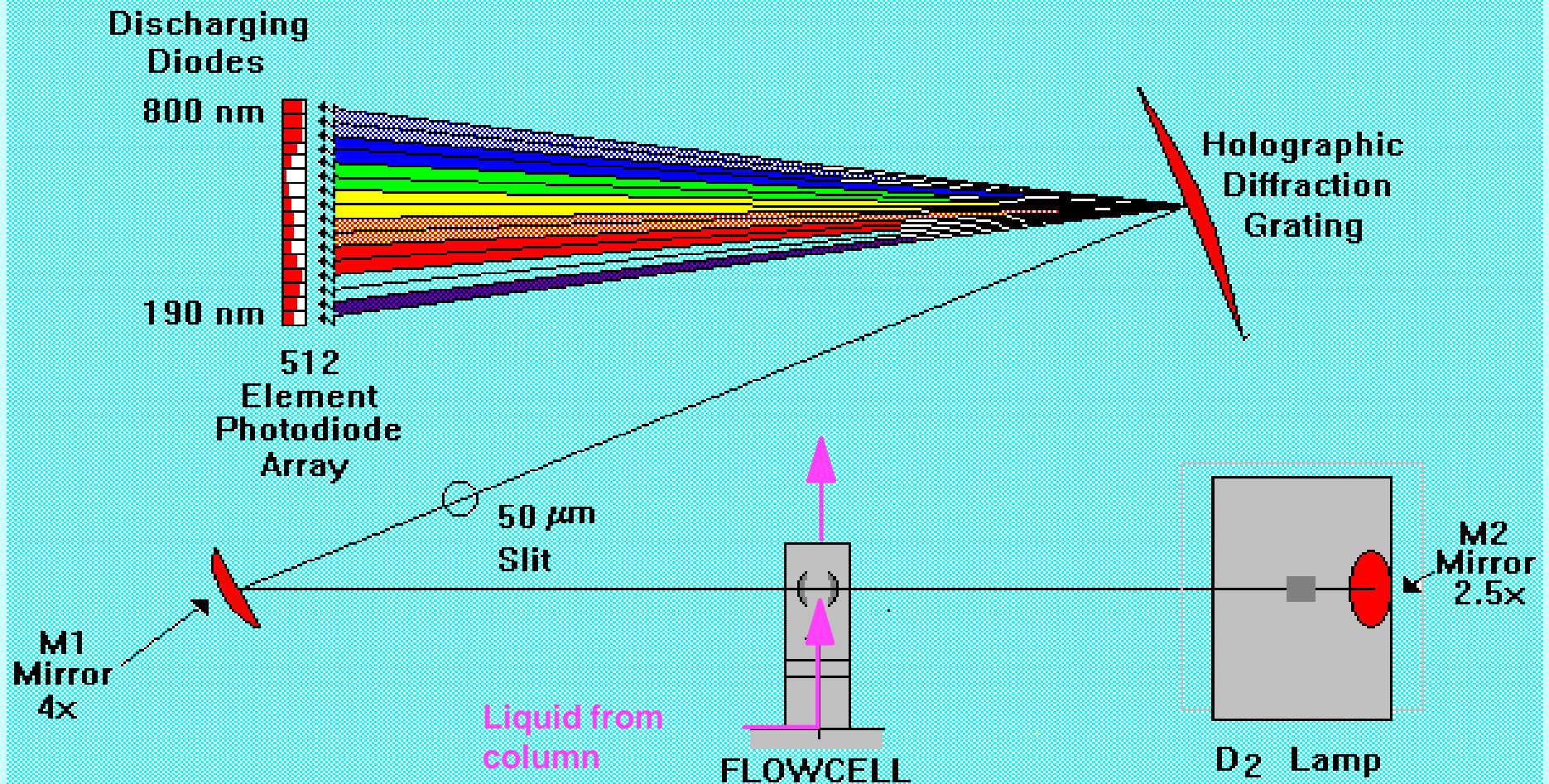


Considerations in Peak Purity Measurements

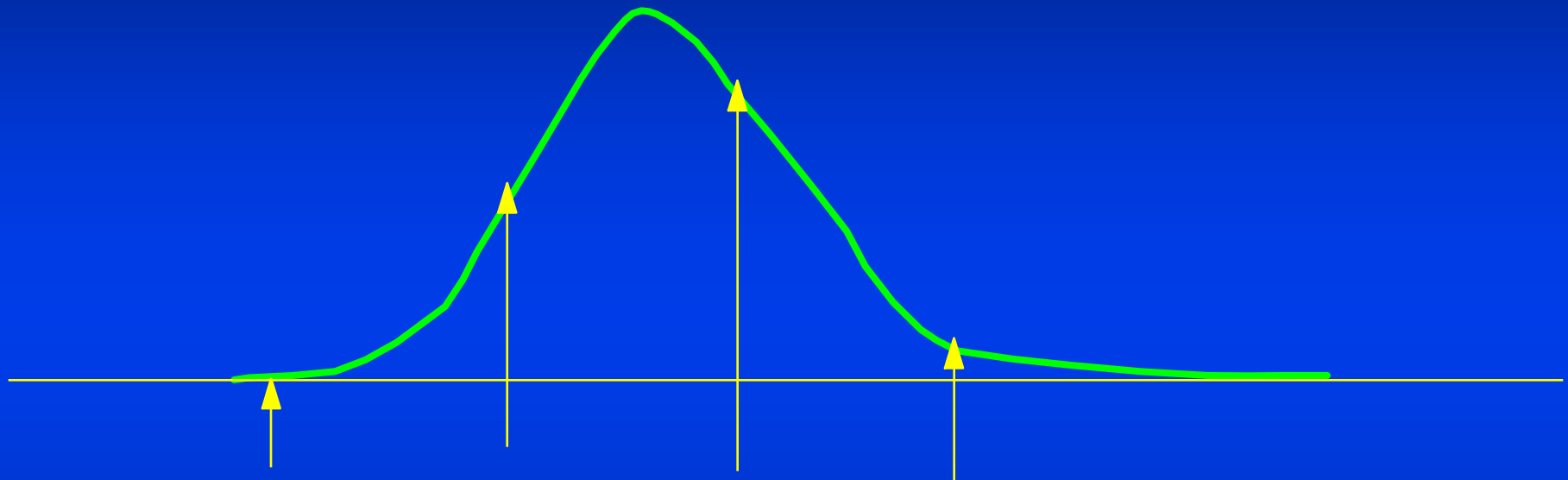
Shulamit Levin, 1999

Principle of Diode Array Detection

Principle of Measurement



The Data is 3D



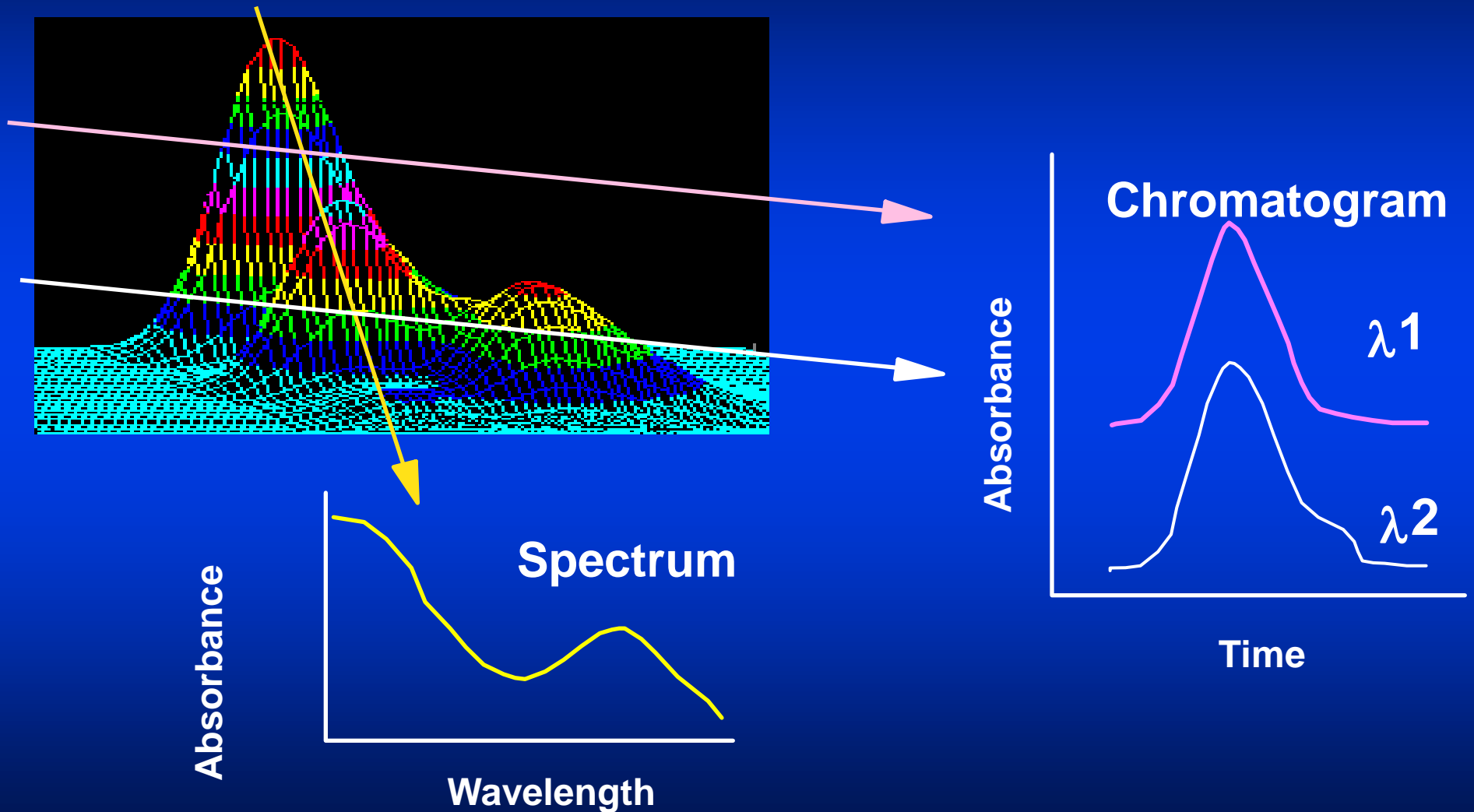
<u>nm</u>	<u>Abs</u>
200	0.00
201	0.01
202	0.02
203	0.03
-	-
-	-

<u>nm</u>	<u>Abs</u>
200	0.00
201	0.01
202	0.02
203	0.03
-	-
-	-

<u>nm</u>	<u>Abs</u>
200	0.00
201	0.01
202	0.02
203	0.03
-	-
-	-

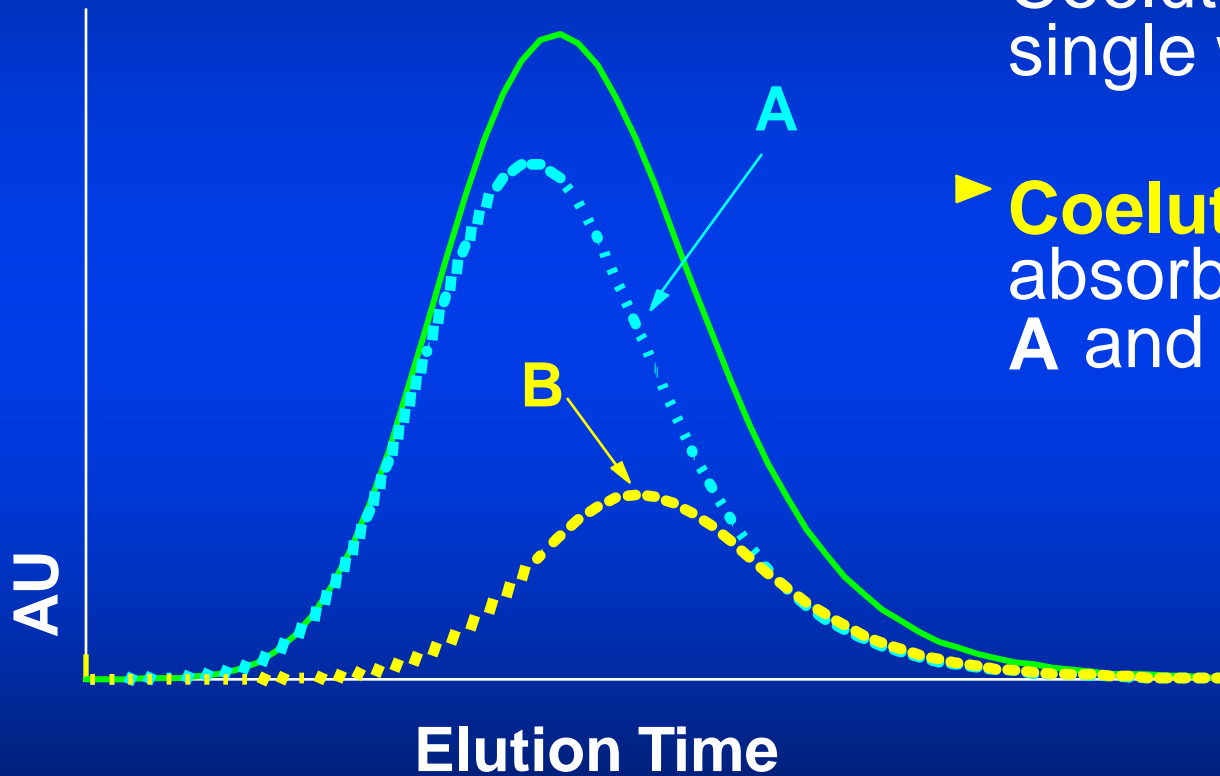
<u>nm</u>	<u>Abs</u>
200	0.00
201	0.01
202	0.02
203	0.03
-	-
-	-

Extraction of 3D Data

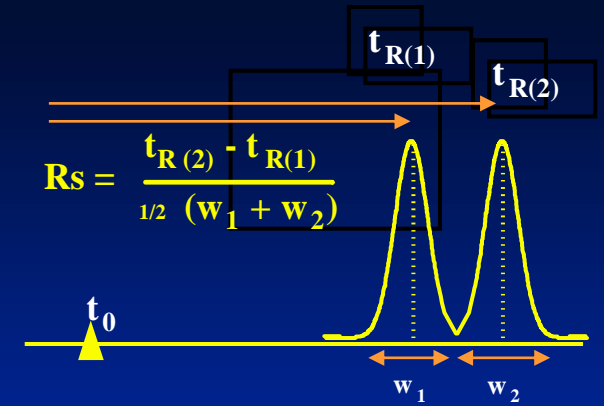
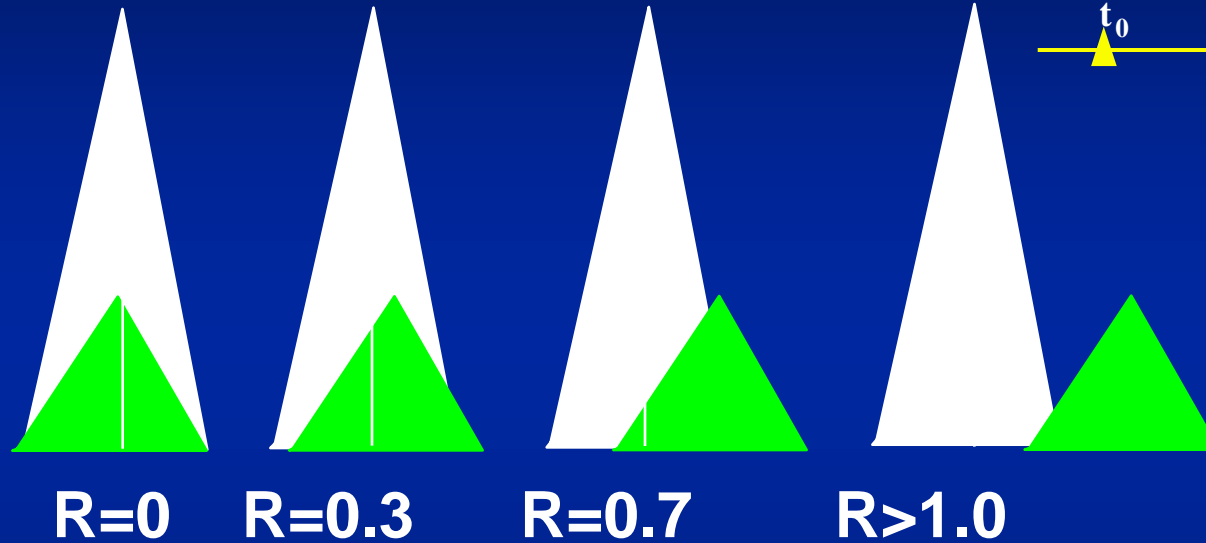


Coelution of 2 Peaks

- ▶ Coelution detection at a single wavelength
- ▶ **Coelution** is the sum of absorbance of 2 peaks A and B



Chromatographic Resolution & Coelution Detection



- ▶ $R=0$ Purity Angle not effective; Match Angle useful
- ▶ $R=0.3$ to $R=0.7$ Purity & Match Angle useful
- ▶ $R>0.7$ Match Angle not useful

Peak Purity and Spectral Matching Principles:

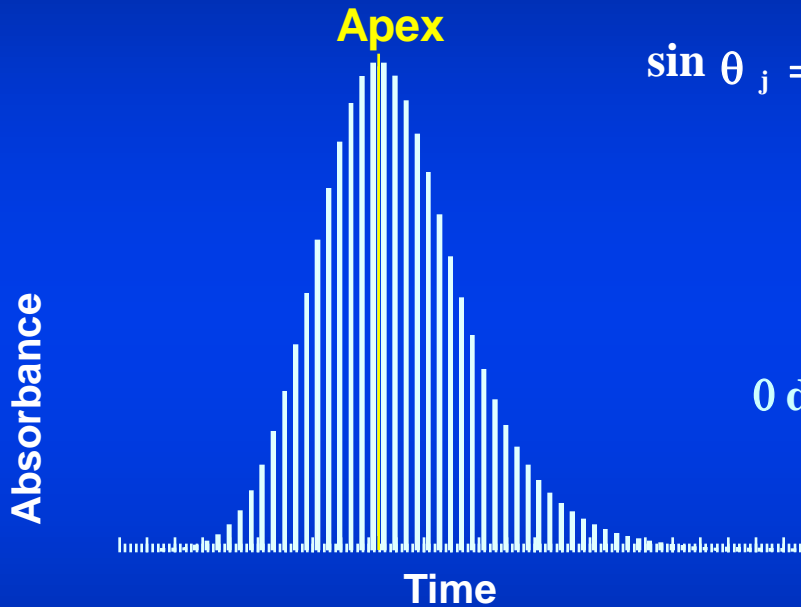
Spectral contrast angle:

$$\sin \theta_j = \frac{\sqrt{\sum_{i=1}^N (B_{ij} - s_j A_i)^2}}{\sqrt{\sum_{i=1}^N B_{ij}^2}}$$

$$0 \leq \sin \theta \leq 1$$

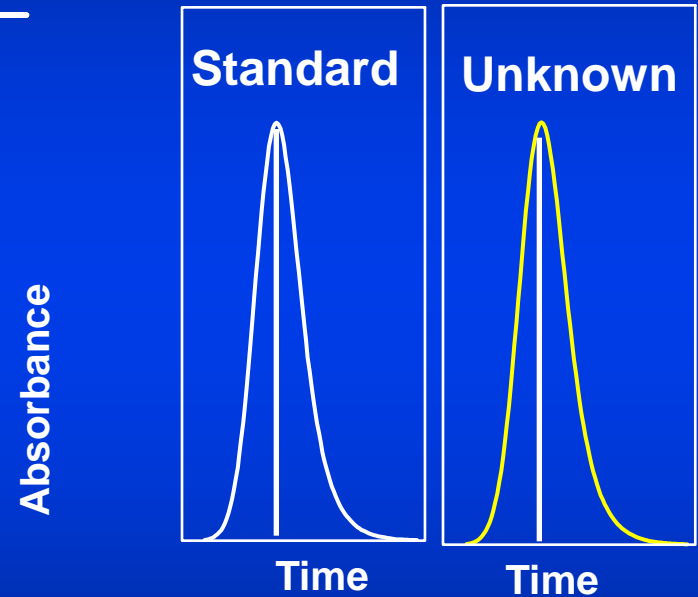
$$0 \text{ deg} \leq \theta \leq 90 \text{ deg}$$

Purity verification



- ▶ Peak Purity analyzes all spectra (minimum 15) within a peak
- ▶ Apex spectrum is the reference spectrum

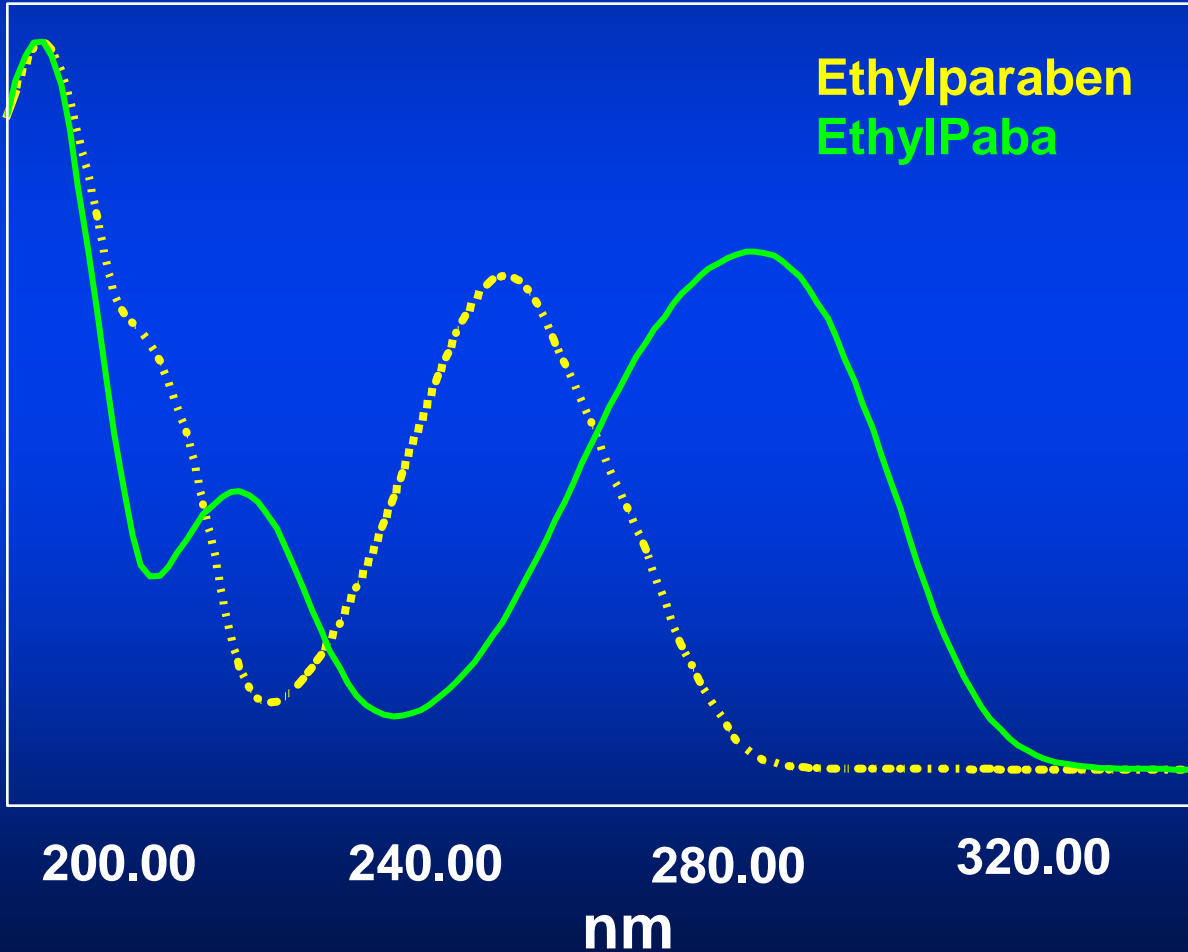
Library identification



- ▶ Matching compares the unknown apex spectrum of the peak with a reference spectrum in a library

