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A METHOD FOR THE DETERMINATION
OF SOLANESOL IN TOBACCO

BY
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A THESIS
SUBMITTED TO THE GRADUATE FACULTY
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JUNE, 1966

DEDICATION

Dedicated to my wife, without whose patience and encouragement this work would not have been possible.

ACKNOWLEDGMENT

On completion of this project I wish to thank Dr. W. Allan Powell. His interest and guidance have made the solution of this problem possible.

The author wishes to express his gratitude to Philip Morris Incorporated for allowing the use of their laboratory facilities to carry out this project and to the managers and director of the Philip Morris Research Center for this encouragement.

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INTRODUCTION

The object of this work was to develop a procedure for the quantitative determination of solanesol in the petroleum ether extract of tobacco. The amount of tobacco extracted into petroleum ether is routinely used to differentiate tobacco types. The extraction procedures have been extensively studied by the Association of Official Agricultural Chemists (AOAC) (20) and reported in the United States Department of Agriculture (USDA) Technical Bulletin # 1186 (11).

There is at present no method for the quantitative determination of solanesol. One procedure (1), which comes closest, involves an infrared measurement of the tobacco extract. Because of the presence of other bands, the results are reported as "solanesol-like substances" rather than solanesol. The reported values are considerably higher than estimates obtained from large scale isolation of solanesol from tobacco.

HISTORICAL

Solanesol was first isolated and named by Rowland, Latimer, and Giles (13). They wrongly characterized it as a pentaterpene alcohol. Erickson et al. (3) showed that solanesol was actually composed of nine isoprene units by attempting to use it to synthesize coenzyme Q₁₀. Using solanesol they produced coenzyme Q₉ (16) and reinvestigation showed solanesol to be C₄₅H₇₄O. Structurally, solanesol is:



The structure was confirmed by Ruegg et al. (14) who did a complete synthesis of solanesol.

I. Measurement Procedures

Methods to determine solanesol were examined. Bilinsky and Stedman (1) used the infrared 10 and 12 micron bands. They reported high values for the solanesol content of tobacco and

felt that other substances were present which could absorb at 10 and 12 microns and therefore give higher results.

Ruegg et al. (14) reported an α and β form of solanesol. The α form does not have an absorption band at 12 microns in a nujol suspension, casting further doubt on the reliability of the infrared procedure.

Von Planta (19) reported that solanesol did not have any ultraviolet bands and only end absorption at 1965 \AA .

Other procedures that could be applied to solanesol, based upon its chemical structure, include cerium nitrate complex (4), alcohol plus vanillin in concentrated sulfuric acid (7), halogenation of the double bonds (9) and various colorimetric methods (8, 17).

A colorimetric procedure developed by Johnson and Critchfield (8) for primary and secondary monohydroxy alcohols has been applied to C_1 - C_{20} alcohols but has not been applied to solanesol. The alcohol is reacted with 3,5-dinitrobenzoyl chloride in pyridine. The ester is extracted from a water solution into hexane. The ester produces a red quinoidal ion having a maximum absorption at 525 m μ in dimethyl formamide made basic with propylenediamine.

II. Separation Procedures

Because tobacco is a complicated mixture of many chemical constituents, considerable separation of the tobacco extract

constituents was required to isolate solanesol. Rowland et al. (13) extracted tobacco with methanol and then with ether. The combined residues were extracted with ether from a water solution. The etherial residue was partitioned between 90% methanol in water and hexane. The hexane solution was chromatographed on a silica gel column and eluted with hexane, carbon tetrachloride, and benzene. A specific fraction of the benzene eluate was chromatographed on an alumina column and treated with 2:1 benzene-chloroform and then 1:2 benzene-chloroform. Other methods of purification are also described using celite, Florisil and carbon (13).

Bilensky et al (1) soxhlet extracted tobacco with Skellysolve B. The Skellysolve B residue was dissolved in 60% benzene in petroleum ether.

The solution was chromatographed on alumina and developed with pure benzene.

Carruthers and Johnstone (2) chromatographed the crude solanesol fractions from green tobacco on alumina and eluted solanesol from the column with ether.

Grossman et al (6) extracted tobacco with petroleum ether. The ether solution was washed with 10% hydrochloric acid and 5% sodium hydroxide. The ether was evaporated off and the residue dissolved in hot acetone. The acetone solution was cooled to 4°C to remove waxes. The dewaxed solution was concentrated to a syrup and the syrup was distilled in a

molecular still. The solanesol was distilled at 300°C and 100-200 μ pressure. Solanesol was isolated by precipitation from an ether-methanol mixture at 4°C.

Stevenson et al. (18) extracted solanesol from a water solution of tobacco by adding ethanol and extracting with ether. The ether residue was dissolved in petroleum ether and chromatographed on an alumina column. The solanesol was eluted from the column with 8% diethyl ether in petroleum ether. The eluate was further chromatographed on silica gel G thin-layer plates.

Mold and Booth (10) isolated solanesol from the particulate matter of smoke by dissolving the particulate matter in diethyl ether and partitioning the ether residue, between water and hexane. The hexane was co-distilled with methanol in vacuo; the material was crystallized from cold methanol. The precipitate was chromatographed on alumina and developed with cyclohexane containing increasing amounts of methanol. The crude solanesol was rechromatographed on alumina and developed with cyclohexane, petroleum ether, and various amounts of benzene in petroleum ether.

Rodgman and Cook (12) separated particulate smoke into various fractions. Solanesol was isolated by washing the traps containing particulate matter with methanol. The methanol solution was acidified and extracted with hexane. The solution was chromatographed on alumina and developed with

hexane, 15% benzene in hexane, and methanol. The methanol eluate was developed on alumina with benzene, 1:1 benzene: ether, ether, 1:1 ether-ethyl acetate, ethyl acetate, 9:1 ethyl acetate:methanol and methanol. Solanesol was eluted in the ether and 1:1 ether:ethyl acetate fractions. The crude solanesol was purified with additional chromatography on alumina and Florisil.

EXPERIMENTAL

I. Reagents

Silica gel, Davison Chemical Corp.

Alumina, neutral, Brockman Grade 1, 80-200 mesh,

Fisher Scientific Inc.

Alumina, neutral, 8-14 mesh, Mathson, Coleman, and Bell

Petroleum ether, 30-60°C, B & A Co.

Pyridine, C.P. or equivalent

Dimethyl formamide, C.P. or equivalent

3,5-Dinitrobenzoyl chloride, Distillation Products Industries

1,2-Propane diamine, Distillation Products Industries

All other reagents were common laboratory reagents of C. P.

quality or equivalent.

II. Apparatus

Chromatographic column - 25 ml. buret

Chromatographic column - 4 mm I. D. tubing

Chromatographic column - 2 mm I. D. tubing

Teflon stopcocks

Wiley Mill

Spectrophotometer - Bausch and Lomb Inc. Spectronic 20

Goldfish extraction apparatus

All other equipment was common laboratory equipment

III. Development of the Method

A. Colorimetric Procedure

After examining several procedures (4, 7, 8, 9, 17), it was decided to further investigate the procedure of Johnson and Critchfield (8) because it was sensitive, specific for aliphatic alcohols, and had been found to be quantitative for C_1 to C_{20} alcohols. The procedure consists of reacting 2 mls of a pyridine solution of the alcohol with 1 ml of a 100 mg/ml pyridine solution of 3,5-dinitrobenzoyl chloride for 15 minutes at room temperature. The reaction is stopped by addition of 25 mls of 2 N HCl. The water also hydrolyzes the excess 3,5-dinitrobenzoyl chloride to the water soluble acid and disperses the ester which is then readily extracted with two 10-ml portions of hexane. The hexane is washed with 5 mls of 5% Na_2CO_3 to remove excess acid, and the hexane solution is dried by passing it through absorbent cotton. The hexane solution is diluted to 20 mls and 1 ml is added to 5 mls of dimethyl formamide. One-half ml of propylene diamine is added to form a red quinoidal ion just before reading the color at 525 m μ on a spectrophotometer.

