Rapid and Direct Iodine Value and Saponification Number Determination of Fats and Oils by Attenuated Total Reflectance/Fourier Transform Infrared Spectroscopy

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A simple, rapid and reproducible method of determining the iodine value (IV) and saponification number (SN) for fats and oils was developed with an attenuated total reflectance/Fourier transform infrared spectrometer and commercially available triglycerides as calibration standards. Partial least squares was used to determine the spectral regions correlating with the known chemical IV and SN values, and the calibration set was augmented with additional standards generated by spectral co-adding techniques. The calibration model obtained was used to analyze commercially available fats and oils with a wide range of IV and SN values, and the results were compared to the values obtained by American Oil Chemists' Society methods. With the spectrometer calibrated and programmed, IV and SN results could be obtained within 2–3 min per sample, a major improvement over conventional wet chemical methods.

KEY WORDS: Edible oils, fats, Fourier transform infrared spectroscopy, iodine value, saponification number.

Fats and oils from a wide variety of sources are important to the food industry and other industrial sectors. In almost every situation, the general characterization of such oils and the monitoring of modifications they may undergo during processing are important relative to their quality, functionality and economic value. Typical analyses that are important include iodine value (degree of unsaturation), saponification number (average molecular weight), moisture content, level of *trans* fatty acids, free fatty acids, peroxide value and 2-thiobarbituric acid (TBA) number. Iodine value (IV) and saponification number (SN) are two of the more common analyses used for characterizing fats and oils, with IV being crucial in monitoring hydrogenation processes. Both of these analyses are tedious, time consuming and expensive, but needed on a routine basis.

The possibility of developing a rapid method for IV determination based on infrared spectroscopic analysis has been investigated by several workers. Arnold and Hartung (1), using a dispersive infrared spectrometer, determined IV for a variety of fats and oils from the ratio of intensities of the olefinic and aliphatic C-H stretching bands in their infrared spectra. The samples were dissolved in CCl_4 and analyzed in an infrared transmission cell. Bernard and Sims (2) developed a similar infrared method except that they analyzed oils in their neat form and used the weak C=C stretching band ($\sim$ 1660 cm^{-1}) to determine total unsaturation. Recently, Afran and Newbery (3) employed Fourier transform infrared (FTIR) spectroscopy and an attenuated total reflectance (ATR) sampling technique for the evaluation of unsaturation in edible oils and compared their results to those obtained by gas chromatography. Similarly to Arnold and Hartung (1), they based their method on the measurement of peak height ratios in the C-H stretching region.

In our laboratory we have been investigating the development of general-purpose quality control methods based on FTIR spectroscopy (4–6). FTIR spectrometers are a major advance over conventional grating instruments in that they have more energy; excellent wavenumber reproducibility and accuracy (laser-calibrated); data manipulation capabilities (subtraction, ratio determination, derivative spectra and deconvolution); and advanced chemometric software to handle calibration development (7,8). Systems such as the Nicolet 8200 series FTIR spectrometers (Nicolet Instrument Inc., Madison, WI) include the analytical software and macro programming capabilities required to automate analytical procedures. FTIR, coupled with an ATR accessory, which simplifies many of the sample handling problems asociated with IR analyses, provides a new approach to quality control applications in the food sector. This paper describes the development of a practical, automated ATR/FTIR method to determine IV and SN simultaneously by the partial least squares (PLS) technique for multivariate analysis of the FTIR data (9) and using pure triglycerides as a basis for calibration rather than pre-analyzed oils.

EXPERIMENTAL PROCEDURES

Calibration standards. Reagent-grade triglyceride standards (purity > 98%) tricaproin (C6), tricaprylin (C8), tricaprin (C10), trilaurin (C12), tripalmitin (C16), tristearin (C18), triolein (C18:1*c*), trielaidin (C18:1*t*), trilinolein (C18:2*c*), trilinolelaidin (C18:2*t*) and trilinoleinin (C18:3*c*) were obtained from Sigma Chemical Company (St. Louis, MO). These triglycerides were chosen to cover a range of molecular weights and degrees of unsaturation. Based on their known structures, their theoretical IV and SN were calculated and used as reference values for subsequent calibration development.

