Infrared Microspectroscopy: Its Applications and Advantages

Honors Thesis (HONRS 499)

Submitted by

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ABSTRACT

Infrared microspectroscopy is a versatile technique for analyzing microscopic and macroscopic samples. The research reported here demonstrates the number of different sampling techniques that can be performed using an infrared microscope accessory. It has been used to characterize tomato cuticle extracts and in conjunction with a visible microspectroscopy to characterize pigments from Byzantine manuscripts.

Part I

SAMPLING TECHNIQUES USING THE IR MICROSCOPE

Introduction

Infrared microspectroscopy is an effective way of determining the composition of a microscopic particulates. This system is capable of obtaining information on samples that are as small as 10 microns. More interestingly, it provides versatility in sampling both macroscopic and microscopic amounts of material. There are four basic types of experiments that can be done using a microscope: transmission, specular reflection, diffuse reflection, and absorption-reflection (1).

Transmission spectroscopy is the most common type of experiment done in infrared microspectroscopy. In this type of spectroscopy, light is sent through the sample and collected from the other side of it. The spectrum is then obtained from the measurement of the amount of light that is transmitted through the sample.

The major problem that arises in transmission spectroscopy is the fact that a sample has an optimal thickness that is dependent upon its components and the nature of the bonding. A particular sample may have the same thickness as another but absorb infrared radiation much

more strongly. If a sample is highly absorbent it needs to be thinner. All samples need to be thin enough that light in the mid-infrared range can pass through them in order for this technique to work. In addition to sample thickness, the diameter is also important. If the sample is too small, diffraction occurs, which can result in improper detection.

Specular reflection is another type of experiment that can be done on the microscope. This type of spectroscopy requires nearly no sample preparation prior to taking the spectrum. Specular reflection experiments are used for thick samples or for samples that must be saved. It is also a valuable experiment for samples that are too difficult to flatten. The sample must be optically thick, and the composition must be uniform throughout the sample. The sample must also be relatively flat on the surface where the scans are being taken.

The theory behind specular reflection is that the angle of incidence equals the angle of reflection. This is accomplished by sending the infrared light to the sample, which then absorbs some of the light and reflects the rest of it. The reflected light is sent back through the upper cassegrainian arrangement and then sent back to the detector by a series of mirrors in the upper portion of the microscope. The resulting spectrum is a combination of absorbance and refractive index spectra. In order to put it

into a form that is more familiar, the Kramer-Kronig program is used.

Diffuse reflection is the third type of experiment that can be done under the microscope. In diffuse reflection the sample is very grainy, which results in scattering the infrared radiation as it is reflected. Diffuse reflection is used for samples that are too thick and too powdery to take either a transmission spectrum or a specular reflectance spectrum. The sample is often mixed with KBr in order to make the sample less concentrated and more likely to diffuse the infrared light.

Absorption-reflection spectroscopy requires a sample that is considered to be a thin film. The thin film is in intimate contact with a reflective surface. The infrared light is sent through the sample and reflected back off of the metal substrate. This means that the light travels through the entire sample approximately twice.

Experiment

The following experiment was performed using a nylon tie obtained from Dennison Manufacturing Co., Fastener Division located in Framingham, Maryland. The sample was not microscopic; however, it is a sample that can be used to illustrate transmission, diffuse reflection, and specular reflection. The absorption-reflection experiment is not conducted here.

Transmission Experiment

The nylon tie is shaved over a microscope slide using a razor blade. A shaving is then flattened, which is accomplished by rolling the tip of the probe over it and applying enough pressure to flatten the sample but not break it apart. The flattened shavings are then put onto a salt plate.

The next step is to put the sample under the microscope and focus it. The adjustable pin hole, or aperture, is then closed around the sample's image. The aperture defines the sample area that is to be scanned. After the sample spectrum is obtained, the sample is then moved out of view, and a background scan is obtained. The next step is to divide the sample spectrum by the background spectrum. This process ratios out the water, carbon dioxide, and other extraneous absorptions. Figure 1 shows the transmittance spectrum.

The number of scans obtained depends on the size of the sample after it is defined. The diameter of the sample should be at least ten microns, in order to prevent diffraction. The aperture is especially important in preventing stray light from going around the sample. The sample should not be too large or the transmitted energy will be too great. However, this size can be defined in order to reduce the amount of transmitted energy.

Specular Reflection Experiment

In the specular reflection experiment, the tip of a nylon tie is cut off and placed on a slide. The nylon tip is placed under the microscope so that its grain is parallel to the beam, and the microscope is focused on the top of the sample. If the grain of the tie is not parallel to the beam the result is that the reflected light is diffused. The sample should be as smooth as possible with few grooves. The adjustable upper aperture is used, and the lower aperture is not needed due to the fact that the light never goes below the stage. Figure 2a shows a specular reflectance spectrum of the tie before the Kramer-Kronig transformation. The spectrum is really not interpretable due to the fact that the spectrum is a combination of an absorbance and a reflective index spectrum. The resulting spectrum is a collection of derivative-like peaks. Figure 2b shows the Kramer-Kronig correction of Figure 2a. This spectrum is now easier to interpret, and even though there are a few minor differences, the specular reflectance and the transmittance spectra have essentially the same peaks.

In the specular reflection experiment, the sample must be large (typically greater than 40 μm) in diameter because the reflected signal is so small and because of instrumental constraints.

Diffuse Reflection Experiment

Diffuse reflection is used for samples that are too thick and too powdery to take a transmission spectrum or a specular reflection spectrum. The sample is often mixed with KBr in order to make the sample less concentrated and more likely to diffuse the infrared light. In this experiment the nylon tie is sanded with sandpaper. The grain of the sandpaper is measured to be between 40 and 80 microns. The sample spectrum is obtained from the sample-coated sandpaper surface and is referenced against the plain sandpaper. Figure 3a shows the diffuse reflectance spectrum before the Kebulka-Munk transformation. The peaks are similar in location to those of the transmission spectrum. Figure 3b shows the Kebulka-Munk spectrum of 3a. The Kebulka-Munk process transforms a diffuse reflectance spectrum into a transmittance spectrum. One can observe that the diffuse reflectance spectrum prior to transformation is quite interpretable. In diffuse reflection, the sample must also be large because of the weak signal involved.

This experiment shows three different techniques that can be used in infrared microspectroscopy. It is important to notice that the different experiments give somewhat different results even though the samples are of the same materials. This is due to the differences in how the light absorbs and how it is reflected.

Part II

IR STUDY OF TOMATO CUTICLE EXTRACTIONS

Introduction

Pesticides are frequently considered to be hazardous to the environment. In addition, many of the targeted pests develop resistance to the old pesticides. As a result, many companies are developing new pesticides, which must meet many government regulations.

One of the important studies that must be conducted on pesticides includes the penetration into a plant cuticle. Tomato cuticles are often used to study a pesticide's ability to penetrate because of the ease in which they are prepared. However, studies done on tomato cuticles may or may not model pesticide penetration in other plants. A first attempt to study the effectiveness of such modelling is to characterize tomato cuticle extractions as a function of maturation and extraction time.

Experiment

Spectra were obtained on a Perkin-Elmer 1760X FTIR coupled to a Perkin-Elmer microscope. The extractions of the cuticular wax from tomato cuticles at four different maturation stages were carried out and given to us by Dow-

Elanco personnel.

The experiment was conducted by placing one milliliter of carbon tetrachloride to each of the sixteen bottles. After dissolving the cuticular wax, 0.10 microliters of the solution is placed onto a KBr plate. This amount varied with the number of the extract for each stage. The infrared transmission spectrum is then taken. The resolution is set at 4 cm^{-1}, and a gain of 1.

Results and Discussion

The IR spectra of tomato cuticles vary with the maturation stage of the cuticle as Figure 4 shows. The differences represent the differences in the chemical composition between stages, provided the extractions were done in a reproducible manner. The differences are slight, but measurable, and most notably occur in the relative intensities of the two carbonyl absorptions.

Secondly, the IR spectra vary with extraction time. The first extract appears to pull off primarily esterified long chain fatty acids, presumably those extracuticular in nature. The second removes phenols and non-esterified fatty acids; the third, long chain fatty acid esters, presumably of an intracuticular nature; and the fourth, alcohols.

Part III

IR CHARACTERIZATION OF PAINTS REMOVED FROM ANCIENT MANUSCRIPTS

Introduction

This study involved the characterization of pigments used in artistic works of various times. Infrared microspectroscopy is very valuable in this type of work as it allows for small samples, which helps to protect the original work. In many of these samples, it is possible to do either the transmission or reflection experiment, and which of these is carried out depends on how absorbing the pigment is.

In this experiment, the red pigments from medieval illuminated Byzantine manuscripts are sampled (2). These manuscripts are housed in Special Collections at the University of Chicago library. The manuscripts range in dates from the 10th century to the 13th century or later. The titles of the manuscripts that have been sampled for this experiment include the Elfleda-Bond Goodspeed Gospel, MS #1054, The Rockefeller-McCormick New Testament, MS #965, the Chrysanthus Gospel, MS #131, and the Greek Gospel, MS #232. All of these manuscripts have been sampled, but the Elfleda-Bond Goodspeed Gospel could not be resampled for

further studies. The manuscripts are written in Greek and describe the life of Christ. In the text, there are illuminations that depict various biblical scenes. It is believed that monks during the middle ages wrote and painted these works of art.

