Simultaneous Determination of Iodine Value and *trans* Content of Fats and Oils by Single-Bounce Horizontal Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

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ABSTRACT: A method for the simultaneous determination of iodine value (IV) and *trans* content from the Fourier transform infrared (FTIR) spectra of neat fats and oils recorded with the use of a heated single-bounce horizontal attenuated total reflectance (SB-HATR) sampling accessory was developed. Partial least squares (PLS) regression was employed for the development of the calibration models, and a set of nine pure triacylglycerols served as the calibration standards. Regression of the FTIR/PLS-predicted IV and *trans* contents for ten partially hydrogenated oil samples against reference values obtained by gas chromatography yielded slopes close to unity and SD of <1. Good agreement (SD <0.35) also was obtained between the *trans* predictions from the PLS calibration model and *trans* determinations performed by the recently adopted AOCS FTIR/SB-HATR method for the determination of isolated *trans* isomers in fats and oils.

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KEY WORDS: Attenuated total reflectance, Fourier transform infrared spectroscopy, iodine value, lipid analysis, *trans* analysis.

Infrared (IR) analysis of fats and oils has traditionally involved dissolution of the sample in a suitable solvent, such as CS_2 or CCl_4, to allow the sample to be injected into a standard transmission IR cell. In recent years, several sample handling approaches that allow IR spectra to be recorded directly from fats and oils in their neat form have been explored. Our group designed a heated transmission flow cell accessory that meets the requirements for rapid, semiautomated analysis of fats and oils (1) and has developed a number of analytical methods that are based on the use of this accessory (2–6). Horizontal attenuated total reflectance (HATR) accessories also have been widely used in the development of Fourier transform infrared (FTIR) methods for the analysis of fats and oils (7–13) because they provide a simple and convenient means of sample handling (14). Oils are simply pipetted onto the surface of an ATR crystal, and premelted fats can be handled analogously provided that the crystal is maintained at a temperature above the melting point of the fat. When only small amounts of sample are available, the single-bounce (SB) HATR accessories that have become available in recent years are particularly useful owing to the small volume of sample required (<50 μL) to cover the surface of the ATR crystal.

Recently, the American Oil Chemists' Society (AOCS) adopted a new IR method for the determination of isolated *trans* isomers in fats and oils that uses an SB-HATR accessory (15). Mossoba and co-workers have described the principles of this method (13), its advantages over the traditional AOCS IR *trans* method, and its potential applicability to the analysis of fats extracted from foods (16). The adoption of this simple and rapid method by the AOCS is timely in view of the U.S. Food and Drug Administration's recently proposed amendment of the regulations on nutrition labeling, which requires that the amount of *trans* fatty acids present in a food be both included in the amount declared for saturated fatty acids and stated in a footnote at the bottom of the nutrition label. Although the principles of the new AOCS IR method can be implemented by using a transmission cell (6), it may be anticipated that most laboratories adopting this method will acquire SB-HATR accessories, since this sample-handling approach is specified in the official method. Accordingly, we felt that it would be of interest to evaluate the broader applicability of the SB-HATR sample-handling technique for the bulk characterization of fats and oils by FTIR spectroscopy. In our previous work with a heated transmission flow cell accessory, we developed an Edible Oil Analysis Package capable of simultaneously determining iodine value (IV), saponification number, and *cis* and *trans* content in a single analysis on a neat fat or oil in less than 2 min (2). The objective of the present work was to develop an SB-HATR method for the simultaneous determination of IV and *trans* content based on the principles established in this earlier study.

EXPERIMENTAL

Instrumentation. The instrument employed for this work was a Bio-Rad Excalibur FTIR spectrometer operating under Mer-