Instrumentation. For this work a Nicolet 8210 Fourier transform infrared spectrometer (Nicolet Instrument Inc.) was used, equipped with a horizontal germanium ATR accessory placed in the instrument optical cavity. The instrument was purged with a Balston dryer (Balston, Lexington, MA) to minimize water vapor and CO_2 interferences. The ATR plate was heated to 60°C (± 0.2°C) by attaching four 25W strips of heating tape onto the plate, with the temperature controlled by an Omega CN4400 temperature controller (Omega Engineering, Stamford, CT).

Calibration. For the calibration, all spectra were collected from 128 scans at 16 cm^{-1} resolution. Prior to calibration or analysis, the ATR crystal was cleaned with a 1% Triton X-100 solution (Sigma Chemical) followed by hexane and dried, and the temperature of the ATR crystal was equilibrated to 60°C. A reference background emittance spectrum was taken of the clean cyrstal and stored to disk. The triglyceride standards were equilibrated to

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62°C in a water bath and applied with a soft-tipped disposable pipette (~1 mL) to the ATR crystal. Individual triglyceride emittance spectra were then recorded and ratioed against the reference background emittance spectrum to obtain the absorbance spectra of the standards, which were subsequently stored to disk. The crystal was cleaned after each standard by aspirating the oil from the crystal, wiping with a soft tissue, washing twice with hexane and wiping dry. The cleaned crystal was checked spectrally to ensure no residue of the previous sample remained on the crystal.

The calibration set was expanded by retrieving selected triglyceride spectra and co-adding them in defined ratios to generate additional spectra representative of triglyceride mixtures for which both IV and SN varied. The IV and SN for each of these co-added spectra were determined based on the relative proportion of each triglyceride used in making up the spectral mixture, and these co-added spectra were used as part of the calibration set (Table 1).

Calibration development was carried out by using the Nicolet PLS calibration and prediction package (Nicolet Instrument Corp.). The calibration routine includes a validation procedure, which provides statistical parameters indicative of the predictive capability of the calibration model (9). The calibration was developed by studying the spectral features of the pure triglycerides and their co-added spectral mixtures for regions that correlated with either IV or SN. The correlating spectral regions were further refined by using the mean difference (MD) and standard deviation of the difference (SDD) between the predicted and theoretical IV and SN as a measure of improved performance of the calibration (10).

Analysis. Thirty-seven fats and oils were investigated in this study, including those from palm kernel, tallow, soya, canola, cottonseed, lard, peanut, coconut, sesame, safflower, sunflower, shortening, olive, corn, linseed and anhydrous butterfat. Most of those listed came from consumer outlets, while the balance were obtained from Canada Packers (Toronto, Ontario) and included commercial specialty fats and hydrogenated oils of various types identified only by a product code. All samples were analyzed in our laboratory in duplicate by the American Oil Chemists' Society (AOCS) standard methods (11). Solid samples were melted, and all samples were equilibrated to 62°C in a water bath prior to FTIR analysis. The samples were applied to the ATR crystal in a similar manner as described for the standards. Spectra of samples were recorded from 32 scans at a resolution of 16 cm^{-1} and run in duplicate.

Prior to analyzing a set of samples, it proved necessary to standardize the analytical conditions to adjust for any differences in the effective pathlength of the ATR sampling accessory or in the spectral baseline relative to the conditions under which the original calibration was performed. This involved recording the spectrum of one of the more common calibration standards (*i.e.*, triolein) and comparing it to the corresponding spectrum in the calibration set to determine appropriate baseline and pathlength corrections, which were applied to all subsequent sample spectra prior to PLS prediction.

To facilitate routine operation of the instrument, the instrument was programmed by macro command language to automate the analysis of oils for IV and SN, leaving the operator only to apply samples to the ATR crystal. A simplified listing of how the program functioned follows:

[1] Enter operator name
[2] Enter date
[3] Enter oil descriptor (batch, type, etc.)
[4] How many samples are your running? = x
[5] Clean cell with Triton X-100
[6] Clean cell with hexane
[7] Cell temperature ok?
[8] Record reference background emittance (R)
[9] Standardize analytical conditions routine [10–14]
[10] Apply triolein standard
[12] Compare to triolein calibration standard
[13] Calculate depth of penetration correction
[14] Calculate baseline correction
[15] X = 1
[16] Apply sample to ATR crystal
[17] Enter sample comment/descriptor
[18] Record sample emittance (S)
[19] Ratio S/R
[20] Apply baseline and depth of penetration corrections
[21] Print sample number, sample comment
[22] PLS IV predict routine–Print IV
[23] PLS SN predict routine–Print SN
[24] Clean ATR crystal with hexane
[25] Cell-clean check routine
[26] C=O<0.0003? continue; C=0>0.0003 GOTO [24]
[27] X = X + 1
[28] IF X<x GO TO [16]

