Use of FTIR to Determine Trierucin, tocopherols and glucosinolates, components important in plant breeding programs.

Khalique Ahmed[1], James Daun[2] and Roman Przybylski[3.]

[1]. Lynn University, Boca Raton, FL. USA.
[2].Canadian Grain Commission, Grain Research Laboratory, Winnipeg, MB, Canada
[3.] University of Manitoba, Department of Human Nutritional Sciences, Winnipeg, MB, Canada

ABSTRACT

Fourier-transform Infra-red spectroscopy (FTIR) has become popular to determine components of oils during processing including free fatty acids, iodine value, peroxide value and trans fatty acids. The relatively low price of FTIR instrumentation, coupled with speed of analysis and the small sample size, makes it an attractive tool for plant breeding programs. Trierucin, an important component for development of high erucic acid rapeseed lines, may be determined in high erucic acid rapeseed with a precision of ±2%. FTIR may also be used to determine either intact or desulfated glucosinolates in seed extracts. The unique aromatic character of tocopherols also makes it possible to determine their content in small sample.

INTRODUCTION

Although Fourier transform infrared spectroscopy is routinely used in chemistry and physics laboratories for characterization, identification and quantitation of materials, it has been only recently that food and agriculture scientists have shown interest in this technique. Some applications of FTIR in food science include determination of iodine value[1], saponification number[1], free fatty acids[2], peroxide value[3] and *cis* and *trans* fatty acid content[4, 5, 6] of edible fats and oils; determination[7] of fat, protein and lactose content of milk; characterization of vegetable oils, butter and margarines[8]; determination of phospholipid content of animal fat[9]; determination of protein content of ground wheat[10]; classification of vegetable oils[11] and determination of adulteration of raspberry purees[12].

Recently, we have been investigating the use of FTIR as a means to rapidly determine some key quality parameters in oilseeds. These parameters are key targets in present and future breeding programs and the development of rapid, relatively inexpensive methods which might allow measurement on small samples is essential. This paper will summarize our findings on the use of FTIR in three areas:

1. Determination of trierucin in high erucic acid oils
2. Determination of tocopherols in oils
3. Determination of glucosinolates in extracts.

EXPERIMENTAL

Materials: Oils were obtained either by extraction from seed samples with petroleum ether or were authentic oils obtained from commercial sources.

Determination of Tocopherols: Tocopherols were analyzed by normal phase HPLC with fluorescent detection[13].

Determination of Glucosinolates: Glucosinolates were determined by HPLC following ISO 9167-1:1992 Rapeseed -- Determination of glucosinolates content -- Part 1: Method using high-performance liquid chromatography.

FTIR Measurements: FTIR spectra were recorded on a BIO-RAD (Cambridge, MA) FTS-135 spectrometer equipped with a KBr beamsplitter and a mercury cadmium telluride (MCT) nitrogen cooled detector. The spectra were recorded in the attenuated total reflection (ATR)

mode with a Horizontal ATR accessory from PIKE Technologies , Inc. (Madison, WI 53719). The accessory was equipped with a ZnSe ATR crystal of a trapezoid shape and was 800 mm long, 10 mm wide and 4 mm thick. The crystal provided an angle of incidence of 45° and was enclosed in a stainless-steel cuvette. To record spectra, about 50 μL of oil was dissolved in 2 ml of hexane and the solution was poured on ATR crystal and allowed to dry. This produced a uniform film on the ATR crystal whose single beam spectrum was recorded and then ratioed against the single beam spectrum of the bare crystal to obtain the absorbance spectrum. Spectra were recorded with a resolution of 4 cm^{-1} and 128 scans were averaged for each spectrum. The spectrometer was not purged during measurements.

RESULTS AND DISCUSSION

Determination of Trierucin. Rapeseed is one of the primary industrial sources of erucic acid (cis-13-docasaenoic acid). An important industrial product, erucamide is produced from high erucic acid rapeseed (HEAR) oil and is used as an antiblock, slip promoting agent in the production of polyolefine films[14]. Currently, there is a great interest in increasing the C22:1 content of HEAR oils from its present level of about 55% to a value of as much as 90%. It is thought that with a greater proportion of fatty acids as C22:1 the market value of HEAR oils in the oleochemical industry will increase appreciably[15, 16]. Traditional lines of high erucic acid rapeseed (*Brassica napus* L.) incorporate erucic acid only into the sn-1 and sn-3 positions of the triacylglycerol[17]. This means that the theoretical maximum for erucic acid content of HEAR oils from such seed is 66%, with values of about 55% being realized by conventional plant breeding techniques. Current approaches involve insertion of genes from other plant species such as *Tropaeolus Majus* L. which direct the incorporation of erucic acid into the sn-2 position of the triacylglycerol.

