Licorice Flavoring—Determination of Sugars by Gas Chromatography and Glycyrrhizin by Infrared Spectrophotometry[1]

R. J. Morris, Jr. and Charles Romano

MacAndrews and Forbes Company
Camden, New Jersey, USA

Introduction

The tobacco industry accounts for about 90 per cent of the licorice flavoring used in this country. The analysis of licorice flavoring supplied to the tobacco industry as an ingredient in casing liquor is based on the scheme proposed by Dr. P. A. Houseman in 1922 (2). The analysis typically consists of the determination of moisture, ash, hot and cold water insolubles, starches and gums, sugars, and glycyrrhizin. Some of these determinations, such as the starches and gums, sugars, and glycyrrhizin, are amenable to an instrumental analytical treatment with the resultant savings in time per analysis. This paper discusses the use of gas chromatography for analyzing sugars and infrared spectrophotometry for analyzing glycyrrhizin in licorice extract.

I. *Analysis of Sugars in Licorice Flavoring Using Gas Chromatography*

The original sugar analysis in the scheme proposed by Dr. Houseman has been revised several times and the most recent revision is an adaptation of the Lane-Eynon volumetric

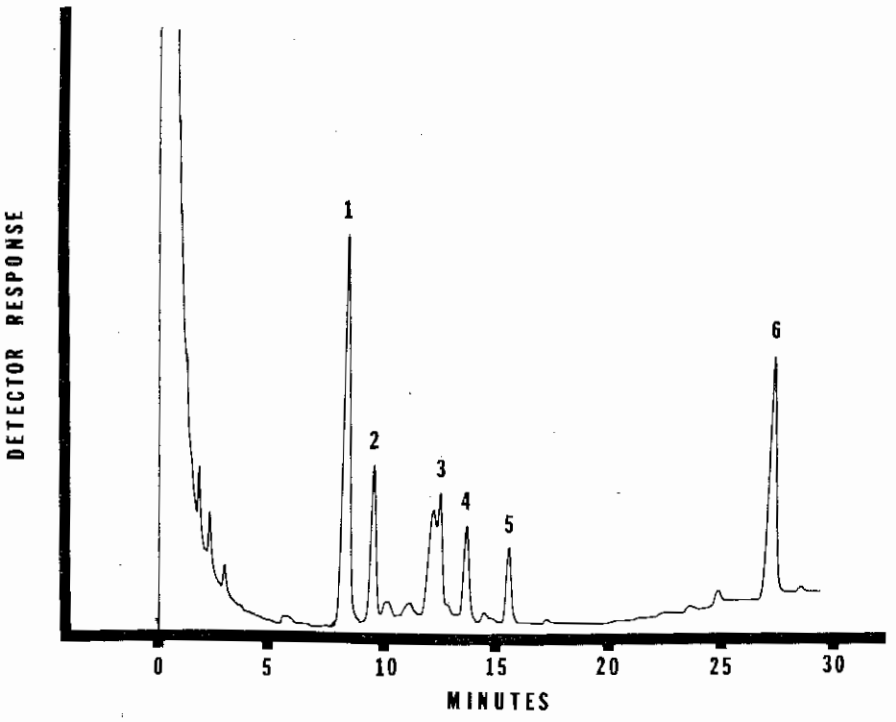

FIGURE 1: Gas chromatogram of silyl ether sugar derivatives of licorice extract. 1. L-rhamnose (internal standard) 2. L-rhamnose impurity 3. Reducing sugar analysis peak 4. α-glucose 5. β-glucose 6. Sucrose.

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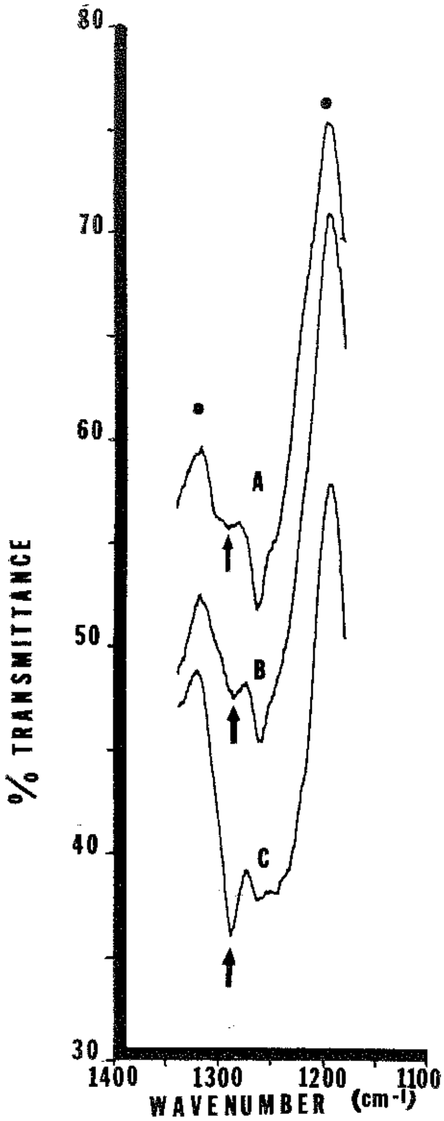

