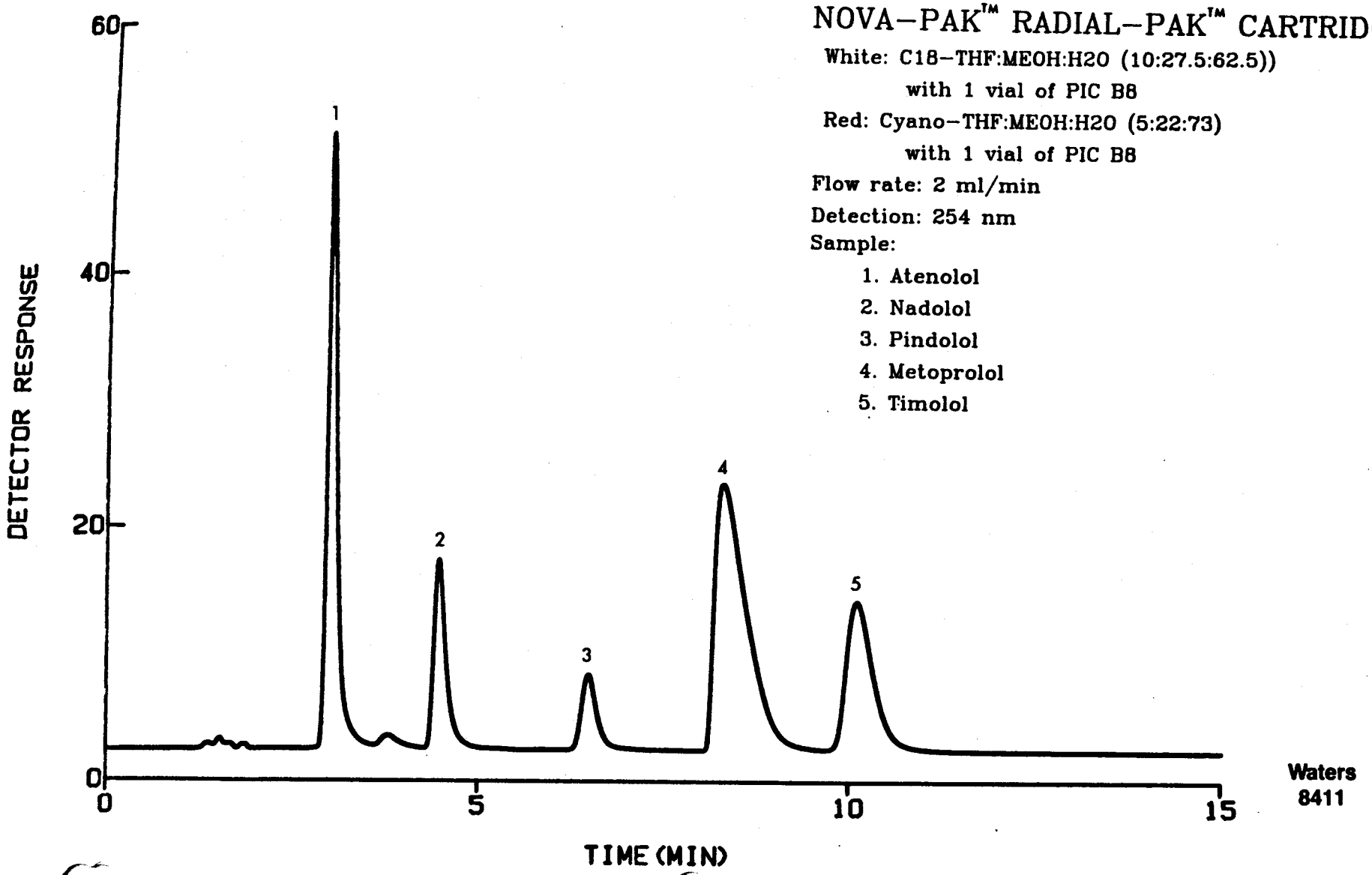


SAMPLE 25µg Cephalosporin C Standard
CARTRIDGE Radial-PAK µBONDAPAK C18, 8 mm x 10 cm
ELUENT 20 mM NH_4OAc, pH 6.2, in 1% CH_3CN H_2O
FLOW RATE 2.0 ml/min
DETECTOR Model 490 Programmable Multiwavelength Detector