The pigments that are being studied are believed to be anthraquinone derivatives. The major dyes that have been considered are kermesic acid, carminic acid, laccaic acid, alizarin and purpurin, which are shown in Figure 5. The kermesic acid, carminic acid, and lac are derived from a various species of beetles. Alizarin and purpurin come from madder plant root, and have also been found to be very common in areas such as ancient Egypt, Persia, and India.

The characterization of the anthraquinone pigments is accomplished by looking at the carbonyl region of the spectrum. The location and presence of the carbonyl absorptions of the molecules are affected by the substituents near it. For instance, an α-hydroxy substituted carbons could result in hydrogen bonding with the carbonyl, which would shift the frequency of absorption. Bloom et al. indicate that this is the case. They show that the number of hydroxy substituents present on the anthraquinone affects the carbonyl frequencies. A summary of the work by Bloom et al. is given in Table 1 (3).

In addition, it is thought that the most common recipe

for making these pigments resulted in an metal complex with the insect extract, which is termed a lake. Therefore, it is important to look in the visible region of the electromagnetic spectrum in order to determine if such a complex has formed, which was done by P. Lang at Miami University in Oxford, Ohio. This complex could also result in shifting of the peaks in the carbonyl region as the metal binds to the carbonyl and to an α-oxygen.

Experiment

The samples were removed from the manuscripts very carefully in order not to do any damage. In the sampling process, a stereoscope is placed over the manuscript, and a fine scalpel is used to lift a small sample of the pigments off. The sample sizes ranged from 4μm to 40μm. These were then transported back to the lab between two microscope slides that had been scraped to rid them of contaminants.

The standards of carminic acid and the carminic acid lake were obtained from Aldrich Chemical Co. The red mercuric sulfide was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. The kermesic acid standard was obtained from D. W. Cameron, who synthesized it in a procedure that had been previously published. The Armenian Red sample was received from Max Saltzman, Institute of Geophysics and Planetary Physics, University of California, Los Angeles. The Armenian Red sample was prepared as an aluminum lake.

The infrared spectra of the samples were obtained using the system described earlier. It is important to take into consideration the type of sample that is being characterized. For example, some of the pigment samples were very powdery and others were more pliable. For the samples of the latter type, it is rather simple to take a transmission spectrum because they make a good thin, large sample. For the powdery samples or for the samples that are highly absorbing, a KBr pellet can be made by mixing a small amount of the pigment with infrared grade KBr in a ratio of about 1 to a 100. This mixture is then pressed into a microscopic pellet using the tip of a metal probe, if the samples are microscopic in nature. For reference samples, where quantity was not as limited samples mixed with KBr were placed in a small die pressed in a hand press.

Results and Discussion

The infrared spectra of the standards show that the absorption patterns vary with the different types of substituents. These results are summarized in Table 2. By comparing the results of these spectra to the pigments it is possible to deduce information about their composition. In addition to looking at the infrared spectra, it is also helpful to look at the visible spectra.

Figure 6 is an overlay of the infrared spectra of carminic acid, carminic acid lake, and the Armenian Red

Lake. It is apparent that they are very similar to one another in the absorption patterns. Armenian red lake correlates very nicely with the carminic acid lake. The absorptions that are labelled at 1254 and 1226 cm^{-1} are due to the C-OH and C-O-C vibrations of the glucosyl substituent (2). The spectra in Figure 7 indicate that there is a difference between the kermesic and carminic acids due to the glucosyl substituent present on the carminic acid but not on the kermesic acid.

The infrared spectra of the samples from the manuscripts give a good indication of the composition of the pigments. In the infrared spectrum of a magenta pigment from MS 131, Figure 8, shows a broad -OH stretching and carbonyl absorptions at 1647 and 1577 cm^{-1}, glucosyl absorptions at 1074 and 1032 cm^{-1}, which is consistent with the formation of a lake with a sugar substituent as compared to the spectrum in Figure 6. Figure 9 shows the visible spectrum of HgS overlayed with a magenta pigment from MS 131. This indicates that the red pigment is composed of red HgS in addition to the anthraquinone lake. In Figure 10, the spectrum of a red pigment from MS 232 is shown. This spectrum indicates that a carminic acid lake is probably the predominant component of the pigment studied. One can again observe the rather broad -OH peak, the carbonyl absorptions, and the glucosyl absorptions present. In the infrared

spectrum of MS 965, page 6, shown in Figure 11, there are definite N-H stretching absorptions along with the amide I (1643 cm^{-1}) and amide II (1555 cm^{-1}) absorptions. This indicates that protein is present, perhaps due to the animal nature (i.e., beetle extract) rather than plant nature of the pigment. When the visible spectra of the red and magenta pigments from MS 965, page 6, are compared to the visible spectrum of the Armenian Red Lake, as in Figure 12, it is apparent that the carminic acid lake is a major component. When the visible spectra of the red pigments from manuscripts 965, page 9, and 232 are compared to one another, Figure 13, they show similar absorptions, indicating a common, but unidentified red pigment (2).

Part IV

SUMMARY

It is evident from the above experiments that infrared microspectroscopy is a very useful and versatile technique for analyzing samples. Firstly, the research reported herein as shown that transmittance, specular reflectance, and diffuse reflectance spectra can easily be obtained using IR microscope and that the information obtained in the different spectra are similar. Sample preparation and factors affecting each experiment have been described. Secondly, we have shown that the IR spectra of tomato cuticles vary with maturation stage and extraction time. Finally, molecular information was obtained on historic paint fragments without visible damage to the manuscript. Spectral evidence indicates the presence of carminic acid lakes in two of the manuscripts studied, and the use of a mixture of vermillion and anthraquinone in a third.

REFERENCES

1. Lang, P. L.; Richwine, L. J. Proper Sampling with Today's IR Instruments, Patricia B. Coleman, Ed., CRC Press, Boca Raton, FL, in press.

2. Lang, P. L.; Orna, M. V.; Richwine, L. J.; Mathews, T. F.; Nelson, R. S. Microchemical Journal, **45**, 1-15, 1992, in press.

3. Bloom, H.; Briggs, L. H.; Cleverly, B. J. Chem. Soc., 1959, 178-185.

TABLE 1

ANTHRAQUINONE STUDIES BY BLOOM, BRIGGS, AND CLEVERLY

Type of anthraquinone (according to α-OH position)	Ketone C=O frequencies (cm^{-1})	
No α-OH present	1678-1653	
1-OH	1675-1647	1637-1621
1:8-$(OH)_2$	1678-1661	1626-1616
1:4- or 1:5-$(OH)_2$	1645-1608	
1:4:5-$(OH)_3$	1616-1592	
1:4:5:8-$(OH)_4$	1592-1572	

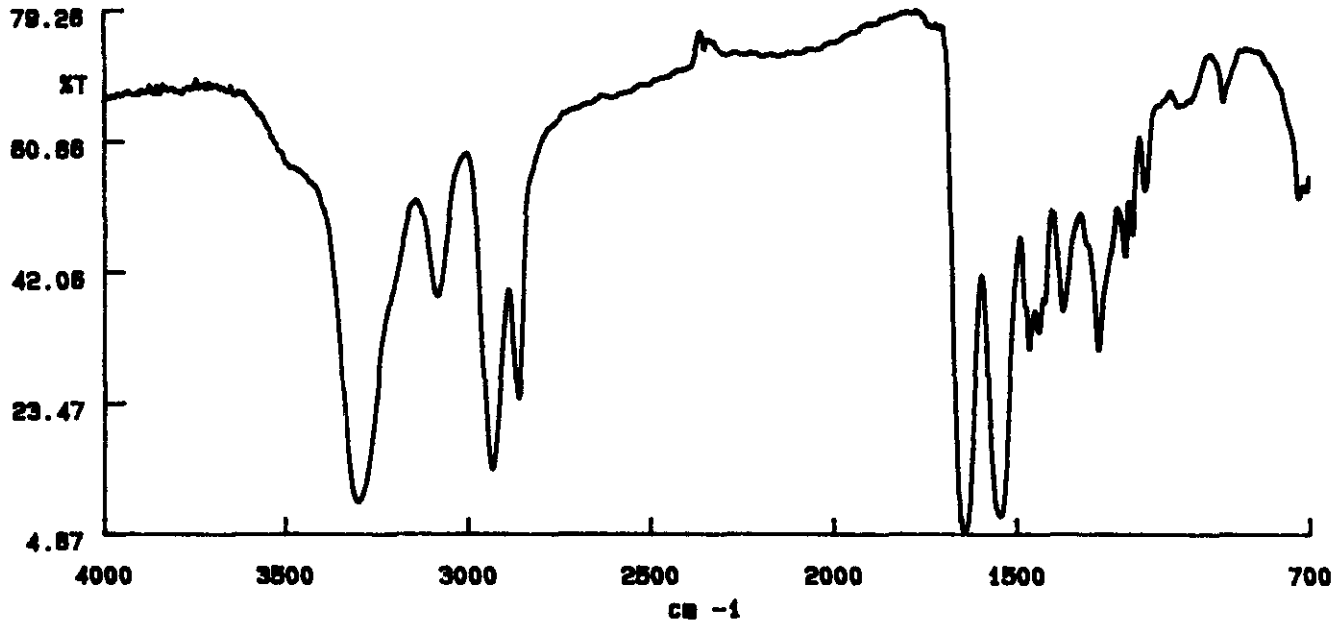
TABLE 2

DIAGNOSTIC ABSORPTIONS (cm^{-1}) OBSERVED IN ORGANIC RED REFERENCES

	Lang et. al.			Bloom et. al.	
	Acid C=O	Ketone C=O	Aromatic C=C	Ketone C=O	Aromatic C=C
Carminic Acid	1713	1666(sh), 1618	1574	_____	_____
Kermesic Acid	1707	1675, 1618	1561	_____	_____
Lac Dye '76	1718(br)	1616	1574	_____	_____
Lac Dye '77	1708(br)	1626	1582	_____	_____
Laccaic Acid	1705(sh)	1630, 1631	1579, 1561	_____	_____
Alizarin		1664, 1634	1587	1658, 1634	1587
Purpurin		1622	1584	1621	1580

