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Rapid Determination of Free Fatty Acids in Poultry Feed Lipid Extracts by SB-ATR FTIR Spectroscopy

S. T. H. SHERAZI,* S. A. MAHESAR, AND M. I. BHANGER

National Center of Excellence in Analytical Chemistry, University of Sindh, Jamshoro, Pakistan

F. R. VAN DE VOORT AND J. SEDMAN

McGill IR Group, McGill University, Montreal, Quebec, Canada

A simple, rapid, and reproducible method has been developed for the quantitative determination of free fatty acid (FFA) content in lipids extracted from poultry feeds by Fourier transform infrared (FTIR) spectroscopy with the use of a single-bounce attenuated total reflectance (SB-ATR) accessory. An FTIR calibration curve was prepared by gravimetrically adding oleic acid (15−37%) to pure refined, bleached, and deodorized (RBD) canola oil and measuring the area of the COOH absorption band at 1710 cm^{-1}. The oil from each of 12 poultry feed formulations was extracted using conventional Soxhlet extraction, and after evaporation of the solvent, the FFA content was determined by the conventional AOCS titrimetric procedure and by the SB-ATR/FTIR method. The SB-ATR/FTIR FFA predictions were related to those determined by the AOCS titrimetric method by linear regression, producing an R value of 0.999 and a SD of ±0.28% FFA. Time-course spectra collected as lipids extracted into hexane indicated that a 15 min extraction was adequate to obtain a representative sample for FFA determination, with further extraction resulting in little, if any, change in the proportion of FFA in the lipid extract. Only a small volume of the hexane extract (~20 mL) yielded sufficient material for the SB-ATR/FTIR analysis. Thus, by shortening the extraction time and taking a small sample so as to reduce solvent removal time, the SB-ATR/FTIR procedure provides a very simple and rapid means of determining the FFA content of poultry feed lipids.

KEYWORDS: Free fatty acid (FFA) analysis; poultry feed oil; SB-ATR; FTIR spectroscopy

INTRODUCTION

In poultry feed formulations, most of the energy requirements are provided by the lipid component, usually ranging from 2 to 6%, which generally consists of triglycerides as well as free fatty acids (FFA). FFAs are more readily oxidized than triglycerides and thus can contribute to the development of rancidity and impact the palatability of the feed. Oxidative rancidity is known to affect fat digestibility (*1*) and cecal activity (*2*) in poultry, and thus, determination of the FFA content is of considerable importance in terms of assessing the overall quality of poultry feeds. The FFA content of fats and oils or lipid extracts can be determined in a variety of ways, including by titrimetric (*3*), colorimetric (*4*), potentiometric (*5*−*7*), chromatographic (gas and liquid) (*8*−*10*), and infrared spectroscopic (*11*−*14*) procedures. Titrimetric procedures are most commonly used for poultry feed lipid extracts and typically involve an exhaustive extraction of ~20 g of the feed using ~200 mL of solvent, followed by solvent removal, weighing to quantitate the oil content, resolubilization, and then titration with base to a visual phenolphthalein endpoint. Although this traditional approach may be satisfactory in terms of accuracy and sensitivity, the development of a simple instrumental methodology that could eliminate tedious titration procedures as well as reduce reagent and solvent use would be highly desirable.

Over the past decade, Fourier transform infrared (FTIR) spectroscopy has proven to be a very useful technique for the rapid characterization of edible oils (*15*−*17*) as well as the study of lipid oxidation (*18*−*20*). Throughout this period, FTIR methodology for the determination of FFA content has evolved substantially (*11*−*14*), particularly in relation to achieving the sensitivity required for the analysis of the low levels of FFA present in refined oils, and an automated system capable of analyzing up to 120 samples per hour has recently been described (*21*). However, this prior work has been primarily directed toward the analysis of bulk quantities of fats and oils, and the need for a simple and convenient FTIR procedure suitable for the determination of the FFA content of small quantities of lipid extracts has not been addressed. On the other hand, in work on the determination of trans fatty acids by FTIR spectroscopy, the single-bounce attenuated total reflectance (SB-ATR) sample-handling technique has come to the forefront as

* To whom correspondence should be addressed. E-mail: tufail_sherazi@yahoo.com.