Figure 4 *Analysis of Cephalosporin C standard.*

NOVA-PAK C18 VERSUS NOVA-PAK CYANO

BETA-ADRENERGIC BLOCKERS

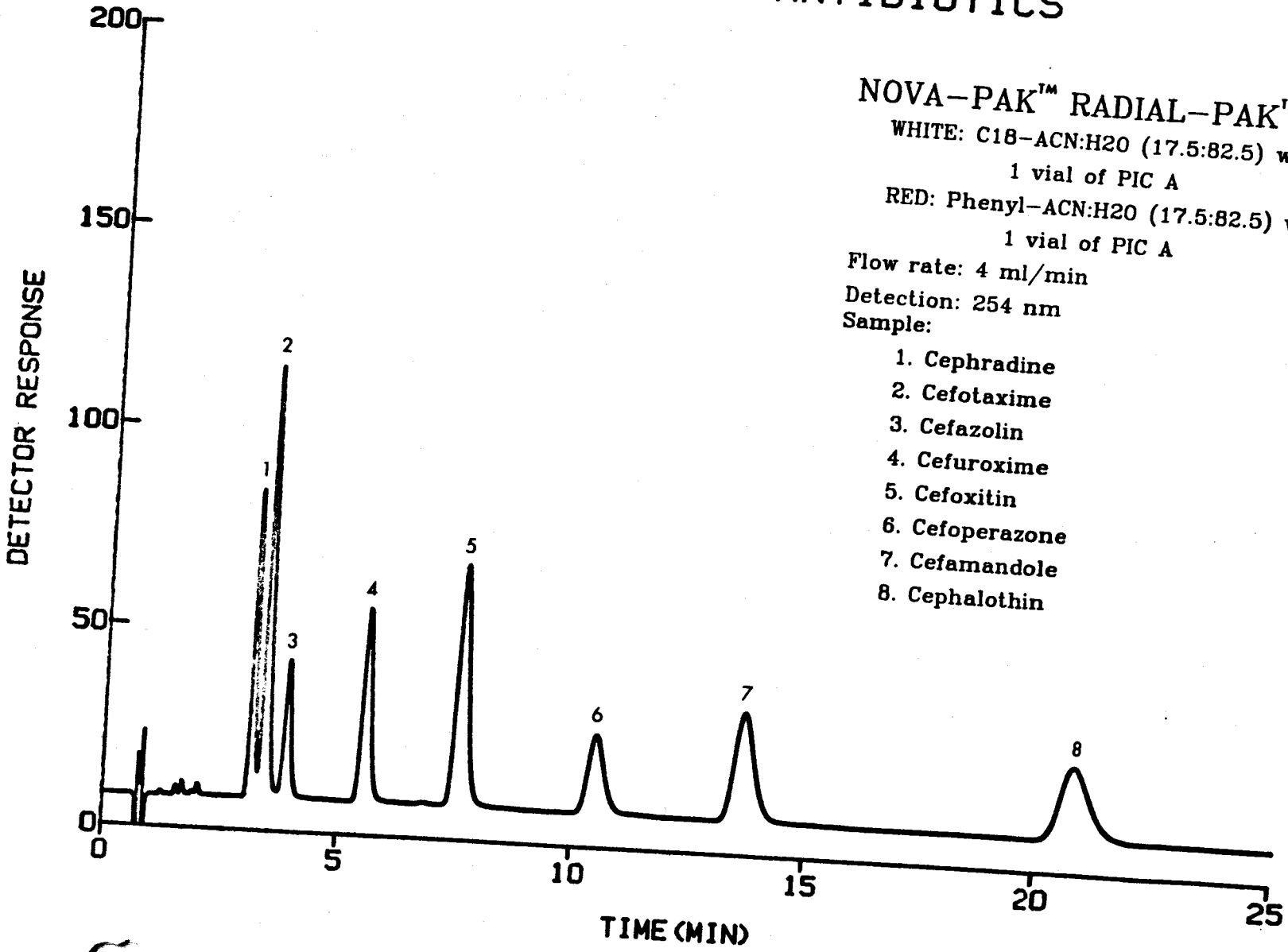


BASIC COMPOUNDS

Waters
8408

NOVA–PAK™

CEPHALOSPORIN ANTIBIOTICS



