Automated Quantitative Analysis of Isolated (Nonconjugated) *trans* Isomers Using Fourier Transform Infrared Spectroscopy Incorporating Improvements in the Procedure[1]

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A method for determining the isolated (nonconjugated) *trans* isomer content of fats and oils was developed using Fourier Transform Infrared Spectroscopy (FTIR), which permits automatic sample analysis and calculation of percent *trans* content. Integrated band areas were used as a measure of the intensity of the band associated with the *trans* C=C double bond. Measured band areas for samples with known percent *trans* content were fit with a second order polynomial, resulting in a correlation coefficient of 0.99998 and a standard error of estimate of 0.11 over the range of 0 to 50% *trans* content. This technique also allows the analysis of neat samples, a significant improvement over current procedures in that it eliminates the need for dilution by volatile solvents and any attendant errors.

The analysis of the *trans* content of lipids by infrared spectrophotometry has progressed over the years. From the initial work showing the practicality of the analysis (1) in 1950, the analysis has undergone several refinements (2-7). These refinements concerned two aspects of the analysis. First, due to a bias error introduced by a broad absorption band dependent on triglyceride structure, the method was changed to the use of methyl esters, which eliminated this problem. Second, the method of computation of the percent *trans* content has been modified over the years. The latter also involved the method used for standardization of the analysis by calibration with known pure *cis* and *trans* samples.

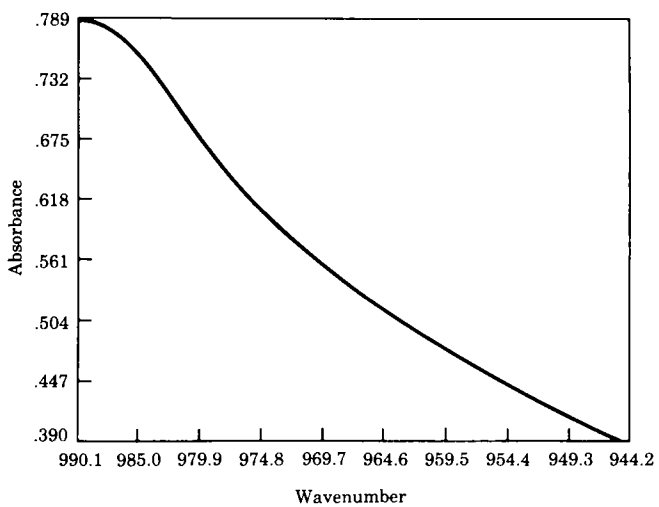

FIG. 1. 100% Methyl linoleate.

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Some early methods (2-4) used the ratio of peak absorptivities at 967 cm^{-1} to determine the amount of *trans* isomers. This method required the analyst to measure the peak heights in transmittance directly from recorded spectra from a hand drawn baseline tangent to the minimum absorbance (maximum transmittance) on either side of the *trans* peak. The transmission peak heights of standards and samples were converted to absorbances using the relationship, Absorptivity (a) = A/bc where A = absorbance, b = cell thickness in cm and c = concentration of solution in g/l. The *trans* content was then calculated from the relationship, percent *trans* = (absorptivity of sample/absorptivity of appropriate standard) × 100.

The most recent refinement (7) has solved the problems of bias for samples containing less than 15% *trans* isomers by employing the use of non-*trans*-containing methyl esters combined with methyl elaidate as standards for the calibration of the method. In addition, to improve reproducibility, the peak heights were measured by overlaying the sample and standard spectra to improve the baseline drawn on the sample spectra by the analyst.

DISCUSSION

In this paper we describe further refinements in the procedure. The major refinement is the use of Fourier Transform Infrared Spectroscopy (FTIR) which allows several modifications to be used providing significant advances to the current state of the art for this analysis (7). Previously, conventional dispersive IR instruments were used for the analysis. An inherent limitation was