The FTIR method was developed in three steps.

1. FTIR spectra of trierucin spiked in the range of 4.5-71% by weight in oil from *B. napus* cv. Mercury containing 55% erucic acid were recorded in the attenuated total reflection (ATR) mode,
2. Spectra were subjected to partial least squares (PLS) analysis to develop a calibration.
3. The calibration was tested for prediction of trierucin in some selected samples using oil from another variety (*B. napus* cv. Turret) which contained about 40% erucic acid.

Spectral Features. Second derivative spectra of trierucin compared with HEAR oil revealed major differences in the C-O stretching region both in the frequencies and intensities of peaks. The second derivative spectra of the C-O stretching region (1300-1000 cm^{-1}) show significant changes in frequency or intensity and are marked with arrows for clarity (Fig. 1). The differences in the C-O stretching region can be rationalized as follows. The C-O groups of trierucin and HEAR oil are attached to the fatty acid chains. In trierucin, all three positions (sn-1, sn-2 and sn-3) of the triacylglycerol are occupied by the 22 carbon erucic acid chain whereas in HEAR oil the 2-positions particularly are occupied by fatty acids of C16 and C18 carbons. These compositional and structural differences are most likely responsible for differences in the intensities and frequencies of C-O stretching vibrations of trierucin and HEAR oil.

The C=O stretching band profile (not shown) in the second derivative spectra of trierucin and mercury also showed minor but reproducible differences in width. This parameter was about 1 cm^{-1} lower for trierucin. The width of the band provides information about the dynamics of molecules. The lower width of the C=O stretching band in trierucin indicate that its acyl groups, composed exclusively of the longer and heavier chains of erucic acid are relatively less mobile than the acyl groups of HEAR which contain erucic acid chains as well as shorter fatty acid chains in the sn-2 position.

FTIR spectra of varying amounts of trierucin spiked in HEAR (*B. napus* cv. Mercury 52% erucic acid) oil were recorded to set up a calibration equation for quantitation of trierucin in HEAR. It is clear from the marked bands in Fig. 2 that the spectral features of the 51 % trierucin mixture are intermediate between spectra of mercury and trierucin. In fact the spectra of mercury-trierucin mixtures shifted towards the spectrum of trierucin as the content of trierucin in the mixtures was increased.

The PLS calibration for prediction of trierucin in mercury was developed by utilization of "leave-one-out" cross validation technique. Eleven samples were used in the analysis which included mercury oil and its mixtures with trierucin. Nine factors were employed in the analysis, however, the best calibration was achieved with six factors. The agreement between the predicted and actual values was excellent. The R^2 value of 0.992, RMSD (root-mean squared deviation) of 2.78 and TE (total error) of 9.24 was produced by the PLS procedure.

PLS Predictions. The established calibration for trierucin was first tested on mixtures of HEAR oil extracted from *B. napus* cv. Turret and trierucin. The Turret oil contained 44% erucic acid, significantly different from the Mercury oil. Five mixtures of Turret oil ranging in trierucin content from 10.5 to 50.7% by weight were prepared for prediction.. The agreement between the two sets of values is good with a RMSD of 1.6% (Table 1). It is interesting to note that although our PLS calibration was established from Mercury-trierucin mixtures, it could still predict trierucin values in Turret-trierucin mixtures. This is encouraging and suggests that our calibration has the potential of being successful in real life samples containing different erucic acid levels. However, we would like to point out that our calibration failed (results not shown) when it was tested on mixtures of *Brassica rapa* cv. Polar oil and trierucin. The cultivar Polar is from a different species from Turret and Mercury and has only 34% erucic acid.