TABLE 1

Theoretical Iodine Values (IV) and Saponification Numbers (SN) for the Triglyceride Standards and Co-added Spectral Mixtures

Triglyceride standards and co-added mixtures	IV	SN
C06:0 (1.0)[a]	0.0	435.6
C08:0 (1.0)	0.0	357.8
C10:0 (1.0)	0.0	303.6
C12:0 (1.0)	0.0	263.7
C14:0 (1.0)	0.0	233.0
C16:0 (1.0)	0.0	208.7
C18:0 (1.0)	0.0	188.5
C16:0 (0.5) + C14:0 (0.5)	0.0	220.8
C18:1*c* (0.2) + C08:0 (0.8)	17.2	324.3
C18:1*c* (0.5) + C18:0 (0.5)	42.9	189.3
C18:2*c* (0.3) + C10:0 (0.7)	51.9	269.9
C18:3*c* (0.2) + C16:0 (0.8)	52.2	205.4
C18:1*t* (0.8) + C12:0 (0.2)	68.6	204.8
C18:2*t* (0.4) + C06:0 (0.6)	69.2	337.9
C18:2*t* (0.4) + C14:0 (0.6)	69.2	216.3
C18:1*c* (1.0)	86.0	190.1
C18:1*t* (1.0)	86.0	190.1
C18:2*c* (0.5) + C16:0 (0.5)	86.5	200.0
C18:2*t* (0.6) + C14:0 (0.4)	103.9	208.0
C18:3*c* (0.5) + C12:0 (0.4)	130.3	212.6
C18:2*c* (0.5) + C18:2*t* (0.5)	173.2	191.4
C18:2*c* (1.0)	173.2	191.4
C18:2*t* (1.0)	173.2	191.4
C18:3*c* (1.0)	261.5	192.7

[a]Denotes ratio of mixture.

RESULTS

Factors affecting the analysis. In the development of this methodology, one of the key problems encountered was

obtaining reproducible spectra due to changes in the characteristics of the ATR crystal over time, the effects of temperature or minor alignment changes caused by switching optical compartment accessories.

In the ATR technique, light from the infrared source is launched at a fixed angle into the ATR crystal and undergoes multiple internal reflection as it travels down the crystal, giving rise to an evanescent wave at the surface of the crystal at each point of reflection (7). The depth of penetration (d_p) of the evanescent wave is a function of the refractive index of the crystal and sample (n_1,n_2), the launch angle (θ) and the wavelength (λ) as described in Equation 1.

$$d_p = \lambda\{2\pi n_1[\sin^2(\theta)-(n_2/n_1)]^{1/2}\} \quad [1]$$

Thus, the effective pathlength of an ATR cell, which is the product of the depth of penetration and the number of internal reflections, both of which vary with the launch angle, depends on the alignment of the ATR accessory and is also affected by temperature. Because the depth of penetration also varies with wavelength, ATR/FTIR spectra exhibit baseline curvature, especially at lower frequencies. A change in temperature by more than 1°C will result in a substantial change in the baseline curvature. These effects, individually or in combination, can seriously affect the reproducibility of quantitative analyses, particularly when the analysis is based on large regions of the spectrum. Once it was recognized that this problem existed, a standardization routine was developed to relate the subsequent analytical conditions (temperature, alignment and depth of penetration) to those used in the original calibration. This was done by recording the spectrum of one of the more commonly available triglyceride standards (triolein) prior to the sample analysis run and comparing the spectrum to that of the same compound in the set of original calibration spectra. The ester linkage carbonyl peak (no. 5 in Fig. 1) is used to determine any depth of penetration changes that have occurred since calibration. Both the triolein calibration spectrum (TCS) and the triolein sample spectrum (TSS) are initially set to zero absorbance at 1600 and 1800 cm^{-1} to remove any offset in either spectrum in this region, and the carbonyl peak height maximum is subsequently measured in the two spectra, and the values ratioed (TCS/TSS) to obtain the depth of penetration correction factor, DPCF. To account for any baseline differences between the calibration run and the analytical run, the TCS spectrum is first multiplied by 1/DPCF and then subtracted from the TSS spectrum to generate a baseline correction spectrum. Subtraction of this spectrum from subsequent sample spectra provides a point-by-point spectral adjustment of the sample spectra to the original baseline conditions; the resulting spectra are then multiplied through by the correction factor, DPCF. Using this standardization procedure, it was found that there were major reductions in the errors resulting from variations in experimental conditions between the calibration and sample runs, providing good run-to-run reproducibility. As all these correction procedures are part of the analysis program developed (see listing in Experimental Procedures under Analysis), they are automatically taken care of and are transparent to the operator.