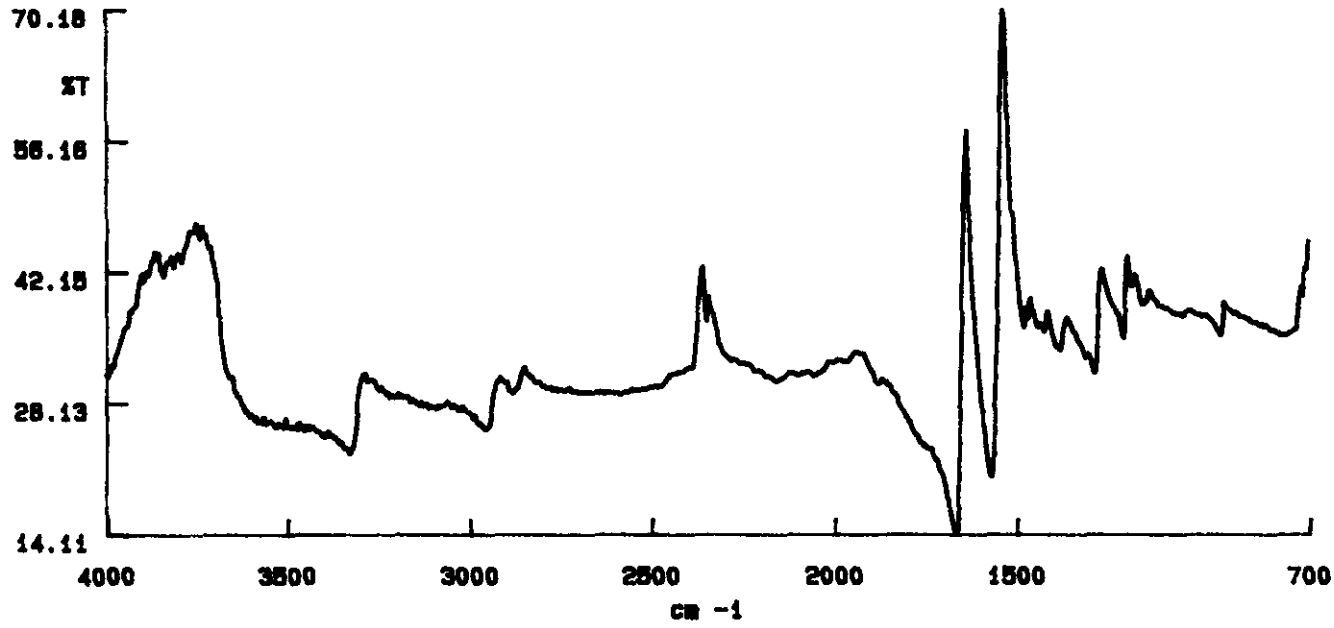
(Lac Dye primarily consists of Laccaic Acid A)

FIGURE 1

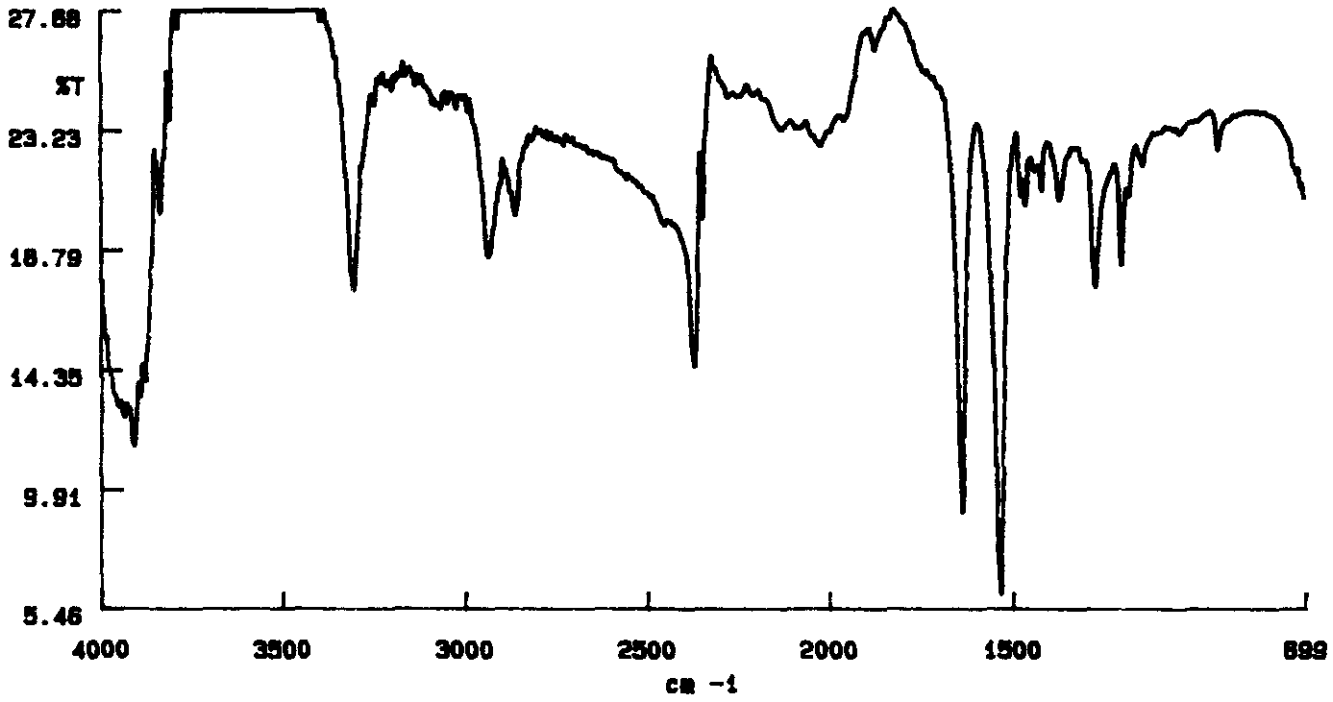


Transmittance spectrum of nylon tie.

FIGURE 2

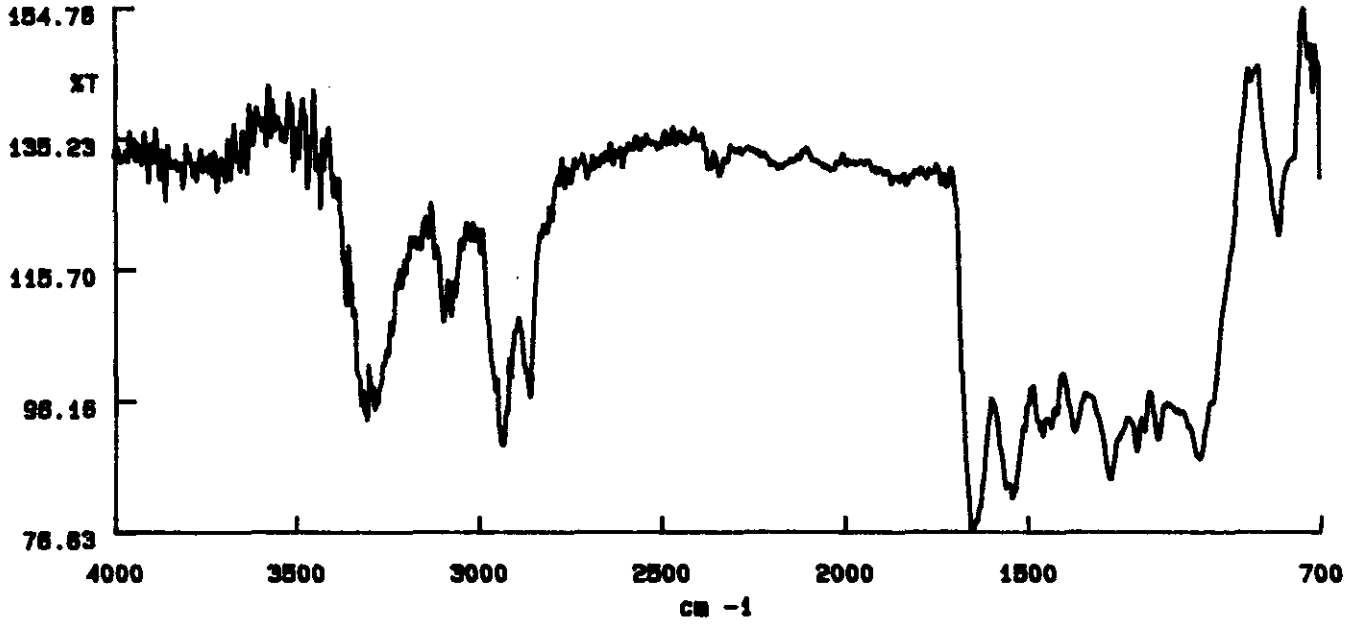


a) Specular reflectance spectrum of nylon tie.