Table 1. PLS Prediction of Trierucin in Turret-Trierucin Mixtures[a]

Actual	Predicted	Difference
10.5	11.3	7%
19.2	20.2	5%
29.0	31.0	6%
36.2	34.0	6%
50.7	52.2	3%

[a] Standard deviation=1.89, R^2=0.994

The calibration was also used to predict the trierucin content in crambe and nasturtium oils. Crambe and nasturtium contain 60% and 80.5% erucic acid, respectively. Our calibration predicted trierucin content of 0.4% in crambe and 75% in nasturtium. Based upon gas chromatography, Carlson and Kleiman[18] reported negligible amount of trierucin in crambe. Thus our prediction of 0.4% trierucin in crambe oil appears reasonable. For nasturtium oil trierucin contents of 53% and 78% erucic acid in the 2-position have been reported in literature. The first value was determined[18] with GC and the second with the hydrolysis of oil with pancreatic lipase[19]. Our value of 75% is in close agreement with the value of lipase method suggesting that FTIR method may give a reading of the erucic acid in the 2 position. Further studies on FTIR spectra of mixed triacylglycerols with erucic acid in the 2 position might clarify this issue.

Tocopherol analysis: Tocopherols may play an important role in breeding programs for new varieties of oilseeds. Studies on soybean[20] and canola, sunflower and flax oils[21] have shown that changes in fatty acid composition are often accompanied by changes in the content and composition of tocopherols. Changes in fatty acid composition have not always resulted in the degree of increased stability expected in new oil types[22]. These findings suggest that future breeding selection programs aimed at developing oils with modified fatty acid patterns will have to include evaluation of tocopherols and possibly other components in order to obtain the maximum stability of the new product.

Unfortunately, rapid methods for determination of tocopherols are not available with the most common method being HPLC.

Development of calibrations for the determination of tocopherols was carried out in a similar manner to that for trierucin. Spectra were obtained from samples of oil from seed and commercial oils with known levels of total tocopherol. Examination of the spectra (Figure 3) showed major differences due to the differences in fatty acid content (shown by arrows) but PLS analysis suggested that tocopherols would best be determined utilizing spectral information from 880 cm^{-1}, to 775 cm^{-1} the region corresponding to substituted benzene rings. This was not surprising given the aromatic nature of tocopherols.

PLS calibration, using 17 samples of different oils with total tocopherols ranging from 200 to 1200 ppm gave R^2 of 0.951 with an RMSD of 66.4 (Figure 4). More work is required to verify this calibration. Also, calibration for individual tocopherols should be attempted.

Glucosinolate Analysis: Glucosinolates are present in many brassica seeds and vegetables. While they are important for developing the pungent and hot flavors of mustard, radish and other brassica crops, they are also antinutritional in nature and have limited the use of meals from brassica oilseeds as protein supplements in animal feeds[23]. As a result, plant breeders worked to lower the glucosinolate level in rapeseed, finally arriving at the "double zero" lines which are generally known as canola today.

Many methods have been developed for glucosinolate analysis[24]. The HPLC method specified by ISO and some spectrophotometric methods involve initial extraction of glucosinolates in aqueous or alcohol medium followed by isolation as desulfoglucosinolates using ion exchange chromatography and sulfatase enzyme. Our research was aimed at determining if FTIR might be used to quickly estimate the amounts of glucosinolates present in extracts before or after desulfation.

Extracts from samples of seed which had previously been analyzed by the ISO method were tested following basically the ISO method: ground seed was dissolved in methanol; lead acetate solution and water was added and the mixture was shaken and centrifuged to separate proteins. The infrared spectra of upper methanol/water layer containing glucosinolates was recorded in the transmission mode after 100 microliters was dried onto a 25 mm CaF2 window following the same procedure as in the case of attunuated total reflection mode (above), with the ATR cell replaced with the transmission cell. A similar method was used to record spectra from samples eluted from ion exchange columns following desulfation with sulfatase enzyme.

Comparison of spectra of extracts before and after desulfation (Figure 5) showed that major bands between 1700 and 1300 cm^{-1} were removed. These bands are probably due to carboxylate symmetric and antisymmetric vibrations from phytic acid. An initial calibration for desulfoglucosinolates was prepared using wavelengths in the 1265 cm^{-1} to 862 cm^{-1}(Figure 6). This suggested that glucosinolates could be determined on desulfated extracts with precision comparable to that of established methods. Further work on crude extracts is being carried out.