Spectral basis for analysis. Figure 1 illustrates three

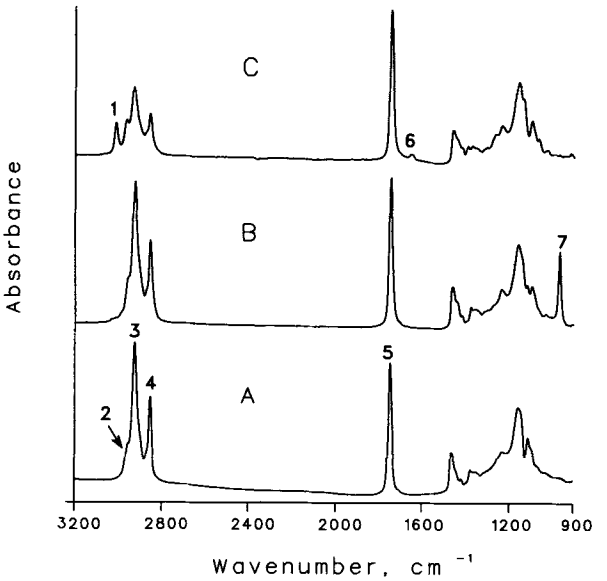

FIG. 1. Attenuated total reflectance/Fourier transform infrared spectra of tripalmitin (A), trielaidin (B), and trilinolenin (C). The labeled peaks are absorption bands that are significant in the determination of either iodine value or saponification number: (1) CH stretch in *cis* HC=CH; (2) CH_3 stretch; (3) CH_2 stretch; (4) CH_2/CH_3 stretch; (5) ester linkage C=O stretch; (6) *cis* C=C stretch; (7) CH stretch in *trans* HC=CH.

superimposed ATR spectra of a saturated and two unsaturated (*cis* and *trans*) triglycerides and notes characteristic absorption bands associated with common lipid systems. In conventional analytical approaches, one normally attempts to relate specific absorption bands to the measure of interest. This approach was investigated and, although found to be workable, had some limitations. The C=O band could be used to determine SN and was excellent for saturated lipids, but the predictive accuracy was reduced somewhat when unsaturated lipids were included in the calibration. In the case of IV, peak height or area worked well for *cis* unsaturation; however, including the *trans* contribution was a problem due to the difficulty of determining an appropriate baseline to measure from, once again limiting the accuracy. If only *cis* double bonds were to be considered, which is an underlying assumption of the published work to date, the results would be adequate. However, it was our intent to be able to measure unsaturation in fats and oils containing both *cis* and *trans* double bonds.

Chemometric software based on partial least squares is an alternative which allows the whole spectrum to be investigated correlationally. The regions that correlate with the measure of interest are readily determined by the chemometric software. PLS analysis indicated that, aside from the traditional regions (C=O, CH, *cis, trans*), the lipid fingerprint region also contained pertinent information, which when included accounted for a major portion of the variation not accounted for in the conventional selected wavelength approach. Although some of the contributing bands were identified, they were difficult to measure accurately. PLS automatically incorporates their contribution into the calibration without these wavelengths being singled out. For these reasons, we ultimately chose to abandon the selected wavelength approach and calibrated both IV and SN with PLS.

Based on the analysis of both the primary and co-added triglyceride standards' spectra, the optimized spectral correlation regions for IV were determined to be 3200–2600 cm^{-1} and 1600–1000 cm^{-1}, while 3200–2600 cm^{-1} and 1850–1000 cm^{-1} were optimal for SN.