In attempting to reproduce the method, difficulty was encountered in obtaining any significant color. The difficulty was traced to the 3,5-dinitrobenzoyl chloride. It apparently had degraded upon standing in a sealed bottle and no longer

reacted with alcohols. Fresh 3,5-dinitrobenzoyl chloride, ordered from Distillation Products Industries, gave a reaction product with alcohols. Another sample of 3,5-dinitrobenzoyl chloride gave a weak color with solanesol. Examination of the 3,5-dinitrobenzoyl chloride showed that the earlier sample of 3,5-dinitrobenzoyl chloride melted in the proper range, 67-69°C, while the latter material melted partially at 67°C, and the remainder did not melt even at 130°C. Recrystallization of the 3,5-dinitrobenzoyl chloride from 40-60°C petroleum ether improved the quality of the older material but did not improve the newer material. Fresh 3,5-dinitrobenzoyl chloride whose melting point was checked and stored in a vacuum desiccator has worked successfully for many months.

During the investigations of the colorimetric procedure, color variability was noticed. It was felt that this could be due to incomplete reaction between solanesol and 3,5-dinitrobenzoyl chloride. When performing the reaction in a 70°C water bath, very little color was obtained.

Because water interferes with the reaction, particular care was taken to insure that all glassware was dry. As an added precaution the pyridine, which had been redistilled as recommended by Johnson and Critchfield was stored over 5A molecular sieves. These precautions gave improved precision.

Increasing the amount of 3,5-dinitrobenzoyl chloride gave increased color at the 150 mg/ml level but not at the 200 mg/ml level. Using 200 mg/ml of 3,5-dinitrobenzoyl chloride required additional pyridine to dissolve the material, additional acid to acidify the water phase, and additional Na_2CO_3 to neutralize the hexane solution. Therefore, it was decided to use 150 mg of 3,5-dinitrobenzoyl chloride.

During the evaluation of the 3,5-dinitrobenzoyl chloride, the reagent was reacted with sufficient solanesol and ethanol to give an insoluble precipitate. The procedure in Shriner, Fuson, and Curtin (15) was followed. The melting point of the solanesol derivative was 51-53°C; literature reference was 57.5-59.5°C (13). The melting point of the ethanol derivative was 89-90°C; literature reference was 93°C (15). Low recoveries were encountered in recrystallizing the solanesol derivative because of its alcohol solubility. Acetone appears to be a better solvent for the recrystallization of the solanesol derivative.

Measured amounts of the ethanol and solanesol derivatives were dissolved in hexane, and the colored quinoidal ion formed in dimethyl formamide. The absorptivity of these compounds calculated as solanesol was somewhat higher than that obtained from solanesol carried through the procedure.

Mold and Booth (10) showed that solanesol was sensitive to photo oxidation and that the infrared spectrum of the oxidation

products were similar to that of solanesol except for the presence of a carbonyl band at 1700 cm^{-1} (Figure 1). Column chromatography of solanesol, which will be described in section C, gave a product free of the carbonyl band (Figure 2). This material was used to determine the absorptivity of solanesol. The results are given below in Table I.

Table I
Solanesol Absorptivity

<u>Sample</u>	<u>Amount Reacted, mg</u>	<u>Absorptivity</u>
Solanesol	6.0	23.8
	3.0	25.0
	2.4	24.0
	1.2	24.5
	0.24	29.7
Ethanol derivative	0.0545	26.6
Solanesol derivative	0.335	25.7
Solanesol derivative	0.134	26.0

Average for solanesol: $24.3 \pm 0.5 = \pm 2\%$ relative error

The 0.25 mg solanesol sample appears to be below the linear concentration range. Johnson and Critchfield reported a linear range for this procedure as 2 to 100 μg hydroxyl concentration.

Hexane has only limited solubility in dimethyl formamide (about 20%); therefore, only 1 ml of the hexane solution of the solanesol-3,5-dinitrobenzoyl chloride reaction product

can be dissolved in dimethyl formamide. However, it was found that the solanesol reaction product can be extracted with the 5 mls of dimethyl formamide from the 20 mls of the hexane solution with a single extraction. Addition of propylene diamine produced a strong color in the dimethyl formamide phase. A second dimethyl formamide extract did not produce a color upon the addition of propylene diamine. The addition of 1 ml of the extracted hexane layer to 5 mls of dimethyl formamide did not produce any color upon the addition of propylene diamine. This is one method of increasing the sensitivity of the procedure twenty-fold, if necessary.

B. Extraction of Solanesol

Many procedures have been described for the extraction of solanesol from tobacco (1, 6, 13, 18). Solanesol is considered to be a contributing factor in tobacco flavor (5) and is a constituent of the neutral fraction of tobacco (1, 6, 13, 17). The AOAC procedure (20) for petroleum ether extractables attempts to evaluate tobacco based upon the amount of neutral material extracted and is routinely used to characterize tobacco. It was therefore decided to use the petroleum ether extraction procedure for solanesol.

Most of the procedures for the isolation of solanesol involve extensive separations, including several column chromatographic steps. The Grossman et al. procedure previously

described in the historical portion of this thesis, appeared well suited.

Thirteen and one-half pounds of bright tobacco were soxhlet extracted with 15 gallons of 30-60° petroleum ether for six hours. The petroleum ether solution was concentrated to four liters with distillation. The solution was filtered. Three grams of precipitate was discarded. The ether solution was extracted three times with 10% HCl, followed by 3 water extractions, then extracted with 3 portions of 5% NaOH and then water extracted again until the water layer was neutral. The petroleum ether layer was filtered through cotton to remove water and evaporated to dryness. The residue weighed 79.13 grams.

The residue was dissolved in hot acetone, cooled to 4°C and filtered. The precipitate was washed with acetone and the residue of 16 grams, consisting primarily of waxes, was discarded. The acetone solution was concentrated to a volume of one liter and used as a standard tobacco extract for future work.

Because of the complex nature of the tobacco extract, it was felt that isolation of the solanesol fraction would be helpful. Attempts were made to precipitate solanesol out of solution, based upon its relative solubility from the acetone solution at -10°C, from an ether-methanol solution at 4°C and

from an acetone-methanol solution at -10°C . These attempts were unsuccessful and the infrared spectra indicated that each sample was still a complicated mixture. Solanesol is water insoluble but very soluble in non-polar and slightly polar solvents; therefore, it was felt that the addition of water to the acetone solution would increase the partition coefficient of solanesol for the non-polar solvents. The 3:1 and 5:1 water-acetone solutions of tobacco extracts were serially extracted with hexane, benzene, petroleum ether, and chloroform. The infrared spectra indicated complicated mixtures. In the attempt to purify them, the solutions were evaporated to dryness, the residues dissolved in ether, methanol added, and the solutions stored in the cold to precipitate solanesol as described by Grossman et al. (6). Complicated infrared spectra were still obtained, none of which were really indicative of solanesol. Using the previously described colorimetric procedure, the heaviest concentration was found in the chloroform extracts. Extracting 50 mls of the acetone solution of tobacco with chloroform after the addition of water and chromatography of the chloroform extract on silica gel gave poor results.

Investigating the difficulties with a solanesol sample of known composition, it was found that the solanesol formed a stable emulsion in water and could not be readily extracted with any of the solvents.

Rowland et al. (13) partitioned solanesol between 9:1 methanol-water and hexane. The procedure was attempted but it was difficult to separate in the presence of acetone. Using a 75% methanol in water solution, improved separation was obtained although the procedure was still not ideal since some emulsion was formed. By adding some NaCl to the acetone-water-methanol solution (1:2:6) extraction was virtually complete with two hexane extractions and the emulsion broke rapidly. The tobacco solution was extracted, and the infrared spectra of the hexane extract strongly resembled solanesol (Figure III). Spectra of the second, third, etc. extracts gave increasingly more complex spectra.

The spectrum of the material remaining in the acetone-water-methanol phase contained most of the alcohols of tobacco (Figure VI).