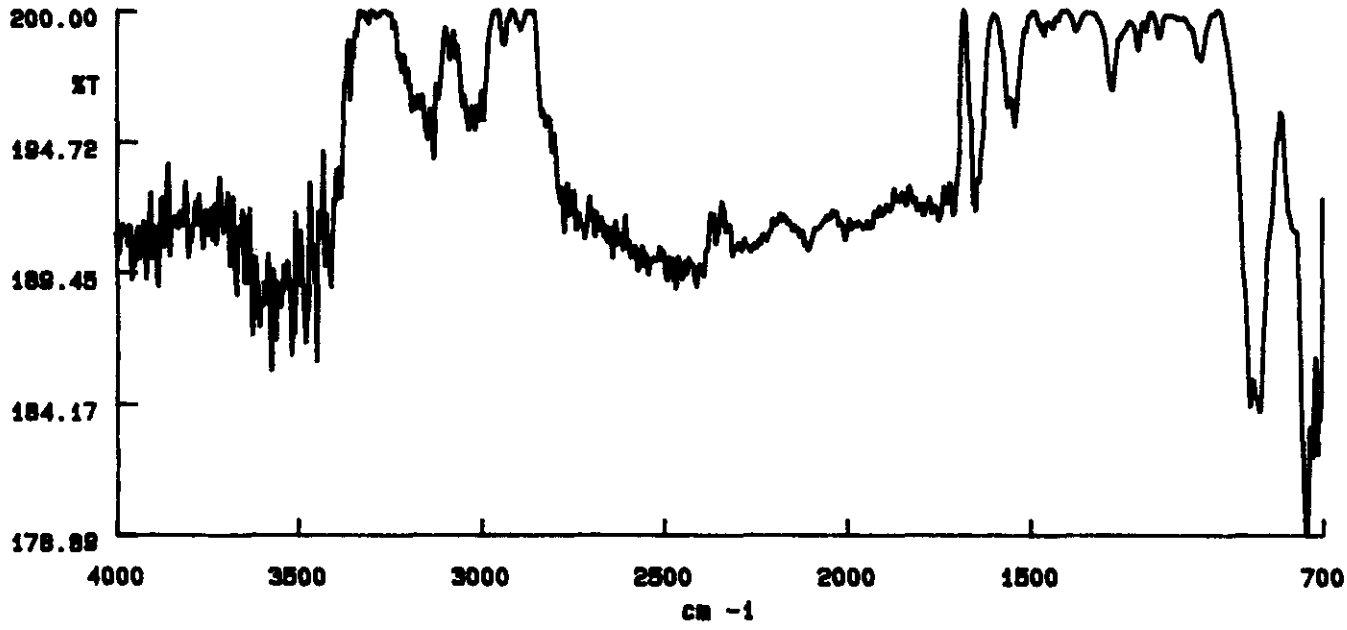


b) Kramer-Kronig correction of specular reflectance spectrum.

FIGURE 3

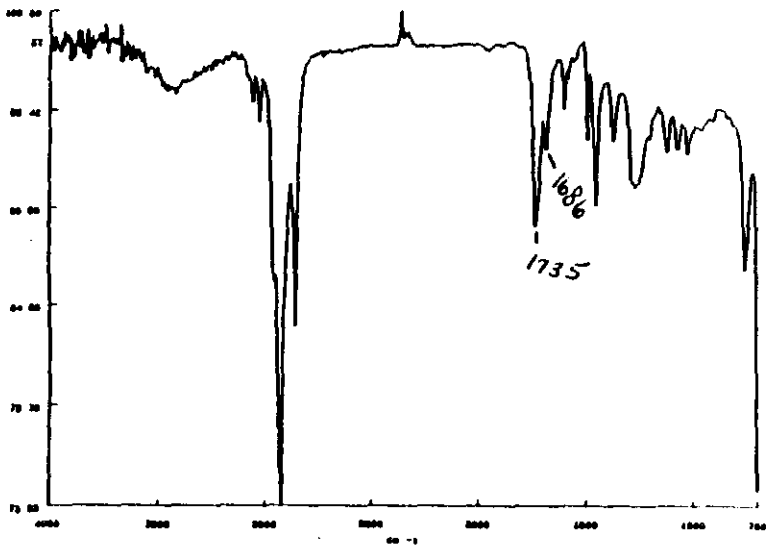


a) Diffuse reflectance spectrum of nylon tie.

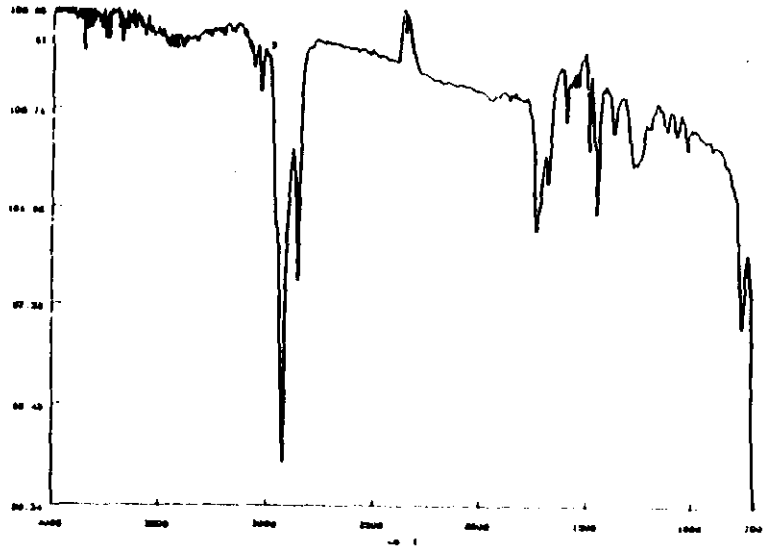


b) Kebulka-Munk transformation of diffuse reflectance spectrum.

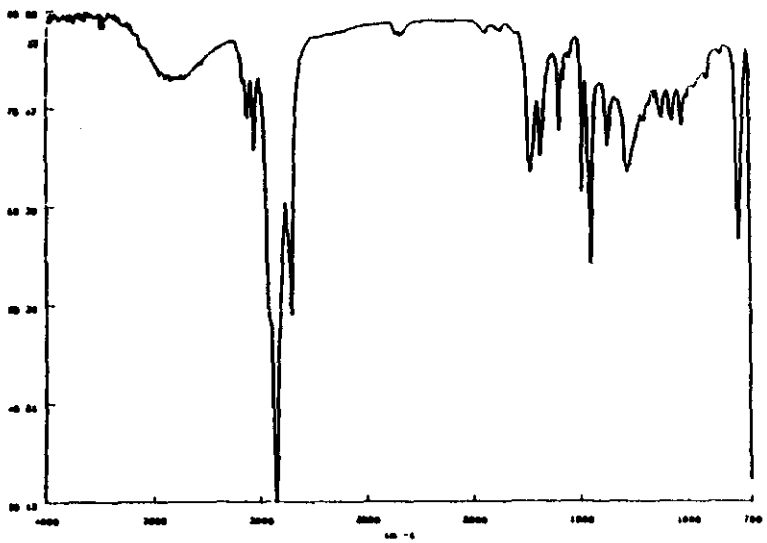
FIGURE 4 Spectra of tomato cuticle extracts that vary with maturation stage