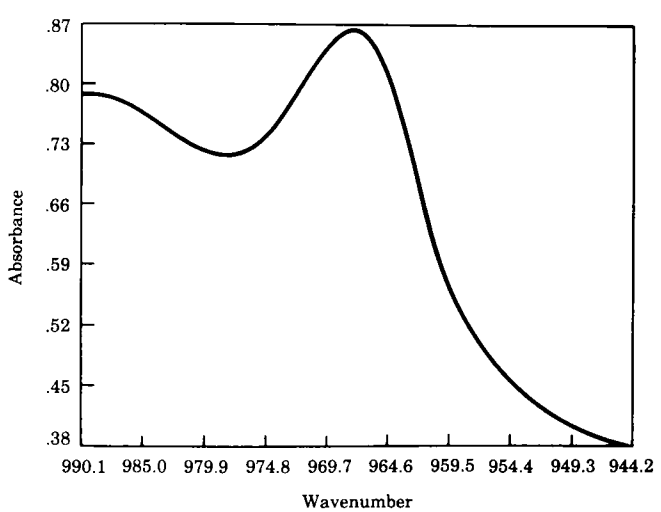

FIG. 2. 20% Methyl elaidate, 90% methyl linoleate.

the requirement for the intensity band of interest (e.g., the *trans* band at 967 cm^{-1}) to be kept between 20–50% T (0.70–0.15 absorbance units). For cells with pathlengths of ~1.0mm, this required the dilution of neat samples in a transparent solvent to about 1% (w/v). Thus, in order to run the analysis, 0.5-g samples were weighed to an accuracy of ± 0.0001 g and diluted. In previous work (1–7), the solvent of choice for subsequent dilution was carbon disulfide due to its lack of absorption at 967 cm^{-1}. The use of carbon disulfide is difficult due to its low boiling point. Evaporation during sample manipulation could lead to significant variations in the measured concentrations of the samples and ultimately to errors in the calculation of the *trans* compositions. One other problem is encountered. When the cell is placed in the sample compartment for analysis, the sample is heated by IR radiation. With volatile solvents, this frequently leads to stratification, vapor or air bubble formation within the cell, mandating a reanalysis of that sample. With thin cells (~0.1mm), FTIR enables the analysis of neat samples (no weighing, no dilution, no bubbles). As long as the cell thickness never varies, all analytical factors cancel out.

As discussed, a solvent was required for dilution of samples to enable the analysis by conventional IR spectroscopy, and concentrations had to be such that the *trans* band absorbed between 20–70% transmission in order to achieve meaningful quantitative data. All previous procedures used dispersive spectrophotometers which, by their design, had diffraction gratings allowing only limited amounts of light to pass through the sample at any particular wavelength. This situation is worsened when attempts are made to obtain higher resolution, necessitating the use of narrower slits and finer ruled gratings. The FTIR uses a Michelson Interferometer, which allows all wavelengths of light to pass through the sample simultaneously. Higher resolution is achieved by increasing the distance traveled by the mirror with no loss of light throughput. Due to this increased amount of light at all wavelengths, the samples can be analyzed neat.

Because FTIR spectrometers are computerized with all data in digital format, area integration is straightforward. This is done and introduces a higher order of accuracy to the analysis than previously possible (Fig. 2). It removes the tedious, subjective and time consuming task encountered with current procedures of manually drawing baselines and measuring the peak heights. The higher order of accuracy is due to two factors. First, noise is reduced by the averaging of multiple spectral scans. The signal-to-noise ratio is proportional to the square root of the number of scans averaged. We used 32 scans per analysis. Second, area integration tends to average out noise over the entire band, but a peak height measurement is subject to whatever noise is superimposed on the signal at that wavelength. When all these factors are combined, the FTIR produces this increased accuracy while providing a significant savings in total analysis time. Our analysis parameters gave a spectrometer total analysis time of 2.5 min/sample at 0.5 cm^{-1} resolution from sample insertion to area integration output.

The computerized FTIR enables easy data storage of all spectra and generated data. Finally, the FTIR computer can transfer data automatically to another computer database via an RS-232 link. This computer, coupled to an auto-sampler, can then act as a master scheduler, sending samples automatically through a sample loop to the FTIR for analysis and accepting results after analysis.