A series of solanesol standard solutions were extracted in several ways and the developed color measured on the individual samples. The complete extraction procedure is:

petroleum ether solution of solanesol

extracted with 10% HCl

extracted with water

extracted with 5% NaOH

extracted with water

filtered through absorbant cotton

evaporated to dryness

dissolved in acetone

stored for 16 hours at 4°C

filtered through Whatman #1 filter paper

diluted with methanol and water

extracted with hexane

filtered through anhydrous Na_2SO_4

The amount of solanesol recovered from the various portions of the extraction procedure are given in Table II below.

Table II

Solanesol Recovery from the Extraction Procedure

<u>Portion of Procedure Used</u>	<u>% Recovered</u>
1. Acid and water washed only	93
2. Base and water washed only	93
3. Acid and base washed	100
4. Stored in acetone at 4°C	96
5. 3 plus 4	100
6. Hexane extracted and filtered through Na ₂ SO ₄	100
7. Entire procedure, 5 plus 6	101

C. Column Chromatography of Solanesol

Most procedures in the literature for isolating solanesol (1, 2, 10, 12, 17, 18) used column chromatography; many used a series of columns to obtain solanesol in a relatively pure form. The column materials included silica gel, alumina, Florisil, Celite, and carbon. Several of these procedures were tested.

Using silica gel and attempting to chromatograph the acetone solution, it was found that separation could not be achieved and all of the sample came through in the acetone effluent. Using the hexane extract, solanesol from an authentic sample

was retained on the column and a hydrocarbon was eluted off with hexane (Figure IV). Solanesol did not come off with 10, 20, or 50% benzene in hexane, but did come off when eluted with 100% benzene. Tobacco extract treated in a similar manner gave complicated spectra and had strong absorption at 1750 cm^{-1} (Figure V). The silica gel used for these columns was not heat activated.

Using heat activated silica gel, the hydrocarbon (Figure IV) came through in the hexane wash and 50% benzene wash. Nothing came over in 100% benzene. One and 10% ether in benzene extracts did not elute any material from the columns. Acetone extraction of the columns gave complicated spectra.

Using activated alumina (Mathson, Coleman, and Bell, 8-14 mesh) and following a similar procedure as described for the silica gel column, similar results were obtained. The hydrocarbon (Figure IV) was eluted with hexane, benzene, and diethyl ether; some came off in each of the serial elutions. An alcohol, but not solanesol, was eluted off with acetone and a sample having a complex spectrum, came off with methanol.

Using 10 grams of Brockman activity grade 1 neutral alumina 80-200 mesh (Fisher Scientific), solanesol was eluted off with 100% benzene. A tobacco sample gave the same results; however, elution of the solanesol off the columns required several hundred ml of benzene.

Repeating the previous experiment, it was found that the solanesol from tobacco came through in the hexane sample while it remained on the column with known solanesol. One set of columns was eluted with hexane, 50% benzene, and 100% benzene. Another set of columns was washed with hexane and 50% ether in hexane. Two hundred and fifty mgs of known solanesol was added to each column. Two hundred and three mgs of solanesol was eluted off with 50% ether (81.4%) but only 110.9 mg was eluted off with 100% benzene. Additional solanesol was eluted off the benzene treated column when washed with ether. The material that was eluted off with 50% ether and 100% benzene was used to determine the solanesol absorptivity described in section A.

It was felt that water may have been present in the hexane extracts and the extracts were therefore dried by filtering them through anhydrous Na_2SO_4 and the procedure was repeated. The solanesol apparently remained on the column although a compound did come through with the hexane that was similar in infrared spectra to solanesol and reverted to the solanesol spectrum after 2 weeks of standing in a vacuum desiccator. The benzene effluents did not look very pure, but the first 50 mls of ether effluent was pure and gave a good infrared spectrum of solanesol. Additional washes with ether gave increasingly more complex spectra.

The previous procedure was again repeated but with less acetone solution of tobacco in order to decrease the overloading of the column and to obtain better separation of the various substances. Known solanesol was also added to the acetone solution of tobacco and to the hexane extract of another sample of tobacco. Solanesol was found in the hexane effluent and hexane wash of the column containing the tobacco samples.

It was felt that the alumina may vary in activity due to the degree of moisture and length of standing. The alumina was therefore activated for 16 hours at 140°C and kept in a desiccator till cool. Fifteen grams were weighed out and immediately placed in a chromatographic column with hexane. The above procedure was repeated with 10-ml aliquots of the acetone solution of tobacco, one 10-ml sample containing 150 mg of 81.4% solanesol added to the acetone solution, another 10-ml sample contained 150 mg of 81.4% solanesol which had been added to the hexane extract, and a third sample consisted of a 10-ml acetone aliquot only. All hexane extracts were dried with Na_2SO_4 . After the samples had been eluted through the columns, the columns were washed with 250-ml hexane, followed by 250 mls of 50% benzene in hexane. Only carbonyls came through in the 50% benzene effluent and the spectra did not resemble solanesol. The columns were then washed with 100% diethyl ether and the weight of the residues were recorded before infrared spectra were run. The results

are given below in Table III.

Table III

Recovery of Solanesol from Tobacco Extracts

<u>Effluent</u>	<u>Tobacco Aliquot Only</u>	<u>Tobacco + Solanesol Added to the Hexane Extract</u>	<u>Tobacco + Solanesol Added to the Acetone Solution</u>
First 20 ml ether	19.9 mg*	Spilled	38.1 mg*
Next 30 ml ether	9.9 mg*	48.2 mg*	44.0 mg*
Next 50 ml ether	75.6 mg**	142.7 mg**	120.1 mg**
Next 50 ml ether	38.8 mg**	41.4 mg**	46.9 mg**
Next 50 ml ether	9.3 mg**	10.2 mg**	13.6 mg**
Next 50 ml ether	4.3 mg**	4.4 mg**	5.5 mg**
Total solanesol	128.0 mg	246.9 mg	230.1 mg
% recovered	0.21%	79.3%	68.1%
Expected recovery	0.3%	81.4%	81.4%
Relative recovery	---	97.5%	83.9%

* Spectra did not resemble solanesol

** Spectra resembled solanesol

The spectra of the ether effluents shown with double asterisks in Table III were not complicated and were identical to solanesol. The column containing 150 mg of known solanesol could not be eluted with ether.

After the elution with ether, the columns were eluted with

acetone and 150.2 mg of known solanesol was eluted off in the first 30 ml of acetone eluate. No additional material was eluted off. Solanesol plus other material was eluted off the tobacco columns with 30 ml of acetone. The weights were as follows: tobacco sample, 62.1 mg; tobacco plus solanesol added to the hexane extract, 60.7 mg; and tobacco plus solanesol added to the acetone solution, 75.9 mg. The spectra were relatively clean but impurities could be seen.

Spectra of additional acetone eluates did not resemble solanesol. An alcohol isolated from these acetone effluents was weighed and run by the colorimetric procedure. The absorptivity of the reaction product was one-half of the absorptivity of solanesol, indicating that the alcohol had a higher molecular weight than solanesol and possible interference by this alcohol would be proportionately decreased.

From the column chromatography of the petroleum ether extract from 61 grams of tobacco, it appeared that the conditions for separating solanesol from other constituents of tobacco had been achieved. Since the petroleum ether extraction procedure (20) recommends extracting 1 gram of sample and a maximum of 2 grams of sample can be accommodated in the Goldfish extraction apparatus, it was decided to "scale down" the procedure.

Using 2 grams of activated alumina in a 4 mm I.D. column, excessive "holdup" and a great deal of diethyl ether was required

to elute solanesol from the column. One gram of alumina did not give good separation because of the small height to diameter ratio of the column. Using 2 mm I.D. columns and 1 gram of alumina, good separations were obtained.

When enough solanesol was available for infrared spectra, it appeared that the solanesol was being converted to an acid as shown by the strong band at 1445 cm^{-1} . After much diligent effort, it was traced to contamination from silicone stopcock grease and not due to any catalytic conversion. All chromatographic equipment was fitted with teflon stopcocks and the succeeding spectra of the solanesol fractions indicated that the solanesol was pure.