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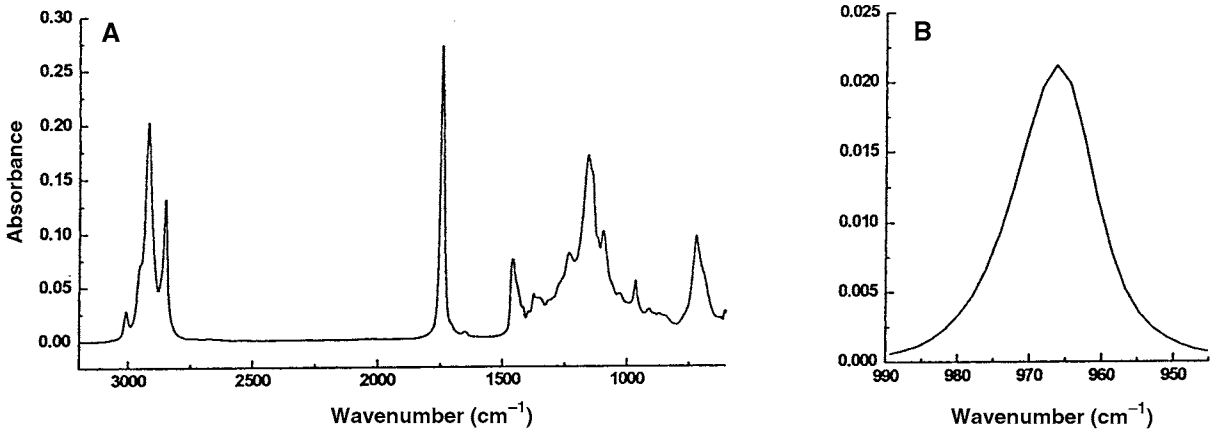

FIG. 1. (A) Fourier transform infrared spectrum of a partially hydrogenated vegetable oil recorded with the use of a single-bounce horizontal attenuated total reflectance (SB-HATR) sampling accessory and ratioed against a background spectrum recorded from the bare ATR crystal; (B) *trans* absorption band in the spectrum of the same sample ratioed against the single-beam spectrum of a *trans*-free reference oil.

lin software (Bio-Rad Laboratories Inc., Cambridge, MA). The spectrometer was equipped with a temperature-controlled SB-HATR sampling accessory (Graseby Specac Inc., Smyrna, GA). The ATR crystal employed was ZnSe, and the temperature of the crystal was maintained at 65°C to allow for the analysis of fats in their liquid state. Both the spectrometer and the optical unit of the sample-handling accessory were purged with a continuous flow of dry air from a Balston dryer (Balston, Lexington, MA) to minimize spectral interferences from water vapor and CO_2.

Development of partial least squares (PLS) calibration models for the prediction of IV and trans *content.* The calibration set consisted of nine pure triacylglycerols (12:0, 14:0, 16:0, 16:1*c*, 18:0, 18:1*c*, 18:1*t*, 18:2*cc*, and 18:3*ccc*; all obtained from Sigma, St. Louis, MO). Each standard was transferred to two 1-mL vials, equilibrated to 65°C in a heated holding block prior to application of the standard onto the surface of the ATR crystal. Spectra were collected by co-addition of 512 scans at a resolution of 4 cm^{-1} and were ratioed against a spectrum recorded from the bare ATR crystal immediately prior to acquisition of the spectrum of the standard. The ATR crystal was cleaned with isooctane after collection of each spectrum and allowed to reequilibrate to 65°C prior to application of the next standard.

Calibration models for the prediction of IV and *trans* content were developed using PLS regression (TQAnalyst; Nicolet Instrument, Madison, WI). Duplicate spectra of each calibration standard were employed in the calibration models. The reference values for the standards were determined on the basis of their chemical structure, with *trans* content being expressed as percent trielaidin. The selection of the spectral regions and numbers of factors to be included in the PLS calibration models was based on minimization of the predicted residual error sum of squares (PRESS) in leave-one-out cross-validation (17).

Calibration: trans *content by AOCS SB-HATR method.* A calibration equation relating *trans* content to the area of the characteristic *trans* absorption band (990–945 cm^{-1}) was derived in accordance with the AOCS SB-HATR method (15). The calibration standards, consisting of triolein/trielaidin mixtures, and the reference oil (a refined, deodorized, and bleached soybean oil) were provided to us as participants in a collaborative study of the method.

Validation. The validation set consisted of 10 samples provided by an oil processor and comprised two samples each of five different oil blends. These samples had all been preanalyzed by gas chromatography (18). For the FTIR analysis, samples were warmed in a microwave oven for 2 min and applied as neat liquids onto the surface of the ATR crystal. Approximately 10 μL of the melted sample was required to cover the surface of the crystal. Single-beam spectra were collected by co-addition of 64 scans at a resolution of 4 cm^{-1}. Cleaning of the ATR crystal between samples entailed wiping off the sample with a lint-free tissue and applying a drop of the next sample and wiping it off.