Spectral Contrast Angle = 53 Degrees

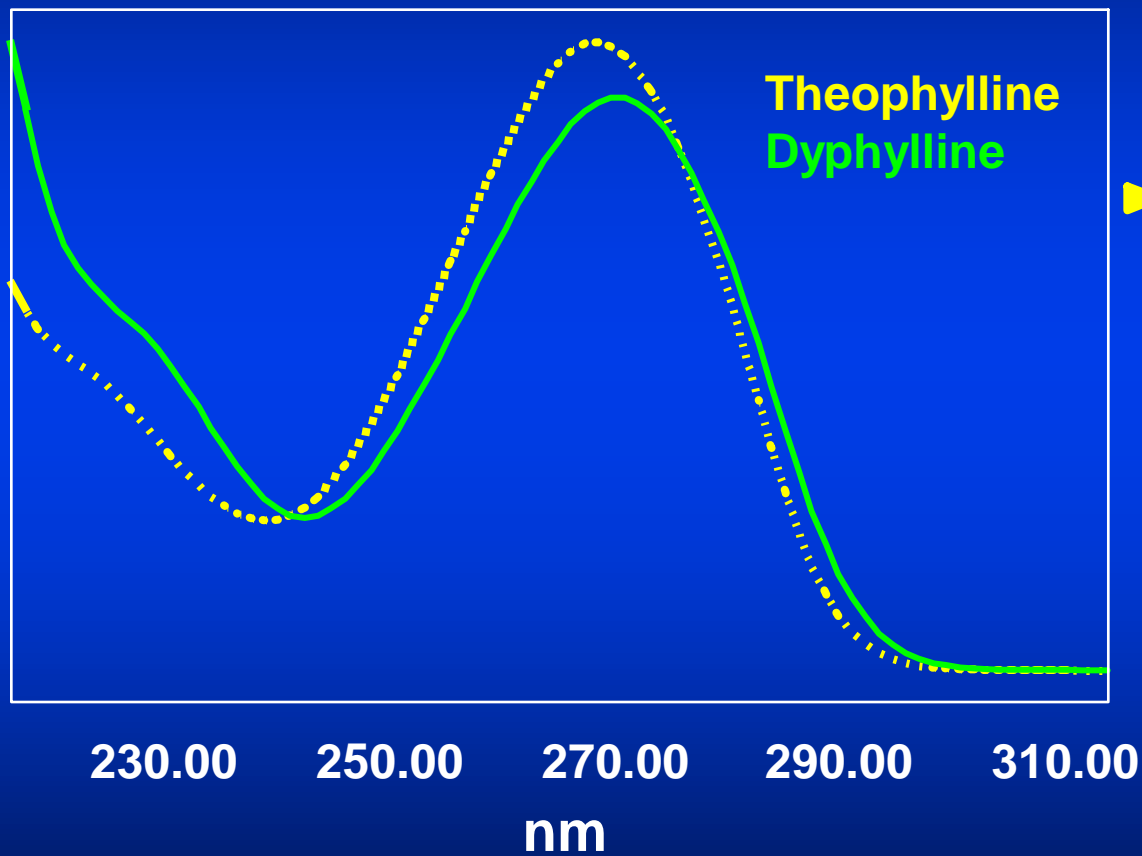
Absorbance



Ethylparaben
EthylPaba

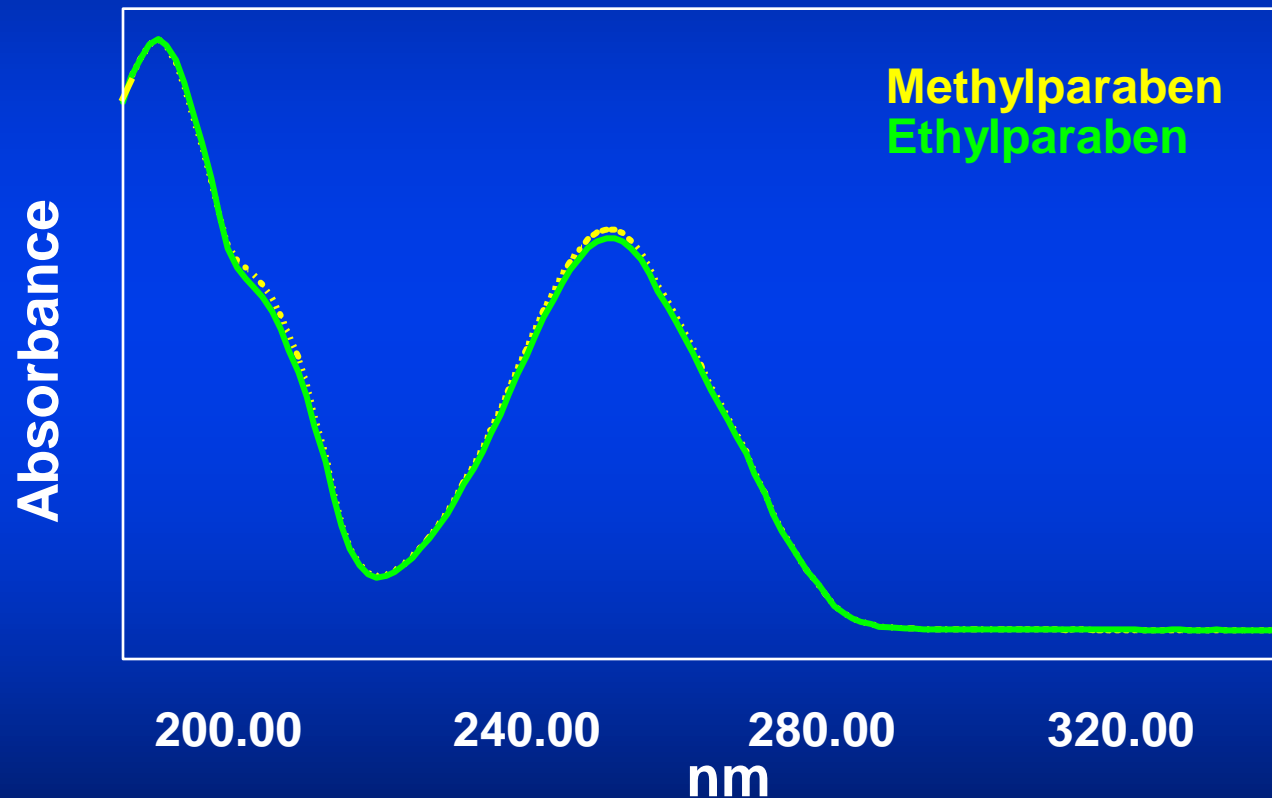
► 53 degrees is a large spectral difference

Spectral Contrast 10 Degrees



► Similar spectra for structurally related compounds

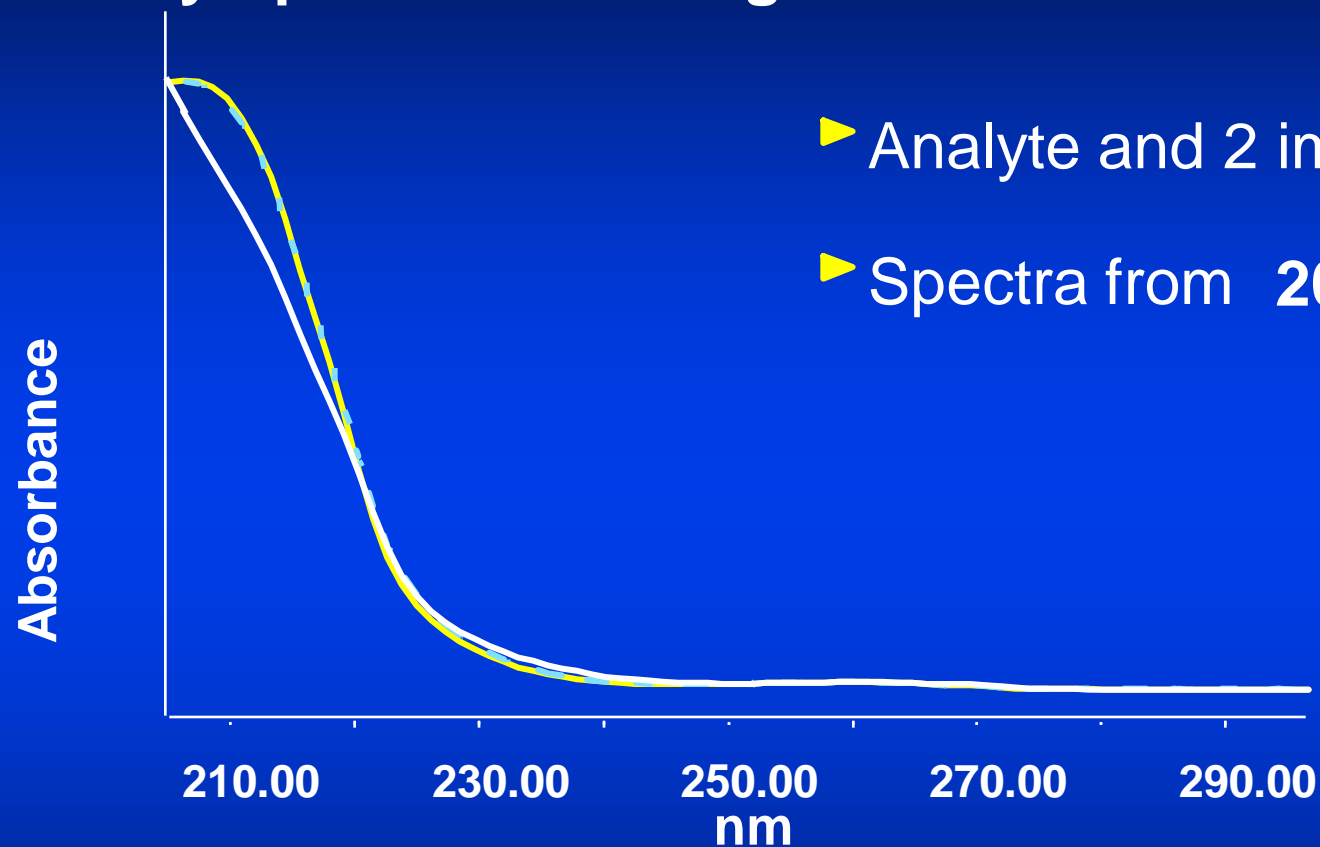
Spectral Contrast 0.5 Degrees



▶ Very similar spectra, CH₂ difference

▶ Spectral Contrast can differentiate these spectra

Very Similar Spectra: Not a Candidate for Peak Purity/Spectral Matching

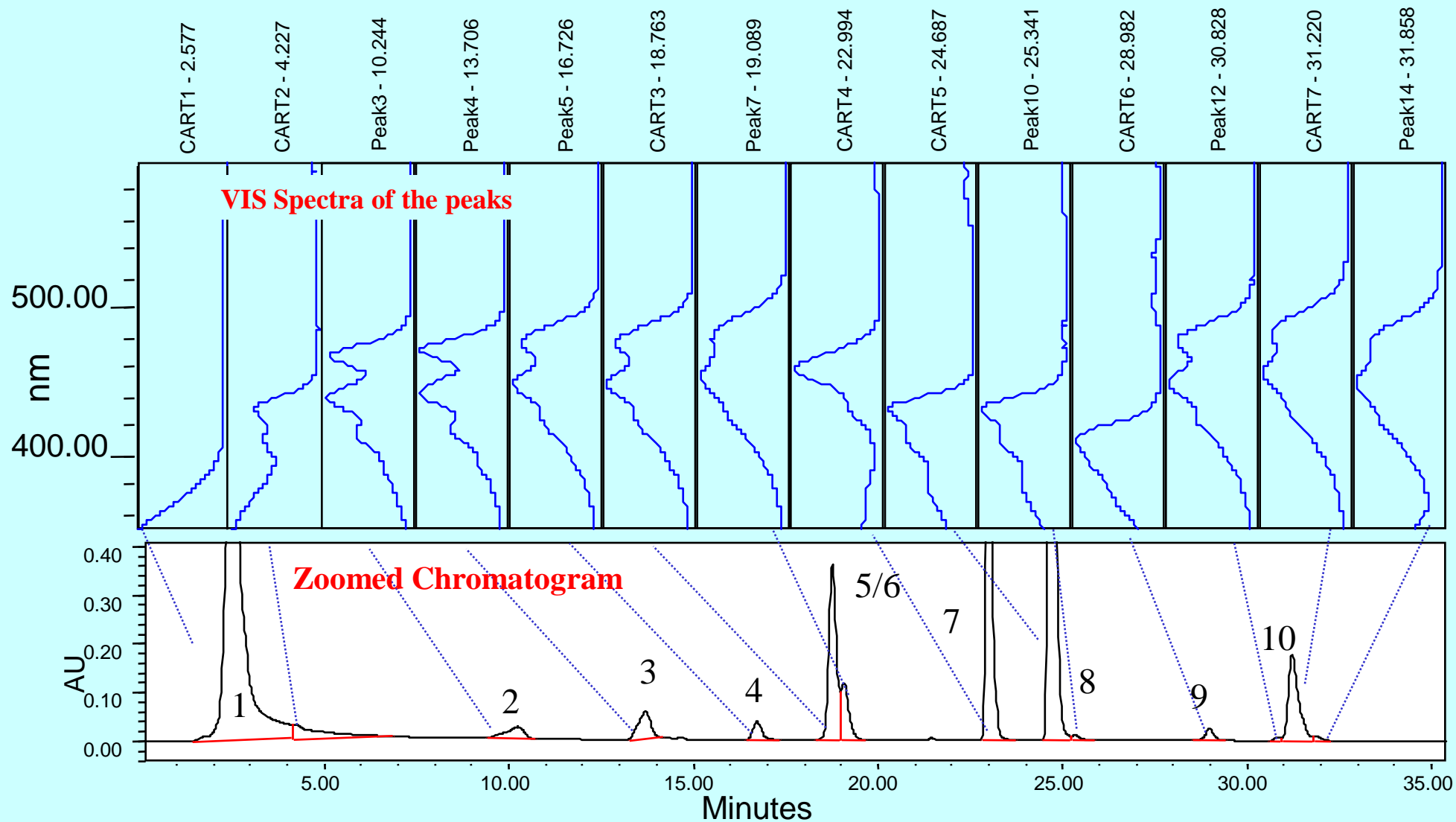


Considerations in Peak Purity Measurements

Questions:

- Is the peak single component peak?
- How many components in a non-single component peak?
- To what extent can the integration events effect the result?
- How indicative is the symmetry of the peak to its purity?
- Can fused peaks be analyzed properly?
- The effect of UV spectrum features on the results?
- The effect of spectral band width on the Peak Purity results

Example: CAROTENOIDS - Extracted from leaves



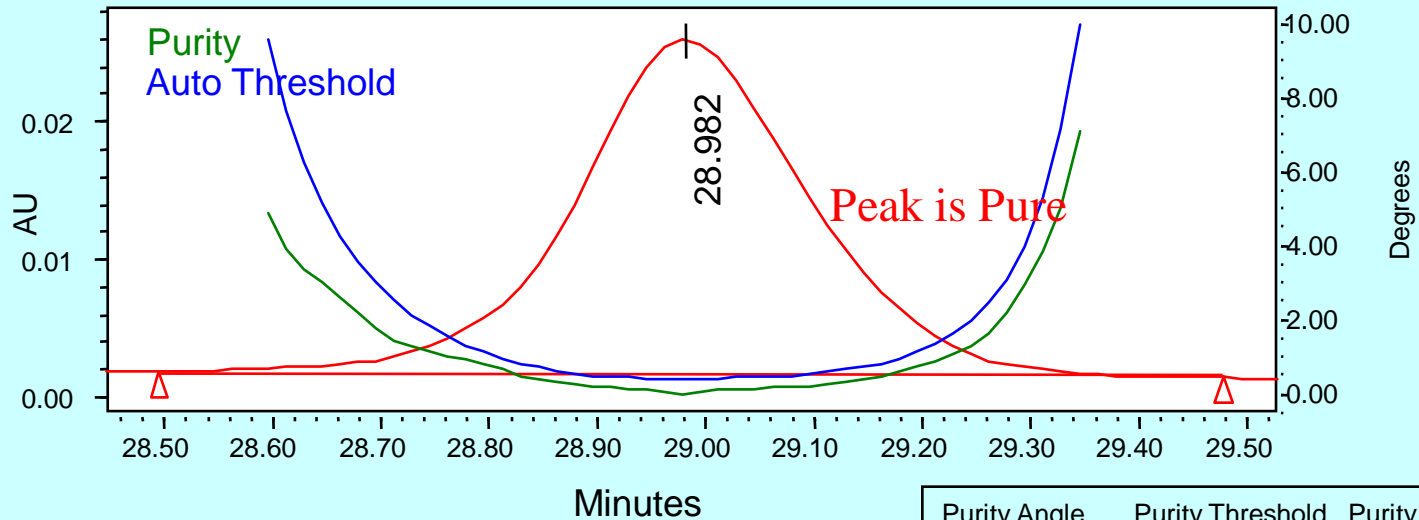
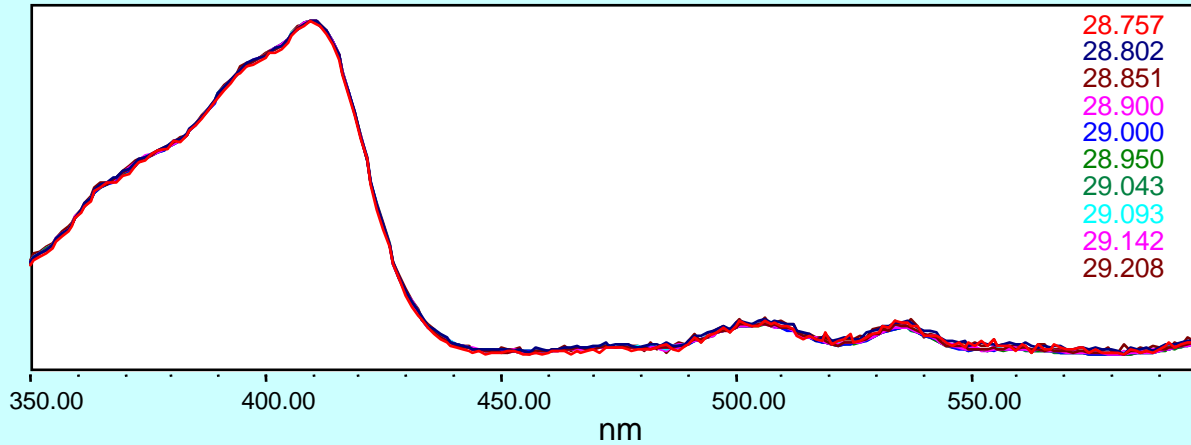
Considerations in Peak Purity Measurements

Questions:

- Is the peak single component peak?
- How many components in a non-single component peak?
- To what extent can the integration events effect the result?
- How indicative is the symmetry of the peak to its purity?
- Can fused peaks be analyzed properly?
- The effect of UV spectrum features on the results?
- The effect of spectral band width on the Peak Purity results

CAROTENOIDS

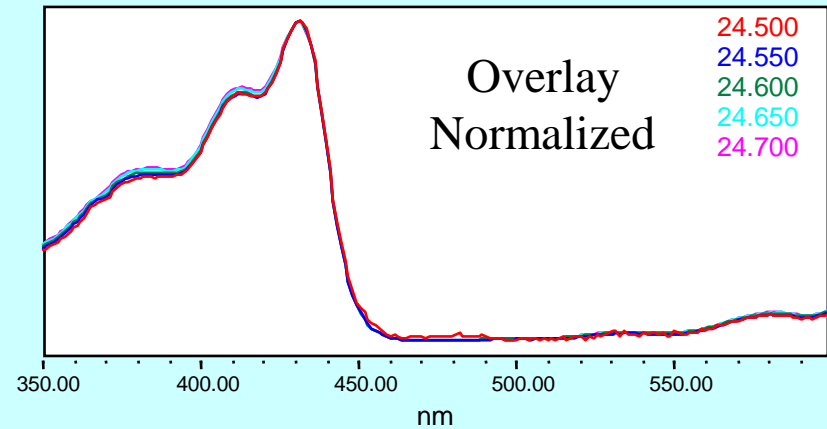
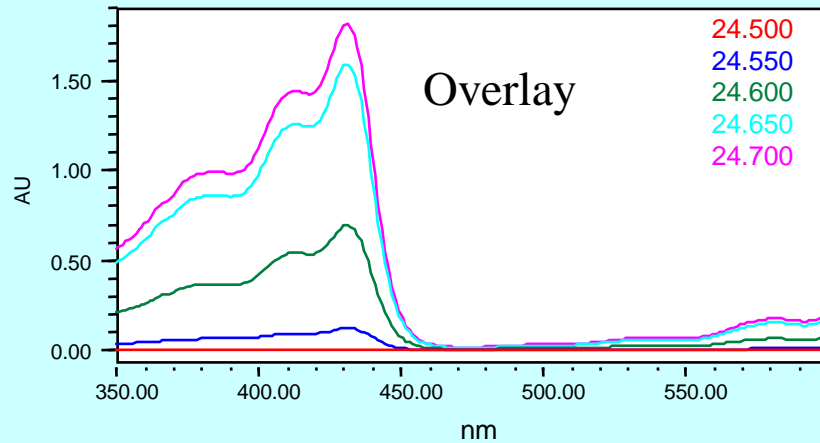
Spectra collected from Peak 9 are all the same after normalization



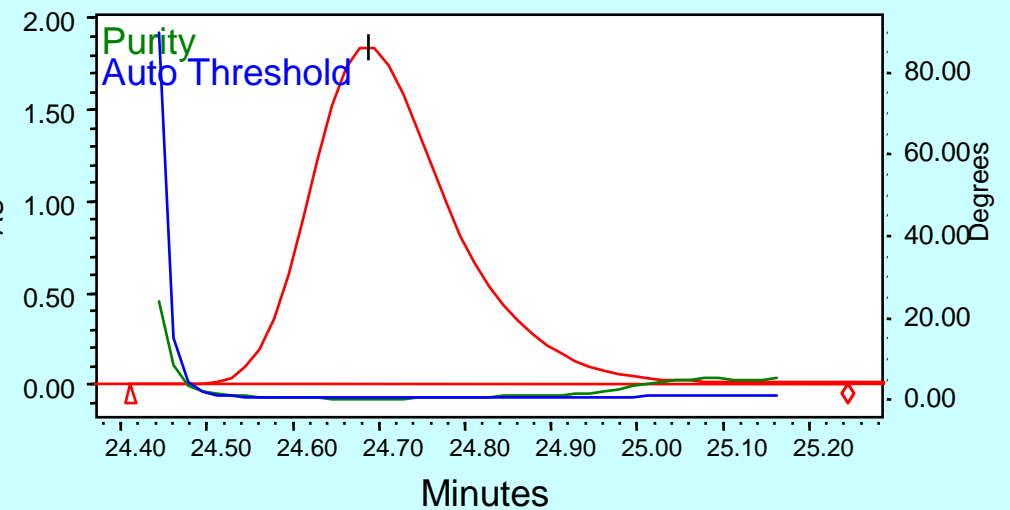
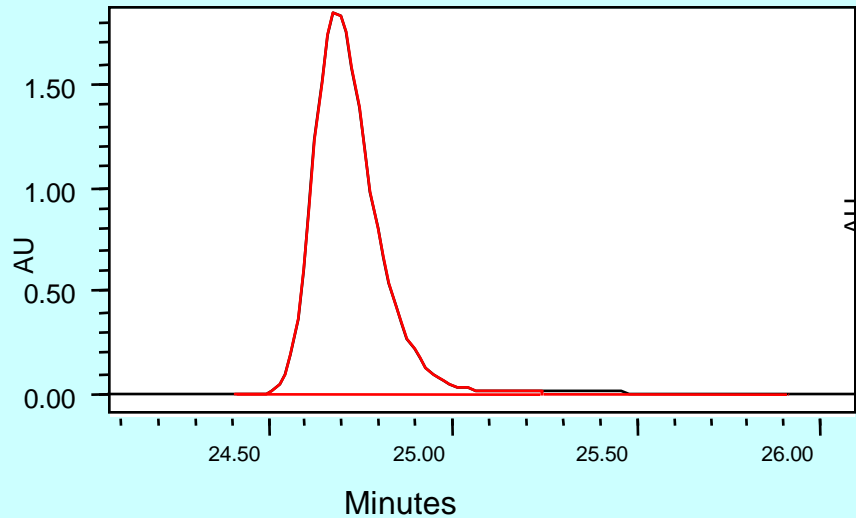
Purity Angle	Purity Threshold	Purity Flag
0.284	0.551	No

CAROTENOIDS

Spectra collected from Peak 8 are all the same after normalization

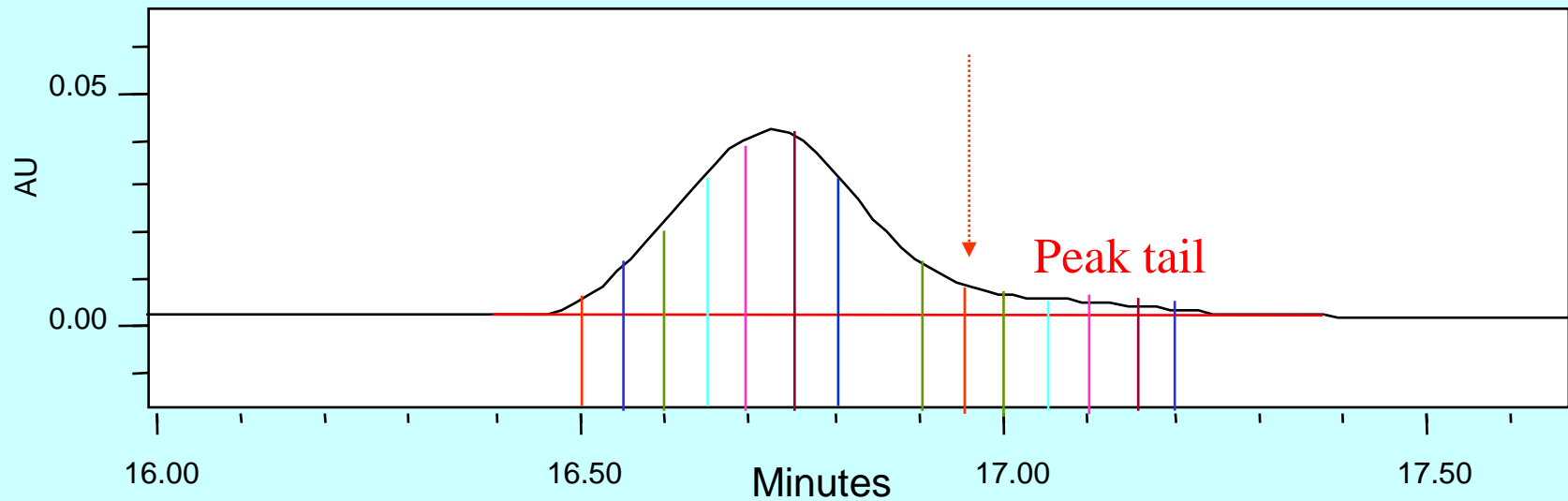
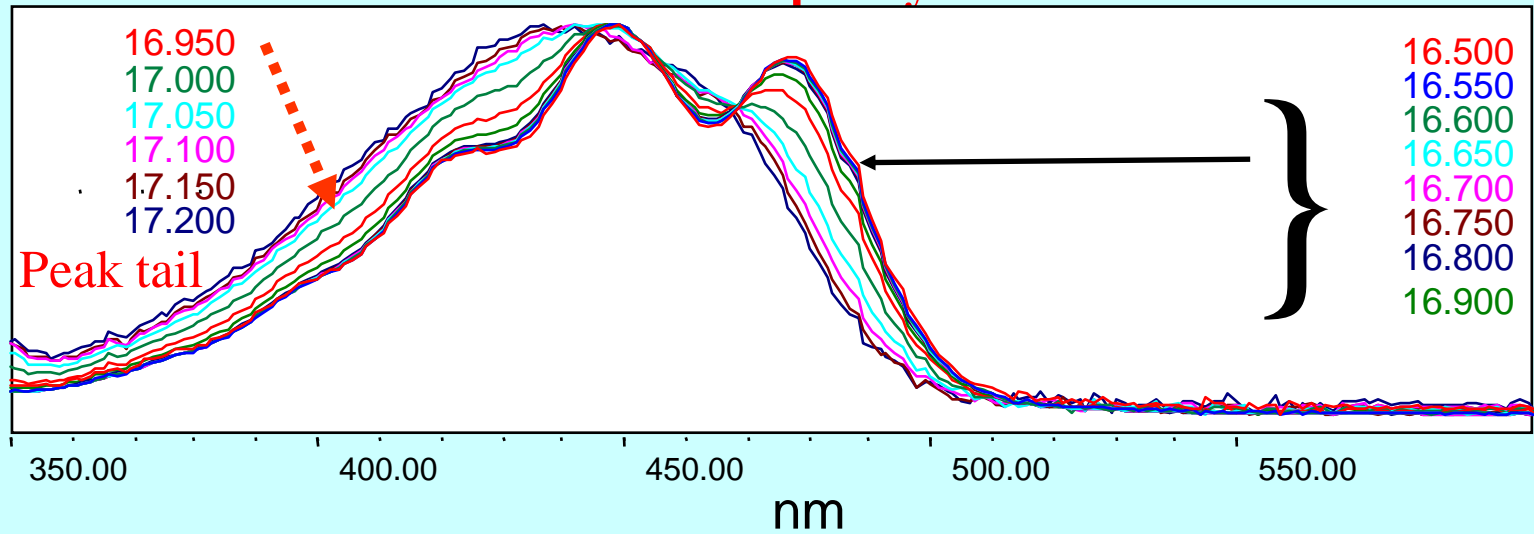