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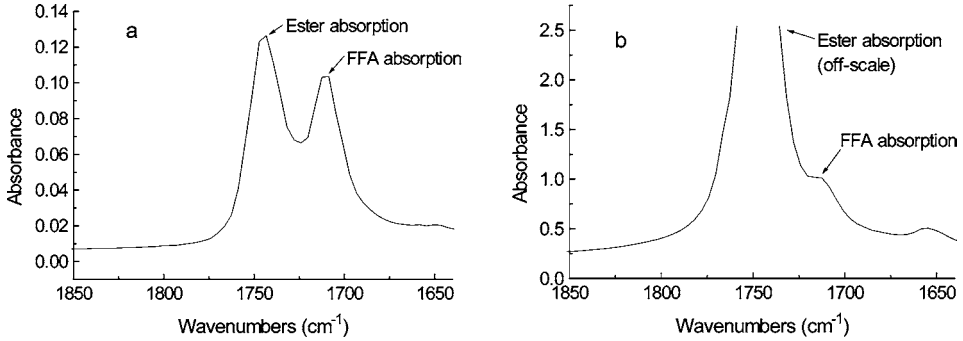

Figure 1. Carbonyl stretching absorption region in the SB-ATR spectrum of poultry feed lipid extract containing about 10% FFA (**a**) and in the transmission spectrum of RBD canola oil containing about 1% FFA recorded in a 200 μm transmission cell (**b**).

Table 1. Hexane-Extractable Lipid Obtained from Duplicate Analyses of 12 Commercial Poultry Feeds after 6 h of Refluxing

	lipid content (%, w/w)		
poultry feed	analysis 1	analysis 2	average
PF-1	3.45	3.52	3.485
PF-2	4.90	5.11	5.005
PF-3	5.20	5.32	5.260
PF-4	3.68	3.71	3.695
PF-5	3.26	3.20	3.230
PF-6	4.53	4.48	4.505
PF-7	4.95	4.81	4.880
PF-8	5.20	5.25	5.225
PF-9	5.33	5.16	5.245
PF-10	5.10	5.14	5.120
PF-11	2.75	2.80	2.775
PF-12	5.18	5.23	5.205

a potential means of analyzing fats extracted from foods for compliance with new food labeling regulations regarding trans fat (*22*). It should be noted that the sensitivity of SB-ATR/FTIR spectroscopy is limited by the inherently short effective path length associated with the ATR phenomenon, and this limitation would make this technique unsuitable for the determination of the low levels of FFA in refined oils. However, we anticipated that this limitation would not prove significant in FFA analysis of poultry feed lipid extracts, given their generally high FFA contents. Accordingly, this paper presents the development of a simple SB-ATR/FTIR procedure by which the FFA content of poultry feed lipid extracts can be accurately and rapidly determined.

MATERIALS AND METHODS

Reagents and Samples. All reagents were of analytical grade. Oleic acid (99%) and sodium hydroxide were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Hexane was obtained from Fisher Scientific U.K. Ltd., and ethanol was purchased from Merck (Darmstadt, Germany). Refined, bleached, and deodorized (RBD) canola oil was obtained locally. Poultry feed samples were collected from industry suppliers, and these feeds were designated as being appropriate for starter broilers. The lipid content of the poultry feeds was determined by Soxhlet extraction of 20 g of ground feed with 300 mL of *n*-hexane for 6 h, in accordance with the procedure described by Anwar et al. (*23*). The solvent was removed under vacuum using a rotary evaporator, and the sample was then placed in a vacuum oven at 105 °C for 15 min, cooled in a desiccator, and refrigerated until further analysis.

Preparation of FTIR Calibration Standards. A set of six standards covering an FFA range of 15−37% was prepared by gravimetric addition of oleic acid to RBD canola oil, previously determined to contain <0.10% FFA by the AOCS titrimetric method (*3*).