Two sets of absorbance spectra were generated from the single-beam spectra of the validation samples. One set was obtained by ratioing the single-beam spectra against a background spectrum recorded from the bare ATR crystal prior to collection of the spectra of the validation set (Fig. 1A) and was employed to predict the IV and *trans* contents of the validation samples from the PLS calibration models developed in this work. The second set was obtained by ratioing the same single-beam spectra against the single-beam spectrum of a *trans*-free reference oil (Fig. 1B) and was employed to determine the *trans* contents of the validation samples by the AOCS SB-HATR method. The between-run precision of the FTIR methods was assessed in terms of mean differences (MD_r) and standard deviations of the differences (SDD_r) for duplicate analyses conducted 1 wk apart. To evaluate accuracy, the FTIR-predicted values for IV and *trans* content were regressed against the gas chromatography (GC) values provided to us by the processor supplying the validation samples.

RESULTS AND DISCUSSION

The primary objective of the present study was to evaluate the suitability of an SB-HATR accessory as a means of sample handling for the determination of IV by FTIR spectroscopy. As in our work on the FTIR determination of IV with the use of a transmission flow cell (2) or a multiple-reflection ATR accessory (7), the IV calibration was devised using pure triglycerides as calibration standards and PLS regression. Extensive validation of the calibrations developed in the previous work has demonstrated that this calibration approach yields "universal" calibrations that are applicable to all triglyceride-based oils (7,19). In addition, the use of a calibration set composed of pure triglycerides has the benefit of eliminating the need for chemical analyses of the calibration standards, as the reference values for the standards are known from their molecular structure, and thus the accuracy of the IR method is not limited by the precision of a reference chemical method. Two spectral regions were found to be optimal in the development of the PLS calibration model for the SB-HATR IV method: 3100–2945 cm^{-1}, which includes the CH stretching absorptions of *cis* and *trans* double bonds, and 2880–2780 cm^{-1}, which encompasses the symmetric CH_2 stretching absorption. Both spectral regions were referenced to a single baseline point at 3200 cm^{-1}. The same set of calibration standards was employed to develop a PLS calibration model for the prediction of *trans* content. The optimized *trans* calibration utilized a single broad spectral region (1110–603 cm^{-1}, referenced to a single baseline point at 1550 cm^{-1}) that includes the *trans*-C=C–H bending absorption at 966 cm^{-1} traditionally employed in IR *trans* determinations. The standard errors of calibration were 0.43 IV and 0.15% *trans*, with the inclusion of six and five loading spectra, respectively, in the PLS calibration models.

For validation of the IV and *trans* calibrations, as well as comparison between the results obtained by the PLS and AOCS *trans* methods, a validation set consisting of two samples of each of five different oil blends was employed. These samples had been preanalyzed by the optimized GC method recently adopted by the AOCS (18) and spanned a wide range of IV (67.8–133.8) and *trans* content (0.3–39.3%). FTIR analyses of these samples were performed in duplicate, 1 wk apart, and the MD_r and SDD_r are summarized in Table 1. The corresponding data obtained by analyzing the same set of validation samples using the previously developed transmission flow-cell method are presented in Table 1 for purposes of comparison. The week-to-week variability of the SB-HATR and transmission methods is seen to be comparable, with the values of MD_r for all parameters being close to zero. The results in Table 1 also indicate that the between-run precision of the PLS-based *trans* analysis is very similar to that of the AOCS SB-HATR *trans* method.

The correspondence between the FTIR-predicted values of IV and percentage *trans* and the GC data provided for the validation samples was examined by simple linear regression analysis. Table 2 presents a summary of the linear and Z-linear (forced through the origin) regression coefficients and the associated statistics. In all cases, the slopes of the regression equations are close to unity, and the correlation coefficients and regression errors demonstrate an excellent correspondence between the FTIR and GC data. Eliminating the intercept in each of these regression equations by Z-linear regression did not result in an appreciable increase in the regression error, indicating that the FTIR predictions are not biased relative to the GC data.