IV. The Developed Procedure

A. Preparation of Reagents

Alumina, 80-200 mesh, Brockman activity grade 1, (Fisher Scientific Inc.), must be activated for at least 16 hours in an oven at 140°C and cooled in a desiccator just before preparing the chromatographic columns.

The alumina column should be washed with 100-ml hexane before chromatographing the sample. The pyridine must be redistilled and stored over heat activated 5A molecular sieves.

B. Procedure for Separation and Determination of Solanesol

The tobacco samples are ground in a Wiley Mill, to pass through a 25 mesh screen, and 1.000 grams of the ground sample

weighed into a Goldfish extraction thimble. The sample is extracted with 60 ml of petroleum ether for 8 hours. At the end of the extraction period the sample is replaced with a solvent recovery thimble and the extract is distilled until free of solvent. The extract is then dried to constant weight in a 100°C oven and cooled in a CaCl₂ desiccator. The weight of the residue is the amount of petroleum ether extractables.

The residue is dissolved in 50 ml of warmed petroleum ether and the solution extracted 3 times with 10 mls of 10% HCl solution. The petroleum ether solution is then washed with 3 or 4 portions (10 ml) of water till the water wash is neutral. The extract solution is then extracted with three 10-ml portions of 5% NaOH solution and again washed with 10 water till neutral. The petroleum ether solution is filtered through cotton to remove water and the sample is evaporated to dryness on a steam bath under a slight vacuum.

The residue is dissolved in 30 ml warm acetone and the acetone solution is stored overnight in a refrigerator at 4°C to precipitate out the waxes. The acetone solution is filtered cold through Whatman #1 filter paper and the beaker and precipitate rinsed with cold acetone.

The acetone solution is reduced to a volume of 10 ml on a steam bath with a slight vacuum. Eighty mls of 75% methanol in water is added to the acetone solution along with

several hundred mgs of NaCl. The acetone-methanol-water solution is extracted 4 times with 25 mls of hexane.

The hexane solution is filtered through 2-3 grams of anhydrous Na_2SO_4 . The Na_2SO_4 is washed with hexane and the hexane solutions are combined. The hexane solution is percolated through a 1 gram column of alumina in a 2 mm I.D. column fitted with a teflon stopcock.

The column is rinsed with 25 mls of hexane after the sample has passed through. The column is next rinsed with 75 mls of 50% benzene in hexane. The solanesol is then eluted from the column with 200 mls of diethyl ether.

The diethyl ether solution is evaporated to dryness on a steam bath under a slight vacuum.

One hundred fifty mg of 3,5-dinitrobenzoyl chloride is added to the residue and the acid chloride and residue are then dissolved in 2 mls of redistilled pyridine. The solution is allowed to stand at room temperature for 15 minutes.

At the end of that time, 25 mls of 2N HCl is added and the solution is transferred to a separating funnel with 10 mls of hexane. The solanesol ester is extracted twice with the hexane and an additional 10 mls of hexane. The combined hexane extracts are washed with 5 mls of 5% Na_2CO_3 and the hexane solution is then filtered through cotton into a 25 ml stoppered graduated cylinder. The cotton is washed with additional hexane and the total volume brought to 20 mls.

One ml of the hexane solution is pipetted into a test tube, 5 mls of dimethyl formamide is added and the test tube is shaken.

Just prior to reading the absorbance of the solution in a Bausch and Lomb Spectronic 20 spectrophotometer at 525 mμ, 0.5 ml of 1,2-propane diamine is added and the test tube is shaken. The absorbance of the sample versus a reagent blank is recorded and the amount of solanesol calculated.

C. Calculations

$$1. \text{ Mg of solanesol} = \frac{\text{absorbance of sample}}{24.3} \times \frac{6.5}{1} \times 20 \text{ (aliquot factor)}$$

$$\text{Mg of solanesol} = \text{absorbance of sample} \times 5.3$$

$$2. \text{ \% solanesol in tobacco} = \frac{\text{absorbance of sample} \times 6.5 \times 20}{24.3 \times \text{wt. of sample (mg.)}} \times 100$$

$$\text{\% solanesol in tobacco} = \frac{\text{absorbance of sample} \times 530}{\text{sample wt. (mg.)}}$$

The value of 24.3 is the absorptivity for solanesol found previously. The absorptivity for ethanol may be substituted, as seen from the results presented in Table I.

V. Results Employing the Method

One and two-gram samples of ground bright tobacco were run by the standardized procedure. The results are given in the following table.

Table IV

Results of Tobacco Analyses

Sample #	Tobacco (grams)	Solanesol Added (mg)	Petroleum Ether Extractables (%)	Solanesol Found		
				Total (mg)	Net (mg)	%
48	2	6	5.8	12.8	5.8*	0.49
49	2	3	5.3	12.2	8.7*	0.44
50	2	1.5	5.5	12.3	10.6*	0.53
51	2	--	5.6	5.6	8.7	0.44
52	2	--	5.5	5.5	6.0	0.30
standard	--	6	--	7.0	7.0	--
42	1	3	6.0	14.0	10.3*	1.03
43	1	1.5	5.4	15.3	11.6*	1.16
44	1	--	5.0	11.5	11.5	1.15
45	1	--	5.5	8.2	8.2	0.82
46	1	--	5.6	9.2	9.2	0.92
standard	--	3	--	3.7	3.7	--

* Mg of solanesol found in tobacco (after subtracting the amount of additional solanesol recovered)

The above samples were all extracted with petroleum ether at the same time and the 2-gram samples were run immediately while the 1 gram samples were stored dry in a refrigerator for 9 days.

The solanesol was added to the tobacco as a petroleum ether solution and the petroleum ether was allowed to evaporate

before extraction was begun.

Literature values for the expected amount of solanesol in tobacco are about 0.3%, based upon large scale extraction of 30 Kg of tobacco.

Apparently there are solanesol compounds in the tobacco extract which degrade to free solanesol upon standing in the dry state.

A 1 and 2-gram sample, that had been extracted at the same time as the previous samples and stored dry in a refrigerator at 4°C, were analyzed 14 days after extraction. The results are given below.

Table V

Tobacco Analyses

Sample #	g. Tobacco Extracted	% Petroleum Ether Extractables	Solanesol Found	
			mg.	%
47	2	5.4	11.5	0.58
53	1	5.4	18.4	1.84

When there was sufficient sample, infrared spectra were run and the material quantitatively rinsed back into the effluent solution. The infrared spectra of the samples were indicative of pure solanesol.

Because of the poor reproducibility within a population, when extracting tobacco with petroleum ether, it was decided to try hexane extraction in the Goldfish apparatus. The

results are given below.

Table VI

Hexane Extraction of Tobacco

Tobacco (grams)	% Extractables	Solanesol Found		
		Total (mg)	Net (mg)	%
1*	6.0	5.4	3.9**	0.39
1	6.2	3.3	3.3	0.33
1	6.1	4.1	4.1	0.41
1.5 mg standard		--	1.5	--

* 1.5 mg solanesol added to the tobacco

** 3.9 = total solanesol found -1.5 mg (amount of solanesol recovered from standard)

From the results, it appears that hexane extraction is somewhat better; and visual observation showed that there appeared to be less material on the column that could interfere with the determination of solanesol, and the possibility of overloading the column is greatly reduced.

Investigating the procedure per se without the possible variability of the petroleum ether extraction step, 10 one-gram samples of tobacco were extracted in the Goldfish extractor. The solvent was not evaporated off but the solutions were combined in a 1 liter volumetric flask and brought to volume with petroleum ether.

Five 100 ml samples (equivalent to the extract of 1 gram of tobacco) were used and 0.98 mg of solanesol standard was added to several samples.

The proposed procedure was followed and the results are given in Table VII below.

Table VII

Analyses of the Petroleum Ether Solutions of Tobacco

<u>Sample #</u>	<u>Solanesol Added (mg)</u>	<u>Solanesol Found (mg)</u>	<u>Net Amount Found (mg)</u>
0.98 mg std.	0.98	0.86	--
2	0.98	5.31	4.5
3	0.98	5.02	4.2
4	--	4.86	4.9
5	--	4.59	4.6
6	--	4.94	<u>4.9</u>

Average = 4.6

σ = 0.3

2σ = 0.6 = 13% rel.