Purity Angle	Purity Threshold	Purity Flag
0.413	0.684	No



CAROTENOIDS

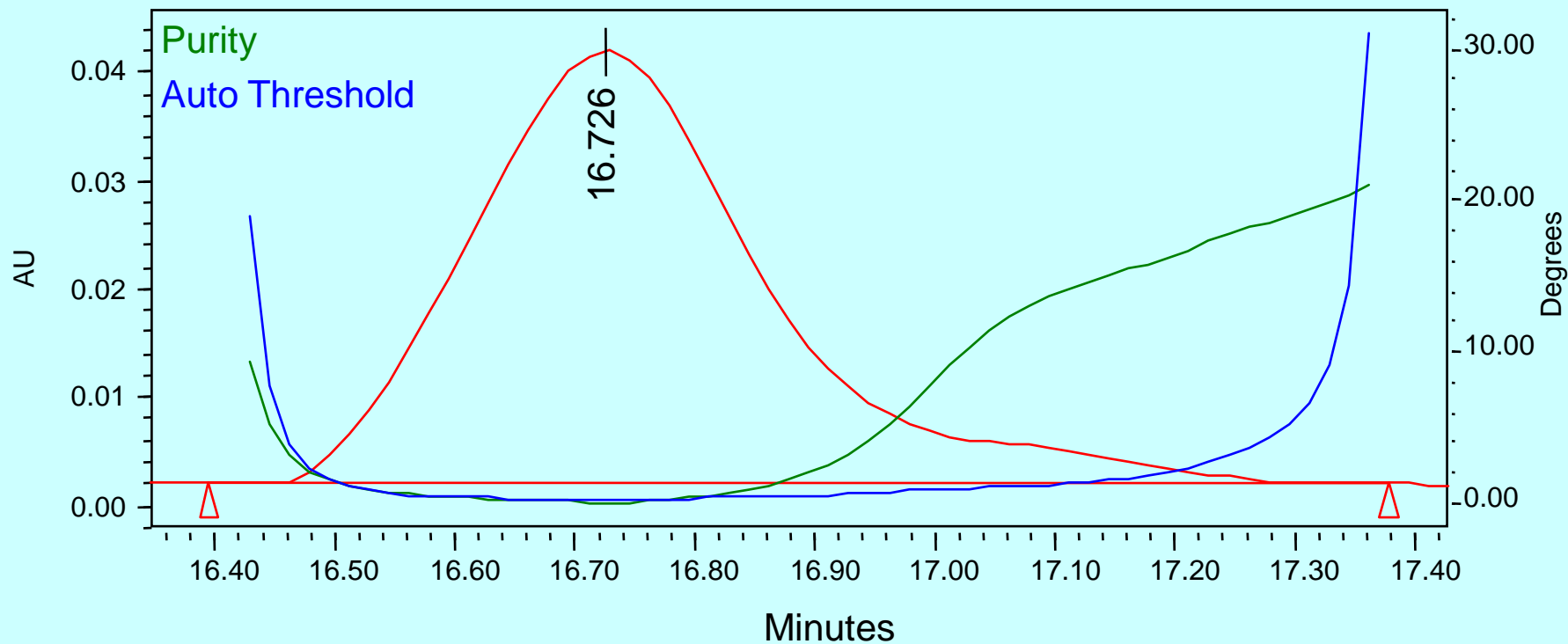
**Spectra Selected from Peak 4: Spectra collected from the Peak's tail are different:
There is an impurity**



CAROTENOIDS

Purity Plot of Peak 4 - Not Pure

Purity Flag	Maximum Impurity	Purity Threshold	Purity Angle
Yes	17.078	0.404	1.885



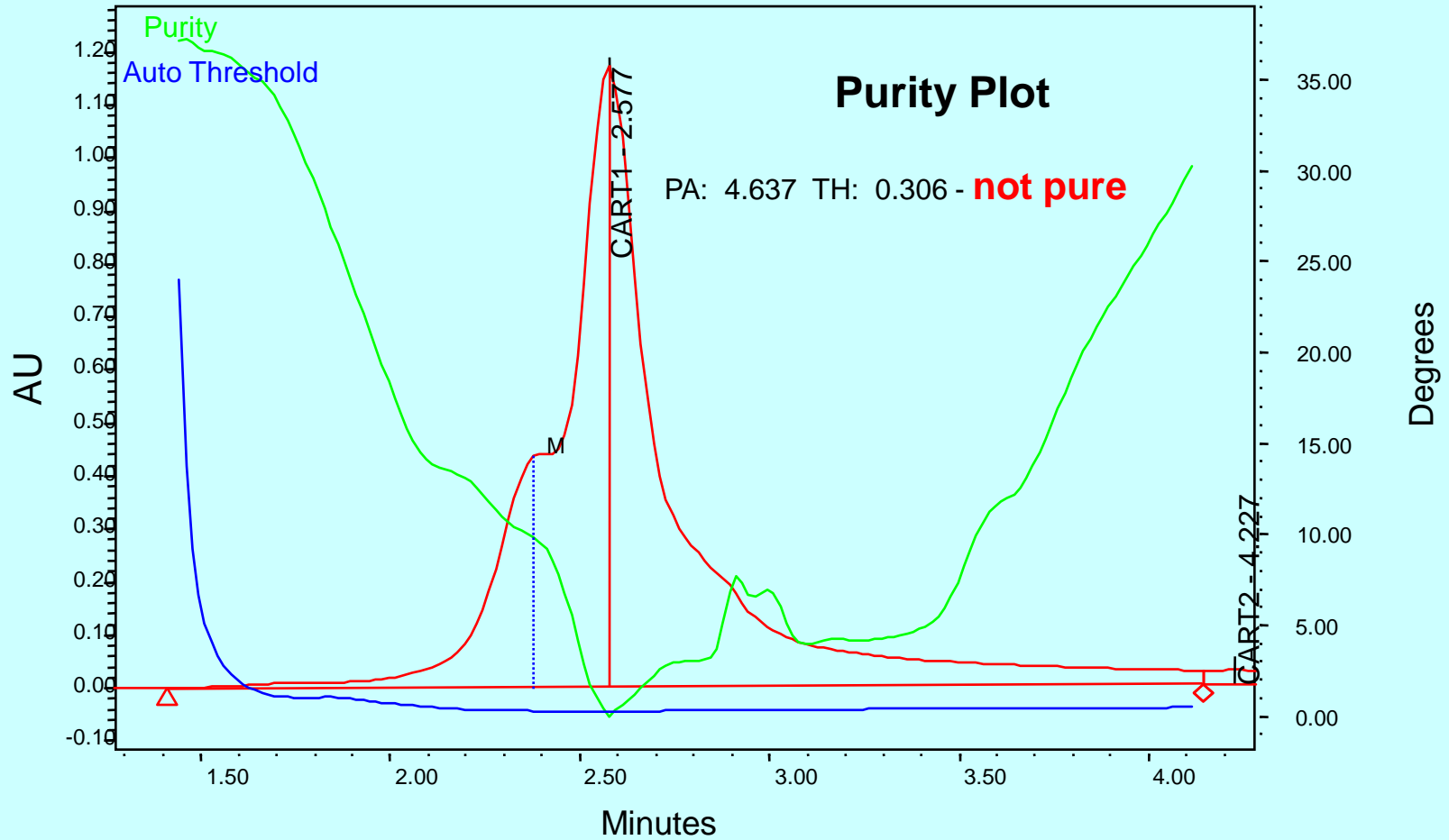
Considerations in Peak Purity Measurements

Questions:

- Is the peak single component peak?
- How many components in a non-single component peak?
- To what extent can the integration events effect the result?
- How indicative is the symmetry of the peak to its purity?
- Can fused peaks be analyzed properly?
- The effect of UV spectrum features on the results?
- The effect of spectral band width on the Peak Purity results

CAROTENOIDS

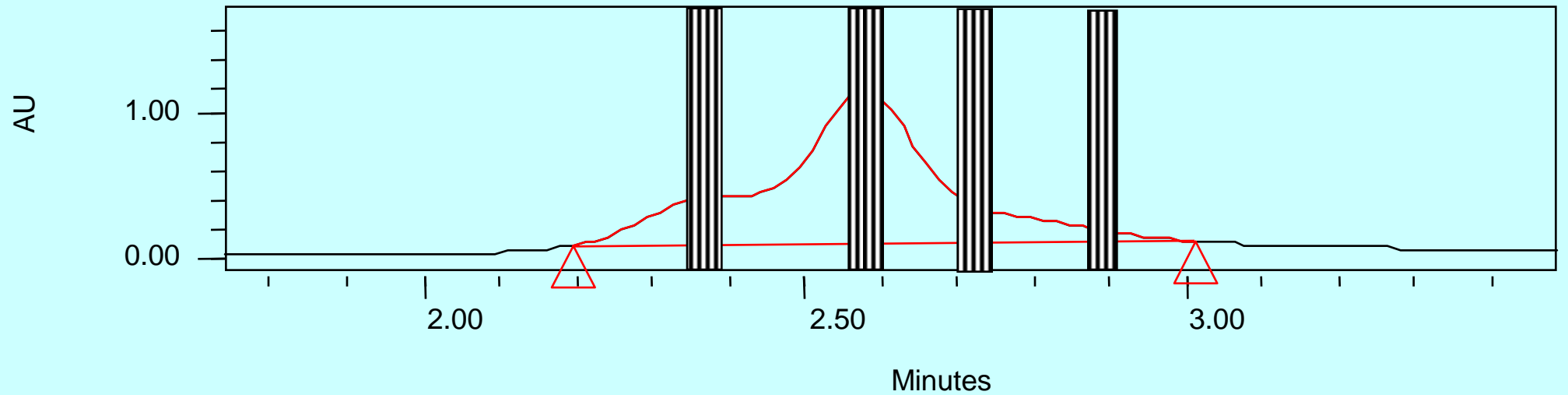
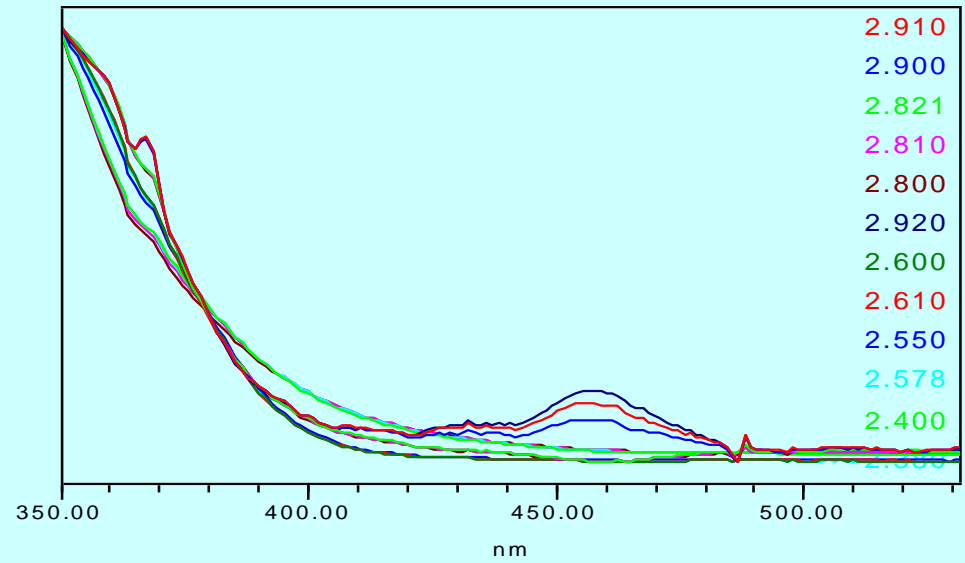
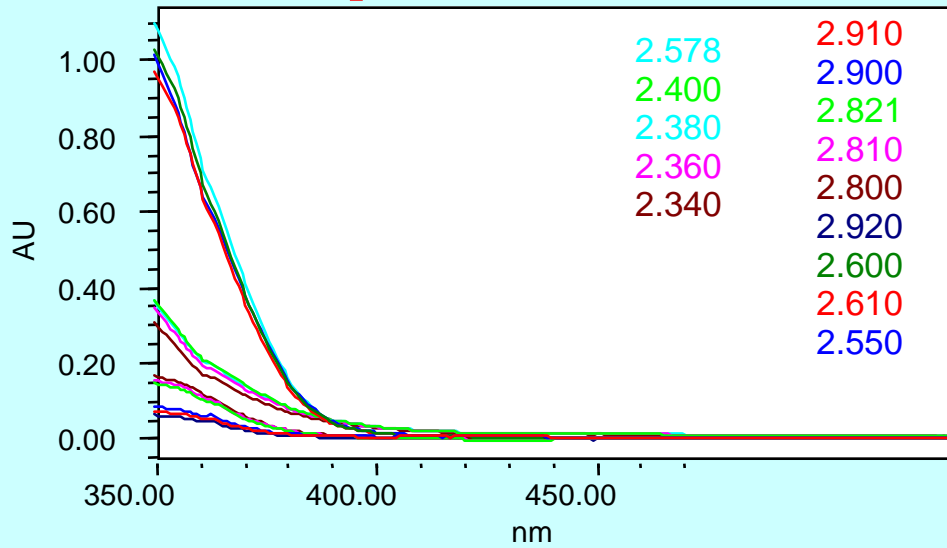
Purity Plot of Peak 1 - Multi-component peak



- Peak
- Purity
- Noise

CAROTENOIDS -

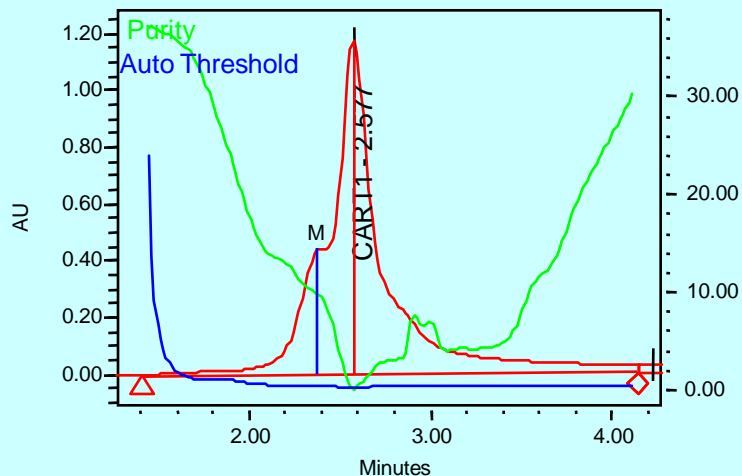
Spectra collected from Peak 1 at various segments of the peak



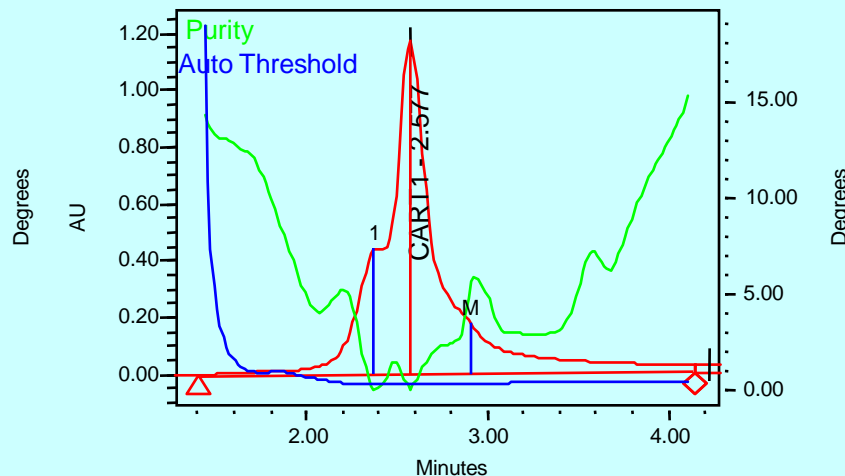
CAROTENOIDS

More than 3 impurities

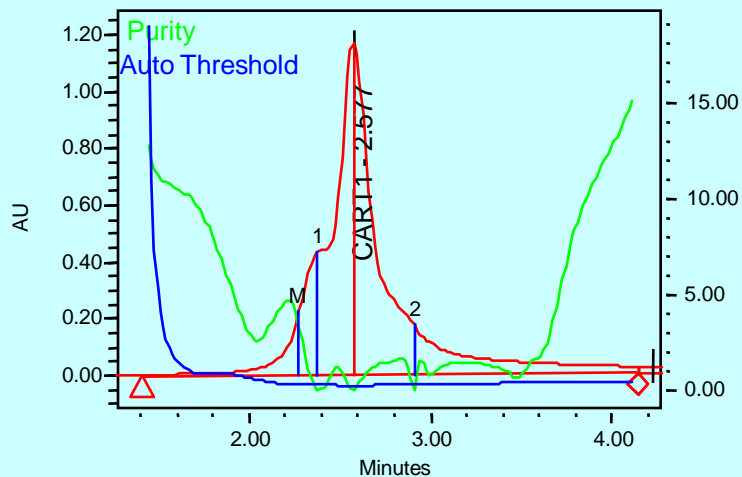
Purity Plot: Multi component detection



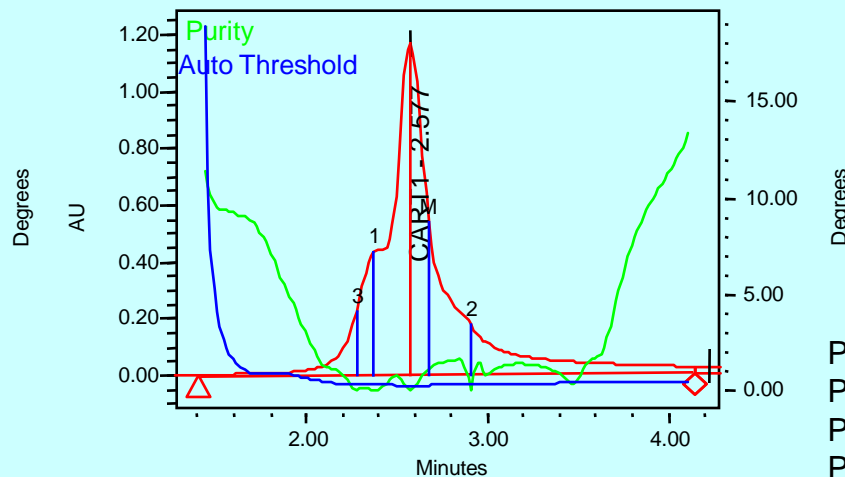
PA: 4.637 TH: 0.306 - not pure 1st impurity



PA: 1.666 TH: 0.308 - not pure 2nd impurity



PA: 1.224 TH: 0.309 - not pure 3 rd impurity



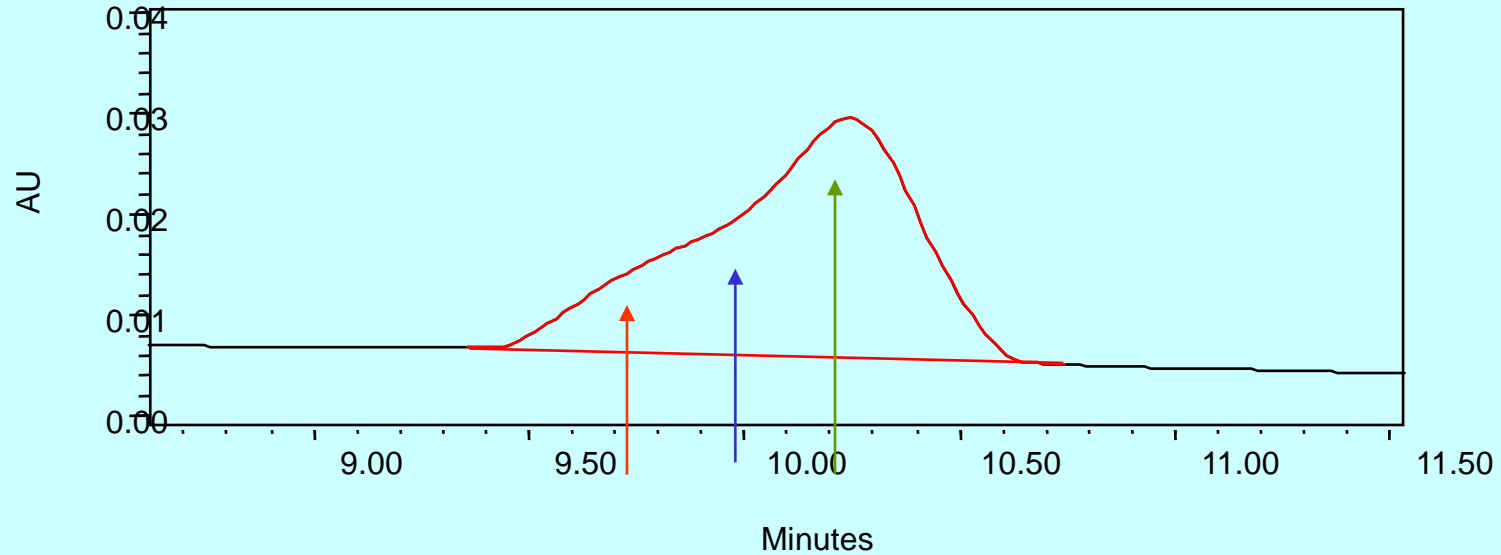
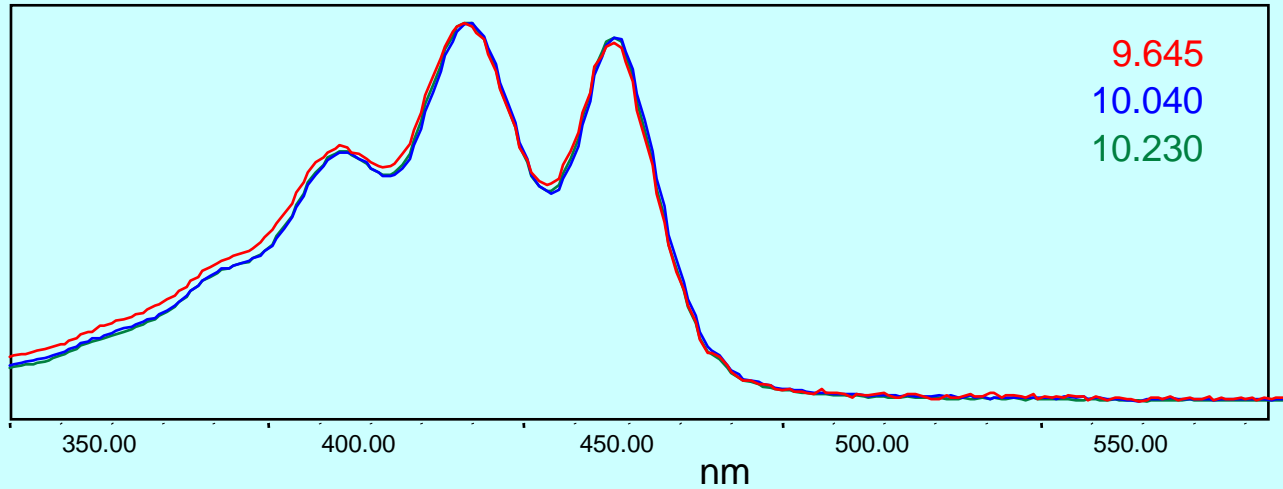
PA: 0.961 TH: 0.309 - not pure: more than 3 impurities