NOVA–PAK™ RADIAL–PAK™ CARTRIDGE

WHITE: C18–ACN:H20 (17.5:82.5) with
1 vial of PIC A

RED: Phenyl–ACN:H20 (17.5:82.5) with
1 vial of PIC A

Flow rate: 4 ml/min

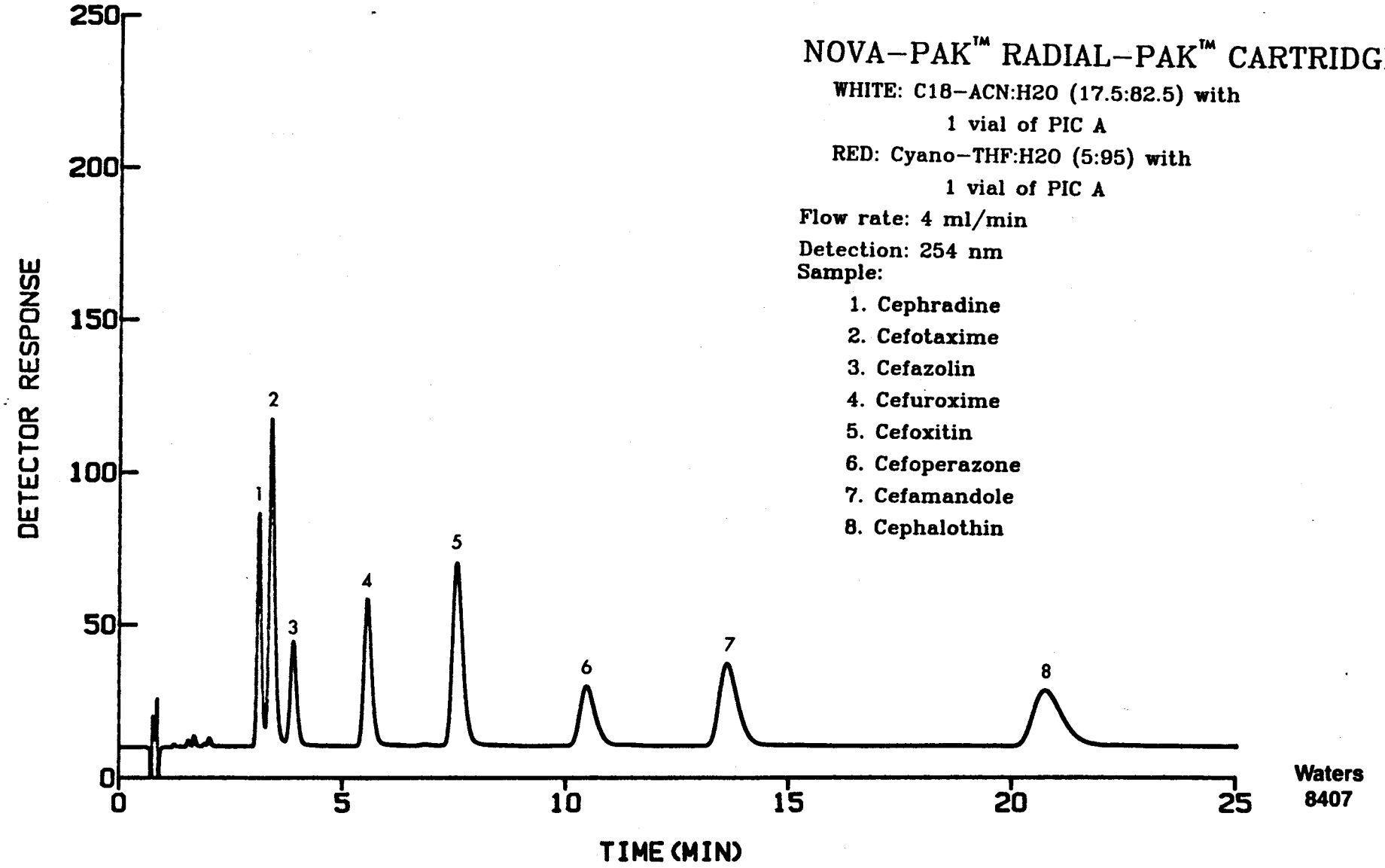
Detection: 254 nm

Sample:

1. Cephradine
2. Cefotaxime
3. Cefazolin
4. Cefuroxime
5. Cefoxitin
6. Cefoperazone
7. Cefamandole
8. Cephalothin

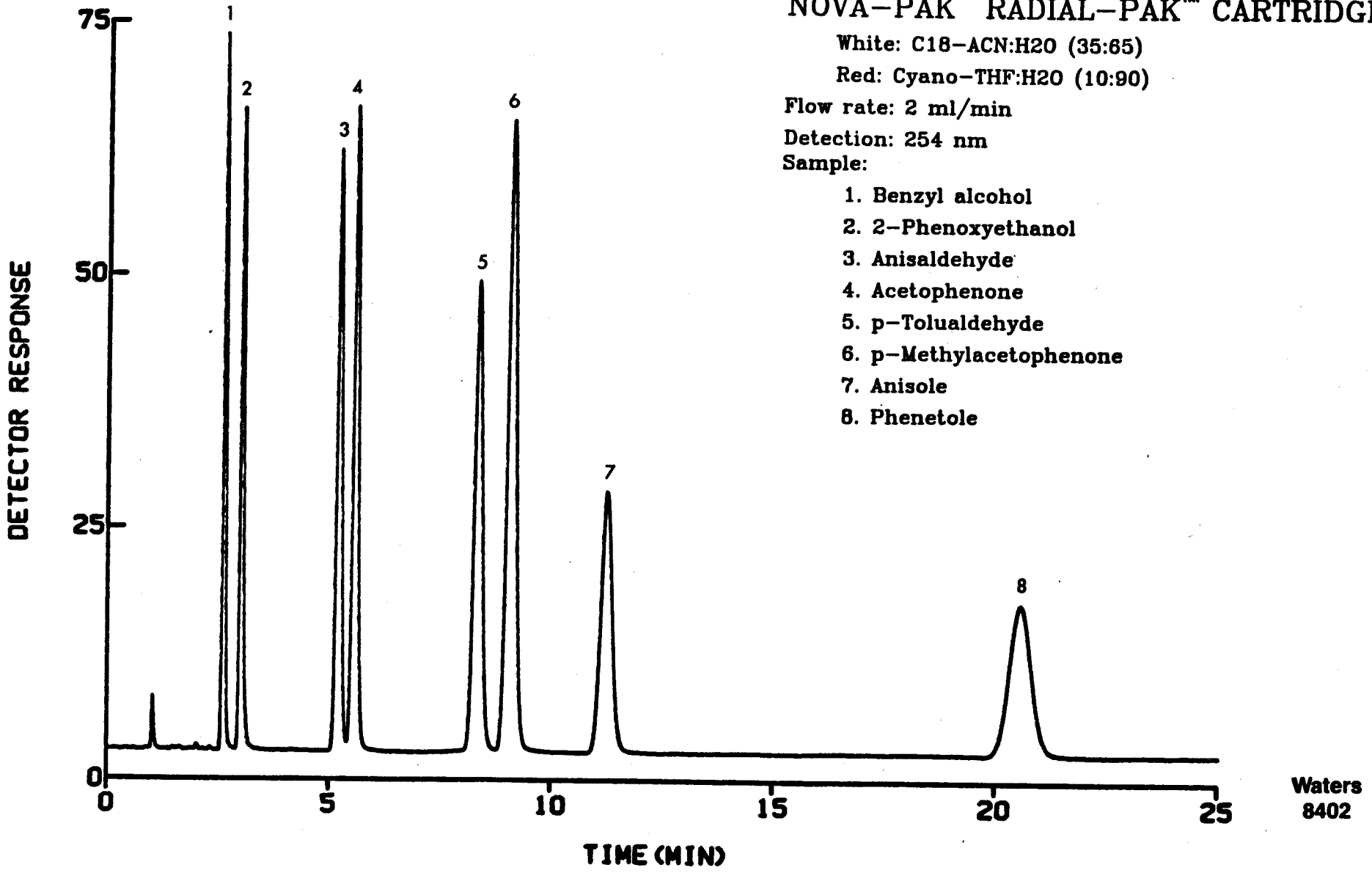