Visual examination of the chromatographic column during the chromatographic development of the sample showed that the samples from the petroleum ether solutions had fewer colored bands than the samples from the petroleum ether residue samples. The depth of the brown band at the top of the column was smaller for the solution samples as compared to the residue samples.

The solanesol was eluted off the column containing the solution samples at a faster rate than with the residue samples. These observations would tend to indicate that changes occur, with respect to the tobacco extracts, upon drying. Although the chromatographic development of the hexane samples resembled the chromatographic development of the petroleum ether solutions, the solanesol was not eluted at a faster rate. These observations would tend to indicate that different substances were extracted with hexane than with petroleum ether.

VI. Discussion

From the results, it appears that any common alcohol may be used to prepare a standard curve for solanesol or to calculate the absorptivity for solanesol for any instrument and optical cell combination employed. The method for calculating the absorptivity of solanesol from another alcohol is:

$$\text{Absorptivity for solanesol} = \frac{\text{absorbance (alcohol)} \times \text{M.W. (alcohol)}}{\text{concentration of alcohol (mg/ml DNF)} \times 631.04}$$

The 631.04 value is the molecular weight of solanesol. M.W. stands for molecular weight.

Petroleum ether extraction of tobacco apparently gives imprecise values for solanesol and although hexane extraction appears to be somewhat better it also suffers from imprecision. It may be virtually impossible to extract solanesol from tobacco unless a more drastic destruction of the individual cells of the

tobacco leaf is employed. The poor reproducibility obtained with the petroleum ether extractables tends to confirm the suspicions of the AOAC Methods Committee (20) that further study is needed in obtaining a reproducible method for petroleum ether extractables in tobacco.

Solanesol is apparently extracted as the free alcohol and also as readily hydrolyzable compounds which appear to decompose on standing in the dry state in the presence of a humid atmosphere. High solanesol values were not obtained from the tobacco extracts stored as an acetone solution which was used to develop the procedure, even though it was several months older than the petroleum ether extracts of the 1 and 2-gram samples of the same bright tobacco. It would therefore appear that there are readily hydrolyzable solanesol derivatives in the extracts. This may be used to advantage, in that free and total solanesol may be determined simultaneously by controlled hydrolysis of the residue.

Visual examination of the extracts, visible bands on the columns and infrared spectra would tend to show that hexane extraction of tobacco does not remove as many substances as petroleum ether. It may also be concluded that evaporating the petroleum ether solutions to dryness produces chemical changes in the residue as seen by the increased number and amount of visible bands on the chromatographic columns. The more rapid elution of solanesol from the columns, although

the columns appeared less contaminated, would indicate that lower molecular weight material was present on the column from the petroleum ether solution than from the residue.

SUMMARY

A method has been developed for the extraction, chromatographic separation and colorimetric determination of solanesol in complex mixtures. The mixture, in an organic solvent is washed with acid, base and water to remove polar material. The solvent is evaporated and the residue dissolved in acetone. The acetone solution is cooled to precipitate out the waxes. The filtered acetone solution is added to a methanol-water mixture, which is insoluble in hexane, with the aid of some sodium chloride the solanesol is extracted into the hexane phase. The hexane solution is dried with some sodium sulfate and the solution is chromatographed on an alumina column. Impurities are removed by washing the column with hexane and then with a benzene-hexane mixture. The solanesol is eluted from the column with diethyl ether.

The diethyl ether residue is dissolved in redistilled dry pyridine and 3,5-dinitrobenzoyl chloride is added and allowed to react at room temperature to form the ester derivative.

A hydrochloric acid solution is added to stop the reaction; to allow the ester to be extracted into hexane; and hydrolyze the acid chloride to the free acid. The hexane is washed with base to neutralize the solution and dried free of water. An aliquot of the hexane solution is mixed with dimethyl formamide and is made basic with propylene diamine to form the red colored quinoidal ion, which is read on a spectrophotometer.

It has been shown that the Johnson and Critchfield method can be applied to a C_{45} alcohol, solanesol, and that the color produced is proportional to the molecular weight of the alcohol and therefore the molar absorptivity is the same. A spectrophotometer can therefore be calibrated using a common alcohol without resorting to extensive use of difficultly obtainable alcohols under investigation.

The results obtained, using the procedure, show that there is uncontrolled variability in the petroleum ether extraction procedure. The presence of substances in the petroleum ether extractables of tobacco that can degrade, when dry, to give free solanesol on standing can also be shown.

The separation procedure could be used for the large scale isolation of solanesol or for the separation of solanesol from other complex mixtures.

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AUTOBIOGRAPHY

I, Ludwig Weissbecker, was born on May 24, 1930, in Spachbrueken, Germany. I immigrated to the United States in 1936 and became an American Citizen in 1942. I attended elementary school in New York City, graduating in 1945. I graduated from George Washington High School in New York City in 1948. I graduated from the City College of the Colleges of the City of New York in 1952 with a B. S. degree in Biology and Chemistry. After graduation, I was employed as a biochemist by Brooklyn Jewish Hospital. In 1953 I was inducted into the U. S. Army and served for a period of two years. After my honorable discharge in 1955, I was employed by American Cyanamid Co. in New Jersey and enrolled for graduate study at the Brooklyn Polytechnic Institute. In 1960 I joined Philip Morris Inc. as a research scientist.