— Peak
— Purity
— Noise

Purity1 Flag: Yes
Purity2 Flag: Yes
Purity3 Flag: Yes
Purity4 Flag: Yes

CAROTENOIDS

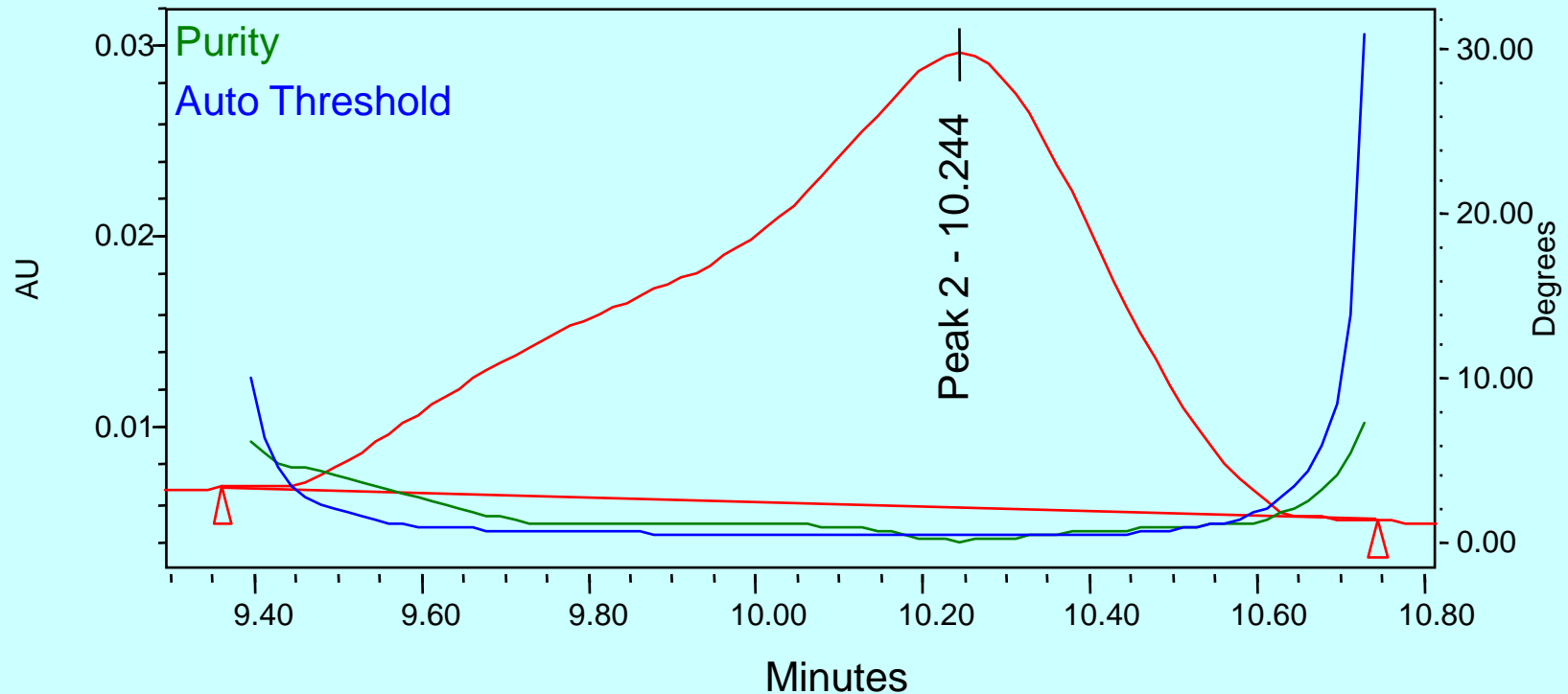
Spectra Selected from Peak 2: 2 impurities



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Purity Plot of Peak 2: 2 impurities

	Purity Angle	Purity Threshold	Maximum Impurity	Purity Flag
1	0.894	0.497	10.045	Yes
2	0.596	0.529	9.645	Yes



Considerations in Peak Purity Measurements

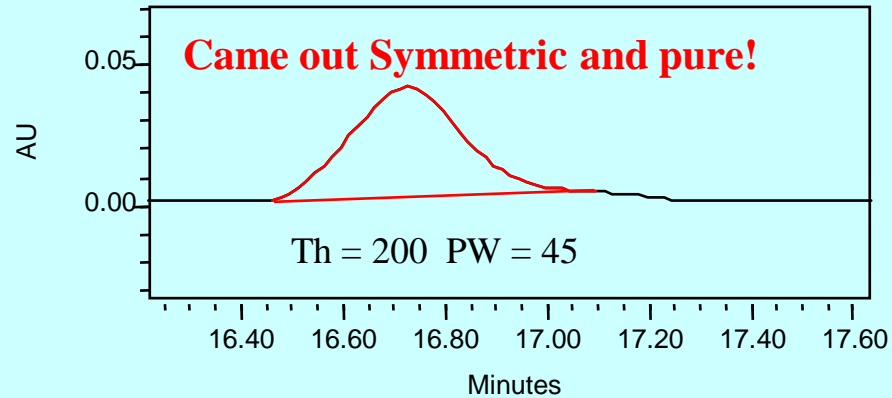
Questions:

- Is the peak single component peak?
- How many components in a non-single component peak?
- To what extent can the integration events effect the result?
- How indicative is the symmetry of the peak to its purity?
- Can fused peaks be analyzed properly?
- The effect of UV spectrum features on the results?
- The effect of spectral band width on the Peak Purity results

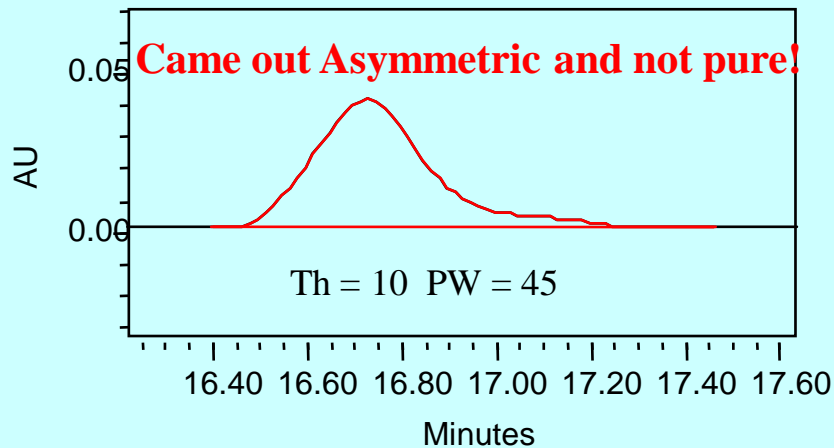
CAROTENOIDS

Effect of Integration Events on Peak Purity Results

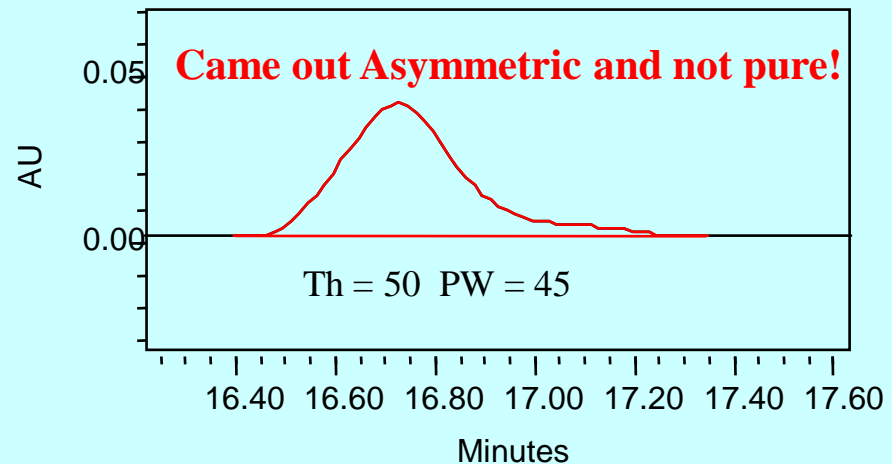
Purity Angle	Purity Threshold	USP Tailing
0.297	0.380	1.057



Purity Angle	Purity Threshold	USP Tailing
2.259	0.410	1.438



Purity Angle	Purity Threshold	USP Tailing
1.682	0.401	1.415

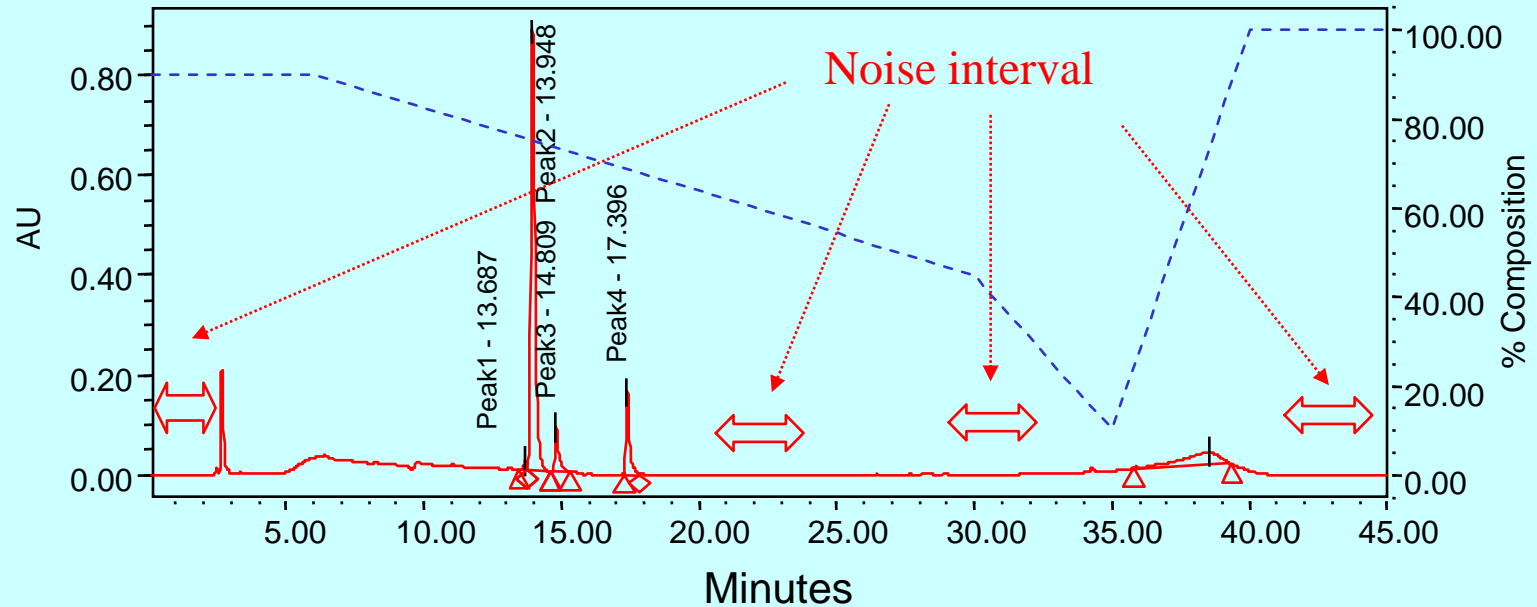


Peptides

Effect of Integration Events on Peak Purity Results

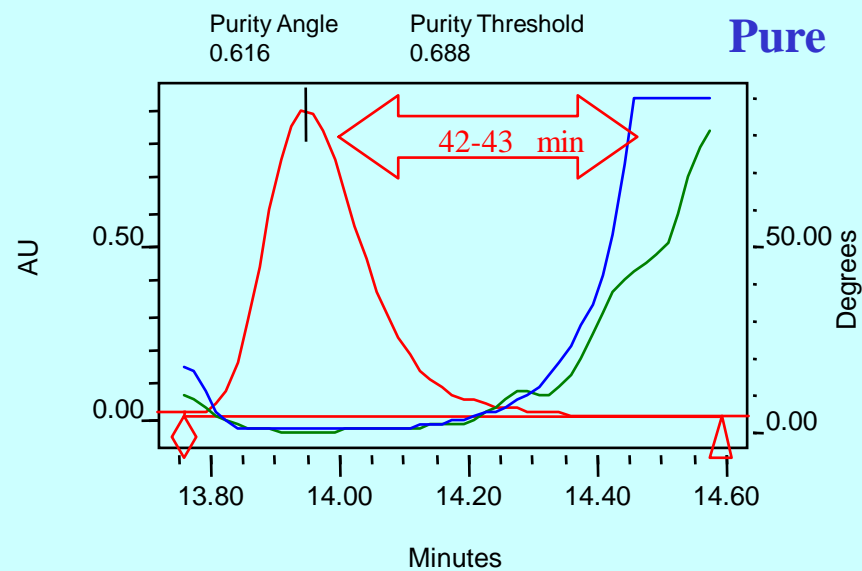
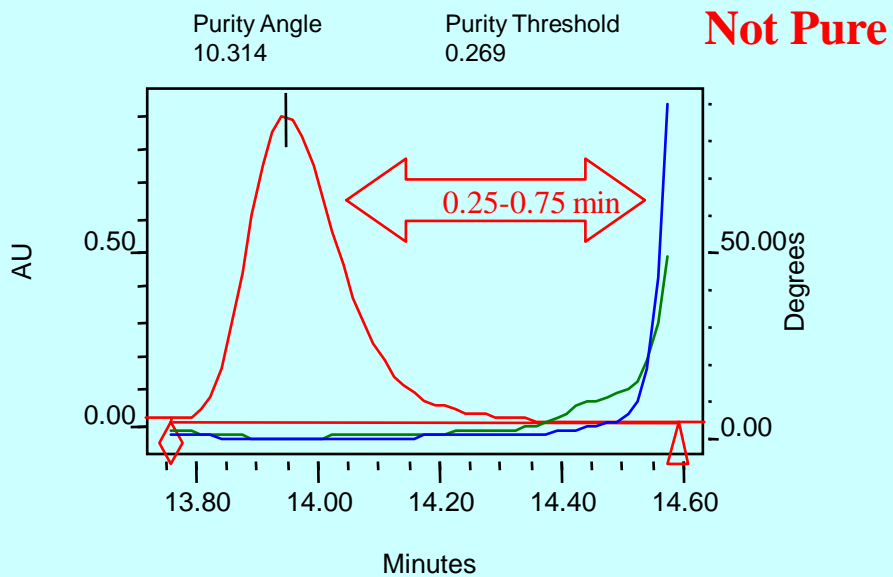
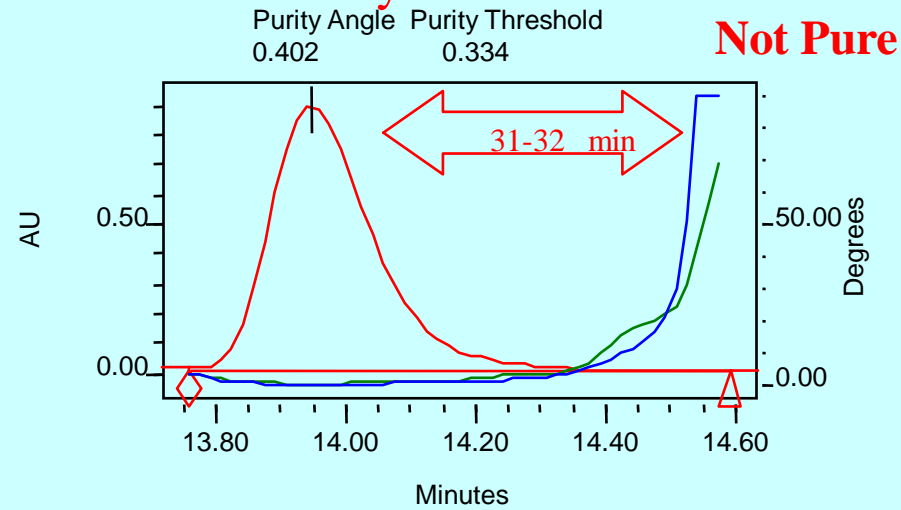
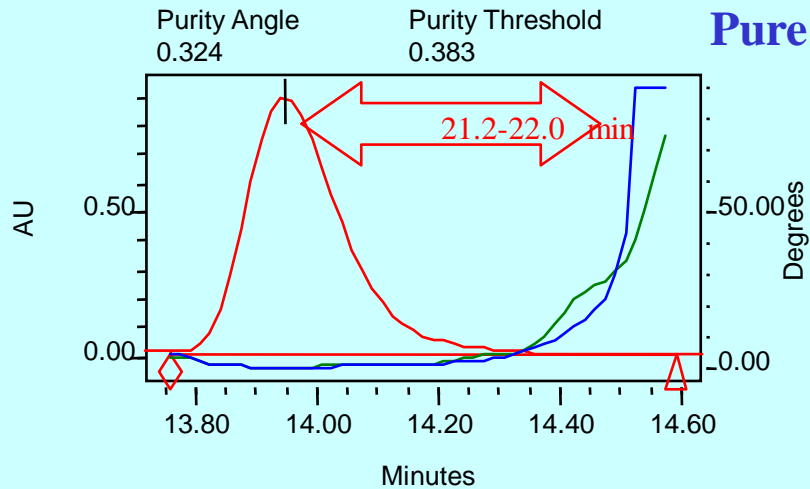
Gradient Table

Time	Flow	%A	%B
	1.00	90.0	10.0
30.00	1.00	45.0	55.0
35.00	1.00	10.0	90.0
40.00	1.00	100.0	0.0
45.00	1.00	100.0	0.0



Peptides

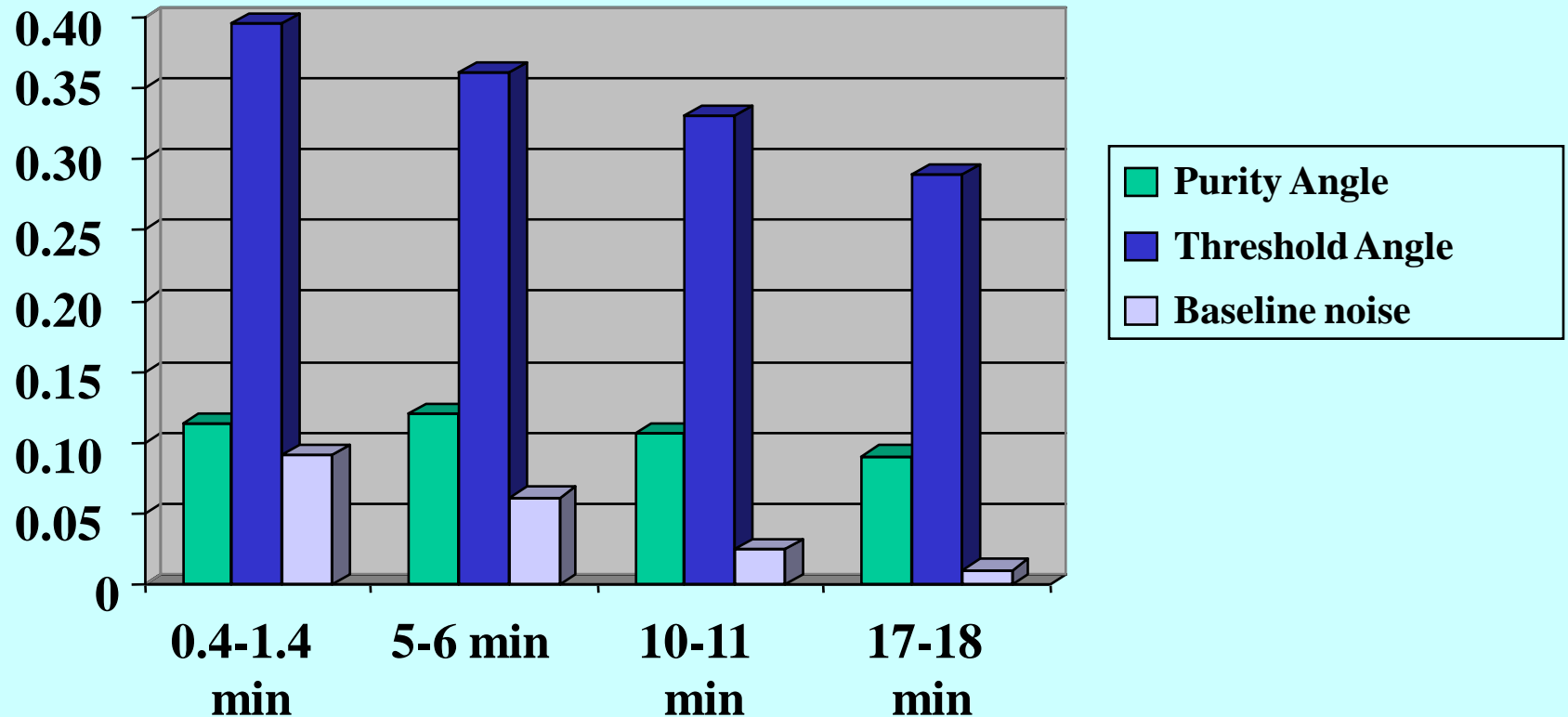
Effect of Noise Interval Selection on Peak Purity Results



Substance G

Noise Interval	Purity Angle	Threshold Angle	Baseline noise (mv)
0.4-1.4 min	0.114	0.395	0.092
5-6 min	0.121	0.361	0.062
10-11 min	0.107	0.330	0.026
17-18 min	0.091	0.289	0.011

The lower the baseline noise, the lower are the values of Threshold and Purity Angles



Considerations in Peak Purity Measurements

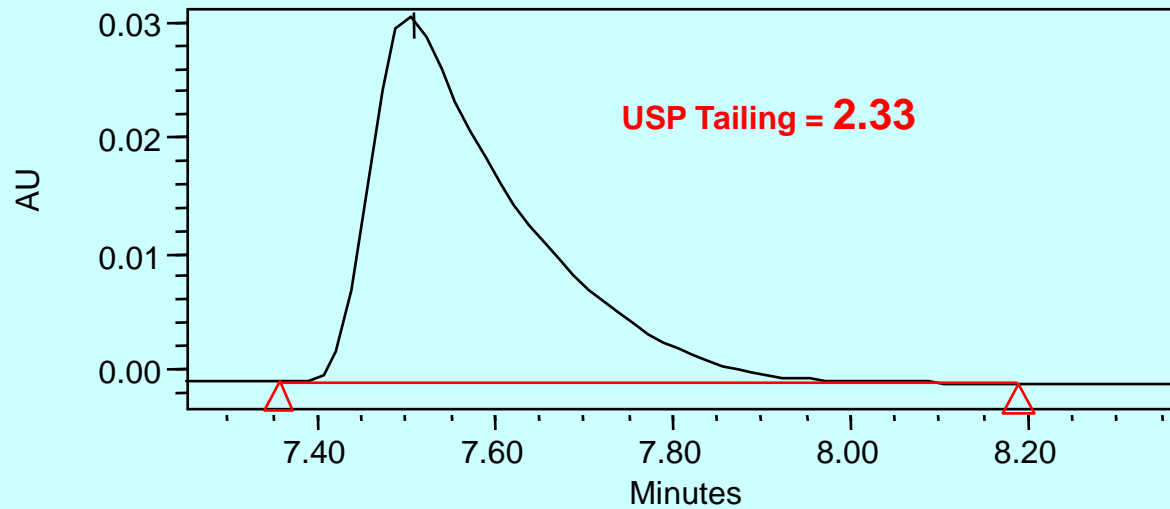
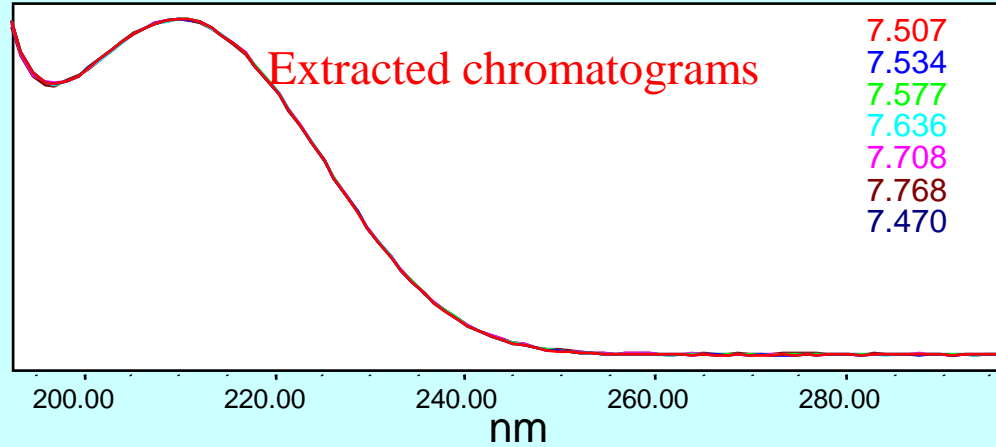
Questions:

- Is the peak single component peak?
- How many components in a non-single component peak?
- To what extent can the integration events effect the result?
- How indicative is the symmetry of the peak to its purity?
- Can fused peaks be analyzed properly?
- The effect of UV spectrum features on the results?
- The effect of spectral band width on the Peak Purity results

Substance G Chromatogram

Peak is asymmetric but pure!