Iodine value. A plot of the predicted *vs.* theoretical IV values, obtained from the PLS calibration based on triglyceride standards, is presented in Figure 2 along with its regression equation and associated statistics. The plot is linear and has a slope close to unity, with a standard error of ± 2.7 IV units. A plot of the means of duplicate FTIR IV predictions obtained for the 37 oils assayed with the PLS calibration *vs.* their corresponding duplicate AOCS mean chemical values is presented in Figure 3. This plot illustrates that an excellent linear relationship exists between the FTIR-determined IV and the chemically determined values. Table 2 compares these data in terms of the MD_r and SDD_r for reproducibility (r) between duplicates of the chemical and instrumental results and the MD_a and SDD_a for overall accuracy (a) between the chemical and instrumental results. In terms of reproducibility, both the chemical and the instrumental results have comparable mean differences, with the IR method having a somewhat lower standard deviation between duplicates. A paired comparison test showed that the chemical and instrumentally derived data were not significantly different (<0.05). In terms of accuracy, the predictions from the instrumental method are ~0.6 IV units higher than the chemical values overall, with the SDD_a being comparable to the standard error obtained for the triglyceride calibration standards (Fig. 2). These results indicate that one can determine the IV by FTIR to within 2.8 units of its chemically determined value with 95% confidence.

Saponification number. A parallel analysis to that described for the IV was carried out for the SN. Figure 4 illustrates the plot obtained of the predicted *vs.* theoretical SN for the triglyceride standards along with its regression equation and the corresponding statistics. Excellent predictions were obtained in this calibration, with a standard error of <1.0 SN units. Figure 5 provides a plot of the FTIR-predicted SN *vs.* the chemical values for the 37 oil samples. Although the SN of the set of analyzed oils covers a broad range (185–253), most of the samples are grouped within a relatively narrow range (~185–190). Dramatic variations in SN are clearly responded to by the FTIR method, *e.g.*, coconut (SN = 253), palm kernel (SN = 243), and anhydrous butterfat (SN = 227); however, the plot in the SN = 185–190 range is relatively noisy. In terms of reproducibility, the overall MD_r for the FTIR and chemical methods were quite similar; however, the SDD_r of the chemical method was substantially poorer (2.050 *vs.* 0.333), indicating that the FTIR method is superior in producing consistent results for any one sample. In terms of accuracy, the FTIR results were ~2.7 SN units higher overall, with an SDD_a of ~2.0 SN units. Although the differences between the chemical and instrumental results were not statistically different ($P < 0.05$), the chemical method definitely appears to be less consistent, making any judgement about the accuracy of the FTIR method limited by the reproducibility of the chemical method. Judging from the excellent standard curve obtained for the triglyceride mixtures, it is likely that the FTIR results are more reliable than the chemical results.

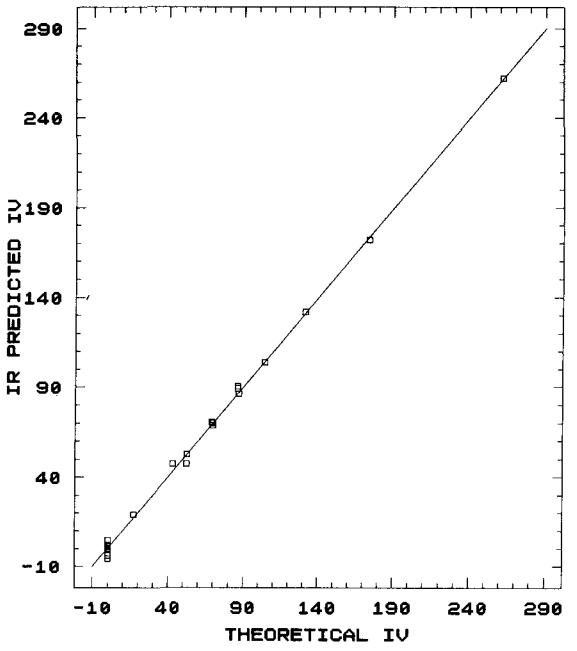

FIG. 2. A plot of predicted (PIV) *vs.* theoretical iodine values (IV) (TIV) for the triglyceride standards. Equation of the line: PIV = 1.0026TIV; R^2 = 0.9993; SE = 2.68. IR, infrared.