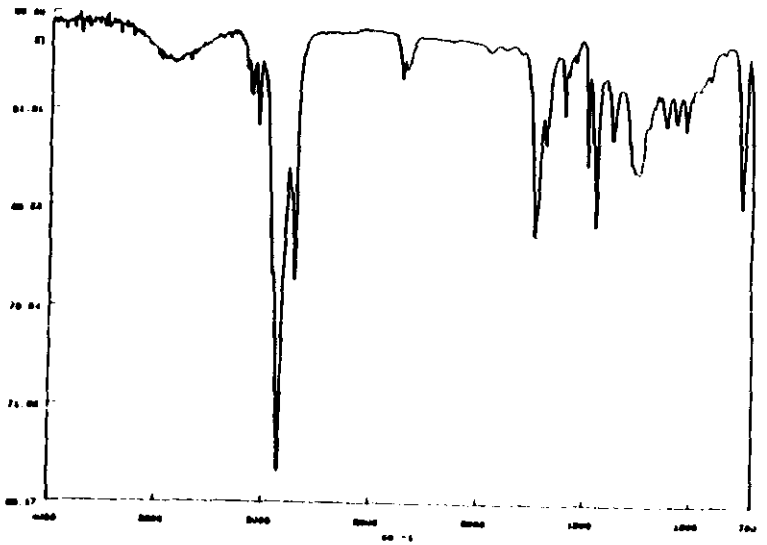
Stage 1 Extract 2



Stage 2 Extract 2

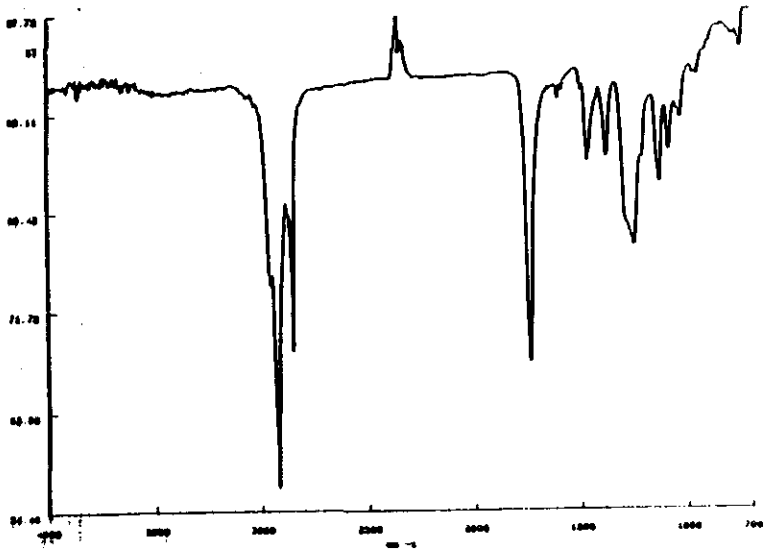


Stage 3 Extract 2

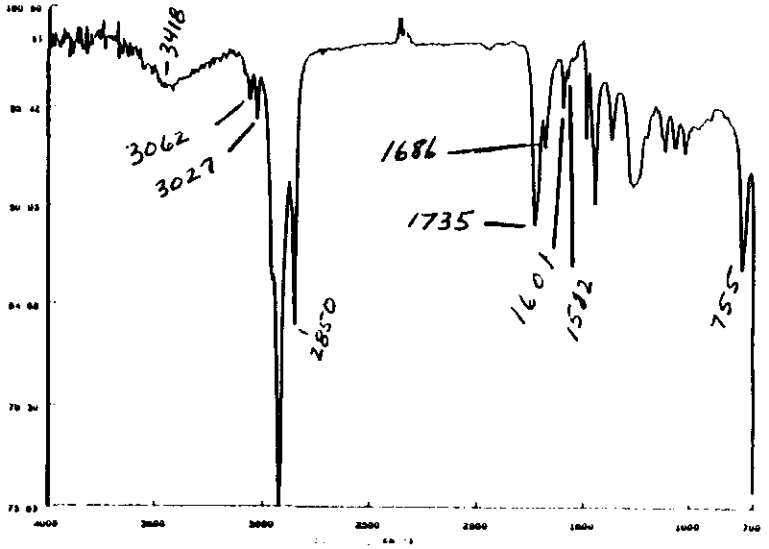


Stage 4 Extract 2

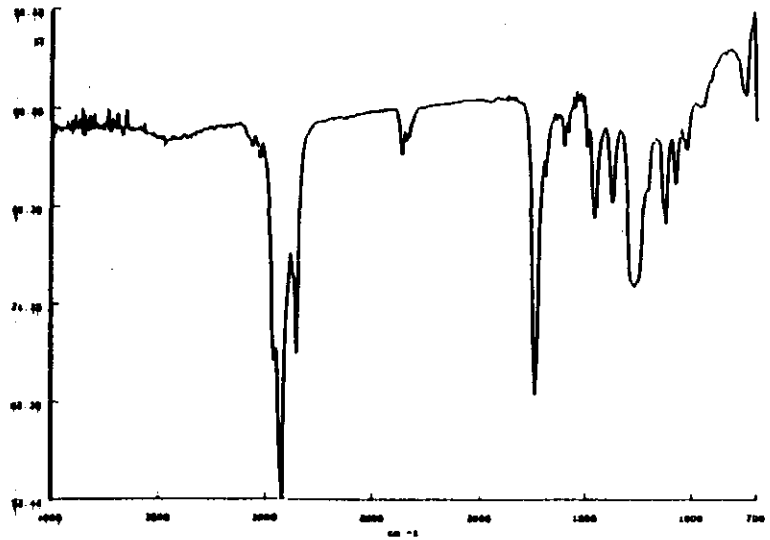
FIGURE 5 Spectra of tomato cuticle extracts that vary with extraction time



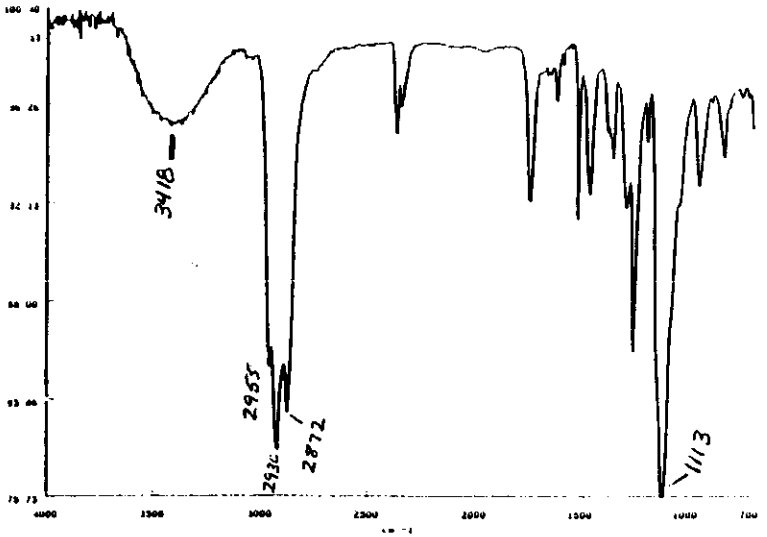
Stage 1 Extract 1



Stage 1 Extract 2



Stage 1 Extract 3



Stage 1 Extract 4

FIGURE 6

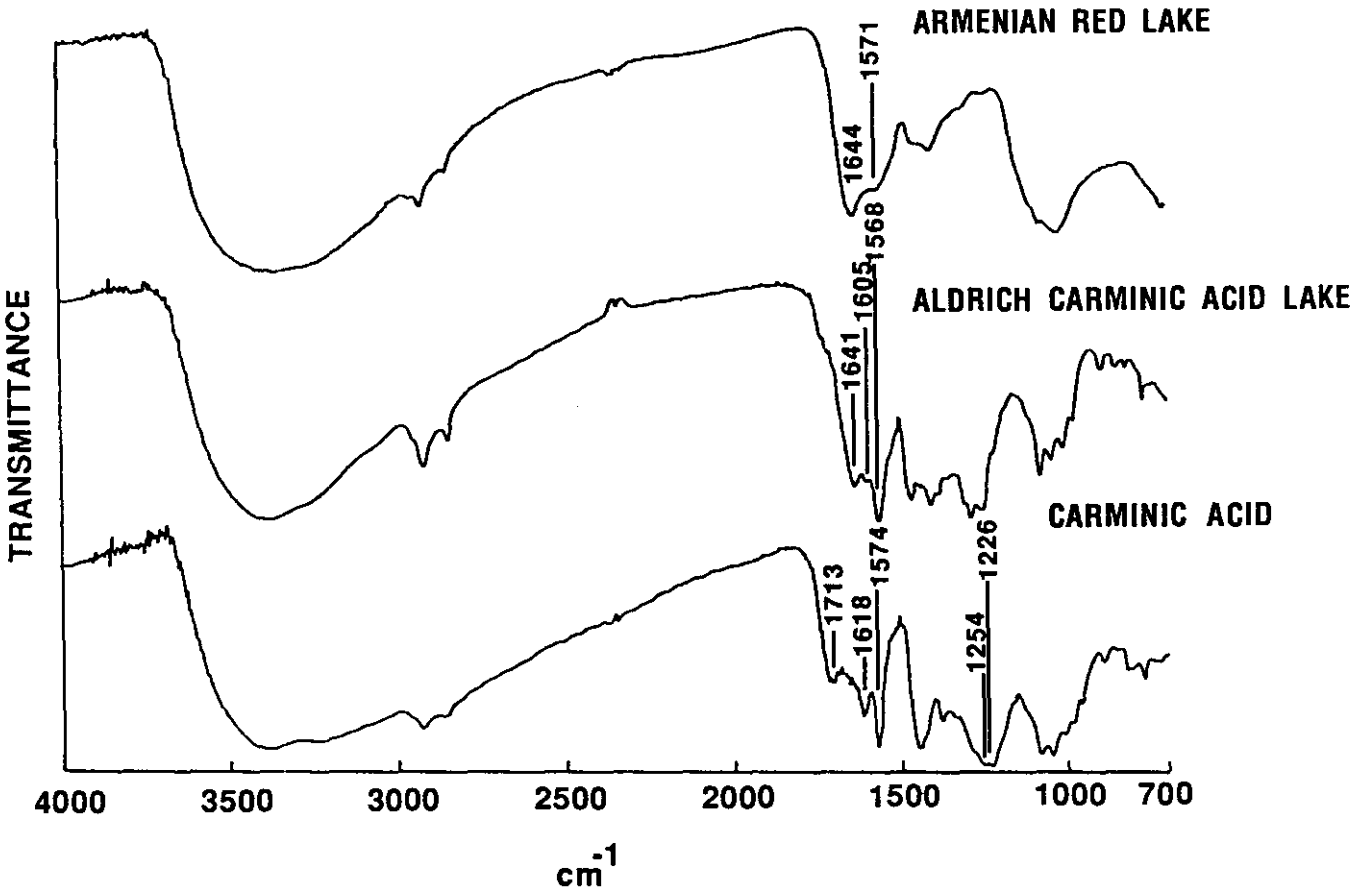

ARMENIAN RED LAKE
1644
1571
1568
ALDRICH CARMINIC ACID LAKE
1641
1605
CARMINIC ACID
1713
1618
1574
1254
1226
TRANSMITTANCE
4000
3500
3000
2500
2000
1500
1000
700
cm-1