Name	Purity Angle	Purity Threshold
GBPN	0.217	0.383



Considerations in Peak Purity Measurements

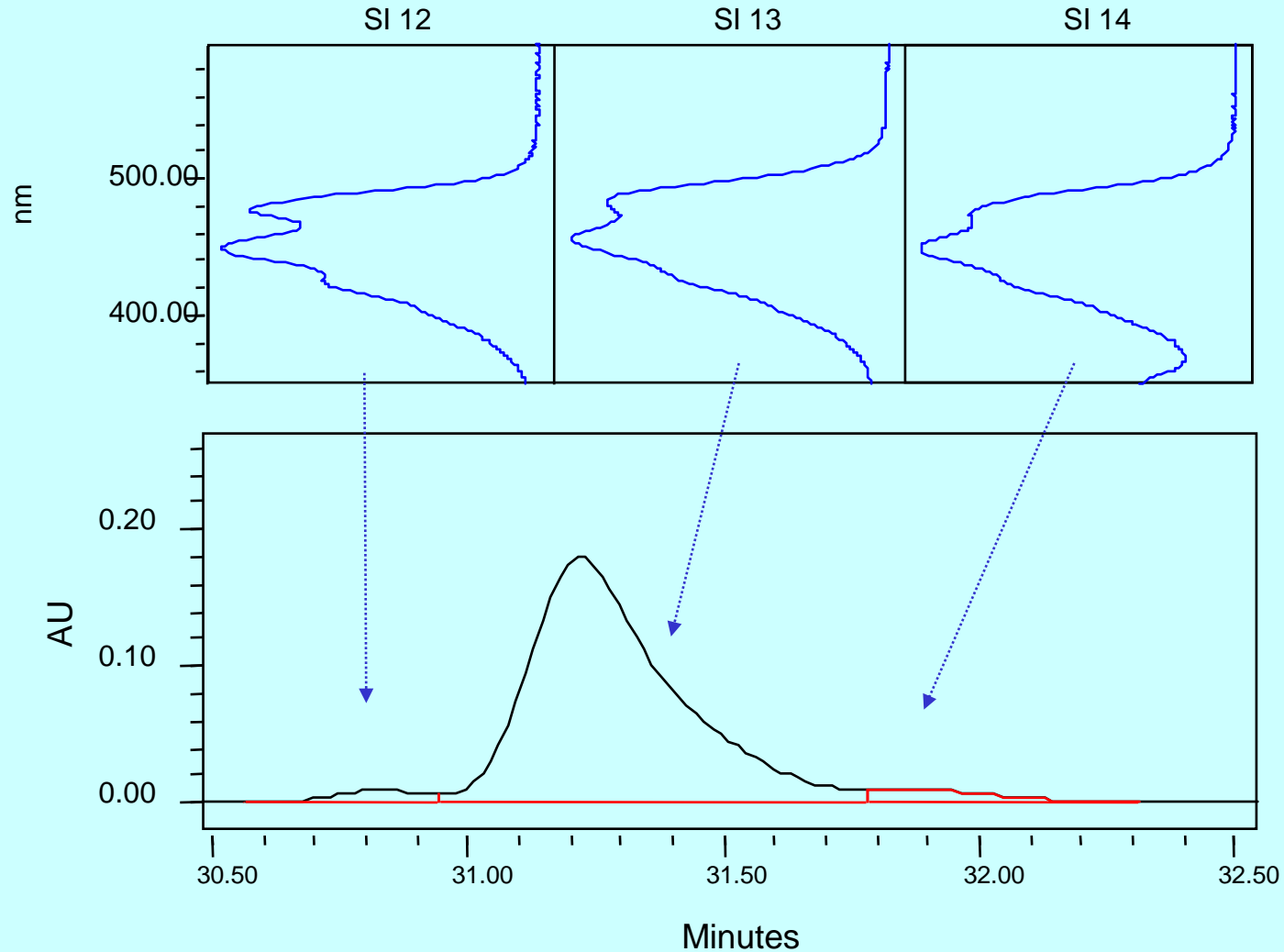
Questions:

- Is the peak single component peak?
- How many components in a non-single component peak?
- To what extent can the integration events effect the result?
- How indicative is the symmetry of the peak to its purity?
- Can fused peaks be analyzed properly?
- The effect of UV spectrum features on the results?
- The effect of spectral band width on the Peak Purity results

CAROTENOIDS

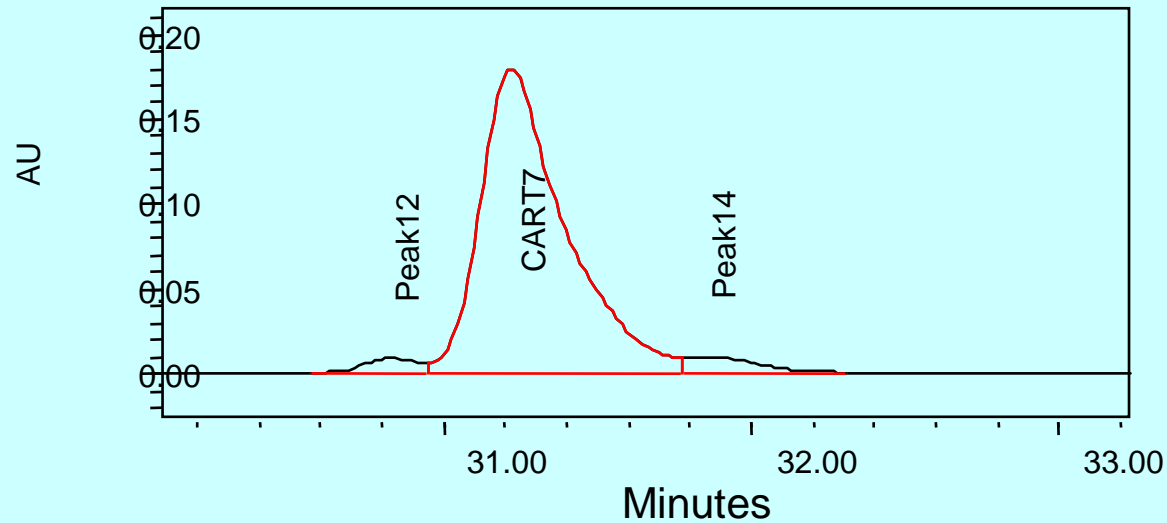
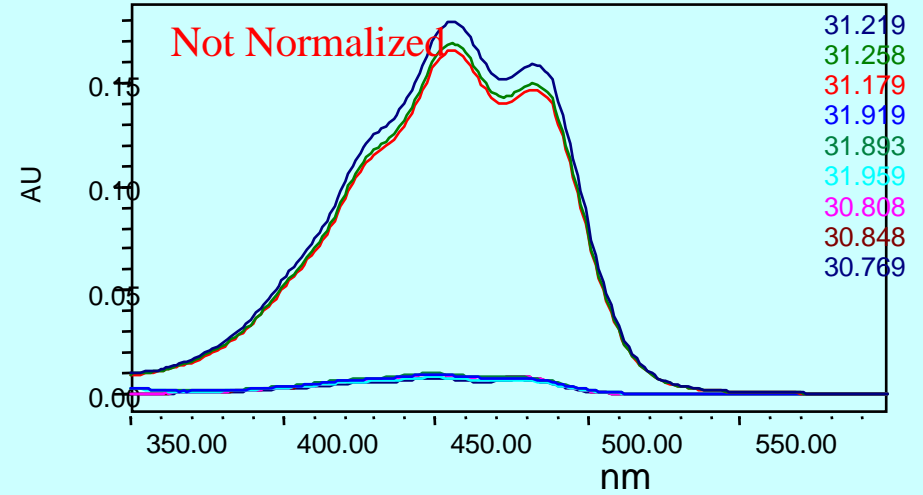
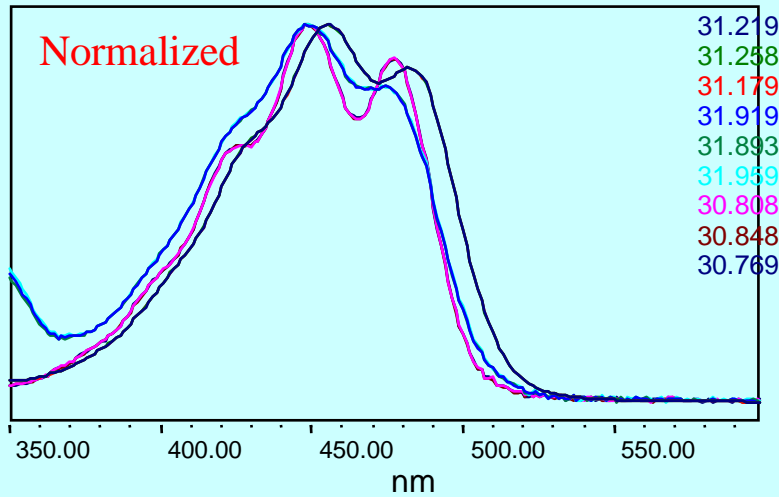
The Answer is YES, the software takes the valley into consideration!

Example: Spectra of Peak 10 and its adjacent peaks



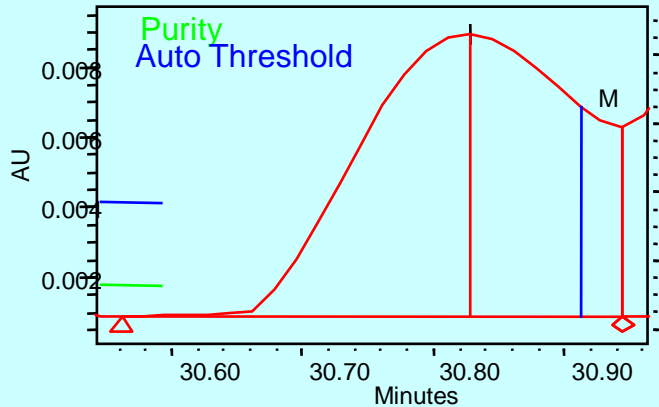
CAROTENOIDS

Spectra of Peak 10 and its adjacent peaks - overlaid

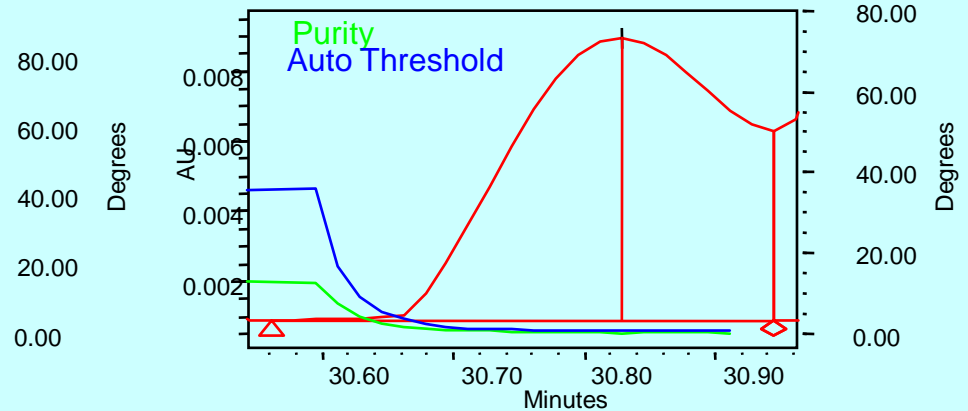


CAROTENOIDS

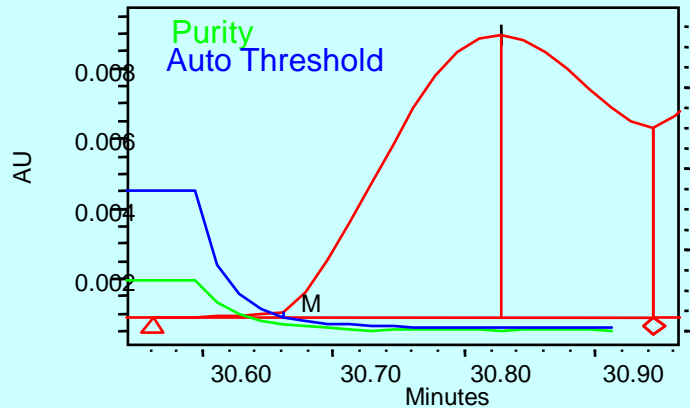
Multi component Purity Plot of the small peak preceding Peak 10 Peak is pure



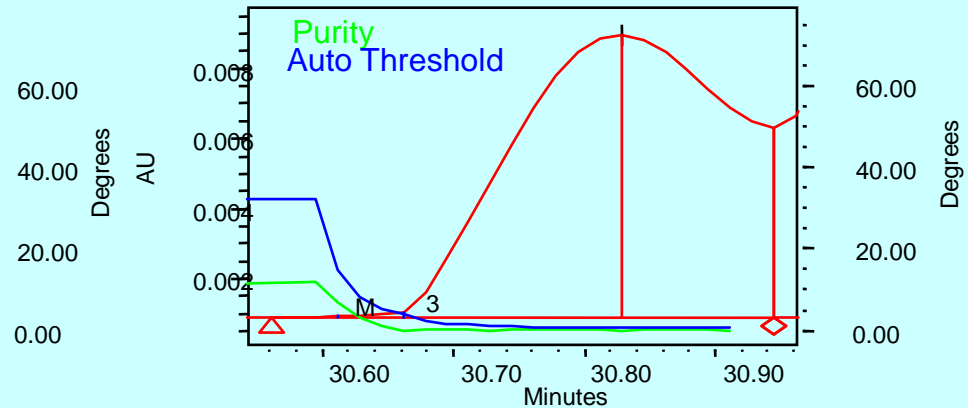
PA: 0.397 TH: 0.882



PA: 0.345 TH: 0.866



PA: 0.293 TH: 0.857



PA: 0.250 TH: 0.837

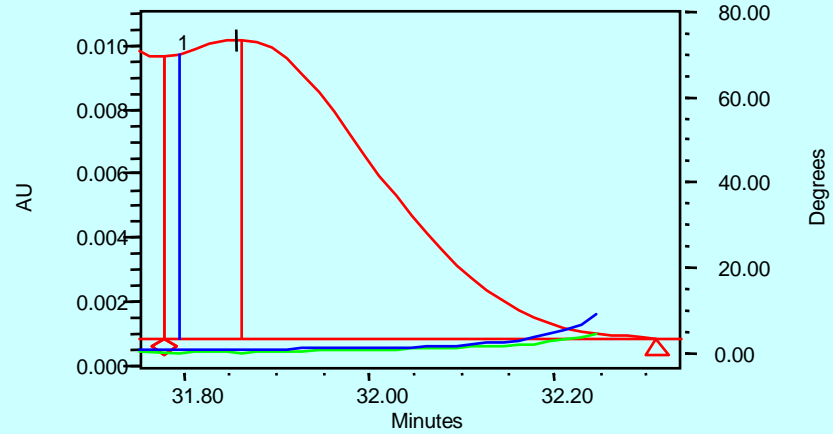
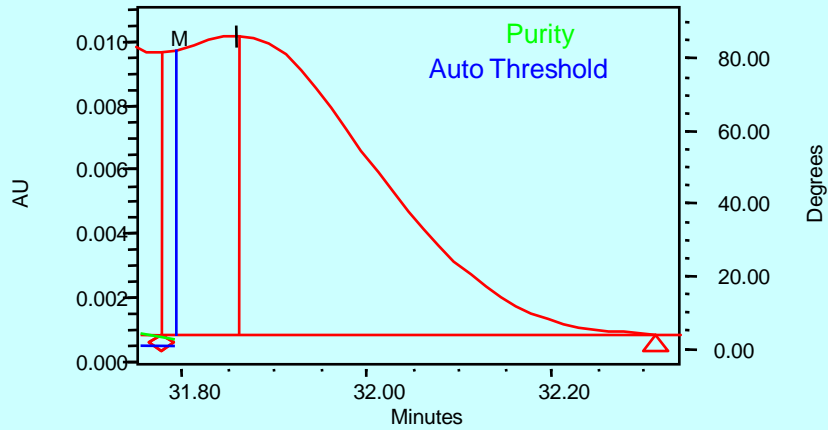
— Peak
— Purity
— Noise

Purity1 Flag: No
Purity2 Flag: No
Purity3 Flag: No
Purity4 Flag: No

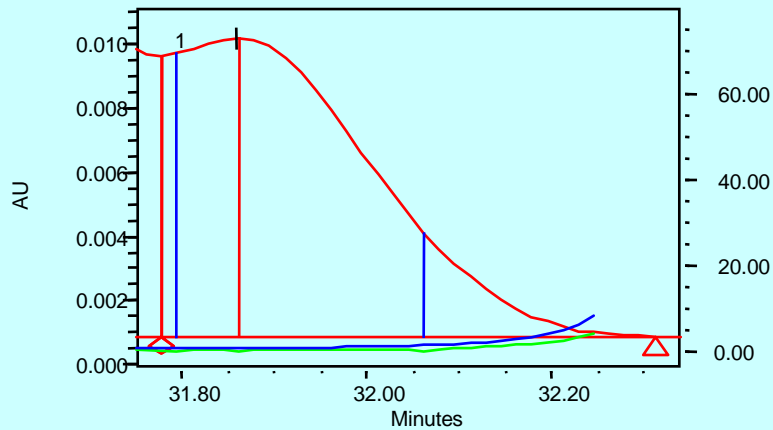
CAROTENOIDS

Multi component Purity Plot of small peak following Peak 10

Peak is not pure

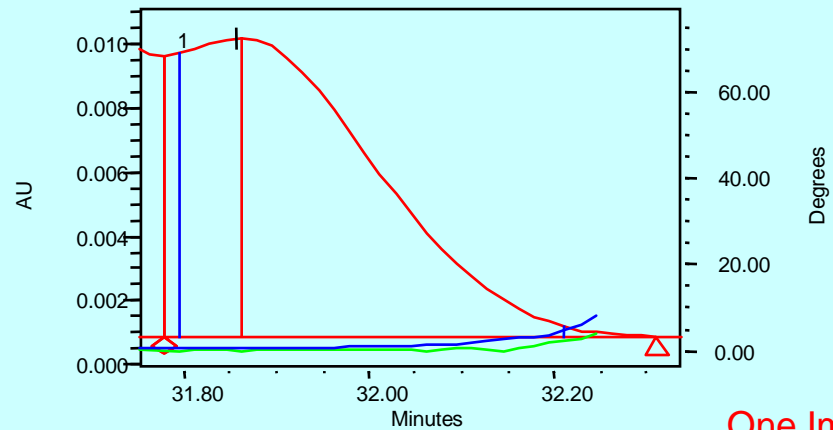


PA: 1.222 TH: 0.809



PA: 0.342 TH: 0.859

PA: 0.526 TH: 0.906



PA: 0.305 TH: 0.839

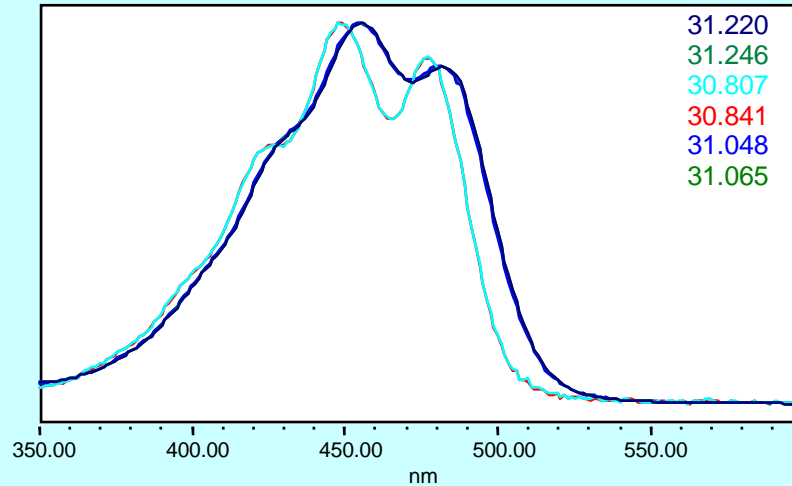
— Peak
— Purity
— Noise

One Impurity:
Purity1 Flag: Yes
Purity2 Flag: No
Purity3 Flag: No
Purity4 Flag: No

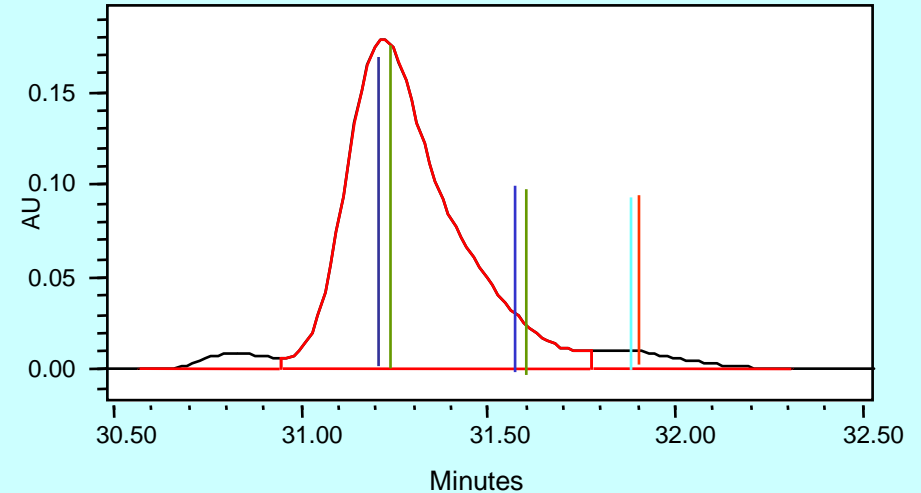
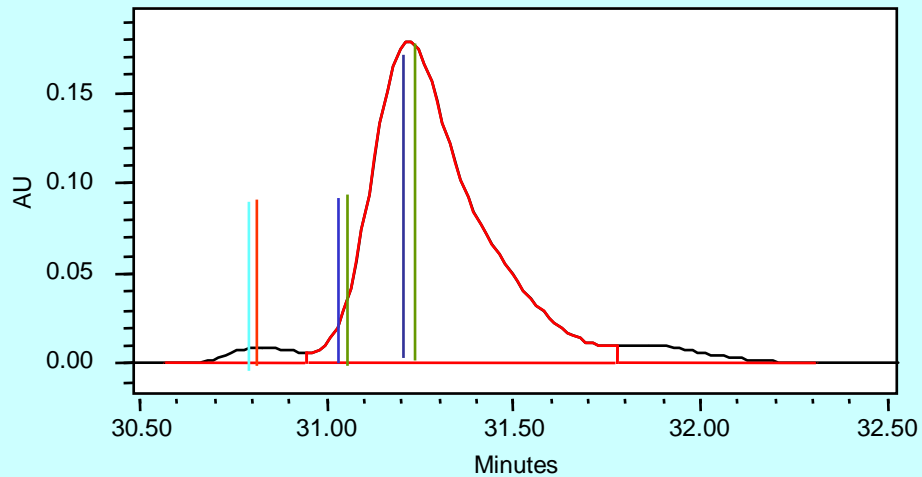
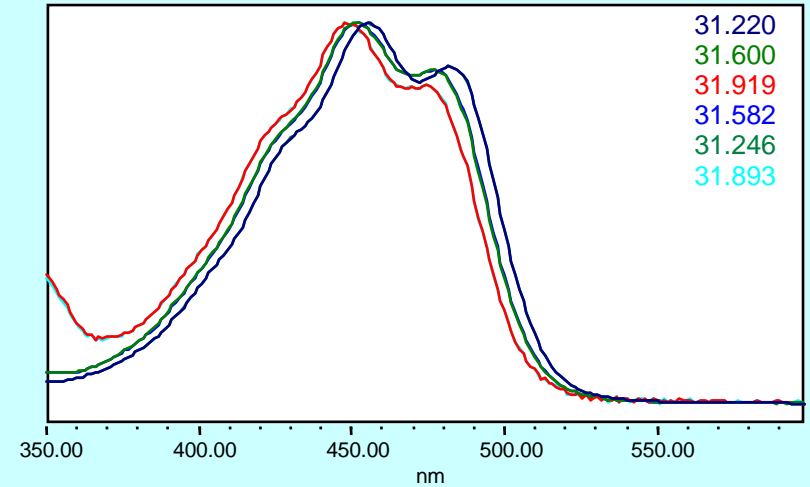
CAROTENOIDS

Spectra from Peak 10 compared to preceding and following peaks

Peak 10 and preceding peak

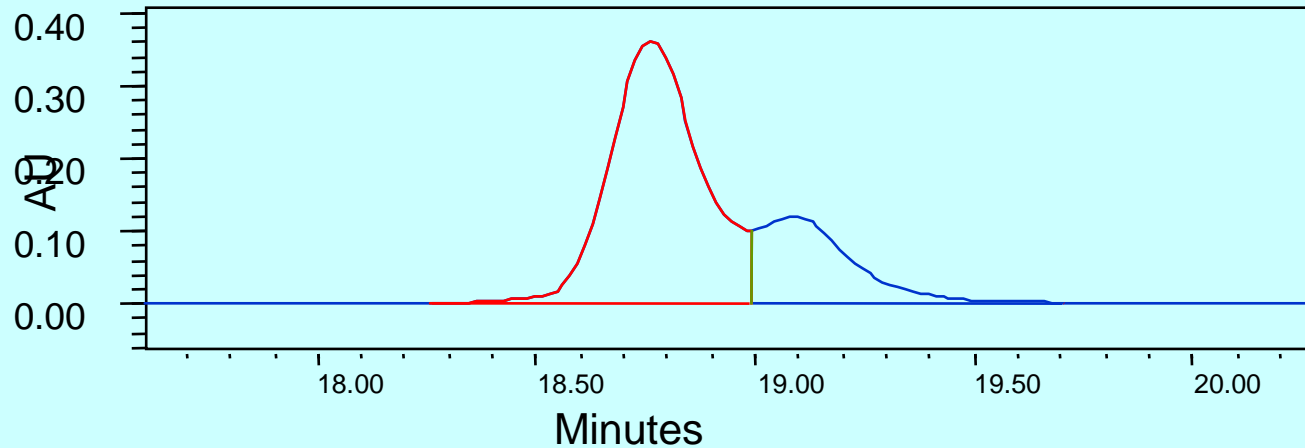
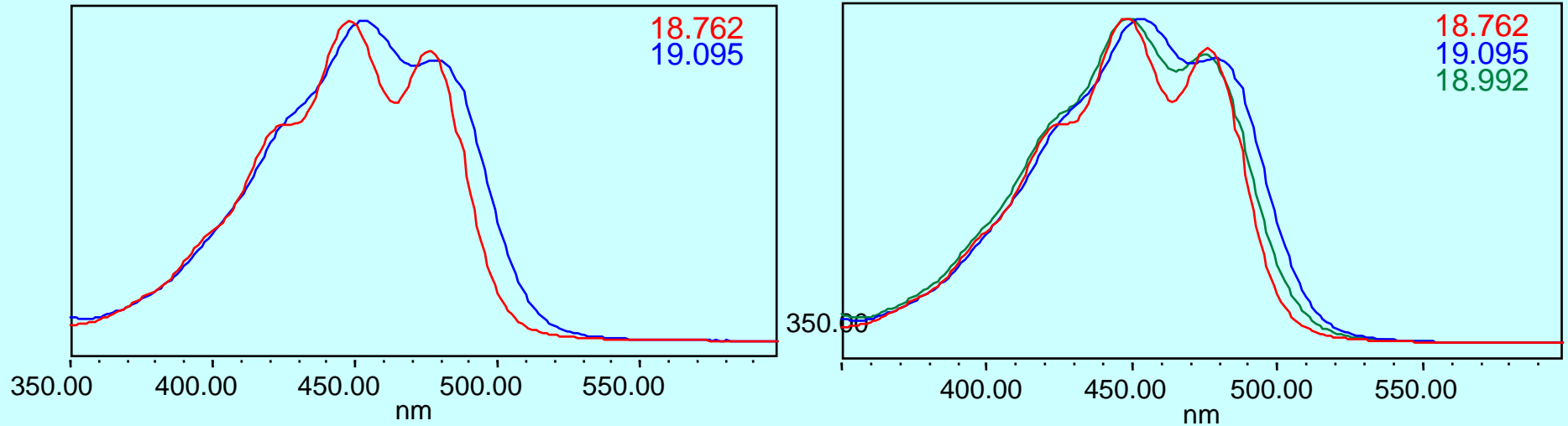