NOVA–PAK™

CEPHALOSPORIN ANTIBIOTICS



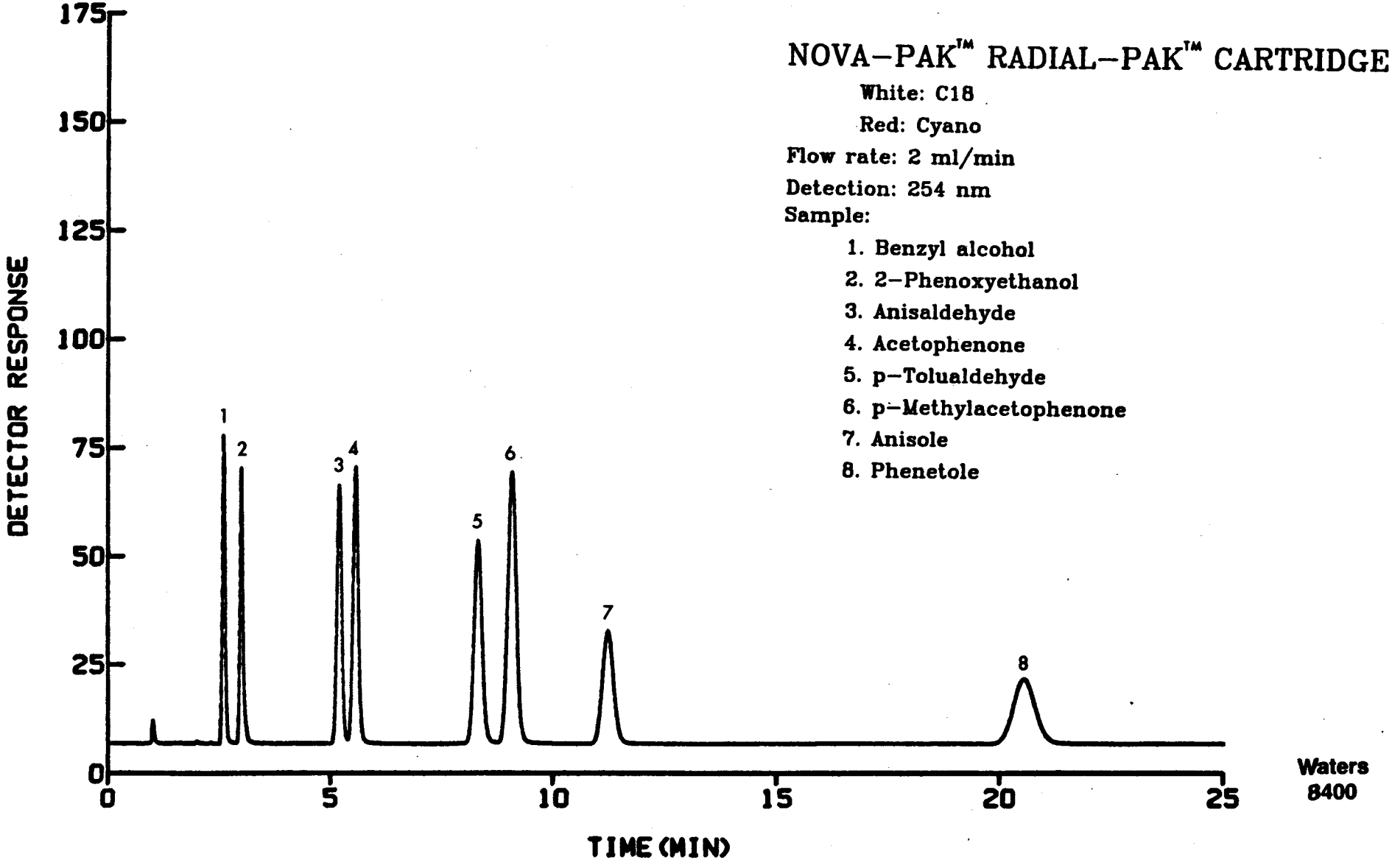
NOVA-PAK C18 VERSUS NOVA-PAK CYANO