FIGURE 7

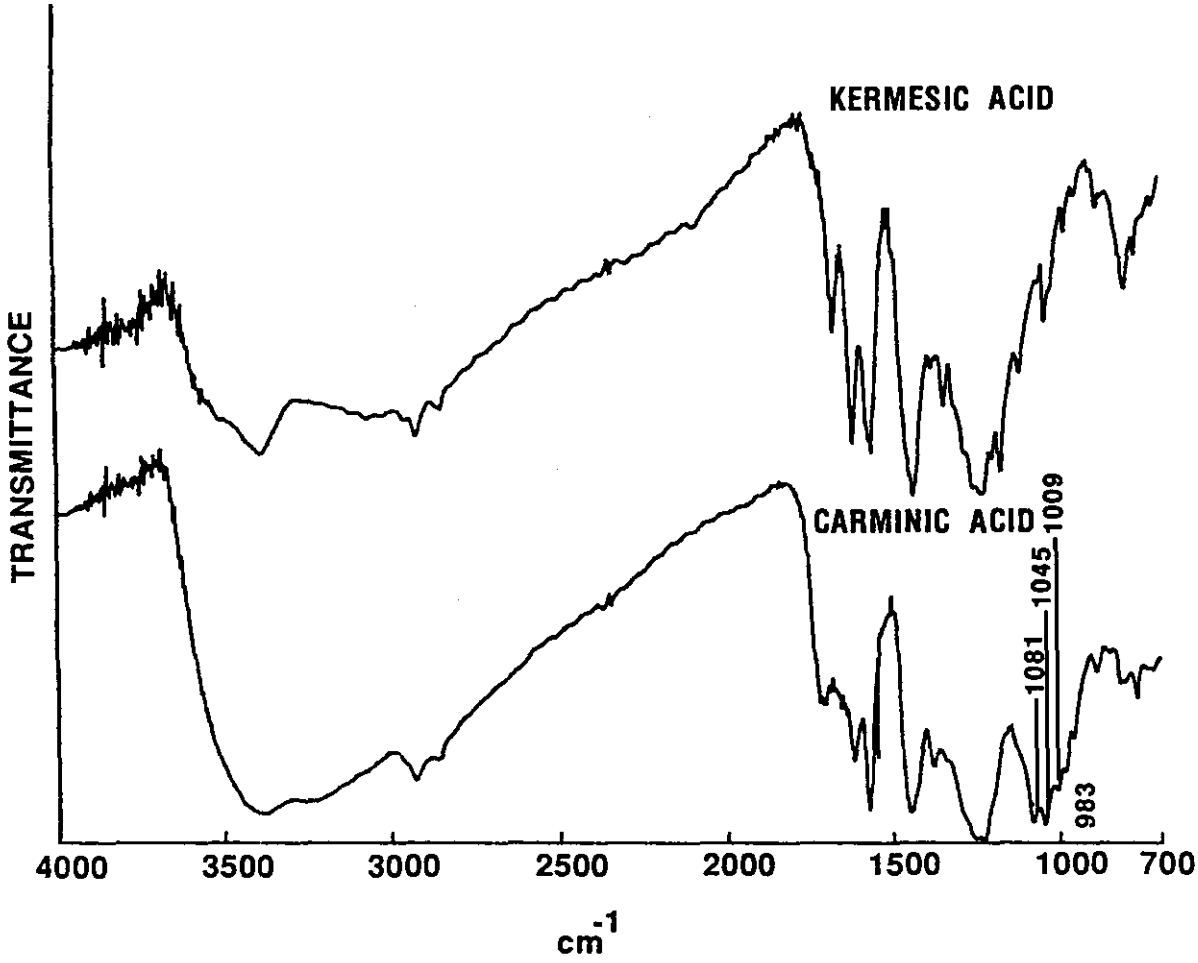

KERMESIC ACID
CARMINIC ACID
TRANSMITTANCE
1009
1045
1081
983
4000
3500
3000
2500
2000
1500
1000
700
cm-1

FIGURE 8

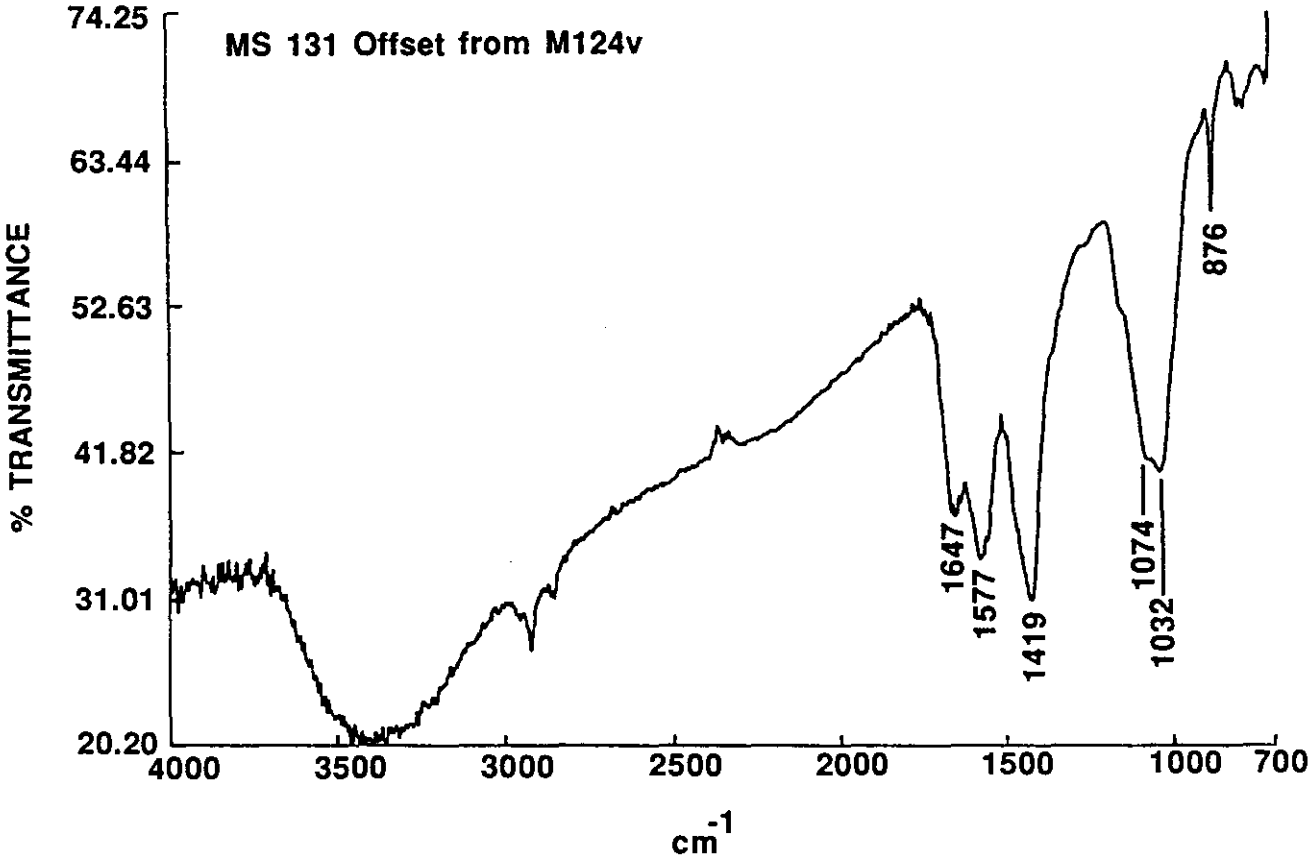

MS 131 Offset from M124v
74.25
63.44
52.63
41.82
31.01
20.20
% TRANSMITTANCE
4000
3500
3000
2500
2000
1500
1000
700
cm-1
1647
1577
1419
1074
1032
876