TABLE 1

Seventeen-Day Reproducibility Data of a Single Sample

Sample description	Prod. date	Area	% *trans*	Path	Run date
SYY 132 36	02/20/86	1.930	6.87	.1034	11/03/86
SYY 132 36 R2R	02/20/86	1.899	6.80	.1034	11/03/86
SYY 132 36 R3R	02/20/86	1.976	6.97	.1034	11/04/86
SYY 132 36 R4R	02/20/86	1.920	6.85	.1034	11/04/86
SYY 132 36 R5R	02/20/86	1.970	6.96	.1034	11/05/86
SYY 132 36 R6R	02/20/86	1.919	6.85	.1034	11/05/86
SYY 132 36 R7R	02/20/86	1.968	6.95	.1034	11/06/86
SYY 132 36 R8R	02/20/86	1.939	6.89	.1034	11/06/86
SYY 132 36 R9R	02/20/86	1.941	6.89	.1034	11/07/86
SYY 132 26 R10R	02/20/86	1.897	6.80	.1034	11/07/86
SYY 132 36 R11R	02/20/86	1.928	6.87	.1034	11/19/86
SYY 132 36 R12R	02/20/86	1.927	6.86	.1034	11/20/86
10.00% ME elaidate std		3.343	10.00		10/01/86
10.00% ME elaidate std		3.380	10.08		02/24/87
10.00% ME elaidate std		3.366	10.05		03/05/87
For SYY 132 series					
Average	6.88				
High	6.97				
Low	6.80				
Standard deviation	0.058				

The analysis was run routinely for a period of nine mo prior to this writing without the need to determine a new calibration spectra. The relative response was checked at least once daily with either a primary or a secondary standard. This demonstrated the greater stability of an FTIR spectrometer than of a dispersive instrument (Table 1). Frequency accuracy is maintained on an FTIR spectrometer by continuous calibration with the accurately known frequency of a reference He-Ne laser.

Our FTIR program was written initially to perform all operations including sample logging, analysis, standardization, integration and *trans* calculation. Subsequently, we found it easier to split these functions into two aspects: (I) analysis and integration, and (II) logging, standardization and *trans* calculation. With this system, Aspect I, analysis and integration, remained on the FTIR program along with a simplified calculation of the percent *trans*. However, Aspect II, logging, standardization and *trans* calculation, was broken out and transferred to another computer. This allowed Aspect II to be expanded easily to include data manipulation, including trending, report writing, graphing and other factors. We chose the Saturn Calc spreadsheet package for use on our DEC PDP 11/73 Computer. Other similar software, for example, Lotus 1-2-3, is available for other computers. The FTIR program provides the integrated value of the *trans* area. This number is then entered into Saturn Calc along with data including sample identification, analysis date, cell thickness, analyst identification and any other necessary information. It then processes the area into percent *trans* composition directly. It will then sort, trend, plot and report the data as needed (Table 1). Finally, this system is optimized for maximizing sample throughput because the FTIR computer is used solely for sample analysis and not as a data processor.

Most fats and oils in general use today, when methyl esterified, are liquids at room temperature and may be analyzed easily in a conventional IR cell. For those few solid samples, a heated cell may be used to facilitate analysis. An inexpensive cell was constructed for this purpose by wrapping a conventional cell first with fiber glass heat tolerant tape and then with a layer of NiCrom resistance wire. The ends of the wire were connected to a Variac to provide adjustable current to heat the cell to the desired temperature.

The technique reported here now lends itself to the possibility of being fully automated. Three previous papers have been presented which automate the methyl esterification and/or sample handling (8-10). By incorporating parts of these procedures into this analysis scheme, a completely autonomous analysis is possible.

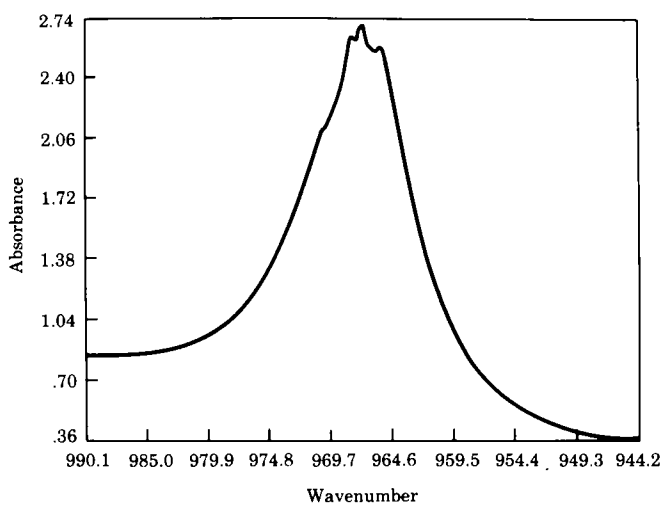

FIG. 3. 50% Methyl elaidate, 50% methyl linoleate.