Peak 10 and following peak



CAROTENOIDS

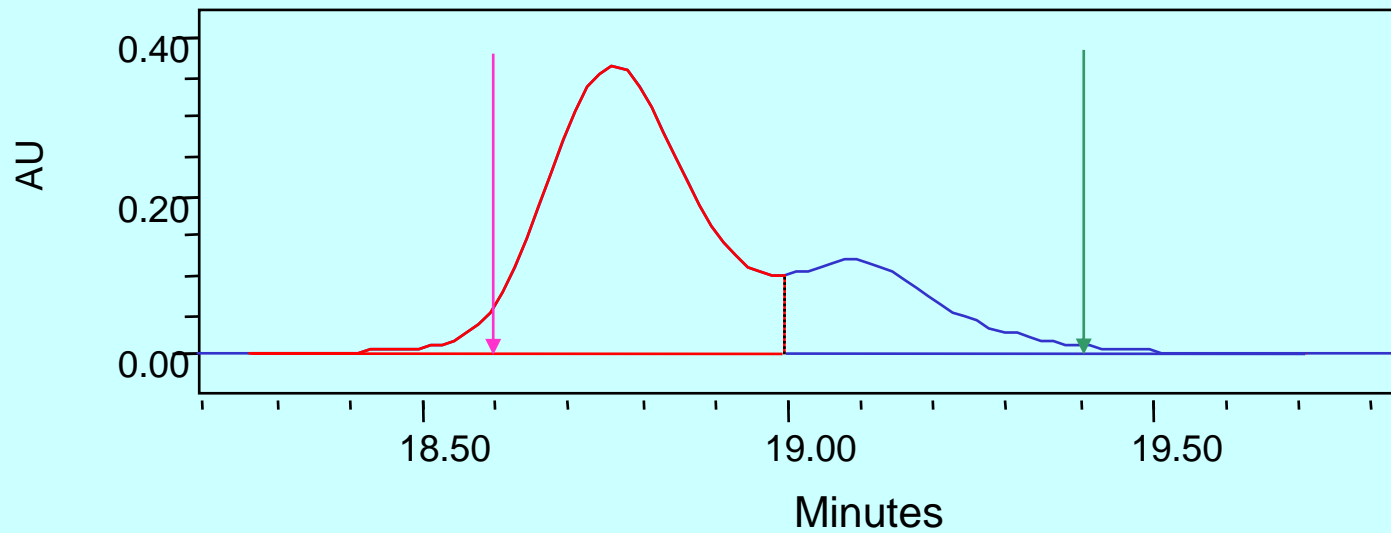
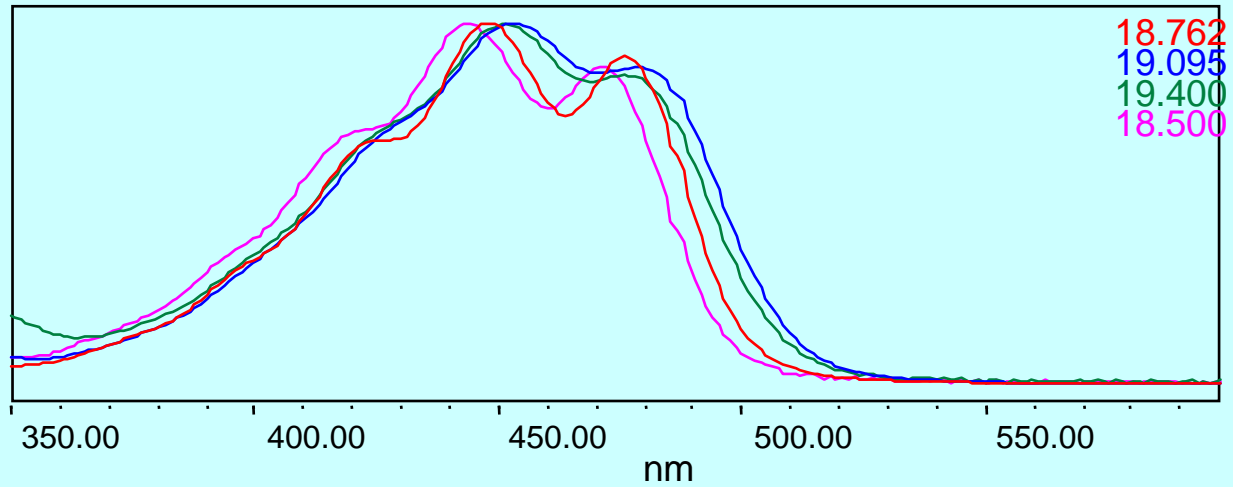
Spectra collected from Peak 5/6 and the intermediate point



CAROTENOIDS

Spectra collected from Peak 5/6:

Front, Intermediate, Apex and Rear points

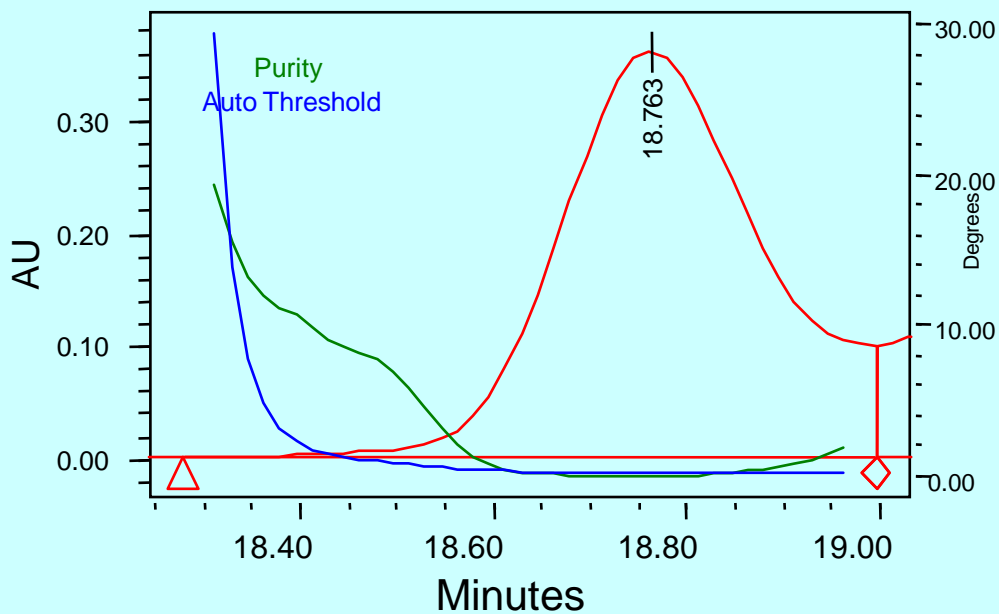


CAROTENOIDS

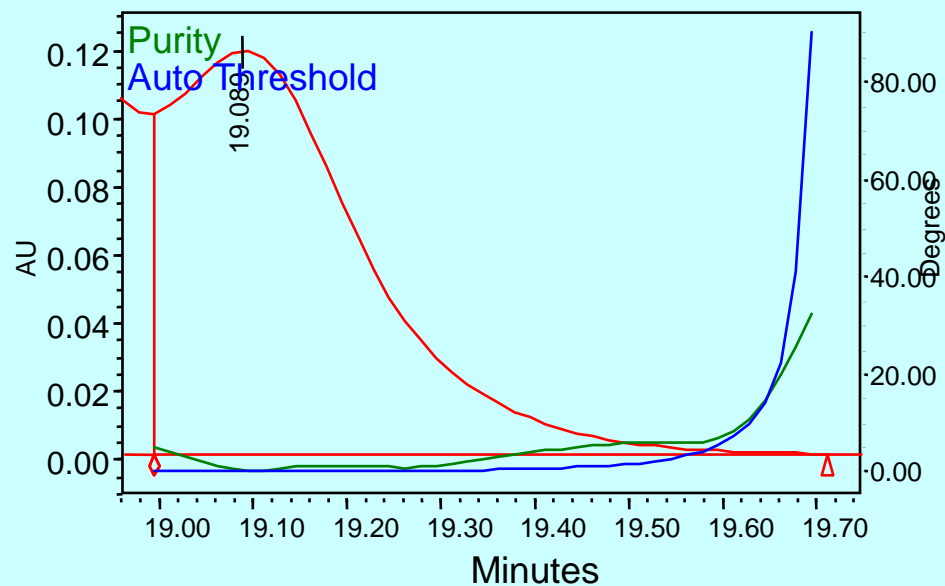
Purity Plot of Peaks 5 and 6

Purity Angle	Purity Threshold	Maximum Impurity	Purity Flag
0.403	0.240	18.962	Yes
0.236	0.243	18.912	No

Purity Angle	Purity Threshold	Maximum Impurity	Purity Flag
2.163	0.260	18.995	Yes
0.385	0.265	19.328	yes



1 Impurity



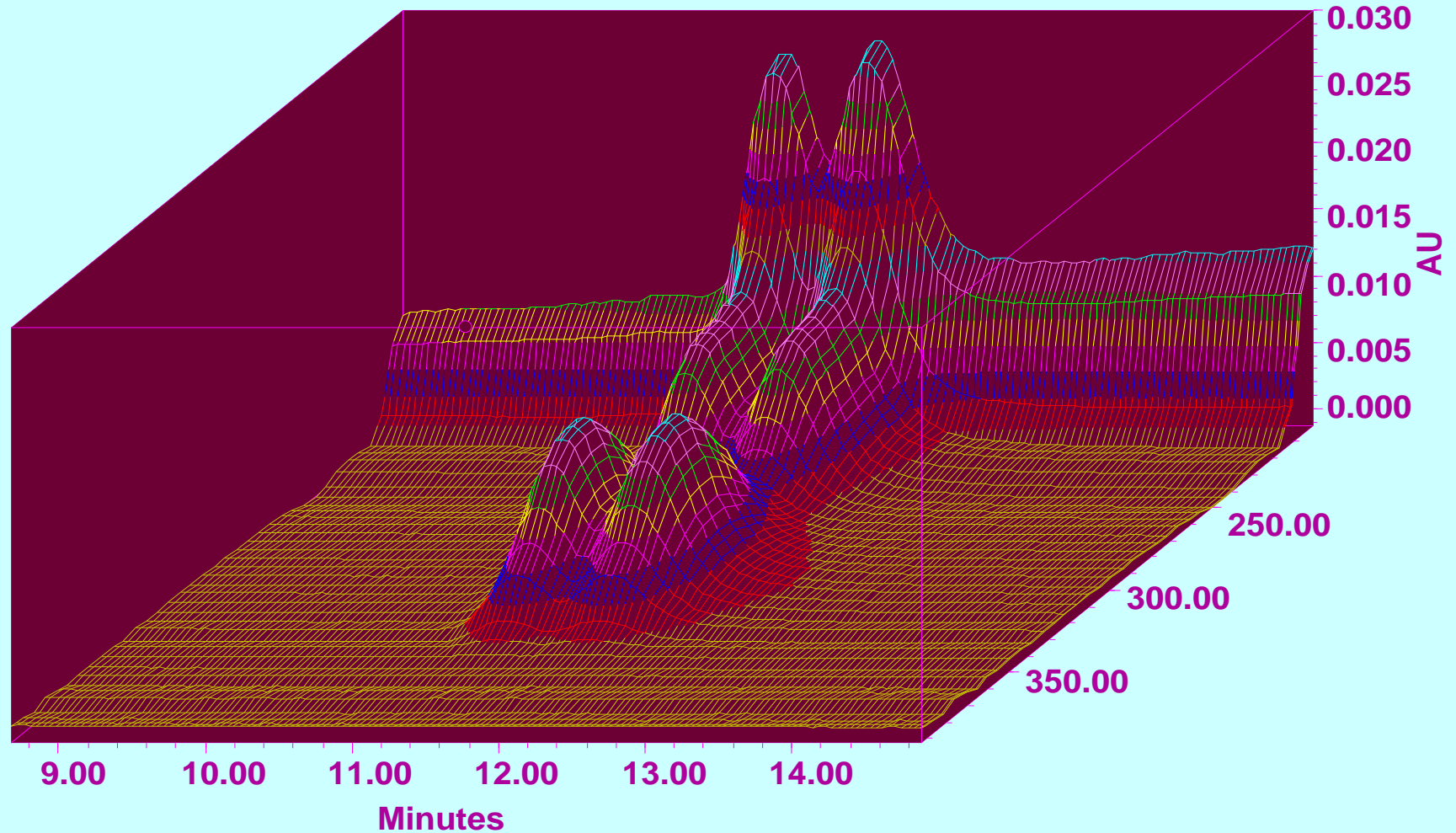
2 Impurities

Considerations in Peak Purity Measurements

Questions:

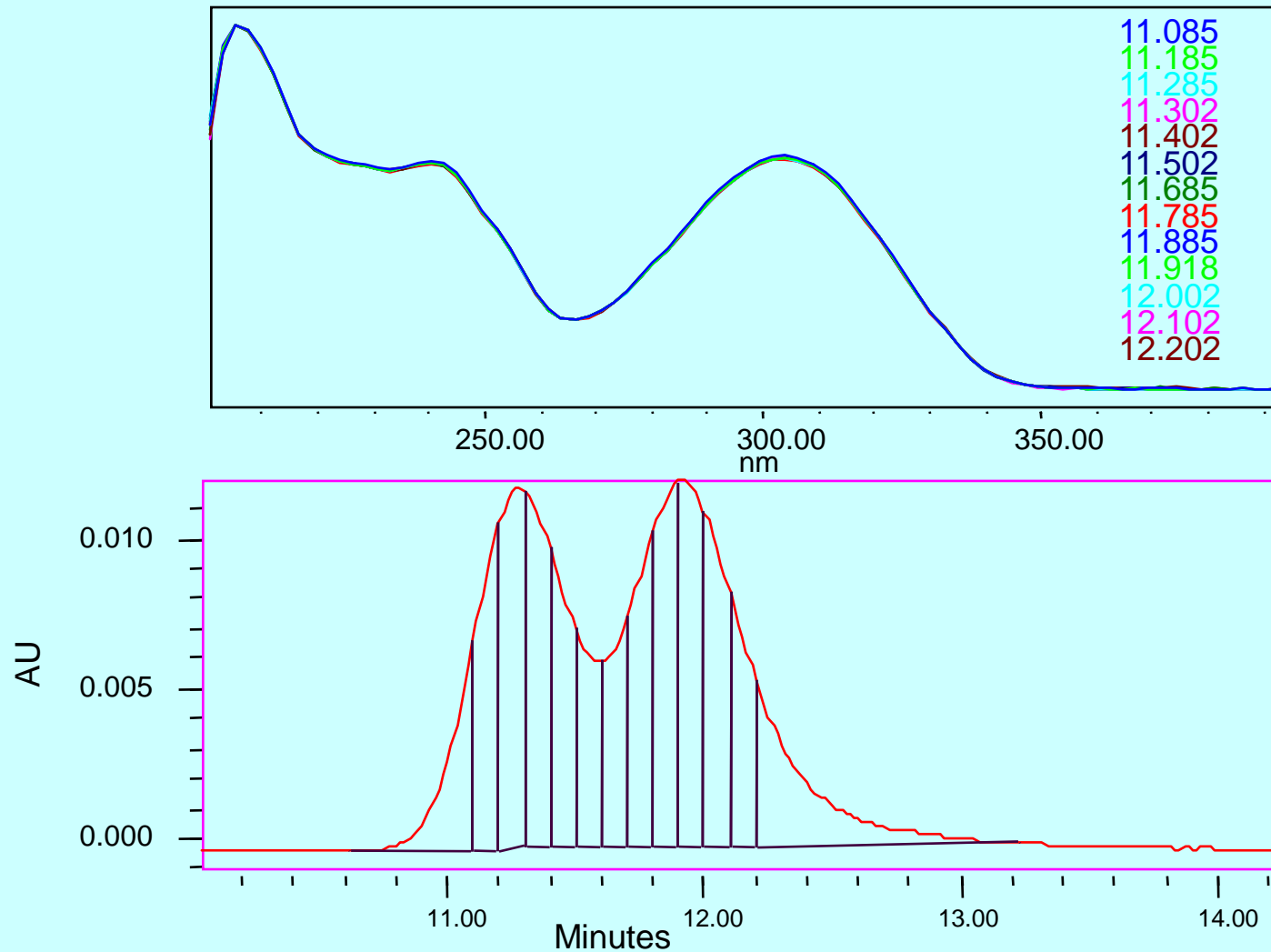
- Is the peak single component peak?
- How many components in a non-single component peak?
- To what extent can the integration events effect the result?
- How indicative is the symmetry of the peak to its purity?
- Can fused peaks be analyzed properly?
- The effect of UV spectrum features on the results?
- The effect of spectral band width on the Peak Purity results

Nucleoside analog's Enantiomers: No Use in Peak Purity Measurements: 3D Plot

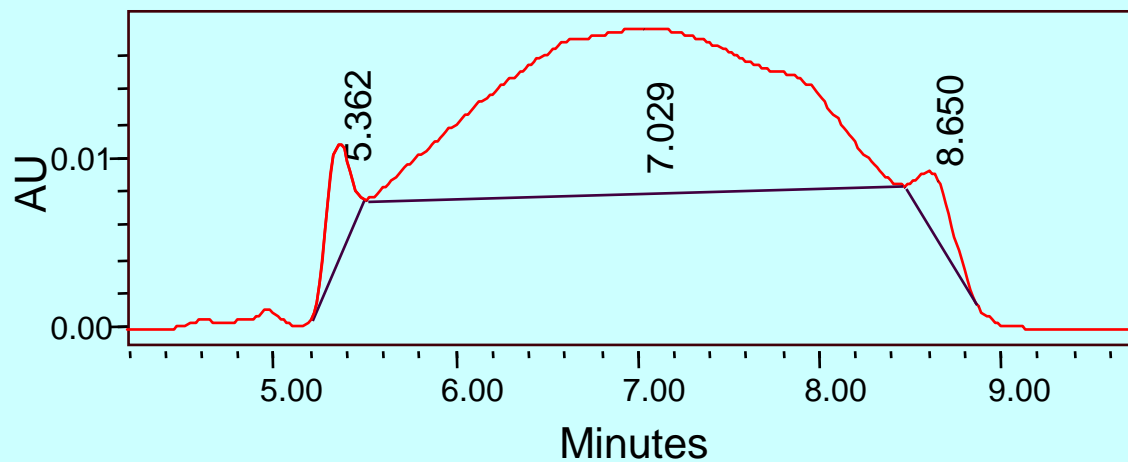
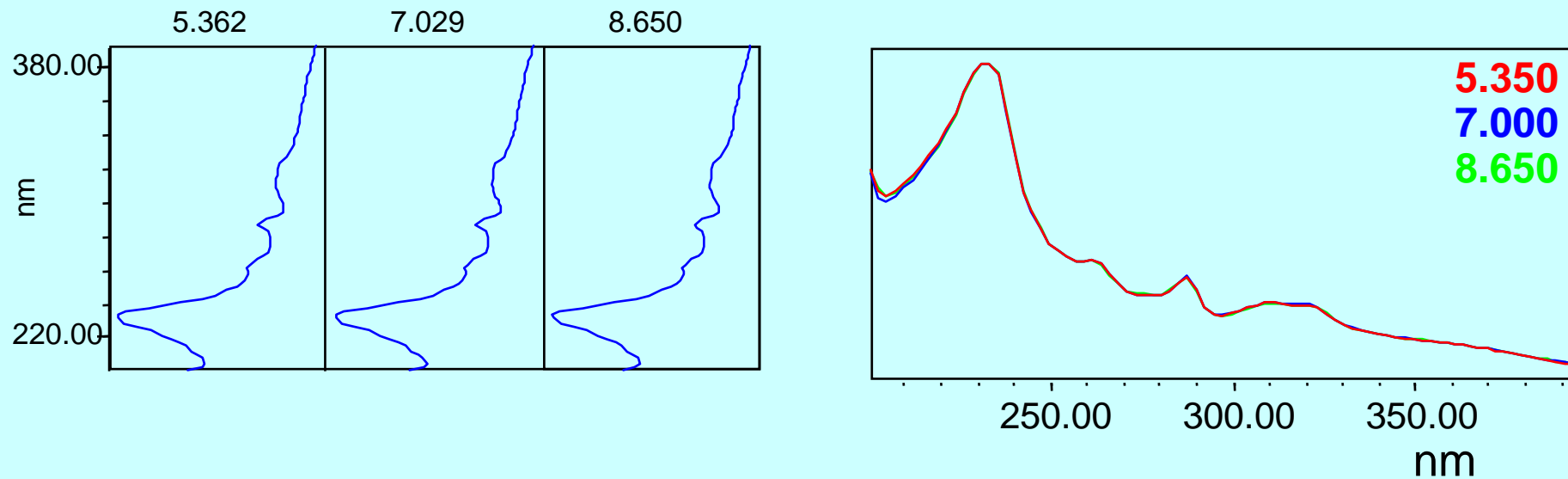


Nucleoside analog' Enantiomers: All spectra are identical

Spectra collected from the two peaks

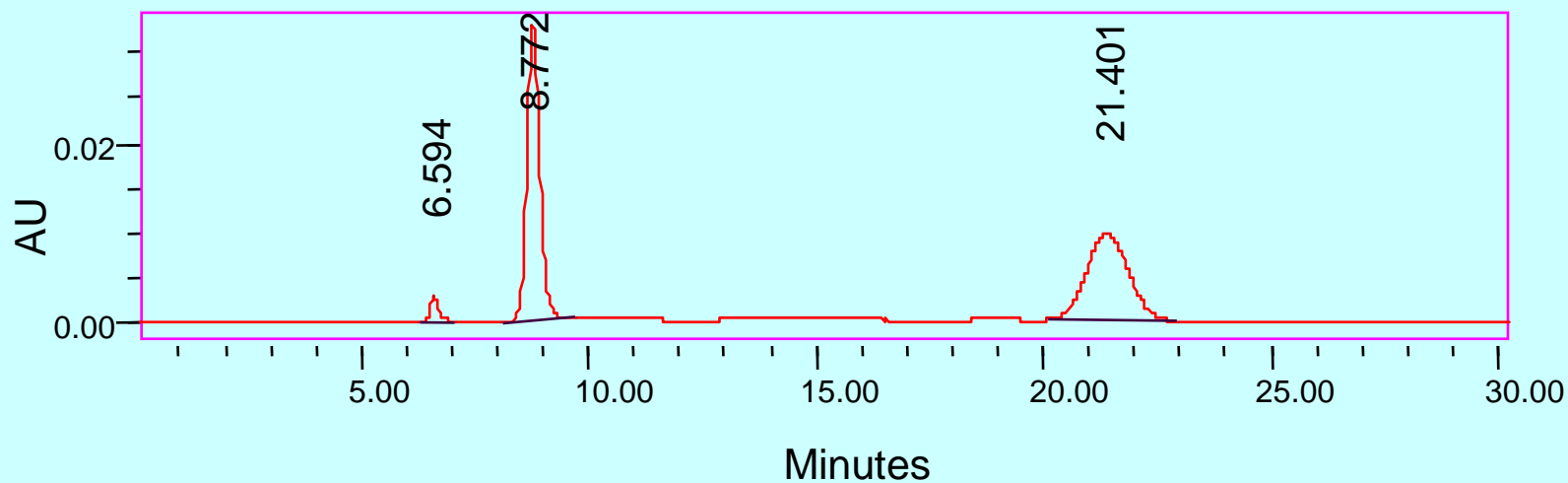
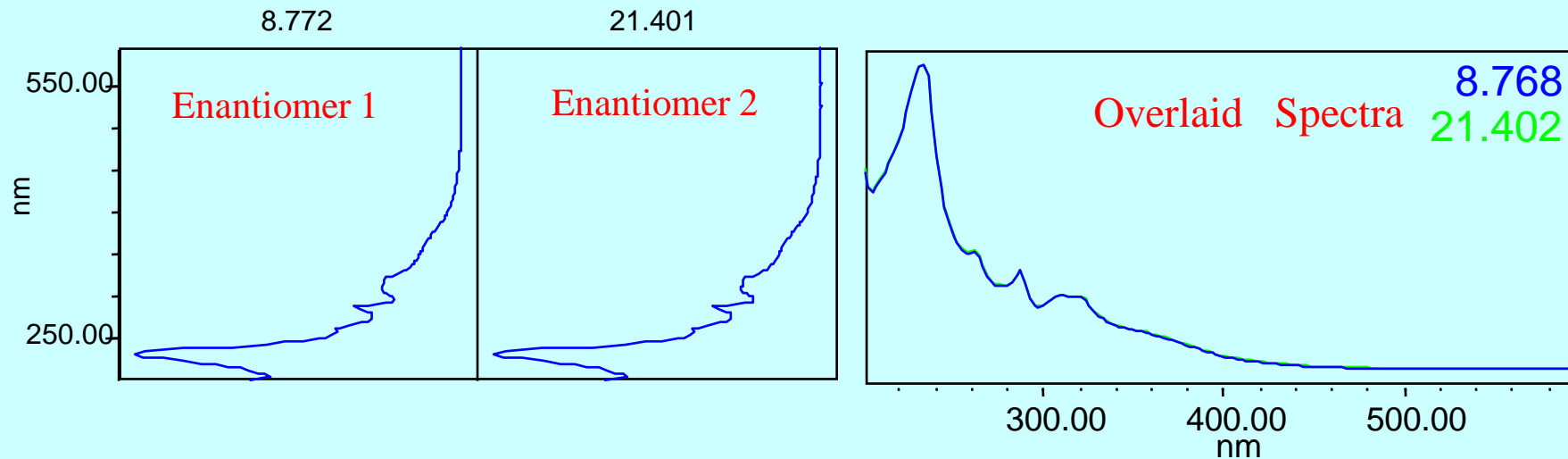