Instrumentation. All infrared spectra were acquired using a Thermo Nicolet Avatar 330 FTIR spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector and KBr optics and controlled by OMNIC software (Thermo Nicolet Analytical Instruments, Madison, WI). An SB-ATR accessory (Spectra-Tech, Shelton, CT) with a removable ZnSe crystal was mounted in the sample compartment of the spectrometer. All spectra were collected by co-addition of 32 scans at a resolution of 8 cm^{-1}. The spectrum of each standard or sample was ratioed against a fresh background spectrum recorded from the bare ATR crystal; prior to collection of each background spectrum, the ATR crystal was carefully cleaned with propanol to remove any lipo- or hydrophilic residues of the previous sample, and the residual solvent was then evaporated using a stream of nitrogen gas.

FTIR Prediction of FFA Content. The SB-ATR/FTIR spectra of the six calibration standards were employed to derive a calibration equation relating FFA content to the integrated area of the ν(C═O) absorption of FFA (1720−1690 cm^{-1}) by a simple linear regression. This equation was subsequently used to predict the FFA contents of 12 poultry feed lipid extracts from their SB-ATR/FTIR spectra, and the predictions obtained were compared with the FFA contents determined by the AOCS titrimetric method (*3*). Taking the latter data as the true reference values, the accuracy of the SB-ATR/FTIR predictions was assessed in terms of mean difference (MD_a) and standard deviation of the differences (SDD_a) as suggested by AOAC International (*24*). The reproducibility of the SB-ATR/FTIR method was compared with that of the AOCS titrimetric method in terms of the mean difference (MD_r) and standard deviation of the differences (SDD_r) between duplicate analyses of each of the 12 samples.

Time-Course Studies of Lipid Extraction. For the time-course studies, Soxhlet extraction was carried out in a flask having a sidearm. As the extraction proceeded, 15 mL samples were withdrawn via the sidearm at selected times and placed in 20 mL test tubes; the flask was then replenished with an equal volume of fresh hexane. The solvent was evaporated from the extracts over a water bath held at 80 °C, and SB-ATR/FTIR spectra of the residual oil were recorded to examine spectral changes over time as the extraction proceeded.

RESULTS AND DISCUSSION

A series of 12 commercial starter broiler feeds was analyzed for hexane-extractable lipids (**Table 1**). The oil contents ranged from 2.75 to 5.33%, providing ∼0.4−1.0 g of lipid. These amounts were more than adequate for SB-ATR/FTIR analysis, as ∼50 μL of oil was sufficient to fully cover the surface of the ATR crystal. The ν(C═O) region in a typical SB-ATR spectrum of a poultry feed hexane lipid extract is presented in **Figure 1a**, showing two bands of comparable intensity characteristic of the carboxylic acid group of FFAs (1710 cm^{-1}) and the ester linkages of triglycerides (1743 cm^{-1}). For purposes of comparison, **Figure 1b** shows the transmission spectrum of RBD canola oil (containing ∼1% FFA) recorded in a 200 μm transmission cell. In the latter spectrum, the FFA ν(C═O) absorption appears as a poorly resolved shoulder on the ν(C═O) absorption band of the triglyceride ester linkage, which is so intense that it is off-scale. From a comparison of the

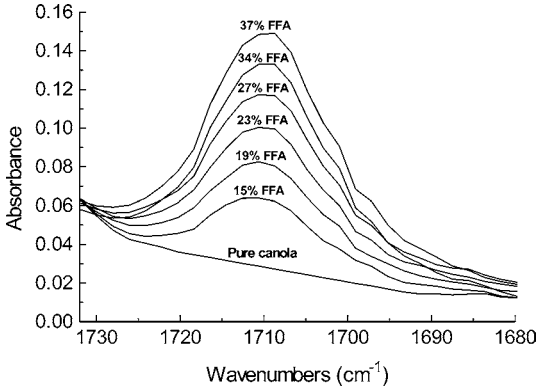

Figure 2. FFA ν(C═O) absorption band in the spectra of RBD canola oil spiked with various amounts of oleic acid.