TABLE 2

Data for Standard Calibration[a]

Sample	Actual percent *trans* composition	Integrated area	Peak height
0% Methyl elaidate[b]	0.00	-1.120	-0.0449
5% Methyl elaidate	5.03	1.170	0.1161
10% Methyl elaidate	10.05	3.345	0.2707
20% Methyl elaidate	20.02	8.075	0.6303
30% Methyl elaidate	30.02	13.030	1.0219
50% Methyl elaidate	49.97	24.440	2.2069

[a]See Ref. 7.

[b]Dissolved in methyl linoleate (w/w).

EXPERIMENTAL PROCEDURES

Equipment. Spectrometer. A Nicolet 20 SXB FTIR spectrometer equipped with a DTGS detector and a Nicolet 1280 Super-Mini computer system with a 280-Mbyte Disk Drive and a 320-Kbyte × 160-Kbyte 24-bit high speed FFT co-processor were used. A DEC PDP 11/73 was used for the Saturn Calc spreadsheet package. All spectra were run at 0.5 cm^{-1} resolution using single sided interferograms and 32 co-added interferograms/analysis. Analysis time per sample was about 2 min 30 sec. Integration for determining the area of the *trans* band was performed between 945-990 cm^{-1}. Baseline correction for integration was "On." (Automatic baseline correction forces the FTIR to compute a tangential baseline between the wavelengths being integrated. This is similar to having the analyst draw a straight line between the valleys on either side of the peak being measured for hand computation.)

Sample cell. A sample cell having a 0.1034-mm pathlength with KBr windows was used. The cell pathlength was checked over a period of nine months, during which time ~700 samples were analyzed in the same cell. We periodically checked the cell pathlength by measuring the interference fringes observed in the infrared spectrum of the empty cell. The variation did not exceed 0.0002 mm. The cell was cleaned by rinsing with dichloromethane three times. The cell was thoroughly dried after each rinse by pulling a vacuum with a drying tube filled fitted to the inlet side of the cell.

trans *Band analysis.* It should be noted that we chose the wavelength band from 945 to 990 cm^{-1} over which to integrate the *trans* absorption for quantitative analysis. This introduces a small negative value and some ripple in the baseline for the 0% methyl elaidate (zero *trans*) reference due to the slight negative curvature of the baseline over that band of wavelengths (Fig. 1).

At 10% methyl elaidate the slight ripple is still present (Fig. 2). The wavelength maxima and minima for integration were chosen to compensate for band broadening observed at higher *trans* content in order that no loss of spectral information be allowed for high *trans* samples. Figure 3 illustrates the effect of this band broadening for the 50% *trans* sample. Because any bow or ripple in the baseline is compensated for by the integration and calculations, very accurate data may be obtained. We felt that a correlation coefficient of 0.99998 justified our approach.

Reagents. Methyl elaidate and methyl linoleate were purchased from Nu-Chek Prep Inc., Elysian, Minnesota. Boron trifluoride-methanol complex was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin.

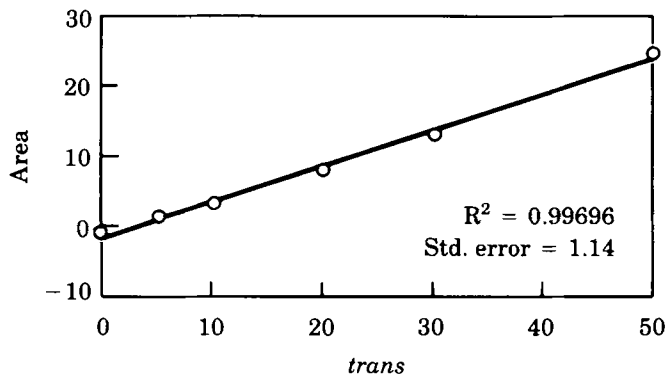

FIG. 4. Percent *trans* by area integration; linear regression.