CONCLUSIONS

FTIR has potential to assist in several areas where rapid analysis of small samples is required. In particular, the method can be applied to the determination of trierucin in high erucic acid oils, the determination of tocopherols in vegetable oils and to the determination of desulfoglucosinolates in extracts from canola seeds. The methodology may be particularly suited for use by breeding programs where small sample size and high throughput must be combined with accuracy and precision.

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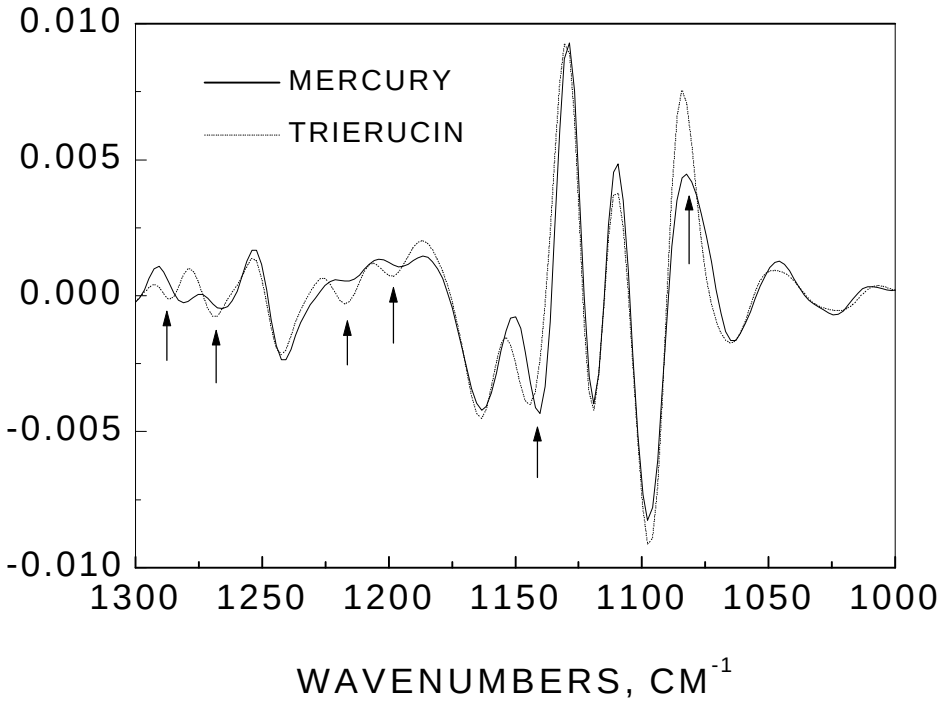

Fig. 1. Comparison of second derivative spectra (Savitsky-Golay, degree=4, points=15) of mercury oil and trierucin in 1300-1000 cm^{-1} region.