FIGURE 2: Infrared spectra of three samples containing different glycyrrhizin concentrations. A—6.5 per cent B—25 per cent C—80 per cent.

A.O.A.C. method (3). This method determines reducing sugars as a group and sucrose individually. The analysis requires about two and one-half hours lapsed time and about one and one-half hours operator time per sample.

A gas chromatographic method has been developed based on the technique of Sweeley *et al.* (4) using the silyl ether derivatives of the sugars. This technique requires about 45 minutes lapsed time and about 30 minutes operator time per analysis.

Experimental

Procedure: About 25 mg. licorice extract weighed to the nearest 0.01 mg. (paste, powder, or granular form) and 1.5 mg. of L-rhamnose weighed to the nearest 0.01 mg. are added to 1.0 cc. pyridine and heated at 95°C for five minutes in a one dram vial. 1.0 cc. hexamethyldisilazane (HMDS) and 1.0 cc. trimethylchlorosilane (TMS) are added to the vial and shaken well. The vial is allowed to stand 5 minutes before injection of 0.15 cc. into the chromatograph. The tan colored insoluble matter of the licorice extract and the white insoluble matter from the HMDS and TMS reaction do not affect the analysis. The L-rhamnose is used as the internal standard.

Apparatus: An F&M Model 720 gas chromatograph equipped with a thermal conductivity detector was used for this work. Gas chromatography was performed on an aluminum coiled column 6 ft. long and 0.25 in o.d. packed with three per cent silicone Gum Rubber (SE-52) coated on 60-80 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.), an acid and alcoholic base washed celatom support treated with dimethyldichlorosilane. This column was programed from 120 to 300°C at 5°C per minute. Helium carrier gas was used at a flow rate of 60 cc. per minute (measured by a soap bubble flow meter) with 40 psig inlet and atmospheric outlet pressures. The injection port was held at 350°C and the detector at 360°C. Over 200 analyses were made on one column without evidence of deterioration of the resolution. Calibration was required once each week.

Results

Calibration of the gas chromatographic sugar analysis is presently based on the Houseman assay so as to be as consistent as possible with the forty-three years of accumulated data. The G.C. method determines reducing sugars as a group using the results of the volumetric method on typical licorice samples as a primary standard and sucrose individually using pure sucrose as the primary standard. The G.C. method is capable, however, of determining each reducing sugar individually when the need arises.

Figure I shows a typical chromatogram of trimethylsilyl (TMS) derivatives of the sugars in licorice extract. Although early work in determining reducing sugars was done by accumulating the total area of all of the peaks grouped near dextrose, experience has shown that a constant calibration factor is obtained using only the peak area of the double peak No. 3 thus eliminating the integration of many peaks. The leading maximum of the double peak is probably a glucuronic acid derivative