OPTIMIZED NOVA-PAK CYANO MOBILE PHASE



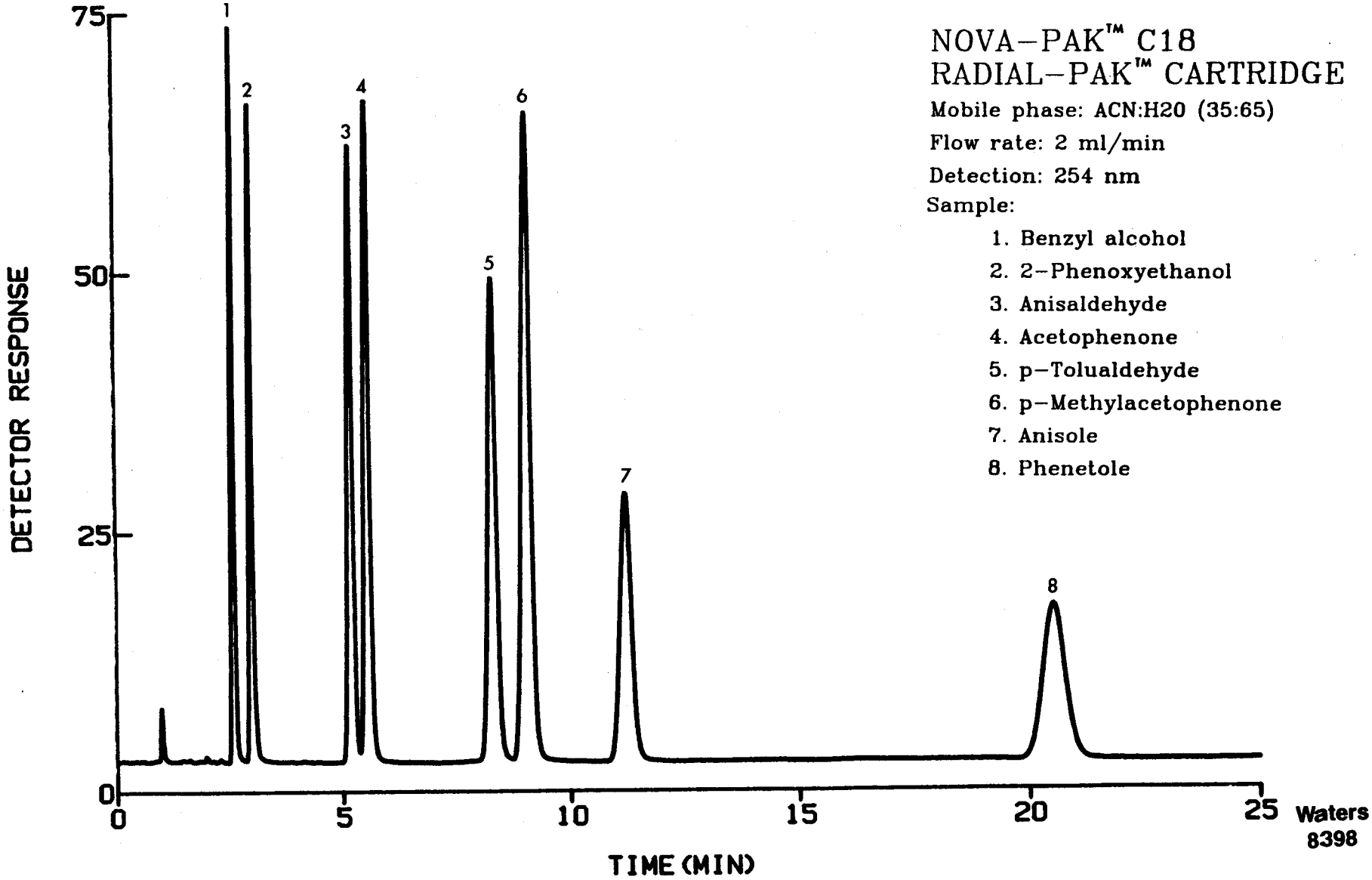
NOVA-PAK™ C18 VERSUS NOVA-PAK™ CYANO

ACN: H20 (35: 65)