TABLE 1
Comparison of Week-to-Week Reproducibility of SB-HATR and Transmission Flow-Cell Methods for the Determination of IV and *trans* Content[a]

	IV		*trans* (%)	
Method	MD_r	SDD_r	MD_r	SDD_r
SB-HATR (PLS-based)	0.42	0.76	–0.19	0.23
Transmission (PLS-based)	–0.64	0.51	0.0	0.47
AOCS SB-HATR	NA	NA	–0.16	0.20

[a]MD_r, mean difference for reproducibility; SDD_r, standard deviation of the differences for reproducibility; n = 10. SB-HATR, single-bounce horizontal attenuated total reflectance; PLS, partial least squres; IV, iodine value.

The results from linear and Z-linear regression of the *trans* predictions from the PLS calibration model against the *trans* data obtained by the AOCS SB-HATR method also are presented in Table 2 and show excellent agreement between the two FTIR methods. The traditional IR method for the determination of *trans* isomers, which is based on measurement of the area of the 966-cm^{-1} *trans* absorption band. (20), requires that samples be converted to methyl esters because of interference from underlying triglyceride absorptions. The spectral ratioing capabilities of FTIR spectrometers provide a means of eliminating the need for this time-consuming sample-preparation step because the interfering absorptions can be removed from the absorbance spectrum of the sample by ratioing the single-beam spectrum of the sample against that of a *trans*-free reference oil of similar triglyceride composition. This principle is employed in the AOCS SB-HATR method, whereas the PLS-based *trans* method mathematically compensates for underlying triglyceride absorptions within the calibration model. The excellent correspondence between the results from the two methods provides indirect experi-

TABLE 2
Regression Analysis Summary[a]

Regression	Abscissa	Ordinate	Intercept	Slope	SD	*R*
Normal	GC IV	PLS IV	–1.33	1.01	0.67	0.999
Z-Regression	GC IV	PLS IV	0.00	0.99	0.73	0.999
Normal	GC *trans*	PLS *trans*	0.42	0.97	0.91	0.998
Z-Regression	GC *trans*	PLS *trans*	0.00	0.98	0.90	0.998
Normal	AOCS *trans*	PLS *trans*	–0.26	0.96	0.29	0.999
Z-Regression	AOCS *trans*	PLS *trans*	0.00	0.96	0.32	0.999
Normal	GC *trans*	AOCS *trans*	0.70	1.00	0.84	0.998
Z-Regression	GC *trans*	AOCS *trans*	0.00	1.03	0.92	0.999

[a]GC, gas chromatography; PLS, partial least squares; *R*, correlation coefficient; see Table 1 for other abbreviations.

mental evidence for the validity of the two different approaches employed to compensate for underlying absorptions. This conclusion is corroborated by the similar good agreement between PLS-predicted *trans* contents and the values from a peak-height calibration based on a spectral ratioing approach that was obtained in earlier work with a transmission flow cell (19).

A noteworthy difference between the results of the latter study and the present work is the improved agreement between the FTIR and GC *trans* data, which we attribute to the use of AOCS Official Method Ce 1f-96 to obtain the GC data for the samples analyzed in the present study. In our earlier work, Z-linear regression of the FTIR data against GC data obtained with AOCS Official Method Ce 1c-89 yielded slopes of 1.12–1.13 (19). In contrast, the Z-regression slopes relating FTIR *trans* to the GC *trans* values obtained with Method Ce 1f-96 are close to unity (0.96 for the PLS method and 1.03 for the AOCS SB-HATR method). Method Ce 1f-96 is optimized to quantify *trans* fatty acids (18), whereas the levels of *trans* isomers in partially hydrogenated oils are underestimated by Ce 1c-89 owing to incomplete separation of the peaks due to 18:1*c* and 18:1*t* isomers (21). Adam *et al.* (22) compared *trans* data from Method Ce 1c-89 and the AOCS SB-HATR method by analyzing three accuracy standards, prepared by gravimetric addition of trielaidin to a *trans*-free oil, and three partially hydrogenated vegetable oils. Based on the good agreement between the FTIR and GC data, which was peer-verified in an independent laboratory, they concluded that the optimized GC procedure provides accurate *trans* values. The similar results obtained in the present study for a larger number of partially hydrogenated oil samples support this conclusion.