Sulfamide Enantiomers: Chiral Inversion at Room Temperature



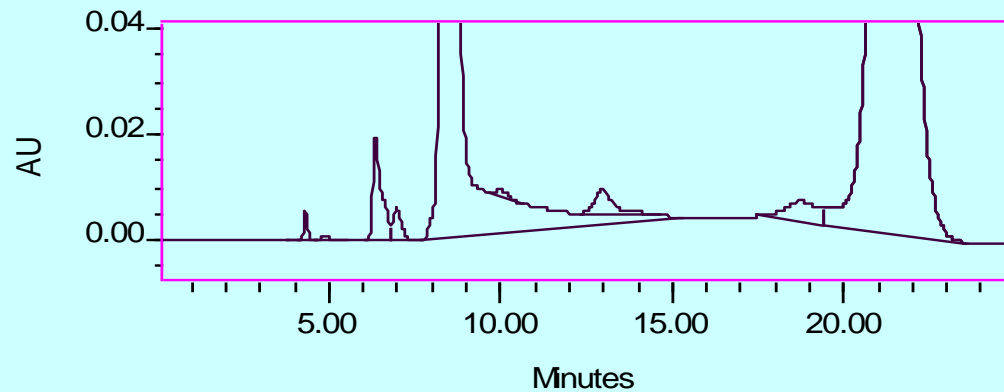
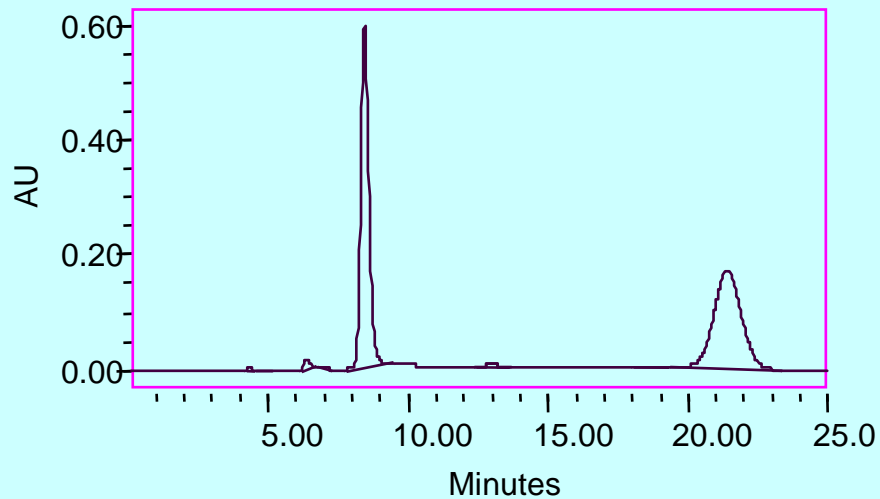
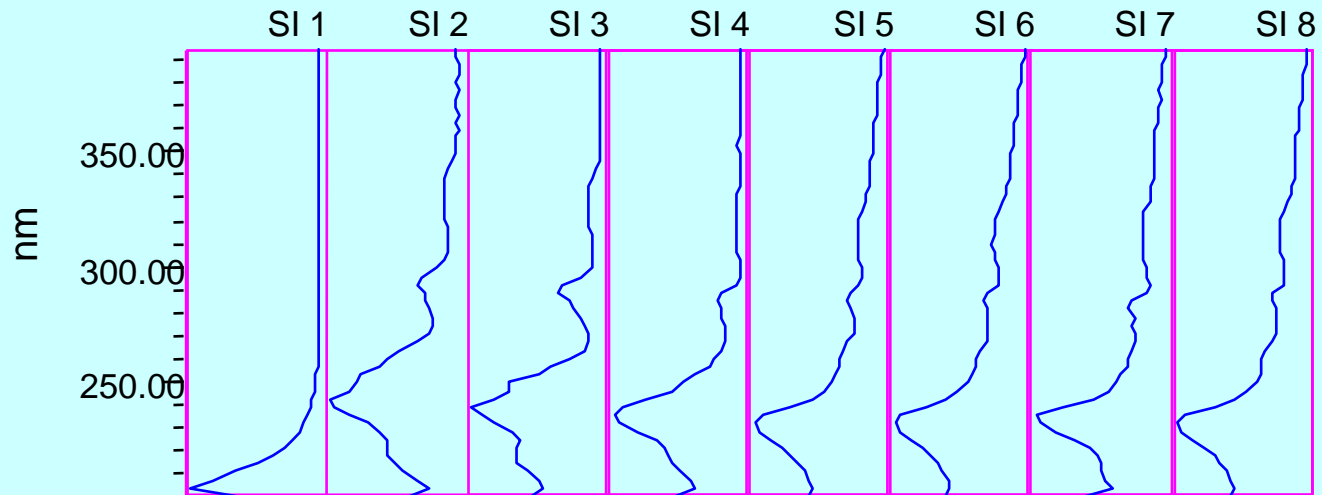
Sulfamide Enantiomers: No chiral inversion at - 0° C

Spectra collected from the peaks



Sulfamide Enantiomers - 0° C at High Concentrations

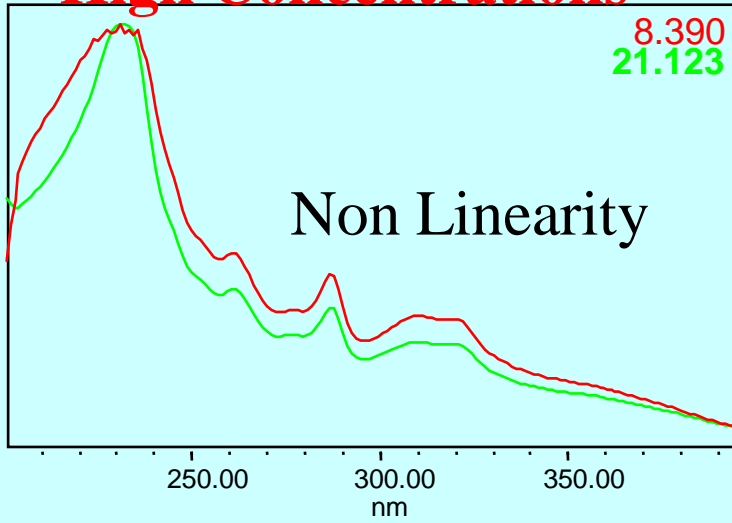
Spectra of all the peaks: enantiomers and impurities



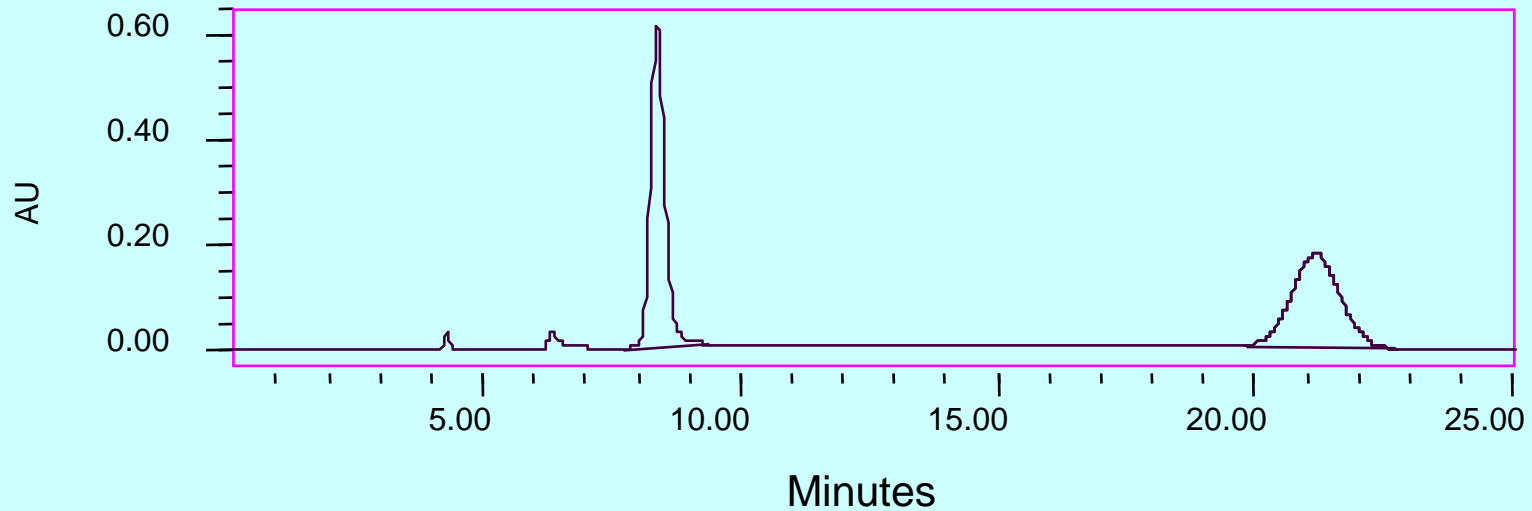
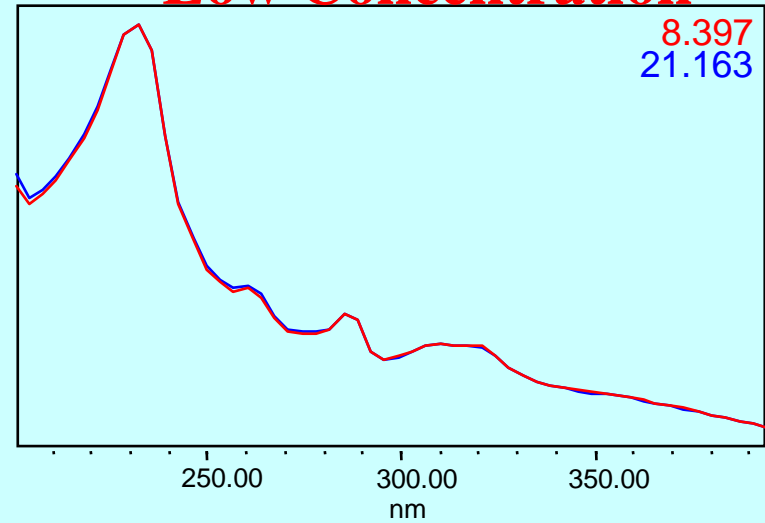
Sulfamide Enantiomers - 0° C

Overlaid Spectra

High Concentrations



Low Concentration

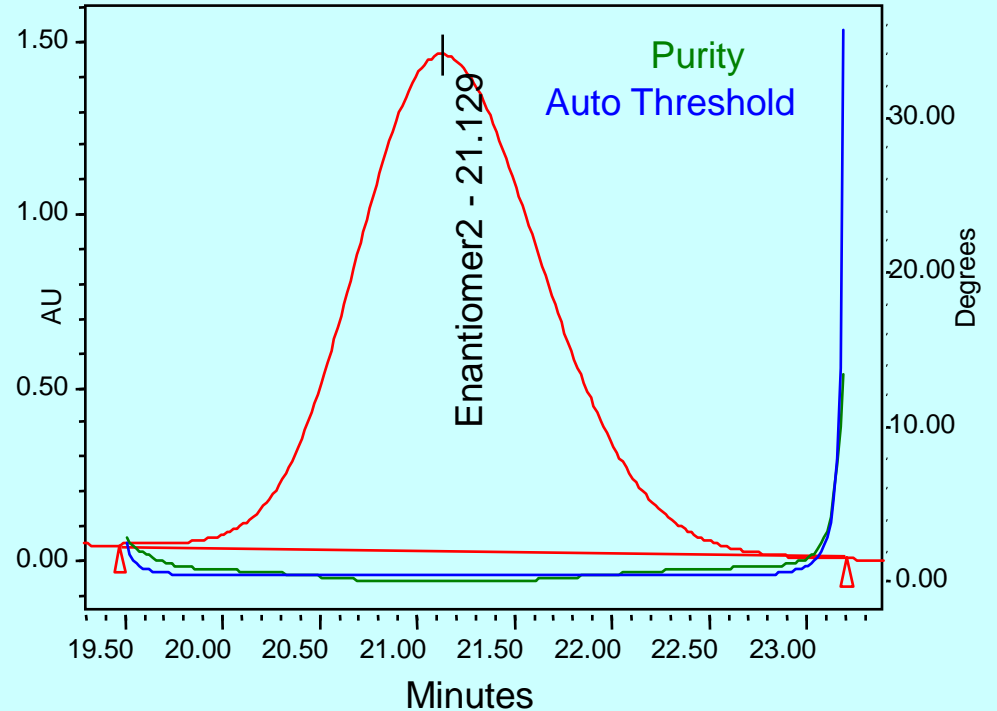
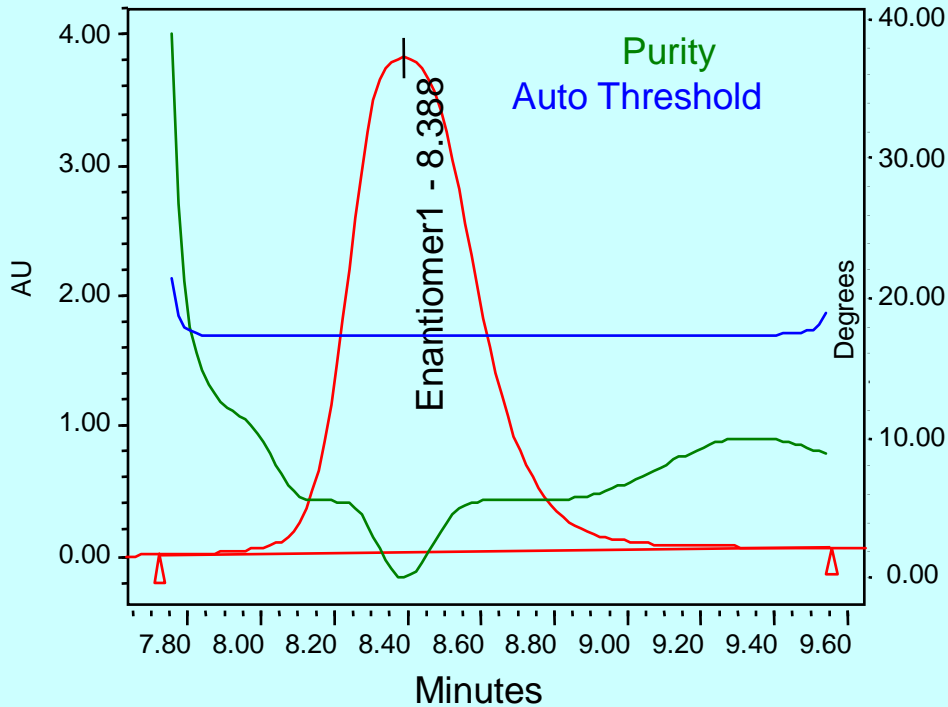


Sulfamide Enantiomers - 0° C

Purity Plot at High Concentrations: Non UV Linearity Effects

Purity Angle	Purity Threshold	Purity Flag
3.465	17.252	No

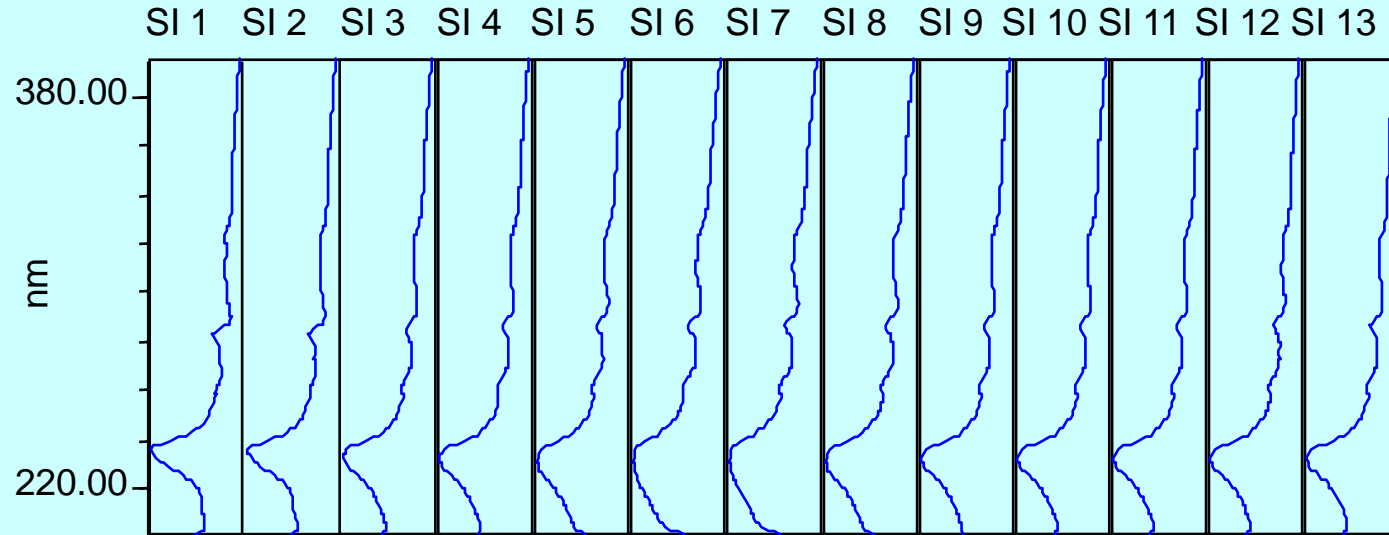
Purity Angle	Purity Threshold	Purity Flag
0.142	0.352	No



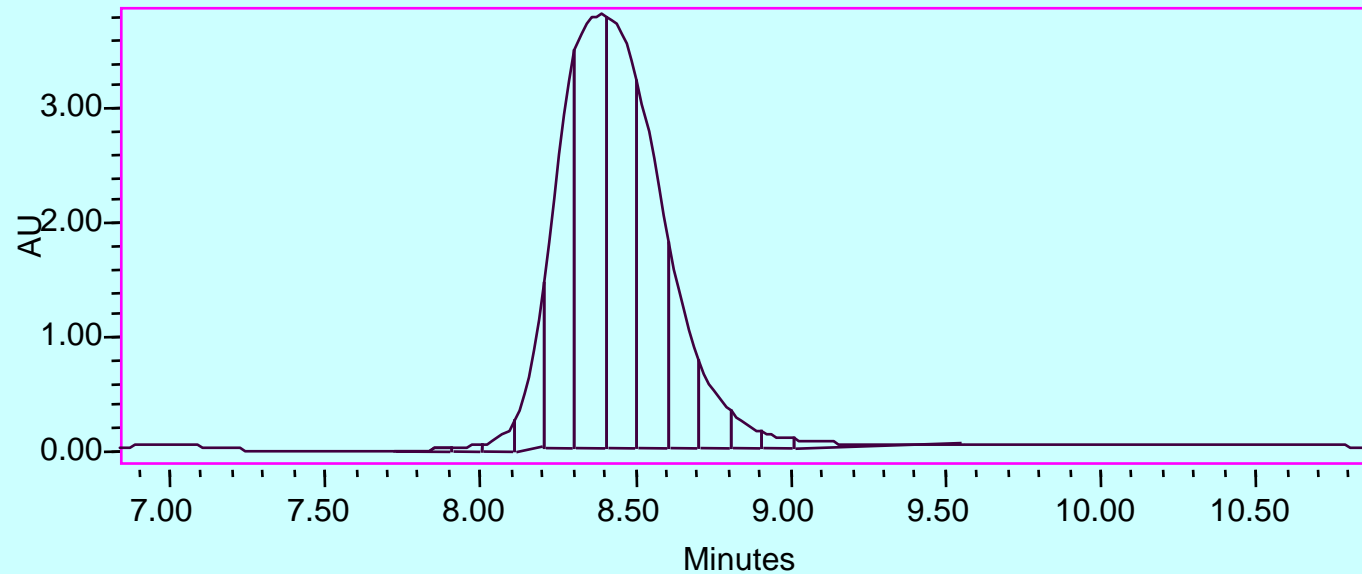
Outside of Beer-Lambert Law

Sulfamide Enantiomers - 0° C

Spectra collected over the peak: Each Concentration is Different in Spectrum



**High Conc.
Non Linear**

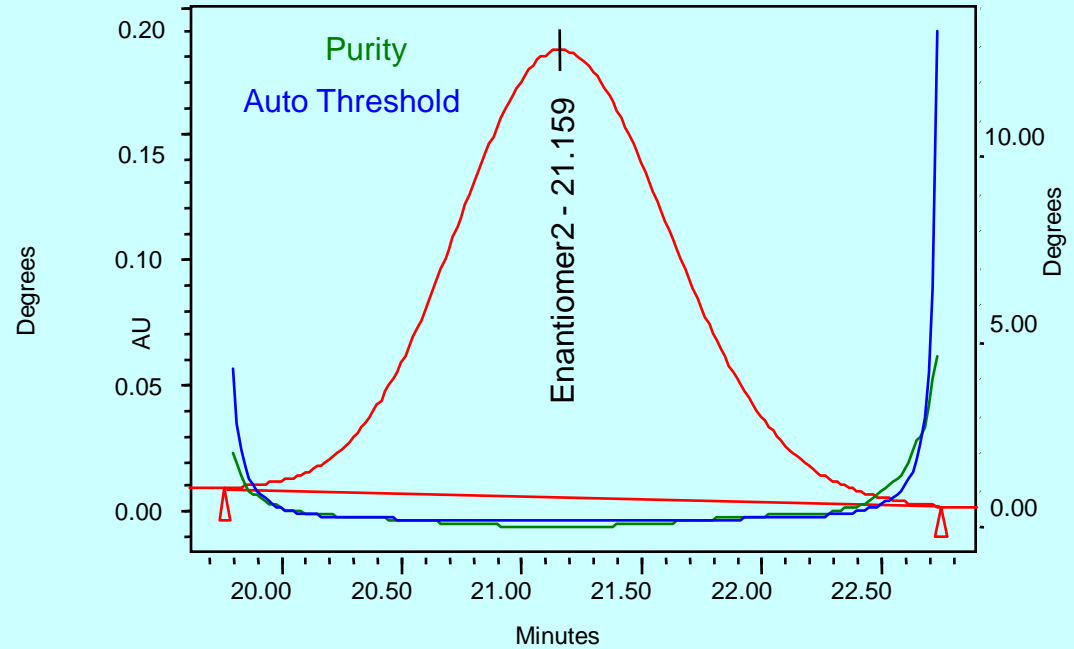
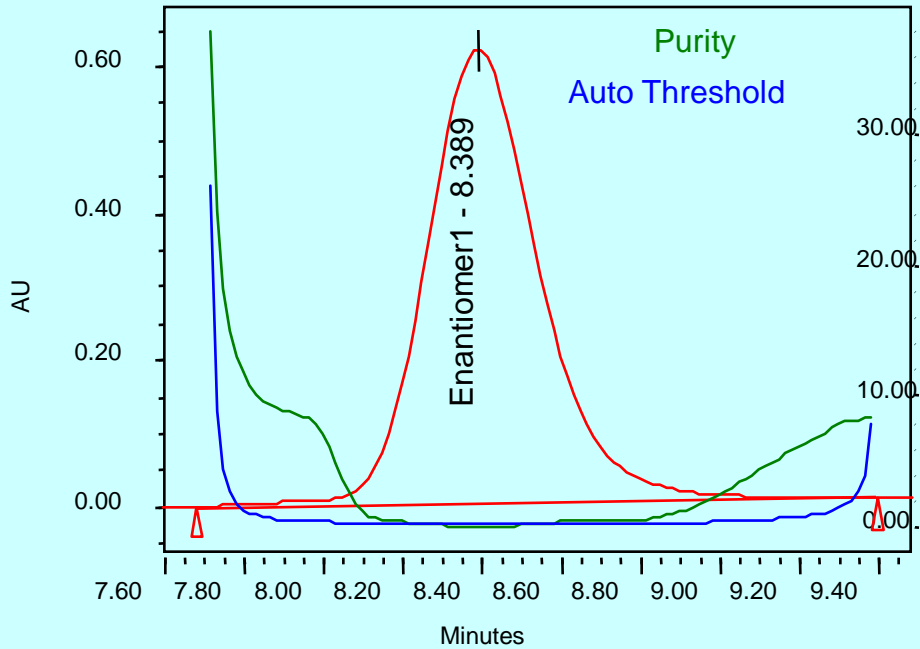


Sulfamide Enantiomers - 0° C

Purity Plot at Low Concentrations: All Normalized Spectra are identical

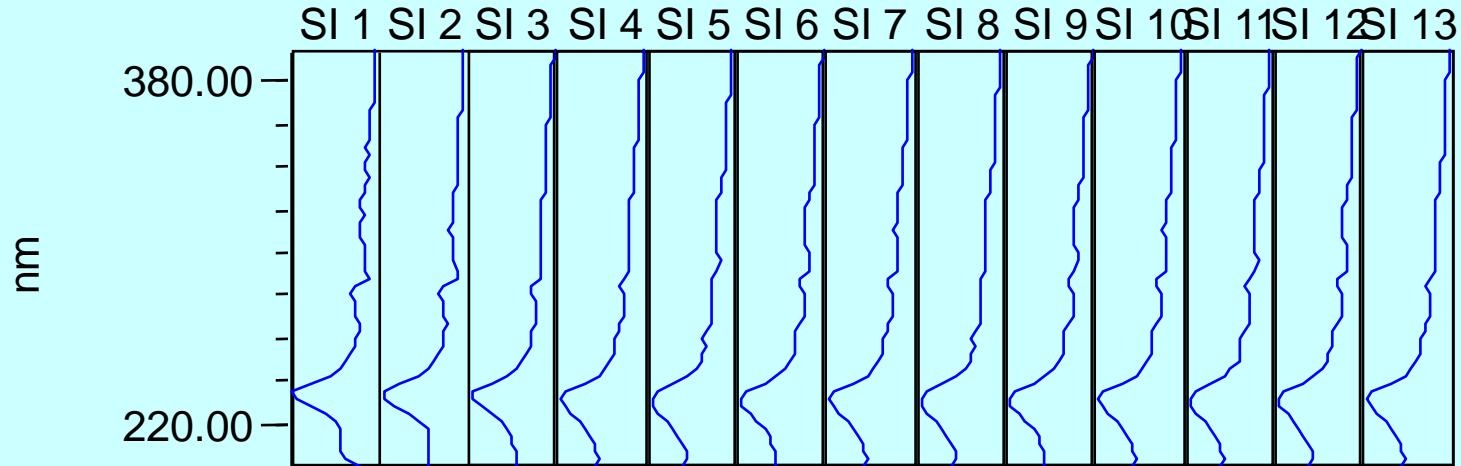
Purity Angle	Purity Threshold	Purity Flag
0.186	0.241	No