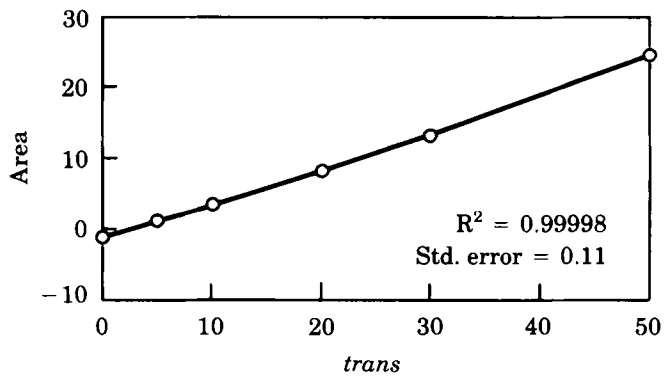

FIG. 5. Percent *trans* by area integration; quadratic regression.

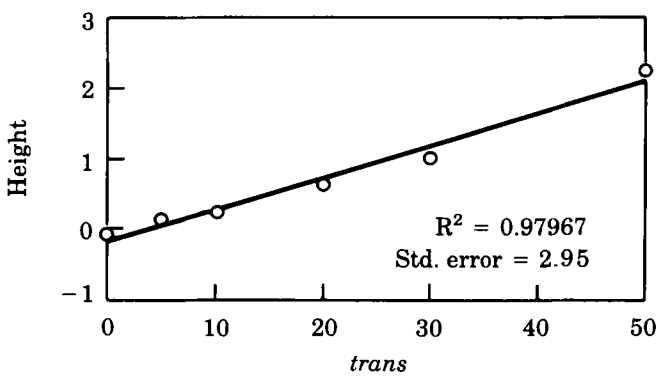

FIG. 6. Percent *trans* by peak height; linear regression.

Methylation procedure. One g of fat to be analyzed was placed in a 25-ml Erlenmeyer flask and fitted with a refluxcondenser. Ten ml of saturated sodium methoxide solution was added, and the mixture was heated while stirring until clear. Five ml of BF3/Methanol reagent was added, followed about one min later by the addition of ca. 25 ml of petroleum ether. The mixture was removed from the heat within 2–5 min. About 30 ml of saturated $NaCl/H_2O$ was added after cooling, and the petroleum ether layer was separated and washed four times with DI water. The petroleum ether containing the methyl esterified sample was dried over anhydrous $MgSO_4$, filtered and evaporated over a hot plate. The sample was placed in a vial under N2 and frozen until analysis.

Standard preparation. The standards, weighed to ± 0.00001 g, were made up as mixtures of neat methyl elaidate and methyl linoleate as in reference (7). (See Table 2 for composition of the standards.) Calibration standards were kept frozen under a nitrogen blanket and did not change in composition over a nine-mo period. (See Table 1 for illustration of the stability.)

Calibration. A least squares linear fit of band area versus percent *trans* content (11) was first tried as the calibration method for this analysis. Data for this calibration is shown in Table 2. The linear fit gave a correlation coefficient (R^2) of 0.99696 and a standard error of estimate of 1.14% *trans*. From a plot (Fig. 4) of the data, it was obvious that there was slight curvature. We then tried using a quadratic equation (12) to fit the data (Fig. 5). Our correlation coefficient increased to 0.99998, and the standard error of estimate decreased to 0.11% *trans*. This standard error of estimate is the root mean square average of the deviations between the predicted line and the actual data; thus, it is a predictor of the accuracy of the analysis. For example, if a sample was analyzed to have between 0 and 50% *trans*, the analysis would carry a one standard deviation uncertainty of 0.11% *trans* over the entire range. Also of interest are the estimates of the error in the coefficients for the calibration equations. In the linear

TABLE 3

Mathematical Relationships

Model: Linear using band area
Equation: % *trans* = 3.21 + 1.957 * Area
Coefficient error: ± 0.64 ± 0.054
R^2 = 0.99696; standard error of estimate = 1.14

Model: Quadratic using band area
Equation: % *trans* = 2.497 + 2.293 * Area - 0.01432 * Area^2
Coefficient error: ± 0.070 ± 0.017 ± 0.00069
R^2 = 0.99998; standard error of stimate = 0.11

Model: Linear using peak height
Equation: % *trans* = 3.7 + 22.0 * Height
Coefficient error: ± 1.6 ± 1.5
R^2 = 0.979; standard error of estimate = 2.9