Table 2. Regression Equations Relating Percent FFA to FFA ν(C═O) Area and Height Measurements Relative to Two Different Baselines

measurement (cm^{-1})	baseline (cm^{-1})	intercept	slope	R	SD
area 1720–1690	1720–1690	6.561	24.231	0.9994	0.316
area 1720–1690	1900–1600	–0.367	13.243	0.9998	0.146
height 1710	1720–1690	–0.294	254.67	0.9996	0.241
height 1710	1900–1600	0.001	254.43	0.9996	0.270

Table 3. FFA Content (Mean ± SD) of Poultry Feed Lipid Extracts Obtained from Duplicate Analyses by SB-ATR/FTIR and Titrimetric Methods

	FTIR % FFA		titration % FFA	
feed sample	mean	SD	mean	SD
PF-1	57.13	0.035	56.45	0.15
PF-2	61.85	0.053	61.50	0.30
PF-3	56.23	0.031	55.70	0.20
PF-4	55.96	0.030	55.70	0.30
PF-5	58.51	0.040	57.90	0.20
PF-6	29.92	0.068	29.45	0.05
PF-7	36.18	0.044	35.75	0.15
PF-8	40.16	0.029	39.60	0.30
PF-9	28.78	0.073	28.45	0.25
PF-10	38.00	0.038	36.70	0.20
PF-11	43.00	0.018	42.65	0.15
PF-12	32.50	0.058	32.00	0.30
	$MD_r = 0.024$		$MD_r = -0.425$	
	$SDD_r = 0.093$		$SDD_r = 0.163$	

absorbance scales in **Figure 1a**,**b**, it is evident that the short effective path length of SB-ATR (~2.5 μm at 1700 cm^{-1}) would not provide sufficient sensitivity for the measurement of the low levels of FFA present in RBD oils. In addition, **Figure 1b** illustrates that measurements of the height or area of the FFA band will be strongly affected by the intensity and the width of the adjacent intense ester ν(C═O) band, which in turn are dependent on the saponification number of the oil, and thus, as discussed in a recent publication (*11*), this band overlap has complicated the development of methods for the measurement of FFA in refined oils. On the other hand, the fairly elevated FFA levels in the poultry feed lipid extracts result in clearly discernible FFA ν(C═O) absorptions in SB-ATR spectra, and under these conditions, the error introduced by band overlap should be minor, such that the peak height or area of the FFA ν(C═O) band should be proportional to FFA concentration. This assumption was substantiated by recording the SB-ATR spectra of RBD canola oils spiked with various amounts of oleic acid, corresponding to FFA concentrations ranging from 15 to 37%. As illustrated in **Figure 2**, these spectra showed a clear,

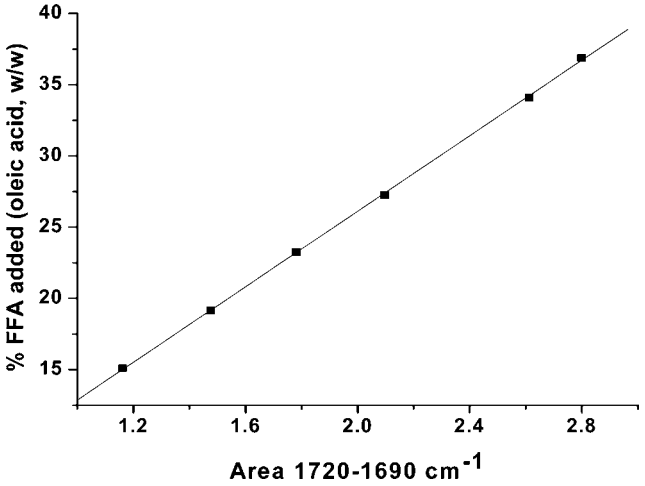

Figure 3. Calibration plot derived from the spectra illustrated in **Figure 2** based on the peak area from 1720 to 1690 cm^{-1} with a baseline drawn between 1900 and 1600 cm^{-1}.