APPENDIX

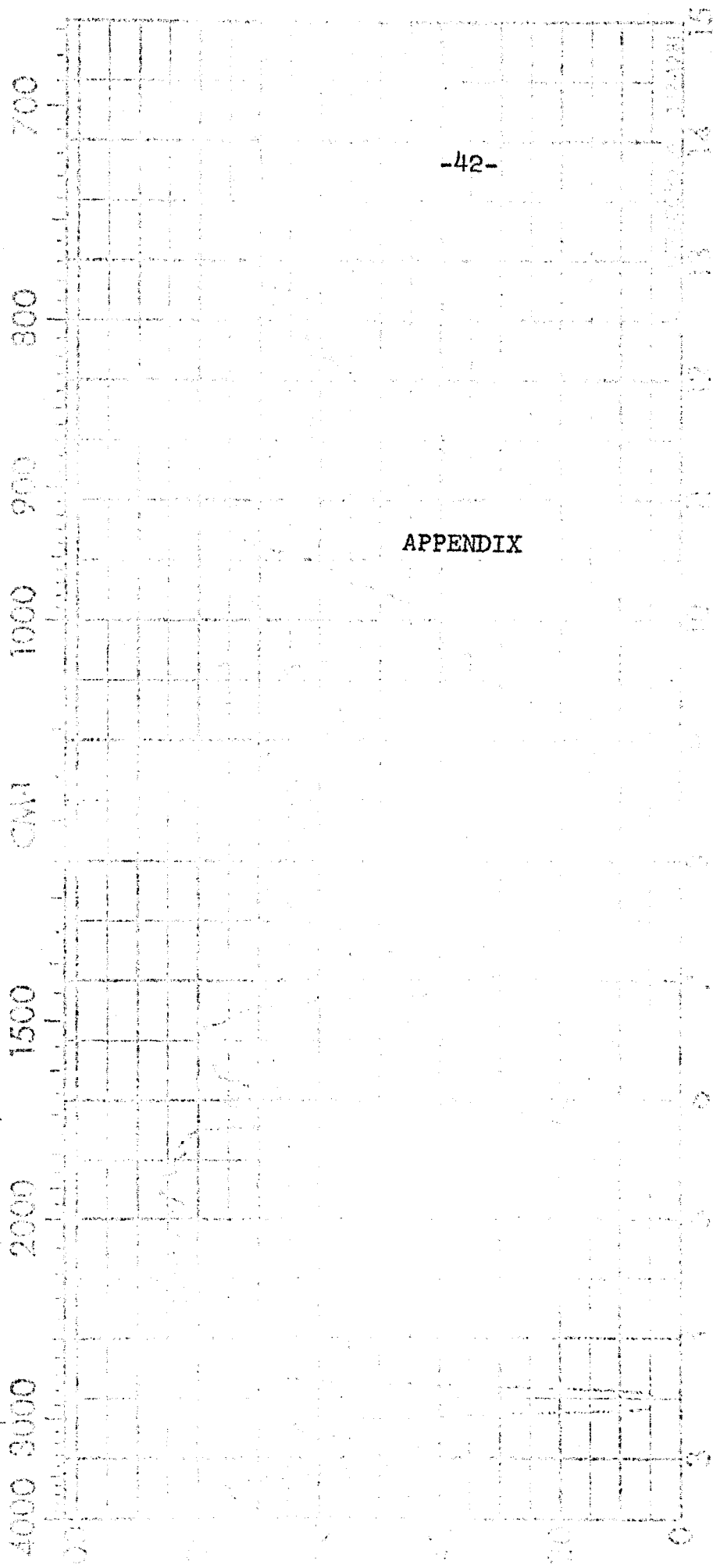


Figure 10. Diagram of the wave of infrared

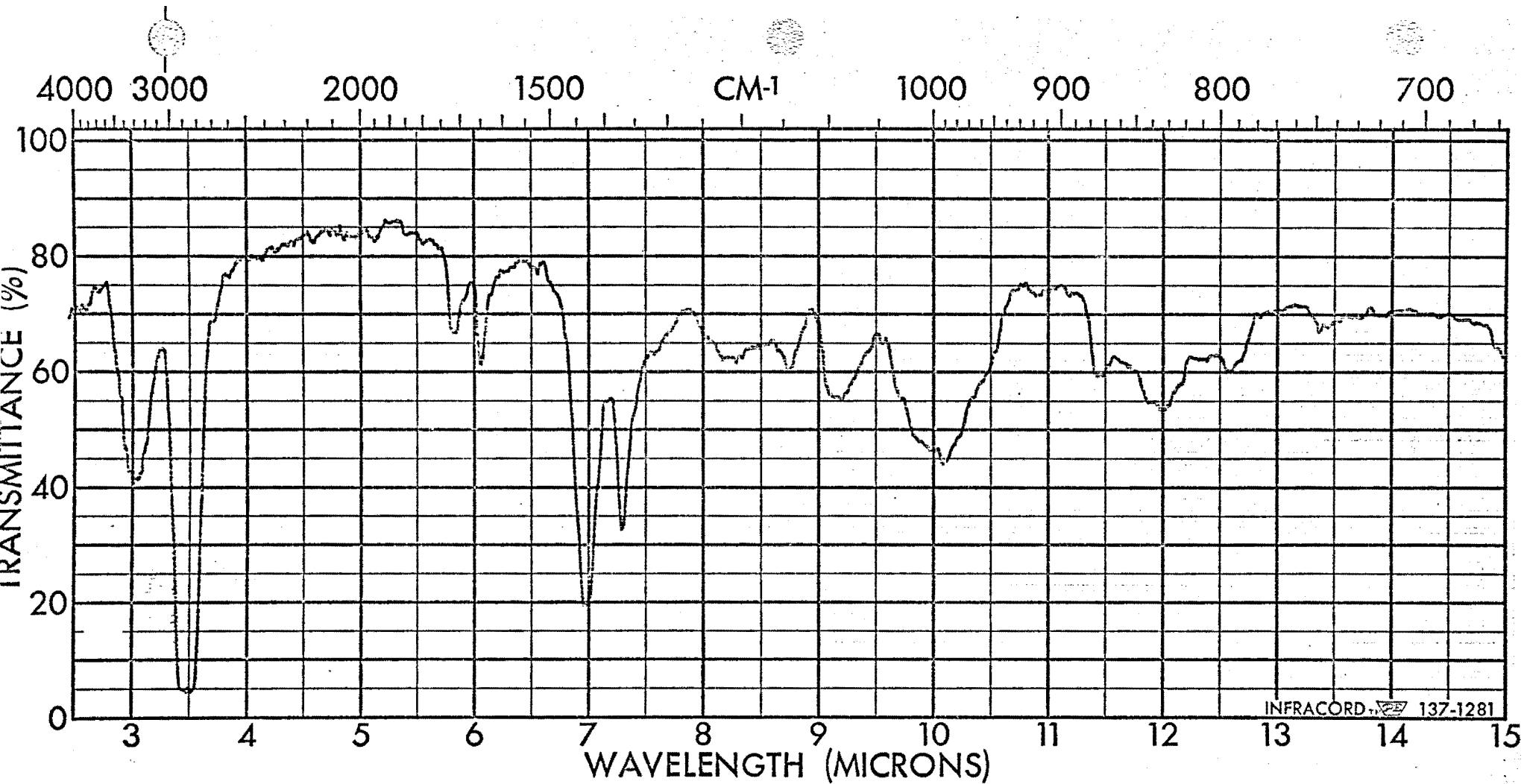
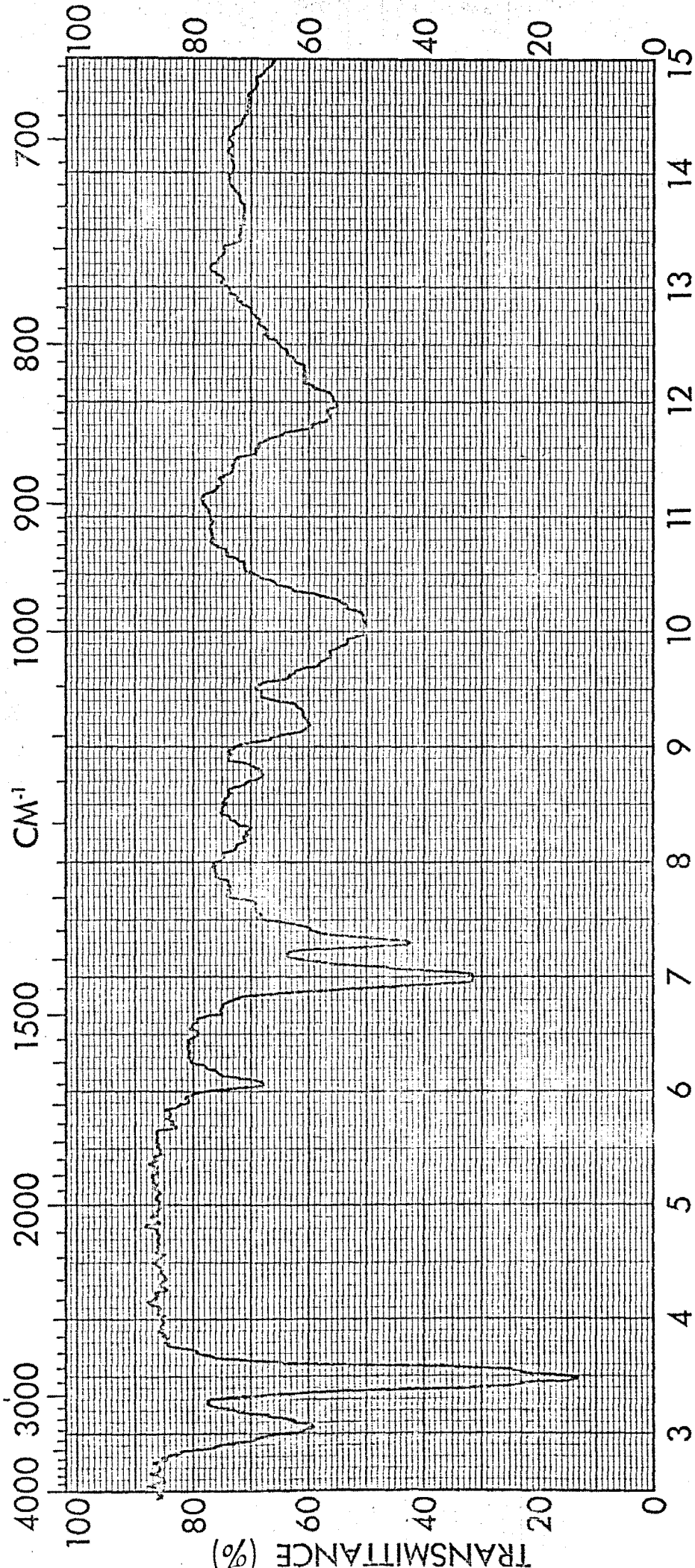