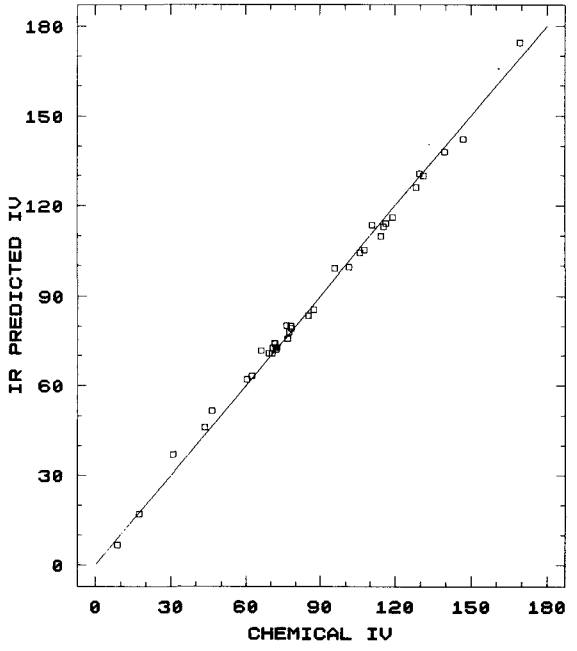

FIG. 3. A plot of infrared (IR) predicted (PIV) *vs.* chemically determined iodine values (CIV) for 37 oils. Equation of the line: PIV = 1.0023CIV; R^2 = 0.9991; SE = 2.87.

DISCUSSION

One assumption made is that the analysis is being made on a relatively clean oil, although this may not always be possible. Impurities such as hydroperoxides, aldehydes and ketones may be present if the oil is oxidized, while free fatty acids can accumulate due to lipolysis during the

TABLE 2

Statistical Comparison of IV and SN Data Obtained by Wet Chemical Methods and FTIR Analysis for 37 Oils[a]

	IV		SN	
	Chemical	FTIR	Chemical	FTIR
MD_r	0.440	−0.550	0.230	−0.106
SDD_r	1.380	0.878	2.050	0.333
MD_a	−0.624		−2.682	
SDD_a	2.804		1.983	

[a]FTIR, Fourier transform infrared; IV, iodine value; SN, saponification number; MD_r, mean difference for reproducibility; SDD_r, standard deviation of difference for reproducibility; MD_a, mean difference for accuracy; SDD_a, standard deviation of difference for accuracy.

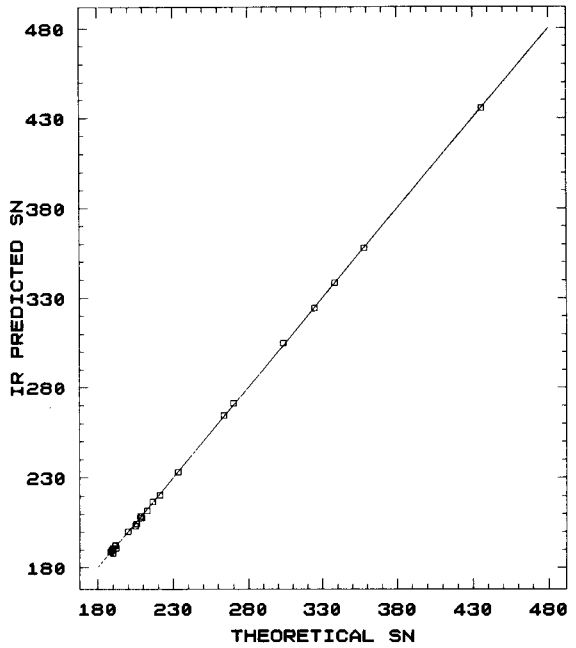

FIG. 4. A plot of predicted (PSN) *vs.* theoretical saponification numbers (TSN) for the triglyceride standards. Equation of the line: PSN = 1.0007TSN; R^2 = 1.00000; SE = 0.93.