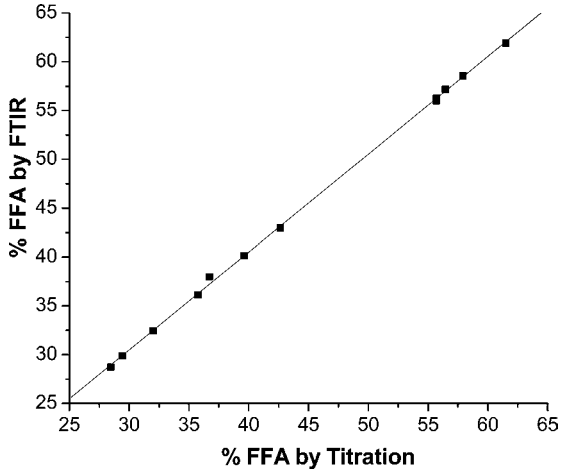

Figure 4. Plot of predicted FFA obtained by SB-ATR FTIR analysis vs titration results.

measurable spectral response to added oleic acid. **Table 2** presents the linear regression relationships obtained from these spectra using both peak height and area measurements, with two different baseline selections. Measurement of the area over the range of 1720−1690 cm^{-1} with a two-point baseline located at 1900 and 1600 cm^{-1} yielded the best linear relationship and was used to derive the calibration plot presented in **Figure 3** and the corresponding calibration equation

$$\% \text{ FFA} = -0.37 + 13.24A_{(1720-1690/B1900-1600)}$$
$$R = 0.9998; \text{ SD} = 0.15 \quad (1)$$

The SB-ATR spectra of the hexane extracts of the 12 commercial poultry feeds listed in **Table 1** were then recorded in duplicate, and eq 1 was applied to estimate their FFA contents. **Table 3** presents a comparison of these results with those obtained from duplicate analyses by the AOCS titrimetric method. Both the mean difference (MD_r) and standard deviation of the differences (SDD_r) for reproducibility were substantially better for the FTIR method than the titrimetric procedure, the FTIR duplicates tending to the same mean without any significant bias ($MD_r = 0.024$ vs −0.425% FFA) with less variability around the mean difference ($SDD_r = 0.093$ vs 0.163). **Figure 4** graphically illustrates that the relationship between the mean values of the duplicate FTIR and titrimetric analyses

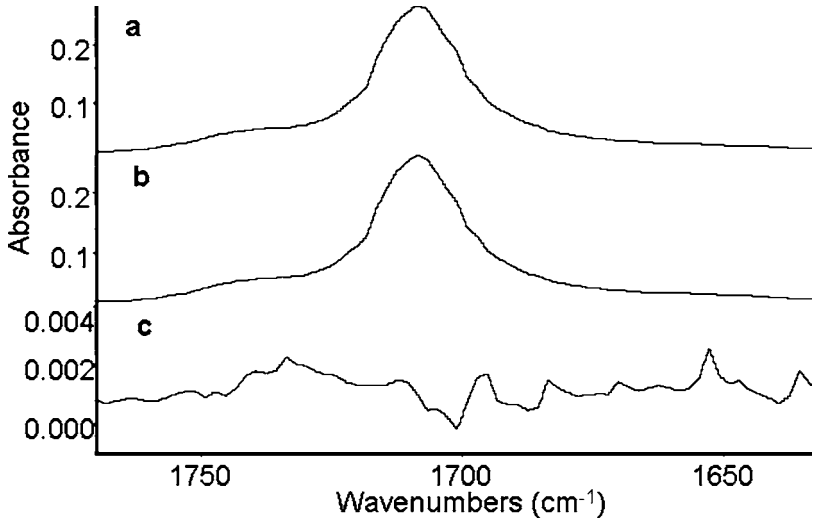

Figure 5. Spectra of lipid extracts after 15 min (**a**) and 3 h (**b**) extraction and difference spectrum obtained by spectral subtraction of panel **b** from panel **a**.