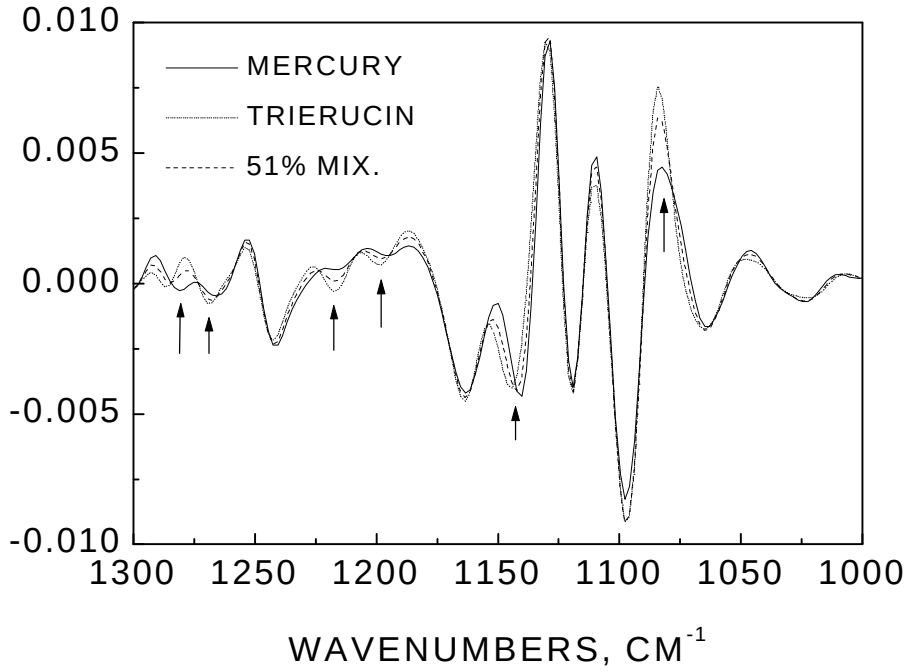

Fig. 2. Comparison of second derivative spectra (Savitsky-Golay, degree=4, points=15) of mercury, trierucin and 51% mercury-trierucin mixture in the 1300-1000 cm^{-1} region.

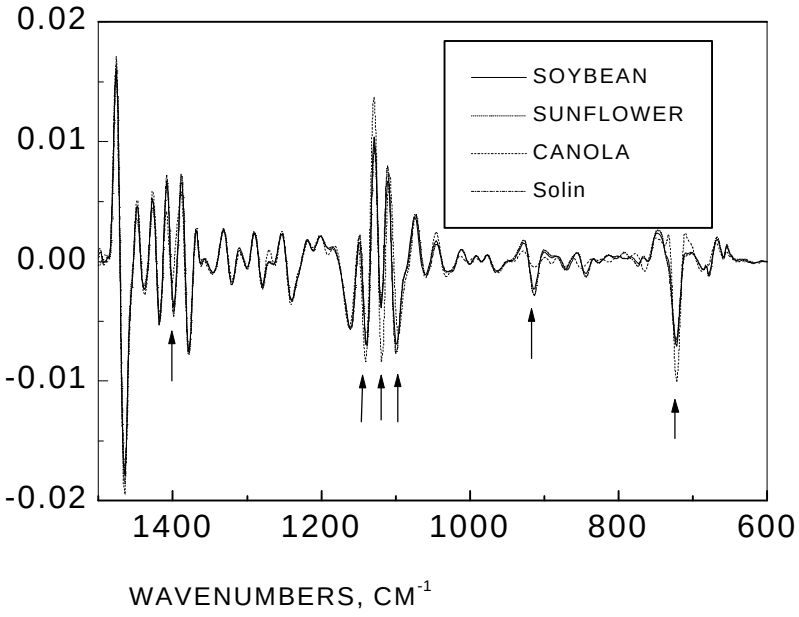

Fig. 3. Comparison of second derivative spectra (Savitsky-Golay, degree=4, points=15) of different vegetable oils.

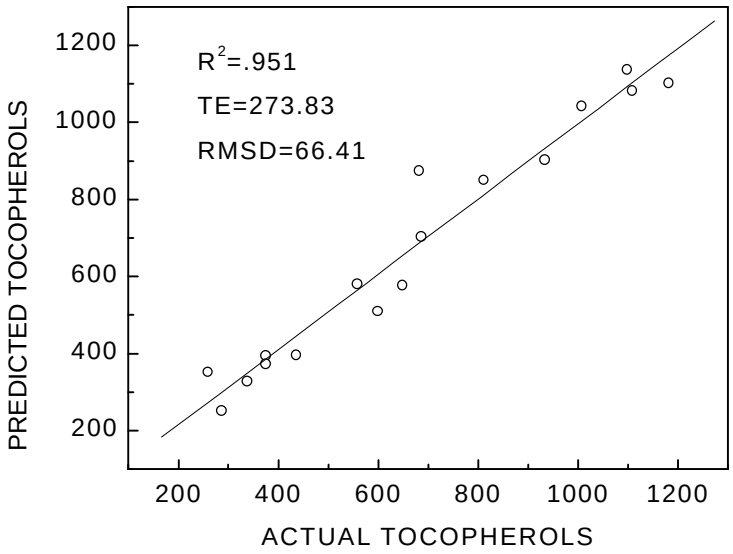

Figure 4. Prediction plot for PLS calibration for tocopherols in vegetable oils (soybean, canola, sunflower and solin).