NOVA–PAK™ C18

AROMATIC NEUTRALS



NOVA–PAK™ C18

NOVA–PAK™ PHENYL

NOVA–PAK™ CYANO

Waters
8396

NOVA–PAK™ C18

HIGH EFFICIENCY

4 micron spherical silica

BROAD SELECTIVITY

neutral, basic and acidic compounds

HIGH REPRODUCIBILITY

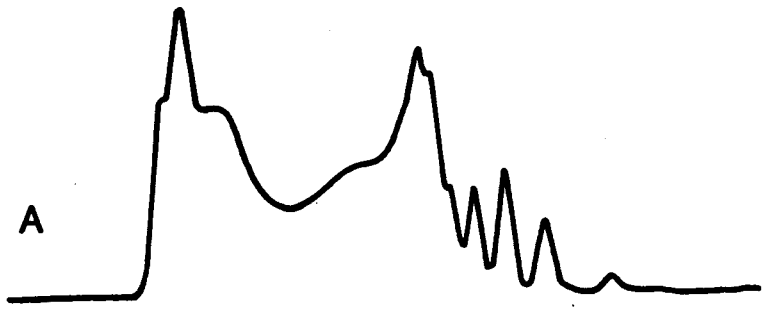
Waters
8394

Four Starch Hydrolyzates

100-500-10^3 Å Column Set

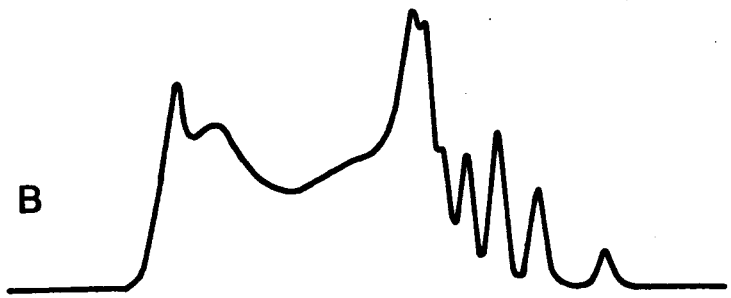
SAMPLE CONCENTRATION: 2%
INJECTION VOLUME: A,B: 45 µl
C,D: 50 µl
COLUMNS: Experimental GPC Columns

FLOW RATE: 0.5 ml/min
MOBILE PHASE: Water with 50 mg/L $CaNa_2$ EDTA
TEMPERATURE: 80°C
DETECTOR: RI 8X

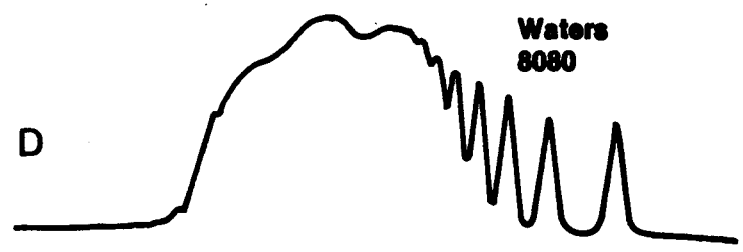


Maltodextrine DE 12

Fro-Dex® 15



Maltrin® 15



Corn Syrup Solids DE 16

Criteria for Good "Biomedical Plastics"

- Narrow MW Distribution
- High Average Molecular Weight
- Purity of Monomers
- Minimize Leachables
- Low Toxicity Additives
  - UV Absorbers
  - Antioxidants
  - Processing Aids
- No Decomposition Products

Acknowledgement

Dr. J.W. King
CPC International
Argo, Illinois