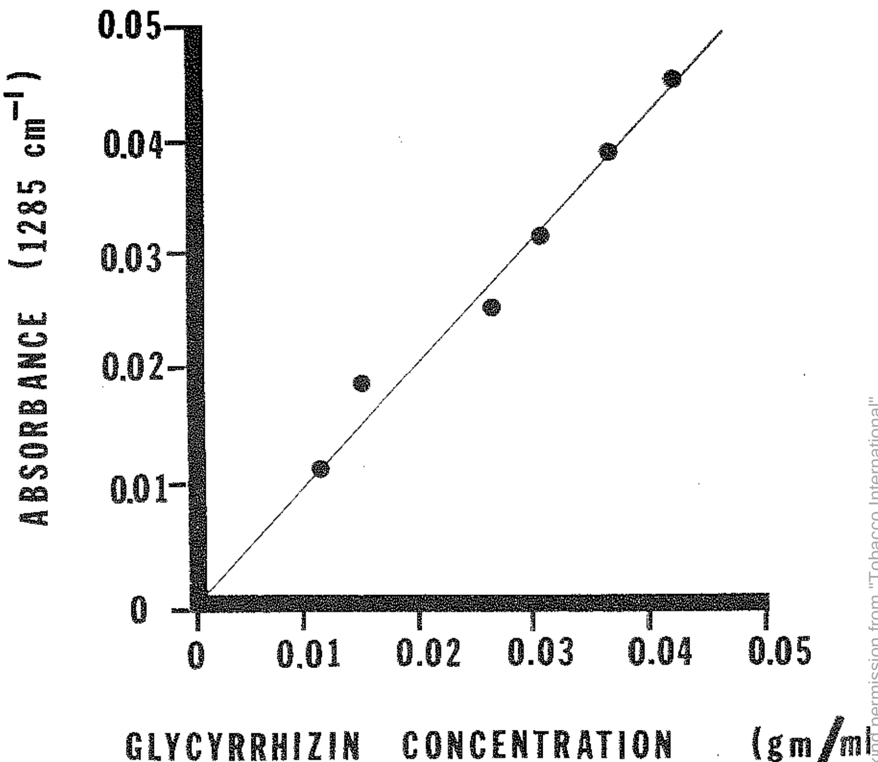

FIGURE 3: Typical working curve for glycyrrhizin analysis.

TABLE I

Analysis of Sugars in Licorice Flavoring

Precision of the A.O.A.C. Method

(P.O.L.E. Lot #5988)

ANALYSIS NUMBER	REDUCING SUGARS ANALYSIS	Δ	SUCROSE ANALYSIS	Δ
1	6.4	-0.2	7.8	-0.4
2	6.5	-0.1	8.5	+0.3
3	6.7	+0.1	8.2	0.0
4	6.4	-0.2	7.8	-0.4
5	6.4	-0.2	8.4	+0.2
6	6.9	+0.3	8.7	+0.5
AVERAGE	6.6		AVERAGE 8.2	
std. dev.	0.2		std. dev.	0.4
rel. std. dev.	3.0%		rel. std. dev.	4.5%

TABLE II

Analysis of Sugars in Licorice Flavoring

Precision of the Gas Chromatographic Method

(P.O.L.E. Lot #5988)

ANALYSIS NUMBER	REDUCING SUGARS ANALYSIS	Δ	SUCROSE ANALYSIS	Δ
1	7.0	-0.2	9.1	0.0
2	6.8	-0.4	8.9	-0.2
3	7.3	+0.1	9.2	+0.1
4	7.1	-0.1	8.7	-0.4
5	7.2	0.0	8.9	-0.2
6	6.8	-0.4	9.0	-0.1
7	7.3	+0.1	9.2	+0.1
8	7.7	+0.5	9.5	+0.4
9	7.5	+0.3	9.4	+0.3
10	7.3	+0.1	9.2	+0.1
AVERAGE	7.2		AVERAGE 9.1	
std. dev.	0.3		std. dev.	0.2
rel. std. dev.	4.2%		rel. std. dev.	2.2%

and the trailing maximum is levulose. Peak areas for reducing sugars and sucrose were determined using a Disc Integrator (Disc Instruments, Inc., Santa Ana, California).

The precision of the volumetric sugar analysis is shown in **Table I.** This table shows results for a licorice extract designated as Powdered Oriental Licorice Extract (P.O.L.E.) and shows a precision of about ±0.3 per cent for reducing sugar and about ±0.5 per cent for sucrose based on six determinations. **Table II** shows the same data for the gas chromatographic method based on ten analyses of the same P.O.L.E. sample. The precision of the gas chromatographic analysis is about ±0.5 per cent for reducing sugars and ±0.4 per cent for sucrose. The gas chromatographic method is more precise for the sucrose analysis than the volumetric method but less precise for the reducing sugar analysis. This is probably because the sucrose gas chromatographic analysis is based on pure sucrose as a primary standard but the gas chromatographic reducing sugars analysis is based on the volumetric analysis as a primary standard thereby reflecting a combination of the inaccuracies in both methods.

Table III shows a comparison of the gas chromatographic analysis and the volumetric analysis for four different licorice products ranging from pure dried and paste forms (P.O.L.E., "Ship" Brand Granules and "Ship" Brand Paste) to the powdered unextracted root (P.S.L.-R.). These data show that the gas chromatographic method is applicable to the most popular forms of licorice extract used in this country.