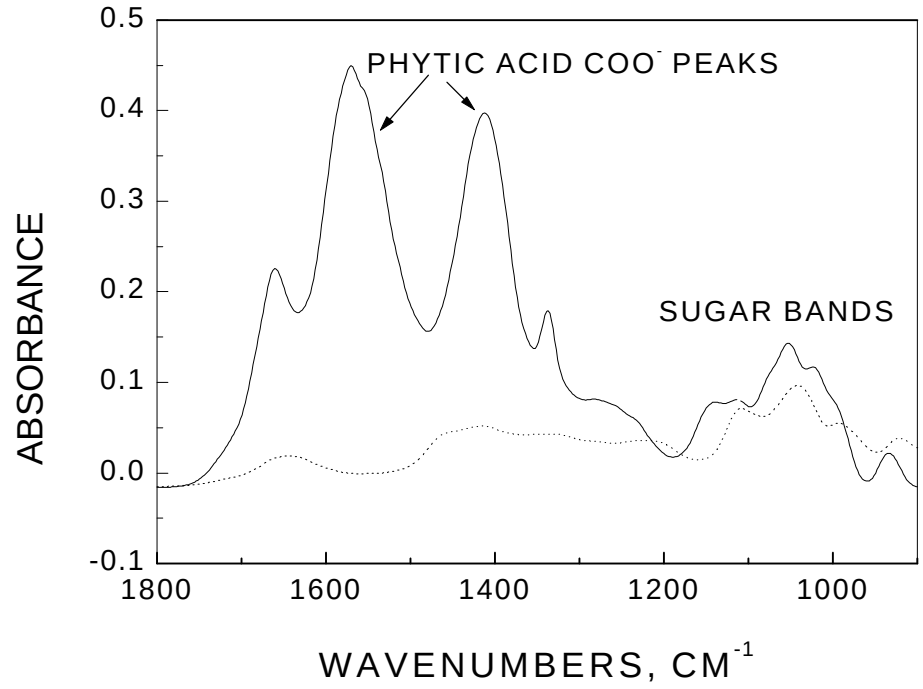

Figure 5. FTIR spectrum of extracts from glucosinolate determination showing strong bands due to phytic acid in the extract prior to desulfation.

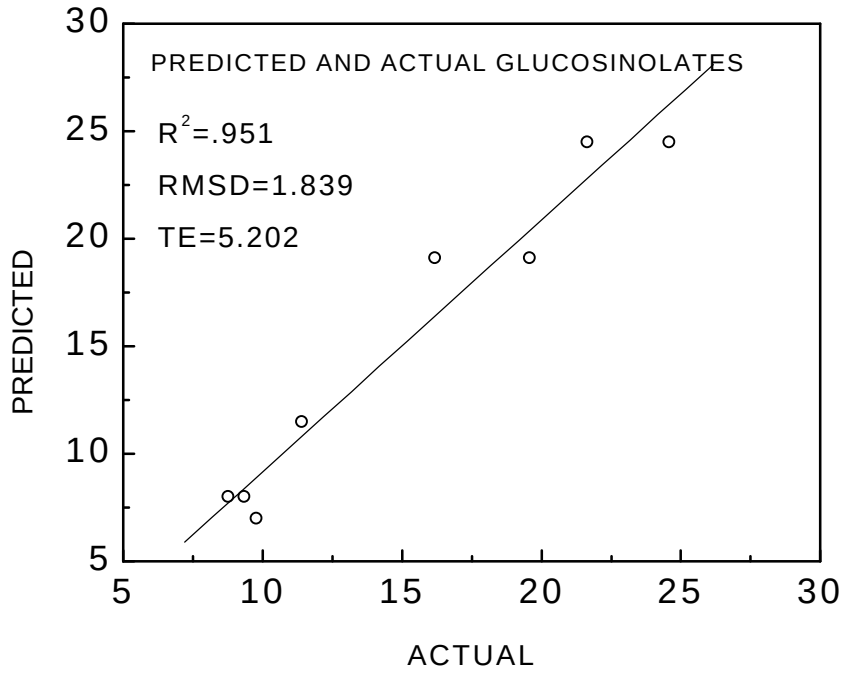

Figure 6. Prediction plot for PLS calibration for desulfoglucosinolates in canola seed.