Figure I. Infrared Spectrum of Solanesol

INFRACORD 137-1281



WAVELENGTH (MICRONS)

Figure II. Infrared Spectrum of Pure Solanesol

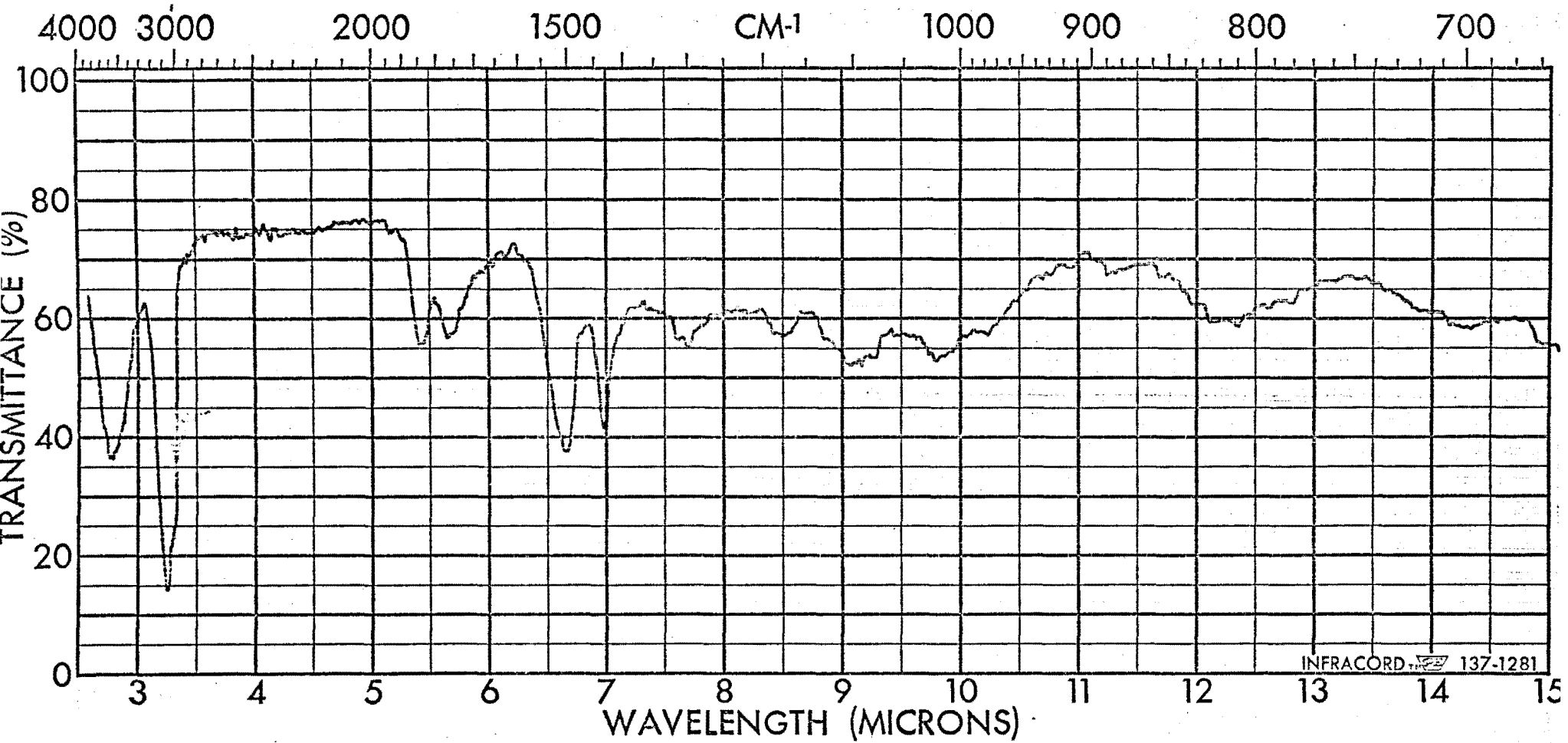


Figure. III. Infrared Spectrum of Hexane Extract of Tobacco Solution

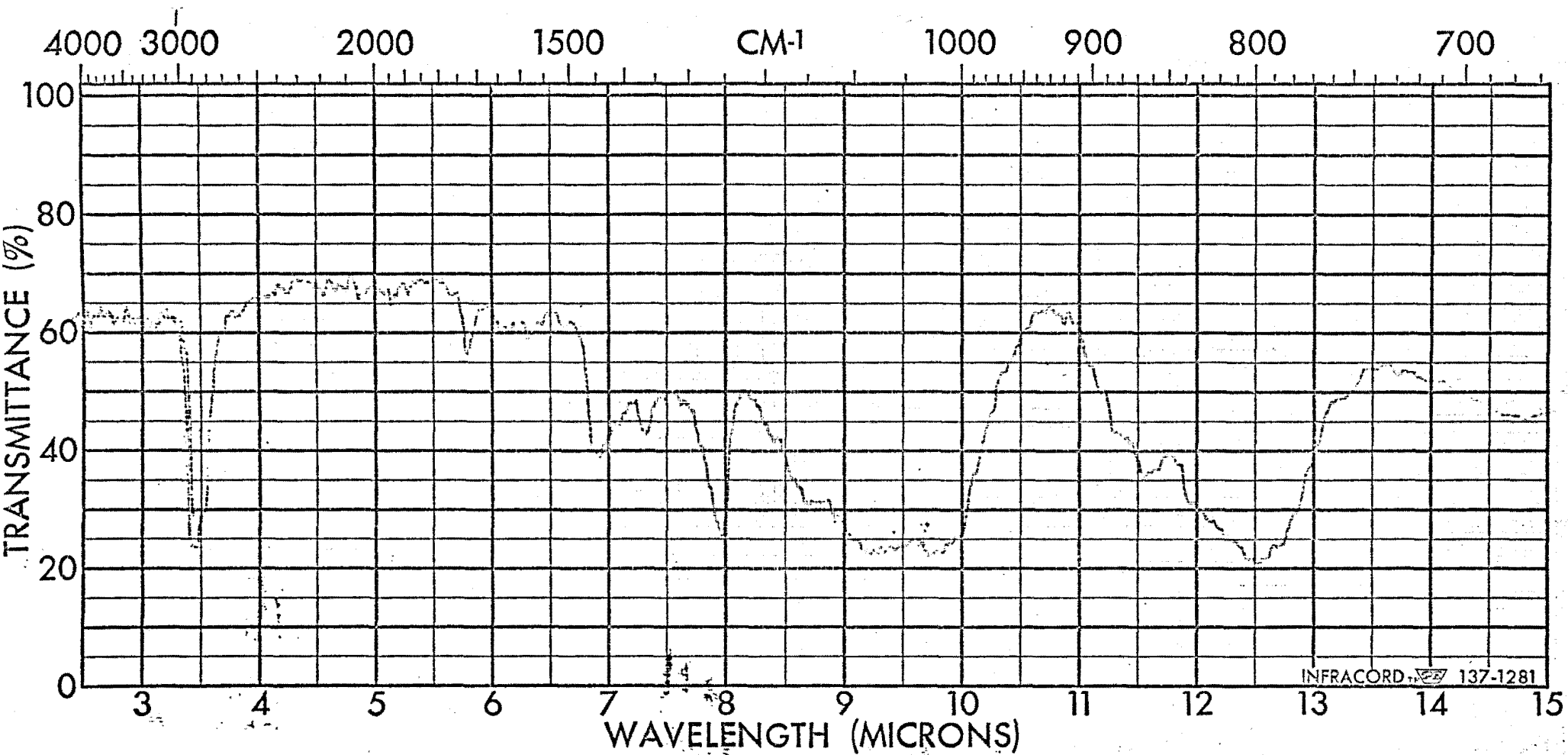