II. *Analysis of Glycyrrhizin in Licorice Flavoring Using Infrared Spectrophotometry*

The analysis which was proposed by Dr. Houseman for determining the glycyrrhizin content of licorice extract consists of precipitating the glycyrrhizin as glycyrrhizic acid and requires about three days lapsed time and two hours operator time to complete an analysis (2). The analysis is essentially an exhaustive precipitation of the glycyrrhizin in two steps, conversion of the acid to the ammonium salt, drying of the precipitate under carefully controlled conditions, and gravimetrically determining the percent glycyrrhizin in the sample. The precision of this method is about ±0.3 per cent.

An infrared spectrophotometric method has been developed using aqueous solutions of the licorice extract and requires about 45 minutes lapsed time and about 30 minutes

TABLE III

Analysis of Sugars in Licorice Flavoring

Comparison of Gas Chromatographic and A.O.A.C. Method

For Various Forms of Licorice

SAMPLE LOT NUMBER	REDUCING SUGARS G.C.	A.O.A.C.	Δ	SUCROSE G.C.	A.O.A.C.	Δ
P.O.L.E. (Powdered Oriental Licorice Extract)						
5995	6.2	6.0	+0.2	8.4	8.8	-0.4
6088	8.6	8.4	+0.2	9.6	9.5	+0.1
5877	8.0	8.5	-0.5	6.0	6.7	-0.7
5928	9.7	9.0	+0.7	8.8	9.1	-0.3
5790	7.9	8.3	-0.4	4.3	5.7	-1.4
5988	7.1	6.7	+0.4	9.0	8.5	+0.5
"SHIP" BRAND GRANULES						
6416	10.3	9.8	+0.5	5.3	6.6	-1.3
6330	9.6	8.9	+0.7	7.8	8.7	-0.9
6352	9.6	10.1	-0.5	7.2	7.7	-0.5
6409	9.9	10.3	-0.4	5.0	6.4	-1.4
6223	9.6	9.4	+0.2	8.7	8.7	0.0
6332	9.3	8.9	+0.4	7.4	7.4	0.0
6200	9.3	8.7	+0.6	8.4	8.6	-0.2
6423	10.5	10.5	0.0	5.1	6.4	-1.3
"SHIP" BRAND PASTE						
6492	8.0	8.3	-0.3	6.3	6.5	-0.2
6484	9.6	10.3	-0.7	5.8	6.7	-0.9
6489	7.7	7.3	+0.4	3.3	3.9	-0.6
6415	6.1	5.6	+0.5	2.4	3.5	-1.1
6422	6.6	6.0	+0.6	2.1	2.9	-0.8
6406	7.0	6.0	+1.0	4.2	4.1	+0.1
6341	6.7	6.0	+0.7	3.0	4.2	-1.2
P.S.L.R. (Powdered Spanish Licorice Root)						
6139	2.7	1.5	+1.2	8.2	6.0	+2.2
6052	1.9	1.5	+0.4	5.7	5.2	+0.5
5918	1.9	1.6	+0.3	6.7	5.3	+1.4
5926	1.7	1.5	+0.2	6.9	5.4	+1.5

operator time for the analysis. The precision of the infrared spectrophotometric method is ±1.2 per cent.

Experimental

Procedure: About four grams of sample weighed to the nearest .01 gm. are dissolved in 15 cc. distilled water in a Vortex mixer (model K-500-21 Scientific Industries Inc.

TABLE IV

Analysis of Glycyrrhizin in Licorice Flavoring

Precision of the Houseman Assay

("Ship" Brand Granules Lot #5988)

ANALYSIS NUMBER	GLYCYRRHIZIN PERCENT	Δ
1	26.3	+0.1
2	26.4	+0.2
3	26.3	+0.1
4	25.9	-0.3
AVERAGE	26.2	
std. dev.		0.2
rel. std. dev.		0.8%

Springfield, Mass.) by agitating for ten minutes. The solution is quantitatively transferred to a 25 cc. volumetric flask, diluted to the mark, and mixed well.

The solution is placed into a 0.5 mm. thickness infrared cell made with windows of Irtran-2. A spectrum is obtained over the 1180 cm^{-1} to 1340 cm^{-1} range, a baseline is drawn from the minimum near 1190 cm^{-1} to the minimum near 1320 cm^{-1}, and the absorbance at the analytical peak near 1285 cm^{-1} is determined.