Model: Quadratic using peak height
Equation: % *trans* = 1.40 + 32.92 * Height - 4.94 * Height^2
Coefficient error: ± 0.11 ± 0.31 ± 0.137503
R^2 = 0.99995; standard error of estimate = 0.16

TABLE 4

Software Program

QUANT MACROS

```
QN1  TO USE, PRESS EXPERIMENT 1 KEY.
     THIS ROUTINE SCANS BACKGROUND AND
     PROMPTS FOR SLOPE AND INTERCEPT VALUES
     IN PREPARATION FOR QN2.

QN2  TO USE, PRESS EXPERIMENT 2 KEY.
     RUNS SAMPLE UNKNOWNS AND CALCULATES
     PERCENT TRANS IN VEG OIL AND STORES SPECTRA
     IN A FILE.

QNS  SUBROUTINE TO SET FTIR OPERATING PARAMETERS
     SCAN BACKGROUND. SUBROUTINE NEEDED
     DUE TO 256 WORD LIMIT ON NICOLET MACROS.

     THE CALIBRATION FOR THIS ANALYSIS ASSUMES
     THAT A QUADRATIC EQUATION IS USED IN ORDER
     TO ACHIEVE THE PUBLISHED CORRELATION 0.99998
       AN EXAMPLE OF ONE OF OUR EQUATIONS WAS:
     %TRANS=2.68+AREA-0.0217*AREA*AREA

!QNS
NSK=8000                          \REQ 8000 FOR SS INTERFEROGRAM
NSD=32                            \DEFAULT # OF SCANS
NSB=32                            \DEFAULT # OF SCANS
NDP=32768                         \REQ FOR 0.5 CM-1 RESOLUTION
NTP=65536                         \2 X NDP
FSZ=45056                         \MUST BE LARGE ENOUGH TO HOLD NDP
TEM=109                           \TEMPORARY MACRO VARIABLE
APT=FL                            \DEFAULT FULL OPEN
BDL=15                            \BEAM DELAY 15 SEC
DBL=NO                            \SS INTERFEROGRAMS
DET=1                             \TGS DETECTOR
DSP=IG                            \DISPLAY OPERATIONS
GAN=1                             \DEFAULT GAIN
PFN=1                             \PARAMETER FILE # DEFAULT 1
VEL=30                            \DEFAULT VELOCITY FOR TGS DET
NPR                               \PERFORM THE 109 X RESET FORM 2
FXF=990                           \INITIAL X FOR INTEGRATION
LXF=945                           \ENDING X FOR INTEGRATION
BAS=YS                            \BASELINE CORRECTION FOR INTEGRATION ON
OMD                               \OUTPUT MESSAGE
OPEN DOOR, CLEAR BEAM FOR BACKGROUND, <RETURN>
PAU                               \WAIT FOR THE <RETURN>
SCB                               \SCAN BACKGROUND
END

!QN1                              \ROUTINE TO SET PARAMETERS SCAN BACKGROUND
QNS                               \CALL FTIR SETUP ROUTINE
OMD                               \OUTPUT MESSAGE
ENTER INTERCEPT FOR CALIBRATION (E.G. 2.68), <RETURN>
VF3                               \VF3 HOLDS THE INTERCEPT VALUE
OMD                               \OUTPUT MESSAGE
ENTER SLOPE (1ST ORDER TERM, E.G. 2.393), <RETURN>
VF4                               \VF4 HOLDS SLOPE VALUE
OMD                               \OUTPUT MESSAGE
ENTER SQUARE TERM (2ND ORDER TERM, E.G. -0.0217), <RETURN>
VF2                               \VF2 HOLDS SQUARE TERM
FXF=4000                          \RESETS SCAN FOR FULL SPECTRUM
LXF=400                           \RESETS SCAN FOR FULL SPECTRUM
END

!QN2
FXF=990                           \INITIAL X FOR INTEGRATION
LXF=945                           \ENDING X FOR INTEGRATION
DFN=10                            \COLLECT UNKNOWNS TO DESTINATION FILE # 10
OMD                               \OUTPUT MESSAGE
INSERT UNKNOWN SAMPLE, <RETURN>
PAU                               \WAIT FOR <RETURN>
```

(Table 4 continued on next page)