is linear, the mean difference (MD_a) and standard deviation of the differences (SDD_a) being 0.53% FFA and ±0.27% FFA, respectively. It is interesting to note that although four of the poultry feed lipid extracts contained FFA levels beyond the range of the FTIR calibration, they still lie on the line, indicating that the calibration equation could be extrapolated to higher FFA values. To evaluate the proportionality between the FTIR and titrimetric methods, linear regression of the data presented in **Figure 4** was performed, yielding the following relationship when the regression was forced through the origin (*Z*-reg):

$$\%\ \mathrm{FFA}_{\mathrm{SB-ATR/FTIR}} = 1.011\%\ \mathrm{FFA}_{\mathrm{titration}}$$
$$R = 0.9997;\ \mathrm{SD} = 0.3\%\ \mathrm{FFA} \quad (2)$$

The slope of the *Z*-reg equation is close to the value that one would expect if there was a 1:1 proportionality between the methods, and based on the reproducibility data, it is clear that most of the regression error was contributed by the titrimetric results. Thus, eq 2 indicates that SB-ATR/FTIR analysis can serve as a simple, convenient, and accurate alternative to titration for the determination of the FFA content of poultry feed lipid extracts.

The FFA analysis of poultry feeds would be further facilitated if the need for a complete and exhaustive lipid extraction of the feed could be eliminated. This possibility is predicated on the assumption that a sample collected after a short extraction time would be representative of that obtained after exhaustive extraction in terms of its FFA content. Because SB-ATR/FTIR analysis requires only ~50 μL of sample, it was possible to investigate this possibility by carrying out time-course studies of Soxhlet extraction of poultry feed samples. Samples were extracted in *n*-hexane, and ~15 mL was collected from the solvent reservoir at ~15 min intervals and replaced with fresh solvent. The solvent was then evaporated from these time-course samples, yielding sufficient material for the SB-ATR/FTIR analysis. **Figure 5** shows the spectrum of the lipid extract after only 15 min of extraction, the spectrum after ~3 h of extraction, and the difference spectrum obtained by subtraction of these two spectra. The difference spectrum shows only a very small (negative) FFA absorption, barely discernible above the spectral noise, indicating that a 15 min extraction would be adequate to obtain a representative sample for determination of the FFA content of the feed lipid by SB-ATR/FTIR spectroscopy. With this shorter extraction time, the amount of solvent that has to be evaporated would also be reduced, as only a small volume of the hexane extract (~15 mL) was required. We subsequently investigated the alternative possibility of performing a simple microwave-assisted extraction, whereby ~6.25 g of poultry feed and 12 mL of hexane were heated in a capped test tube in a conventional microwave oven for six 10 s intervals. The weight of the residue following removal of the solvent corresponded to a lipid extraction efficiency of 59% relative to exhaustive Soxhlet extraction of the same feed sample, and its SB-ATR/FTIR spectrum again was found to be representative of that of the fully extracted lipid. Thus, an FFA value (as a percentage of total lipid) can be obtained rapidly by this very simple procedure, although an exhaustive Soxhlet extraction is still required if the total amount of lipid in the feed is to be determined.

The results of this study demonstrate that there are substantial benefits in terms of speed, precision, and accuracy to be gained from the use of SB-ATR/FTIR spectroscopy for the determination of FFA in poultry feeds. Previous work related to FFA analysis by FTIR spectroscopy has focused mainly on the analysis of low levels of FFA, effectively precluding the use of the SB-ATR technique owing to its short effective path length (and hence limited sensitivity). In the case of poultry feed lipids, where FFA levels tend to be high, SB-ATR/FTIR spectroscopy has been shown to be a simple, convenient, and accurate alternative to the standard AOCS titrimetric method for the determination of FFA. Using this approach, no sample preparation beyond evaporation of the extraction solvent is required, and the FFA content of poultry feed lipid extracts can be determined within 2 min using less than a drop of the neat lipid extract. In addition, it has been demonstrated that the spectrum obtained following a 15 min hexane extraction is comparable to that of an exhaustive hexane extract, which affords the possibility of a large decrease in total analysis time.

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