Purity Angle	Purity Threshold	Purity Flag
0.093	0.206	No

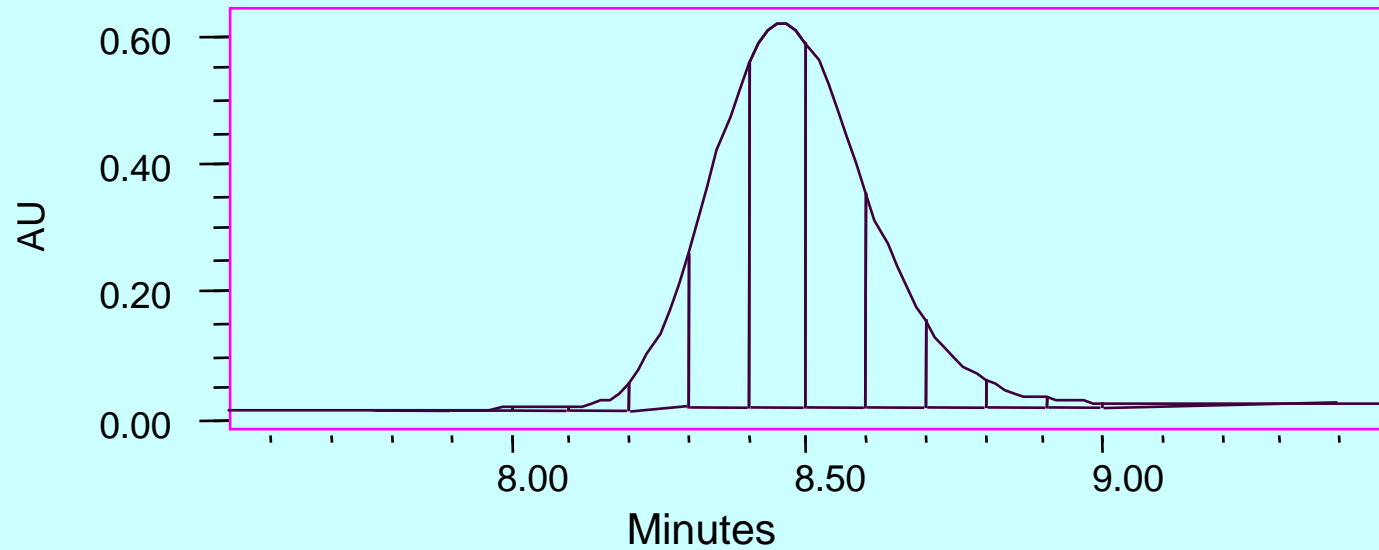


Sulfamide Enantiomers - 0° C

Spectra collected over the peak: All are identical

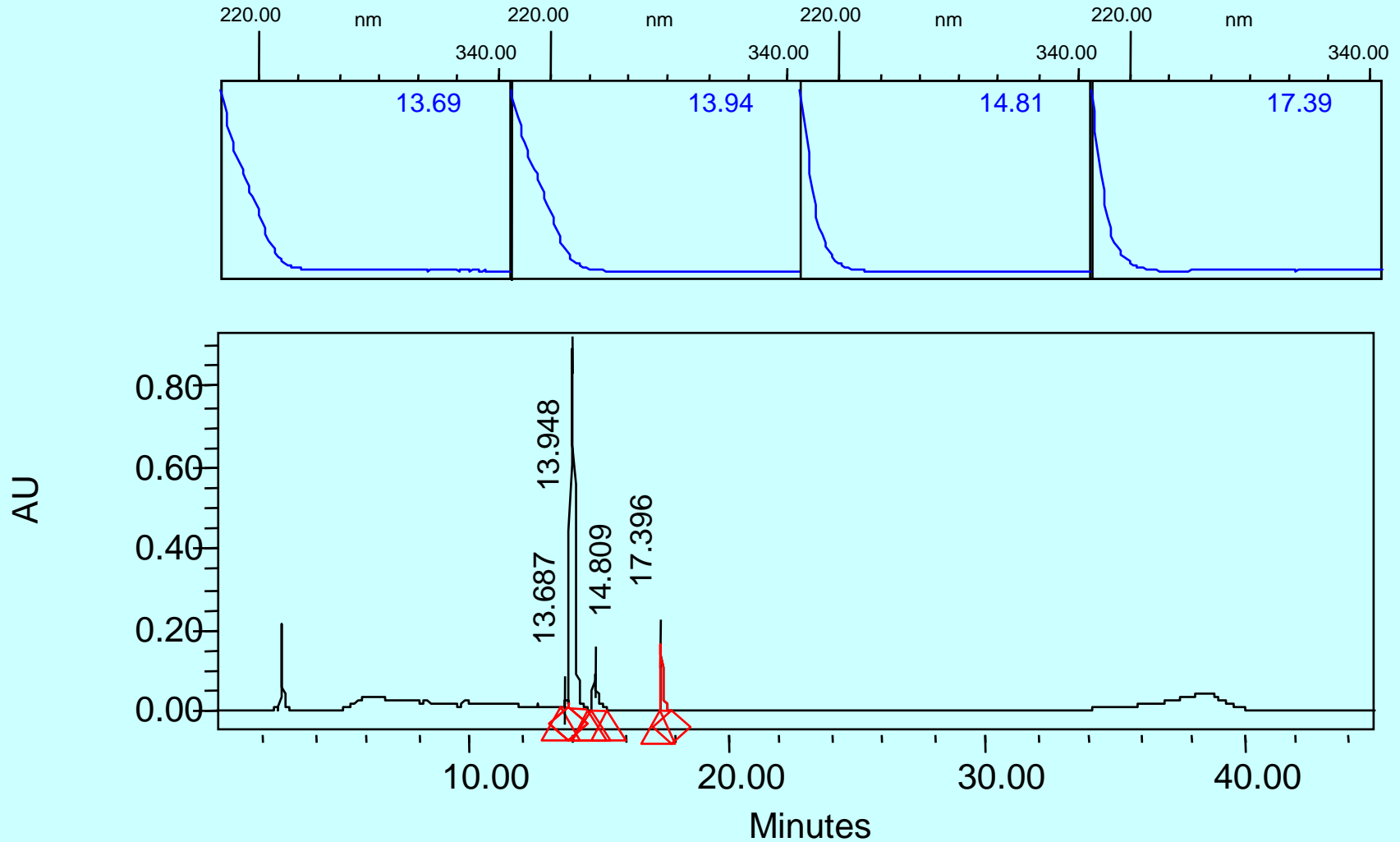


**Low Conc.
Linear**

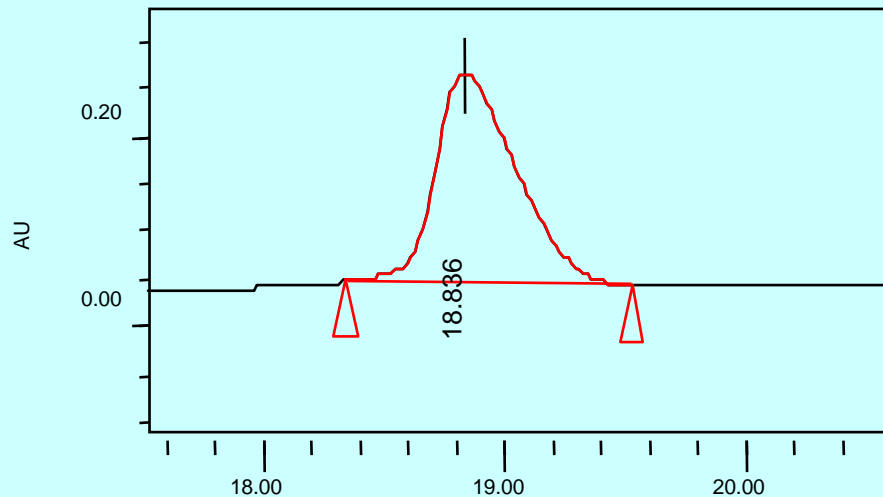
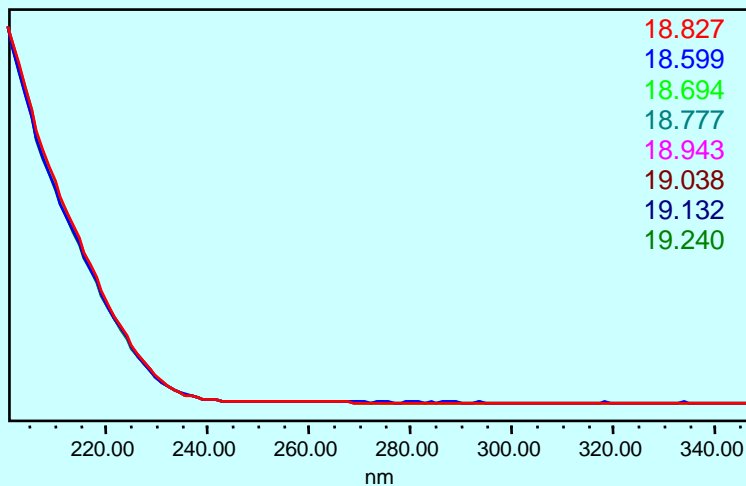


Peptides with no UV Chromophores

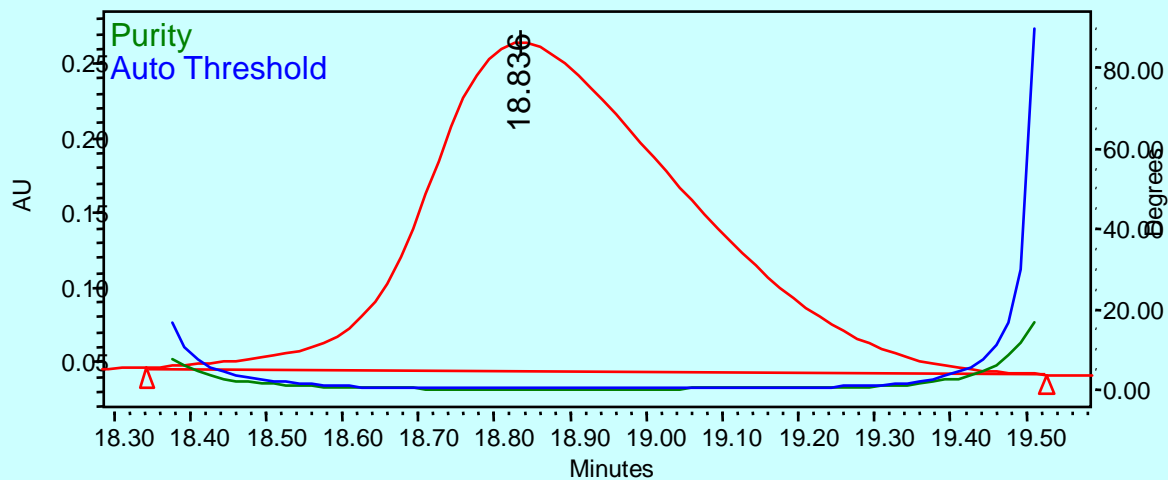
Effect of UV Spectrum Features on Peak Purity Results



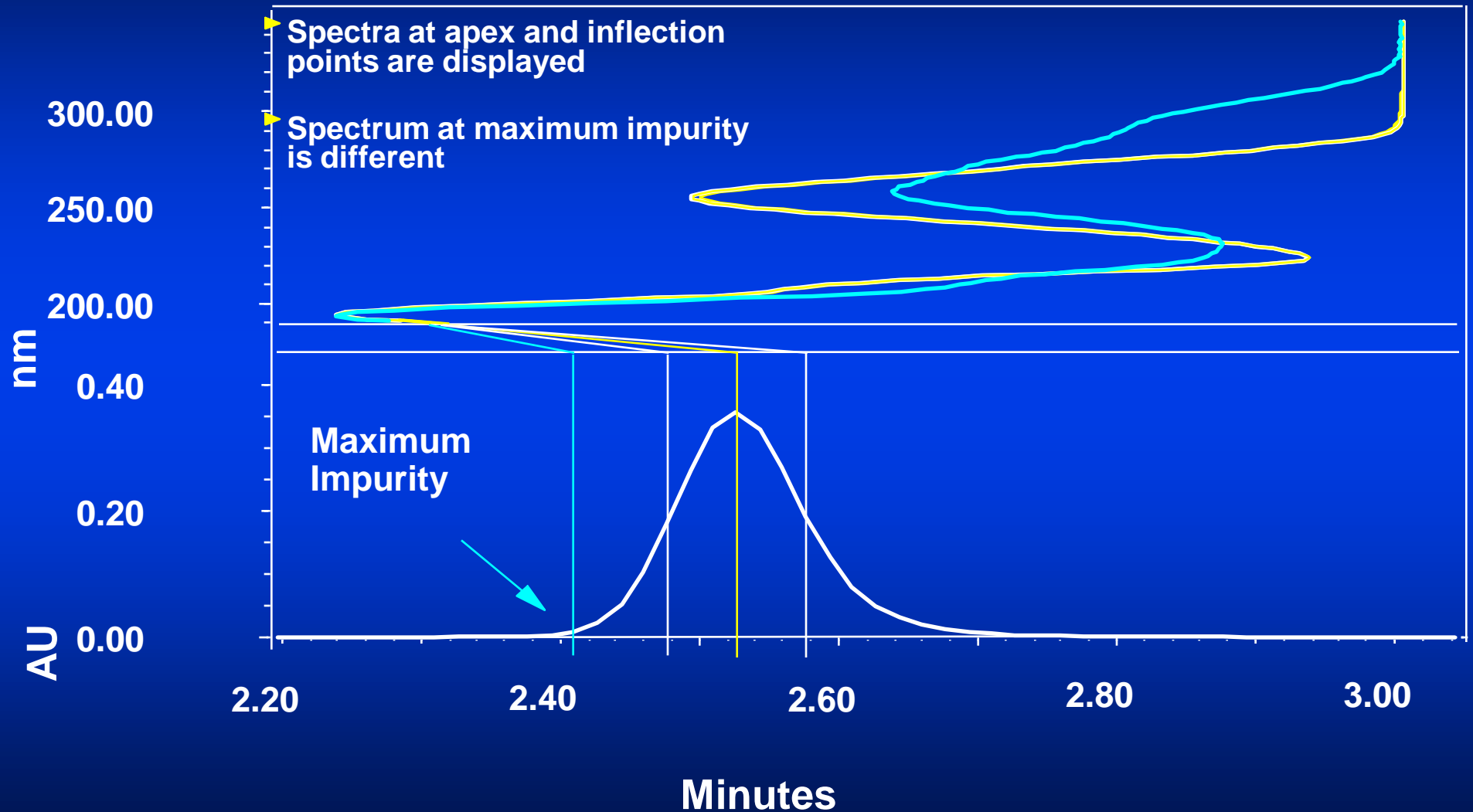
Peptide Peaks with no UV chromophore are always Spectrally PURE



Purity Angle	Purity Threshold	Purity Flag
0.183	0.356	No



Impurity's Spectrum MUST be different to be detected



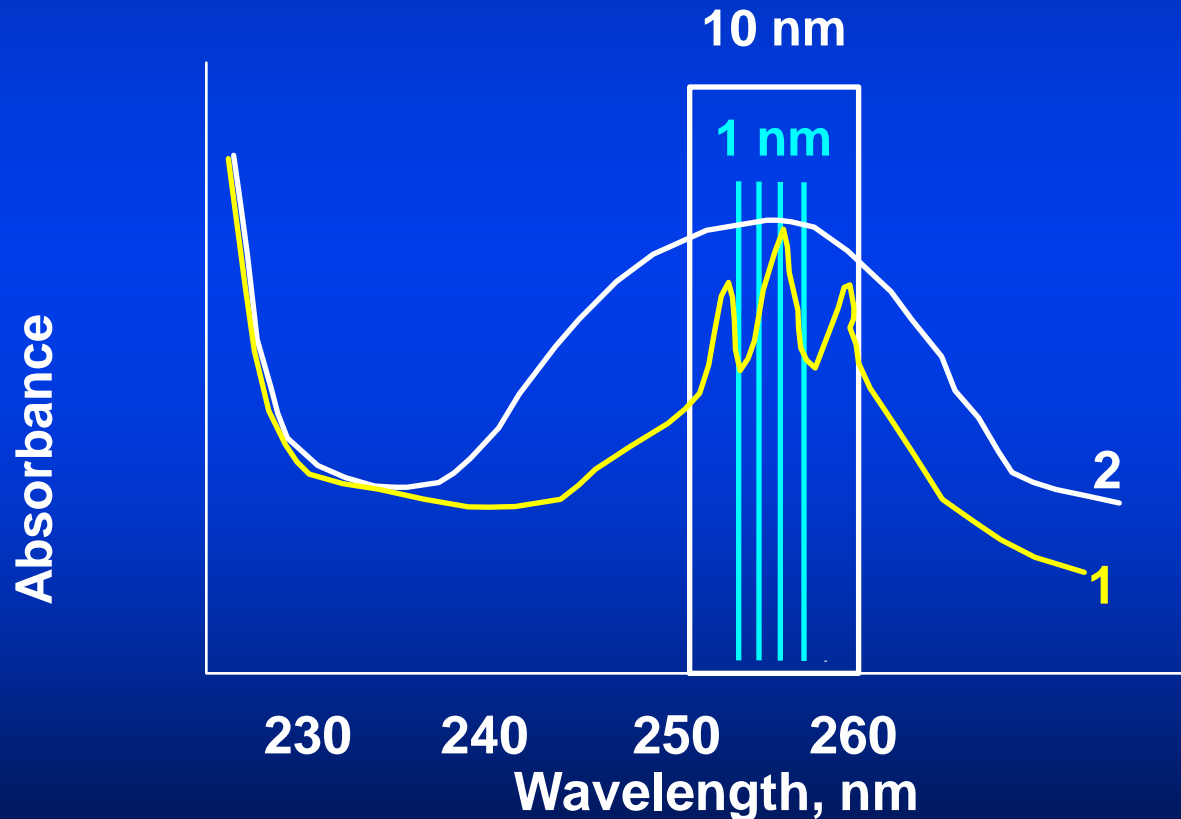
Considerations in Peak Purity Measurements

Questions:

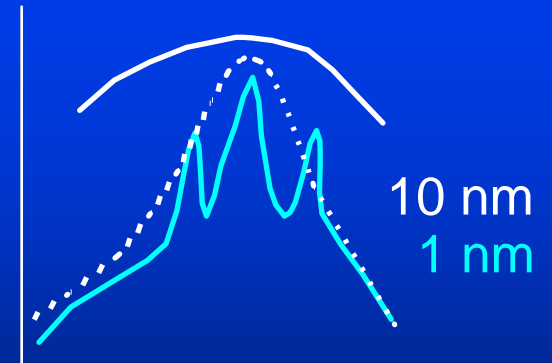
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Bandwidth & Spectral Resolution:

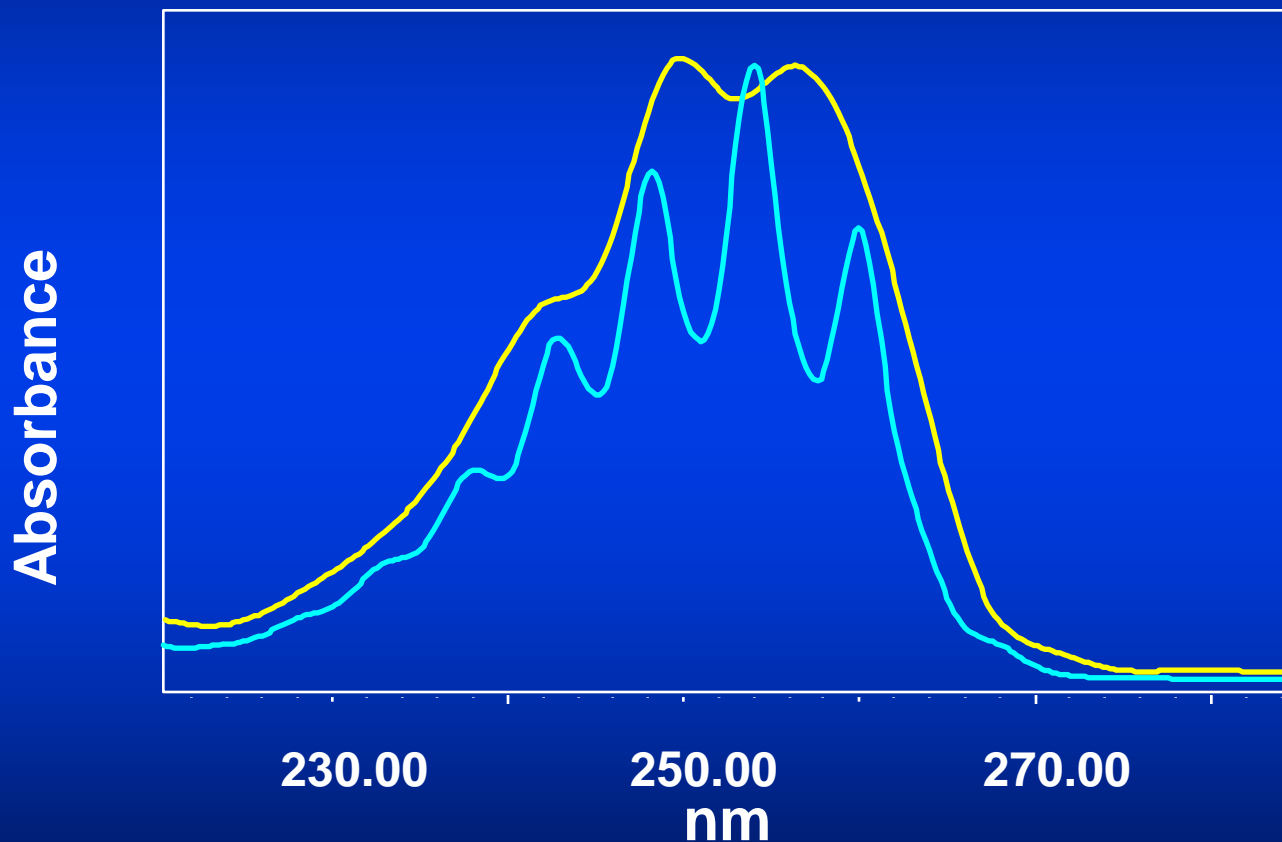
Spectral Resolution Should be minimal as possible to observe fine features of the UV Spectrum



▶ Bandwidth determines spectral resolution



Spectral Resolution - 1.2 nm vs. 3.6 nm

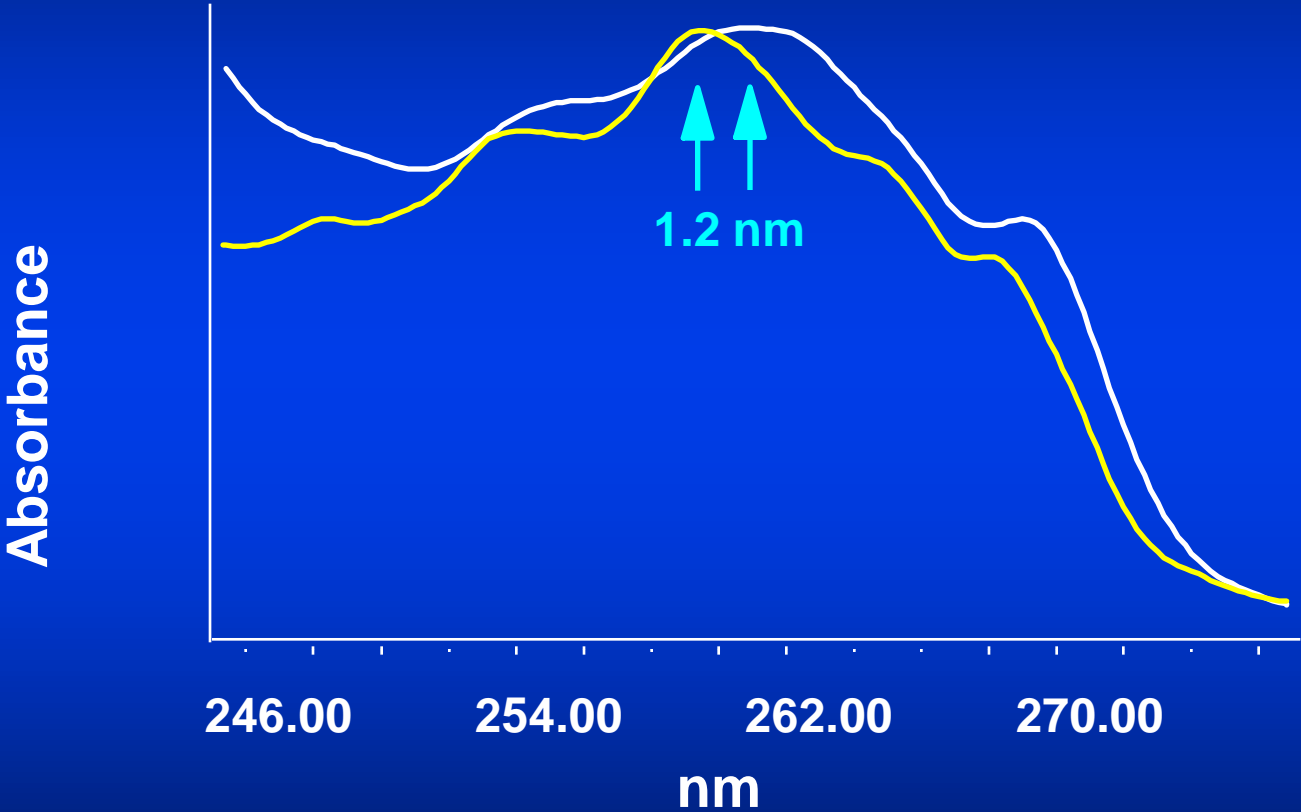


▶ **Benzene**
spectra

▶ Less resolution
at **3.6 nm** vs.
1.2 nm

▶ UV maxima
shifted

Spectral Fine Structure



▶ Analyte and one impurity spectra from 245 to 275 nm

▶ 1.2 nm resolution

Substance G

Baseline Noise at 2 different Spectral Bandwidth

Resolution =1.2 nm

Conc.	Purity Angle	Threshold	Baseline Noise
0.25	0.3430	0.8055	0.083
0.5	0.1455	0.3370	0.077
0.8	0.1065	0.3275	0.025
1	0.1195	0.3295	0.256

Resolution =2.4 nm

Conc.	Purity Angle	Threshold	Baseline Noise
0.25	0.4245	0.8015	0.081
0.5	0.1365	0.5090	0.064
0.8	0.0920	0.2945	0.028
1	0.1030	0.3080	0.132