TABLE 4 (cont.)

```
SCD                                  \SCAN UNKNOWN TO DESTINATION FILE
RAD                                  \RATIO DESTINATION AGAINST BACKGROUND
ABD                                  \CONVERT TO ABSORBANCE
OMD                                  \OUTPUT MESSAGE
ENTER SAMPLE TITLE, <RETURN>
TID                                  \ACCEPT TITLE FOR UNKNOWN
ASD                                  \AUTOSCALE SPECTRUM
DSD                                  \DISPLAY UNKNOWN'S SPECTRUM (990-945)
OMD                                  \OUTPUT MESSAGE
ENTER FILE NAME, <RETURN>
PDD                                  \SAVES SAMPLE SPECTRA IN FILE
SMD                                  \INTEGRATE UNKNOWN AREA
OMD                                  \OUTPUT MESSAGE
AREA OF THE TRANS PEAK IS:
PRN FCD                              \PRINT INTEGRAL VALUE
VF0=VF4*FCD+VF3                      \CALCULATE %TRANS (LINEAR COMPONENT)
VF0=VF2+FCD*FCD+VF0                  \CALCULATE %TRANS (2ND ORDER COMPONENT)
TIQ                                  \OUTPUT TITLE
OMD                                  \OUTPUT MESSAGE
%TRANS CONCENTRATION OF SAMPLE IS:
PRN VF0                              \PRINT CONCENTRATION OF UNKNOWN
FXF=4000                             \RESETS SCAN FOR FULL SPECTRUM
LXF=400                              \RESETS SCAN FOR FULL SPECTRUM
END
```

equation the intercept had an error of ± 0.64, while in the quadratic model it had dropped to ± 0.07. Likewise, the first order term error dropped from ± 0.054 to ± 0.017. This, along with the standard error of estimate, can be taken as indicators of the quality of the fit of the equation to the data.

Although we used band area data from the beginning for all of our work, for comparison purposes we also examined the effect of using peak height data for the calibration, as has been done in all previous papers. Our peak height data used a digitally computed baseline between 990 and 945 cm^{-1}, and the peak height was taken digitally from the FTIR at the peak maximum. The peak height was then subtracted from the computed baseline at that maximum wavelength. Thus, our peak height data was much more precise than that from a manual measurement made from recorder tracings. Table 3 shows the various calibrations we considered. From this table one can see that a linear calibration based on peak height gave a standard error of 2.95% trans. Predictions of *trans* content from previous papers would have been no better because they either did not attempt to produce a nonlinear model equation for the calibration or used simple data plots. A quadratic regression can account for the curvature that is present with both the area data and the peak height data. Although the peak height data has more curvature in the calibration than the area data (Fig. 6), a respectable calibration with a standard error of 0.16% *trans* may be obtained from a quadratic fit. This surprisingly high correlation for peak height data was due in part to the stability of the FTIR instrument, and the fact that signal averaging reduced the noise at the peak maximum. In the final analysis, however, a quadratic fit of band area data produced the best calibration. The 0.11% *trans* standard error for area is 31% better than the 0.16% *trans* standard error for peak height.

FTIR Macro Programs. All operations on the Nicolet 20SXB FTIR are performed via three-letter commands typed on the keyboard. Commands and parameters may be strung together to form simple macro programs. These macros are accessed in turn, by new three-letter codes. Hence, a single macro program can set up operating parameters, acquire and file spectra, perform some limited calculations and prompt the user for sample information. To permit this analysis to be performed by lab personnel not familiar with the FTIR, we wrote the macro programs presented in Table 4. These macros were assigned to two "experiment" keys on the keyboard. This allows any analyst to execute a macro program merely by pressing a designated experiment key. On our system, the Experiment 1 Key sets the operating parameters for performing all analyses and then collects a background single beam spectra. All sample single beam spectra are then "ratioed" against the background to give true transmittance or absorbance spectra. This procedure is necessary because an FTIR is a single beam spectrometer. The Experiment 2 Key performs a *trans* area measurement, prompts for a sample name and file name to permanently store the spectra (allowing later reanalysis if desired) and computes the percent *trans* using the calibration values determined separately for the standard mixtures and then entered in Experiment 1. This essentially reduces the analysis to a two-button operation once the calibration has been obtained.

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