FIGURE 9

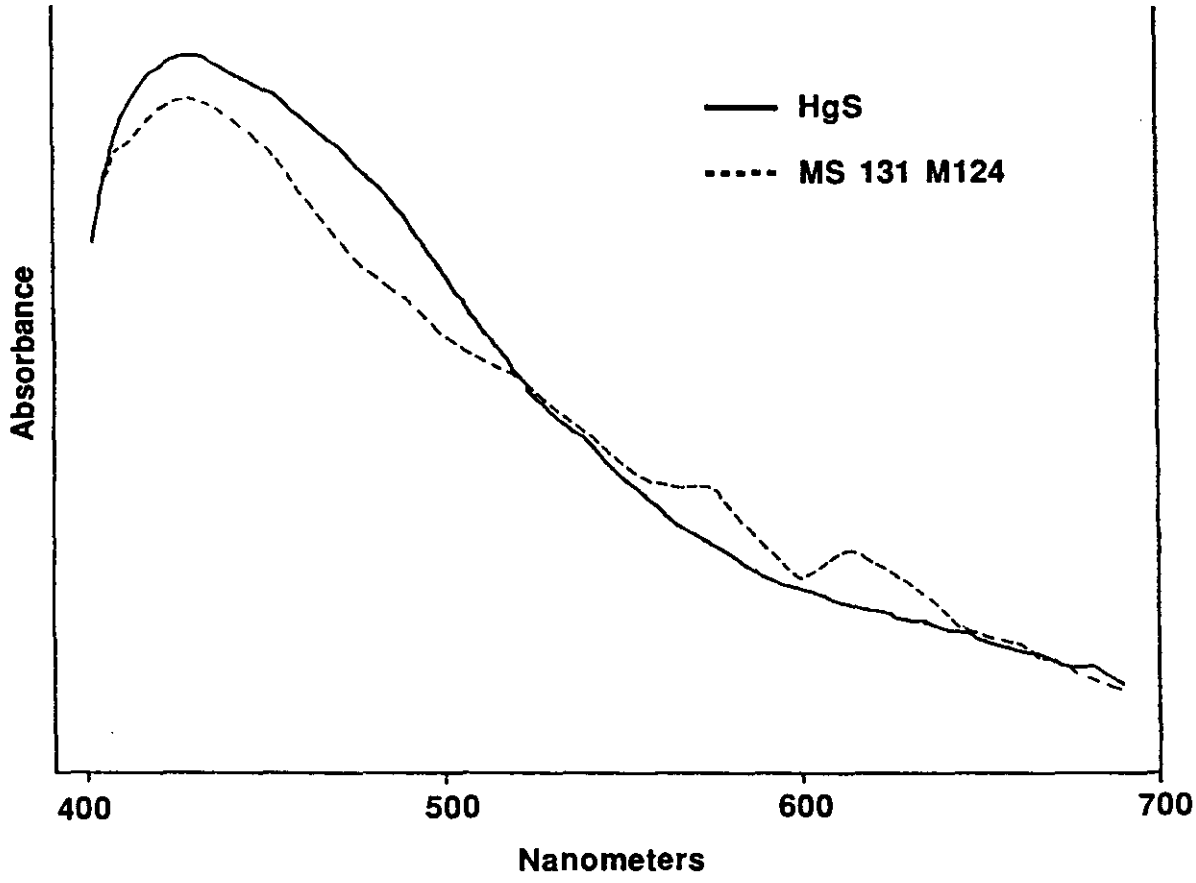


FIGURE 10

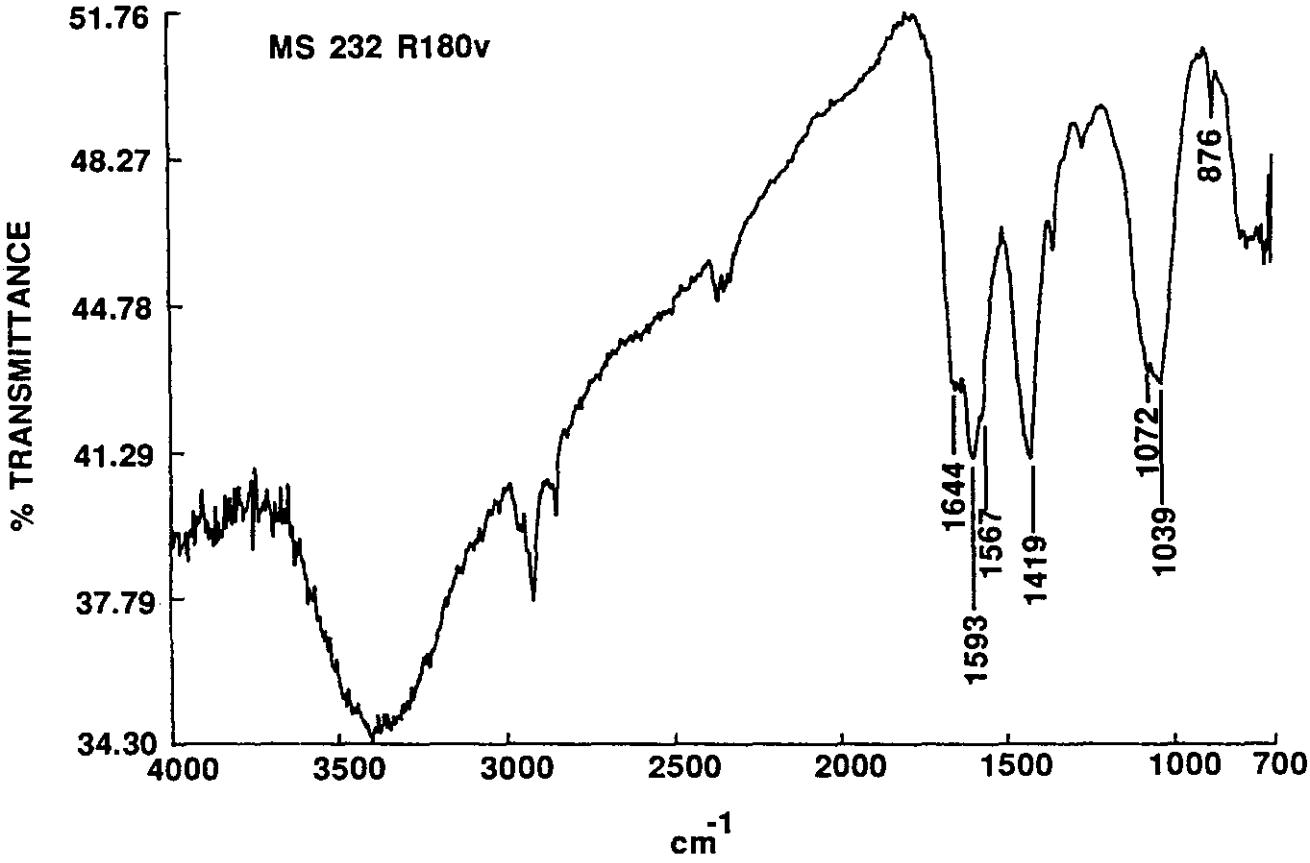

MS 232 R180v
51.76
48.27
44.78
41.29
37.79
34.30
% TRANSMITTANCE
4000
3500
3000
2500
2000
1500
1000
700
cm-1
1644
1593
1567
1419
1072
1039
876