TABLE V

Analysis of Glycyrrhizin in Licorice Flavoring

Precision of the Infrared Spectrophotometric Method

("Ship" Brand Granules Lot #6467)

ANALYSIS NUMBER	GLYCYRRHIZIN PERCENT	Δ
1	23.2	0.0
2	23.0	+0.8
3	23.6	+0.4
4	22.0	-1.2
5	24.4	+1.2
AVERAGE	23.2	
std. dev.		1.0
rel. std. dev.		4.3%

Using the absorbance, the gms. of glycyrrhizin per cc. solution is determined from a previously prepared calibration curve and the percent glycyrrhizin is calculated in the usual manner.

Apparatus: A beckman IR-9 infrared spectrophotometer was used for this work. The instrument conditions are as follows:

Optical Mode	Double Beam
Reference Beam	Air
Scanning Speed	20 cm^{-1} per minute
Amplifier Gain	2.0 per cent
Period	8 Sec.
Slit	3.5 mm. at 1200 cm^{-1}, programmed
Ratio SB/DB	1.0 near 85 per cent at 1200 cm^{-1}
Chart Speed	0.4 inches per minute
Scale Expansion	30 per cent to 80 per cent T expanded to full scale

Results

Again, as in the case of the sugar analysis, the glycyrrhizin analysis is presently based on the Houseman assay so that the backlog of experience with the values from that assay is not rendered useless. A colorimetric procedure for glycyrrhizic acid was recently reported by Cundiff (1). Although this technique is excellent for determining glycyrrhizic acid, it is not applicable to a total glycyrrhizin analysis because the term glycyrrhizin has come to mean that portion of licorice extract which is acid insoluble. Glycyrrhizin, therefore, contains glycyrrhizic acid and other water soluble natural extractives which should be accounted for in any total glycyrrhizin analysis.

Figure 2 shows typical infrared spectra of samples containing from about six per cent to about 80 per cent glycyrrhizin. The maximum near 1285 cm^{-1} is used as the analytical peak. These spectra were prepared using the electronic scale expansion noted in the Apparatus section and are presented only to show qualitative band shape. A typical working curve is shown in **Figure 3.**

The precision of the Houseman precipitation assay is shown in **Table IV.** This table shows results for a licorice extract designated as "Ship" Brand Granules and shows a precision of ±0.3 per cent on four determinations. **Table V** shows the same data for the infrared analysis but on a different lot of "Ship" Brand Granules. The precision is

TABLE VI

Analysis of Glycyrrhizin in Licorice Flavoring

Comparison of Infrared Spectrophotometric and Houseman Assay Methods

For Various Forms of Licorice

SAMPLE LOT NUMBER	GLYCYRRHIZIN I.R.	GLYCYRRHIZIN HOUSEMAN	Δ
"SHIP" BRAND GRANULES			
6467	23.2	24.1	-0.9
6484	21.8	23.1	-1.3
6478	23.0	24.0	-1.0
6479	21.8	23.2	-1.4
6492	20.8	24.0	-3.2
"SHIP" BRAND PASTE			
6482	19.5	20.7	-1.2
6481	18.2	17.7	+0.5
6489	21.8	20.2	+1.6
6486	20.7	19.9	+0.8
6480	19.9	17.2	+2.7

±1.2 per cent based on five analyses. Again, the infrared analysis absorbs the inaccuracies of the precipitation method which is used as a primary standard. The precision of the infrared method suffers further from the lack of definition in the analytical peak as shown in **Figure 2** and the variation of glycyrrhizin composition which undoubtedly occurs in different blends of roots of different origin.

Table VI shows a comparison of the infrared spectrophotometric results and the Houseman assay results for "Ship" Brand licorice extract in the dried granular and in the paste forms. These data show that the infrared method is applicable to the most popular form of licorice flavoring within the quoted precision limits and work is now under way to evaluate the method on other forms of licorice extract.

Conclusion

Instrumental analytical methods have been presented for the analysis of sugars and glycyrrhizin in licorice extract.

The lapsed time savings which accrue from using these methods instead of the classical licorice analyses ranges from almost two hours per sugar analysis to over two days per glycyrrhizin analysis. Although the precision of the new methods does not quite match that of the classical methods, it is felt that the savings in lapsed time and operator time per analysis is ample justification for their use in the modern analytical laboratory.

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