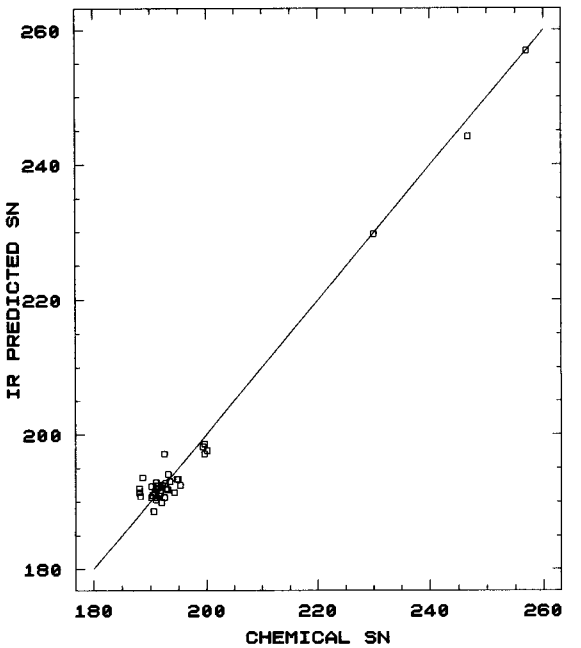

FIG. 5. A plot of infrared (IR) predicted (PSN) *vs.* chemically determined saponification numbers (CSN) for 37 oils. Equation of the line: PSN = 1.0139CSN; R^2 = 0.9999; SE = 2.04.

extraction process. Free fatty acids would be the main concern and levels >2% may introduce some error, while aldehydes and ketones produce weaker signals on the shoulder of the C=O band (1700-1720 cm^{-1}). The presence of any obvious peaks in this region indicates the presence of significant levels of free fatty acids and/or aldehydes. Free fatty acids are readily removed from an oil by silica gel/activated carbon if excessive quantities are suspected to be present, and their contribution can be assessed by ratioing the sample against an absorbent-treated oil. Hydroperoxides are not a problem as their absorbances are weak and they do not absorb within any of the regions being measured. Oxidation and thermal stress also cause a steady shift of *cis* to *trans* over time. However, these changes are accounted for by the IV method. As such, the method can tolerate a reasonable level of impurities but cannot accurately handle excessively oxidized or lipolyzed oils.

What makes our approach unique relative to other FTIR methods developed for IV determination (1-3) is not only that the method works well for SN as well as IV, but that the calibration was determined solely through the use of pure triglyceride standards rather than by calibrating against pre-analyzed oil samples. Unlike previous infrared methods described in the literature, this method avoids the need to dilute with solvents (1) or the flow problems encountered in using a narrow-pathlength transmission cell with viscous oils (2). The ATR/FTIR approach of Afran and Newbery (3) was somewhat along the same lines as the method described here; however, it did not provide results directly in terms of IV and did not consider SN. Furthermore, our method is based on multivariate analysis of spectral data covering broad frequency ranges, rather than peak height measurements at one or two selected frequencies. Such an approach has been shown to provide superior precision in FTIR quantitative analysis (12) and allows the full information content of the infrared spectrum to be exploited.

The use of triglyceride standards has the distinct advantage of eliminating the error associated with the chemical reference method, provides a true theoretical value to calibrate against and avoids substantial analytical work in setting up a calibration. Another useful aspect in this regard is that the weight-average molecular weight of the fat or oil can be determined directly simply by substituting the molecular weight of the triglyceride standards for the SN in the calibration data set and calibrating on that basis. Saponification number has been used as a practical substitute or estimate of molecular weight because the latter could not be determined directly. This limitation can be obviated by using triglyceride calibration standards.

The method developed here provides a practical procedure that can be implemented in a straightforward manner for quality control applications in the fats and oils

industry. The average time of analysis to produce both IV and SN data for fats and oils is ~2 min per sample. With the instrument programmed, the operator is only required to apply the sample and respond to the instrument prompts. An important aspect of our procedure is the automated standardization of instrument conditions to those under which the original calibration was carried out. This standardization routine can be called upon at any time during the analysis to verify that consistent results are being obtained and any deviations (*e.g.*, due to temperature change) are corrected. It may also be possible to transfer calibrations from instrument to instrument through the application of this standardization routine with a smaller set of triglyceride standards than would be required for recalibration (13,14). As implemented, this ATR/FTIR method provides a simple, practical, cost-effective and reagent-free means of rapidly analyzing large numbers of fat and oil samples for SN and IV.

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