FIGURE 11

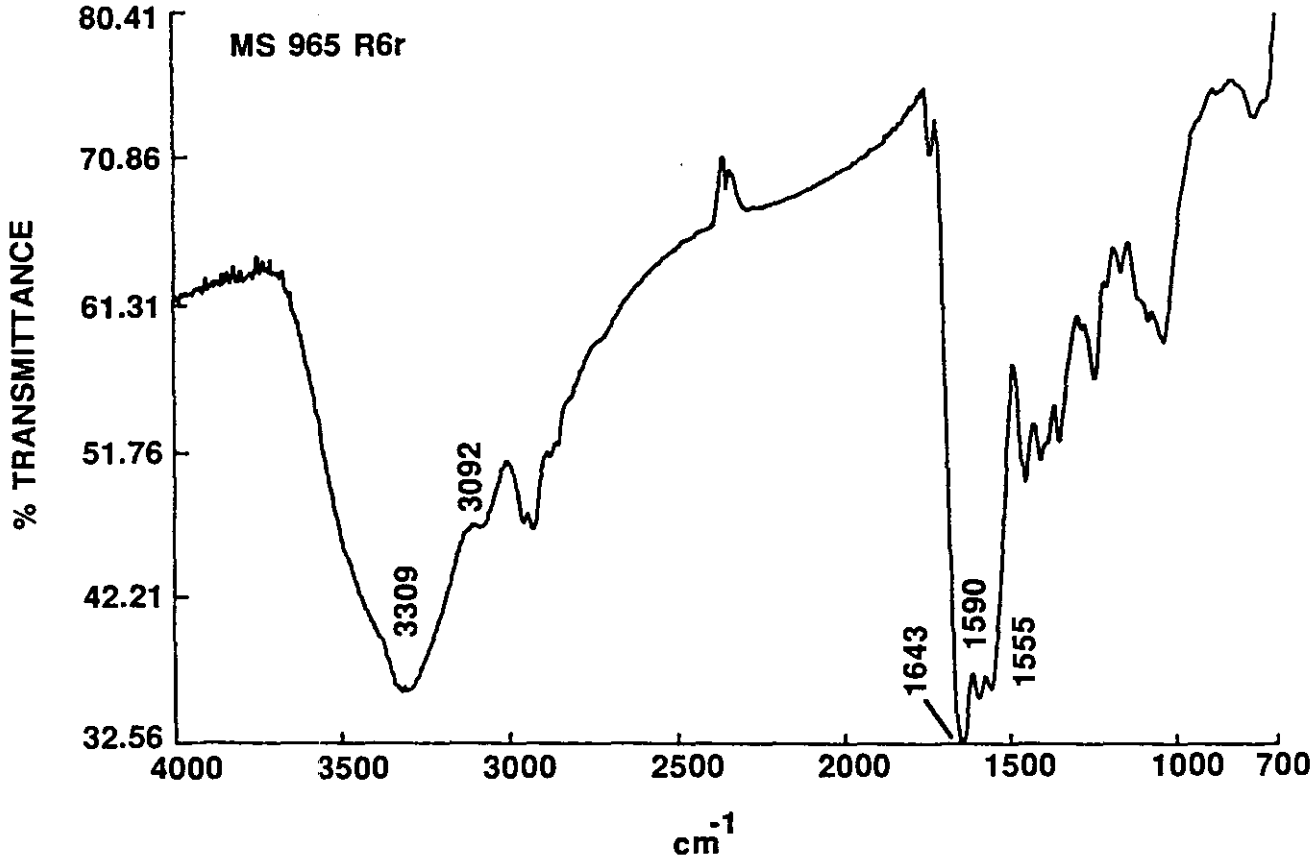

80.41
MS 965 R6r
70.86
61.31
51.76
42.21
32.56
% TRANSMITTANCE
3309
3092
1643
1590
1555
4000
3500
3000
2500
2000
1500
1000
700
cm-1

FIGURE 12

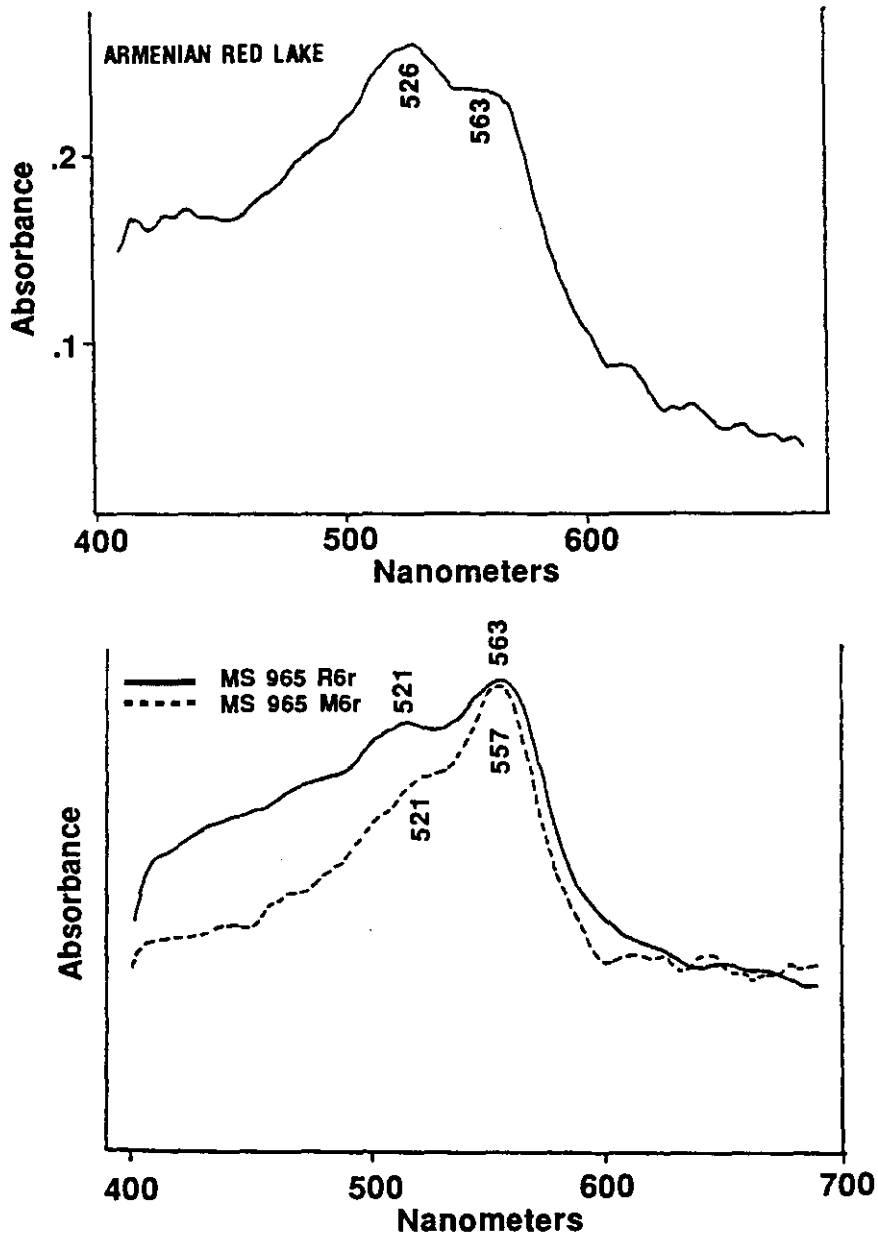
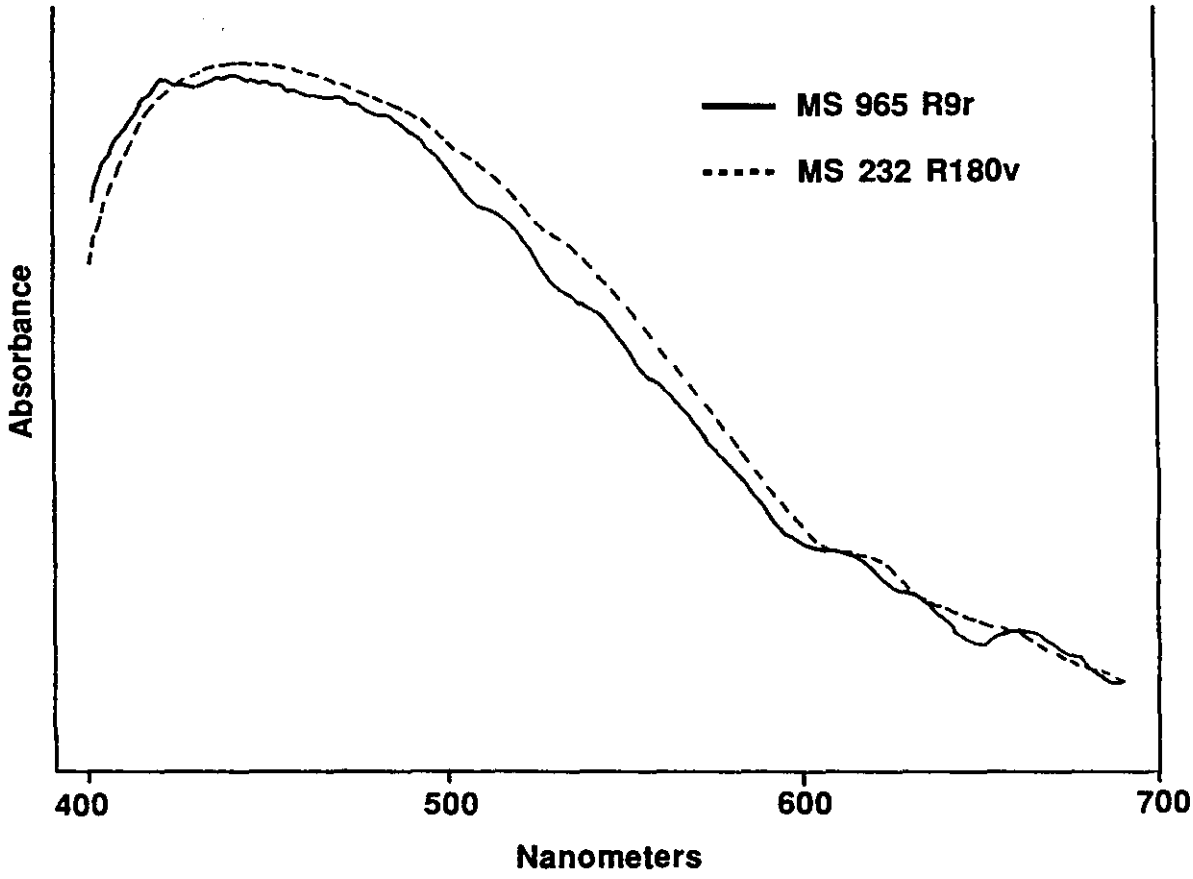

ARMENIAN RED LAKE
526
563
Absorbance
.2
.1
400
500
600
Nanometers
MS 965 R6r
MS 965 M6r
521
563
557
521
Absorbance
400
500
600
700
Nanometers

FIGURE 13


MS 965 R9r
MS 232 R180v
Absorbance
400
500
600
700
Nanometers