Figure IV: Infrared Spectrum of Hydrocarbon Impurity in Solanesol

INFRACORD 137-1281

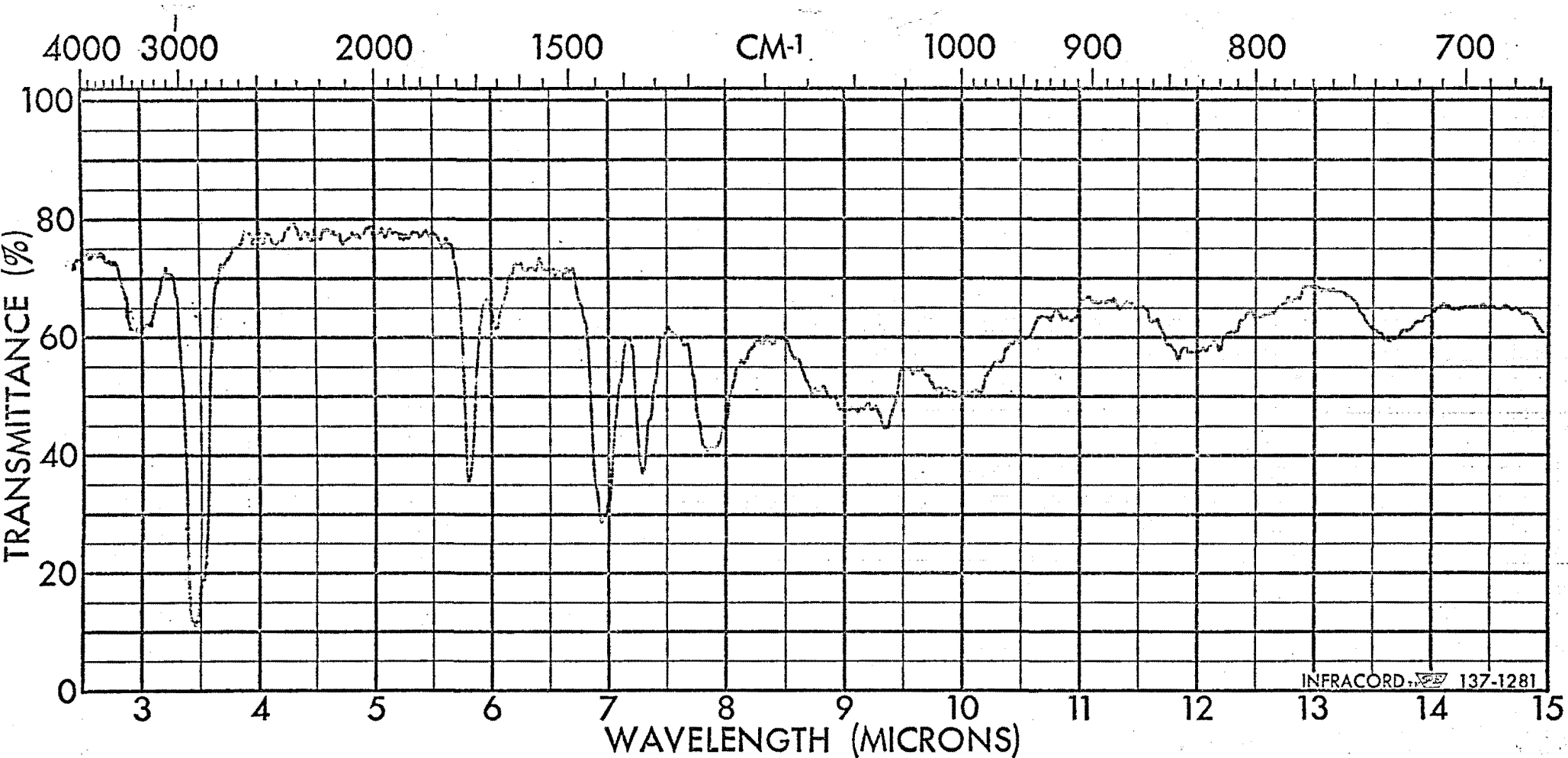


Figure V. Infrared Spectrum of Chromatographic Column Tobacco Effluent

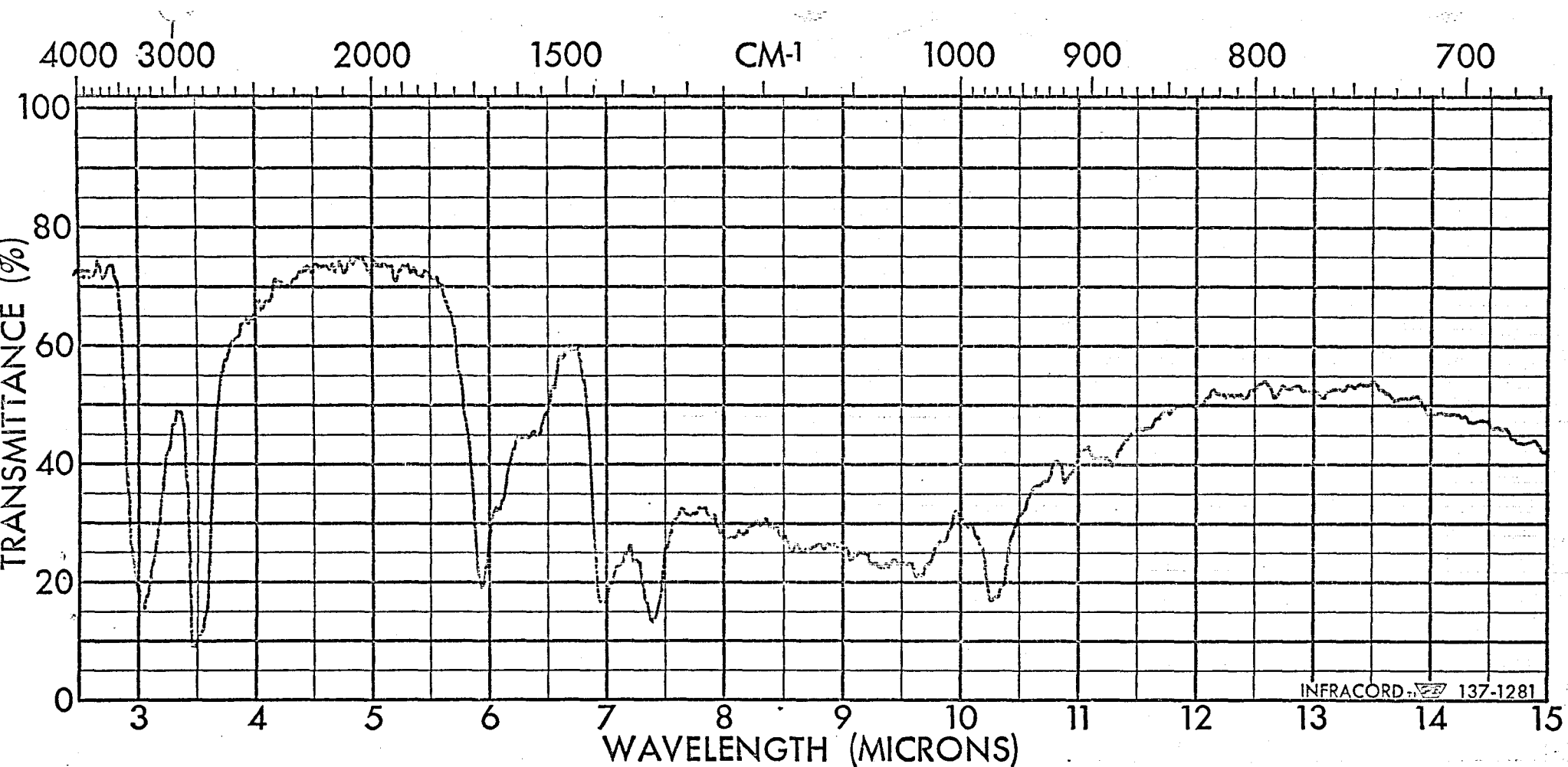


Figure VI. Infrared Spectrum of Hexane Insoluble Residue of Tobacco Solution