The results of the present study indicate that SB-HATR is a suitable sample-handling technique for the FTIR determination of not only *trans* content, as reported by other workers (13,22,23), but also IV. It is noteworthy that the SB-HATR method developed in this work is based on a multivariate calibration approach, as opposed to the simple univariate calibration employed to predict *trans* content in the earlier work. Because minor spectral perturbations can have a major effect on the performance of multivariate calibrations (24) since the calibration models are based on broad spectral regions rather than a single peak, the use of a multivariate calibration approach imposes more stringent requirements on spectral reproducibility than a univariate calibration. For this reason, we had anticipated that the SB-HATR sampling technique might be problematic owing to its short effective pathlength as well as certain limitations of the ATR technique that we had noted in working with a multiple-reflection HATR accessory. In particular, ATR spectra are highly sensitive to temperature variations because the effective pathlength is affected by changes in the refractive index of the sample or the ATR crystal in a wavelength-dependent manner (14). However, by comparison with a multiple-reflection ATR device, the SB-HATR accessory proved to be more amenable to precise temperature control owing to the much smaller surface area of the ATR crystal. In addition, the temperature fluctuations that result from evaporative cooling during cleaning with solvent were avoided in the present work by adopting the cleaning procedure described in the AOCS SB-HATR *trans* method, whereby the ATR crystal is cleaned by wiping it with a drop of the next sample. The good between-run precision and accuracy obtained in this study provides evidence that this simple procedure is sufficient to avoid significant cross-contamination. We noted that there was a tendency for a film to build up on the heated ATR crystal over time, presumably due to the formation of oxidation products, but this film was found to be readily removed by cleaning the crystal with distilled water. In this regard, it is important to note that the ATR crystal should be scrupulously cleaned with an appropriate solvent (e.g., isooctane) and water prior to recording a background spectrum from the bare crystal because any residual sample or contaminants on the surface of the ATR crystal will make a substantial contribution to the background spectrum. Based on our experience, we recommend that a background spectrum be collected daily prior to the analysis of samples and after every 20 analyses.

The results of this study indicate that FTIR analysis of neat fats and oils to determine their overall degree of unsaturation as well as *trans* unsaturation can be achieved with the use of either a heated SB-HATR accessory or a heated transmission flow-cell accessory. The SB-HATR sample-handling technique is simple and convenient, as it requires only that the sample be poured or spread on the surface of the ATR crystal, and is particularly advantageous when the amount of sample is limited, the sample volume required being <50 μL. As suggested in very early work (25), ATR also may be useful in monitoring hydrogenation processes, whereas the presence of catalyst particles in oil samples can be problematic in transmission flow-cell measurements owing to possible clogging of the cell and light-scattering effects. On the other hand, the limited sensitivity of the SB-HATR technique, due to its short effective pathlength, makes it unsuitable as an alternative to transmission-based techniques in applications that require the measurement of components present in oils at low concentrations, such as the determination of peroxide value or free fatty acid content.

Although additional intra- and interlaboratory validation studies are required to assess the robustness of the IV method developed in this work, we suggest that the principles of this method can serve as the basis of an official method for the determination of IV by FTIR/SB-HATR spectroscopy. Apart from the benefits of speed, simplicity, and amenability to automation associated with FTIR analysis (1), such a method would have the advantage of utilizing the same instrument/sample-handling configuration as the recently adopted AOCS SB-HATR method for the determination of *trans* isomers. Because FTIR spectra are collected as single-beam spectra and are subsequently digitally ratioed against a background spectrum to produce absorbance spectra, the use of different background spectra in the PLS-based IV method (open-beam spectrum as background) and the official *trans* method (refer-

ence oil spectrum as background) does not preclude using the same single-beam spectrum of the sample for both determinations, as was done in the present work. Accordingly, an analytical package could be developed for the simultaneous determination of *trans* content and IV, utilizing the presently accepted official *trans* method and a "universal" PLS-based IV calibration. With the availability of such a package, the FTIR/SB-HATR technique could be readily implemented in quality control laboratories and would provide a rapid, simple, and cost-effective alternative to GC analysis in situations in which only IV and *trans* data are required.

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