An Automated Gas-Liquid Chromatographic Method of Measuring Free Fatty Acids in Canola

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An automated method for measuring free fatty acids (FFA) in canola seed was developed by using gas-liquid chromatography (GLC). The results from the GLC method were linearly related ($r^2 = +0.98$) to those obtained with the traditional method involving Soxhlet extraction followed by a titration. In a nested experiment of over 11 different seedlots, the extraction and injection errors were 0.11 and 0.018%, respectively, of the total variation. The variation attributed to sampling within a seedlot was twice the variation attributed to extraction and injection errors combined. A seed sample size of at least 10 mL was needed to prevent the standard deviation from increasing as the FFA mean increased. The GLC method was precise and rapid, and also identified which fatty acids were being cleaved from the oil, but a linear adjustment improved accuracy.

KEY WORDS: *Brassica napus*, canola, free fatty acids, gas-liquid chromatography, rapeseed.

The production of spring canola (*Brassica napus* L.) and the related canola crushing industry has increased in Ontario over the last decade. Canola seed production now averages 30,000 Mg from 18,000 ha (hectares) (1). The crushing plants in Ontario presently import most of their canola seed from western Canada. The opportunity exists for the Ontario crushing plants to process more Ontario-grown canola, but the canola seed must produce an oil that meets all industry specifications. Spring canola seed grown in Ontario frequently produces oil with levels of free fatty acids (FFA) higher than the 0.3% (3 mg/g) normally found in oil from canola grown in western Canada (2). In 1988 and 1991, the oil from the lower grades of Ontario-grown canola exceeded the 1% (10 mg/g) upper limit (3,4) acceptable to the oil refining industry.

The reasons for the high levels of FFA in the canola oil are not well understood, but the severity of the problem varies with location and year and is related to environmental stress during the seed-filling period. In 1988, the FFA levels measured from several cultivars and locations varied from a low of 0.14% to a high of 12%, but most values were below 4%. Investigation of this problem required the analysis of FFA levels in a large number of samples. The standard method adapted from Ke and Woyewoda (5), and similar to the method used by the Canadian Grain Commission, consists of a hot extraction to remove the oil from the ground seed and titrating the extracted oil back to a specific pH with NaOH. This titration method is labor-intensive, slow and difficult to automate. Therefore, an automated method was developed based on gas-liquid chromatography (GLC), which permitted the analysis of large numbers of samples in a relatively short time. This method was adapted from a procedure to analyze FFA in blood serum (6). In this paper, the GLC method for canola is described and evaluated.

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MATERIALS AND METHODS

Plant materials. Numerous individual seedlots harvested from field plots in the Ontario Canola Co-operative Variety Trials were used in these experiments. The seedlots represented several genotypes, years and locations, and were selected to provide a wide range of FFA values.

Extraction of FFA. A 10-mL sample of seed was taken from each sample bag. Each sample was ground in a Moulinex coffee grinder and dried in a forced air oven for at least 8 h at 80°C. After drying, a subsample of 200 mg of ground seed was placed in a 10-mL test tube. (If the FFA levels in the seed samples were below 1%, 400 mg of ground seed were used instead of 200 mg.) An internal free fatty acid standard was prepared by dissolving 1 g of heptadecanoic acid (C-17) in 1 L heptane. One mL of heptane containing the internal standard was added to the test tube. Next, 4 mL of solvent made up of 40 parts isopropyl alcohol and 1 part 1 N H_2SO_4 were added. The test tube was vigorously shaken and left standing for 15 min. Then 2 mL heptane and 3 mL deionized and distilled water were added, and the test tube was vigorously shaken again. A rapid separation into two distinct layers occurred with the FFA and heptane in the top layer. A sample from the top layer was removed with a pipette and placed in an autosampler vial prior to injection into the gas chromatograph.

Machine analysis. The FFA levels in samples were measured on a Perkin Elmer (Norwalk, CT) 8420 capillary gas chromatograph with a 100-sample AS-300 autosampler and a flame ionization detector. After split injection, the FFA were separated on a megabore column (30 m long, 0.53 mm i.d., stationary phase thickness, 1.0 μm; catalog number J125-3232, J&W Scientific DBFAP column, Folsom, CA). Helium was the carrier gas. The injection and detection temperatures were 270 and 330°C, respectively, the pressure on the column was 152 kPa and the oven temperature program started at 220°C with a ramp rate of 1°C min^{-1} to 225°C. The temperature was held at 225°C for 3.5 min, followed by a ramp rate of 5°C min^{-1} to 230°C. The oven stayed at 230°C for 1.5 min and was then cooled.

Peak identification and calculation of FFA. Individual FFA peaks were identified by means of purified standards of palmitic, stearic, oleic, linoleic and linolenic acids obtained from Sedary Research Laboratories (London, Ontario, Canada). Standards were run individually and the retention times of the standards were compared with the retention time of the peaks from the samples. After peaks were tentatively identified, standards were mixed together in concentrations that reflected concentrations found in the samples extracted from the canola seed. The retention times of the peaks from the canola seed and the combined standards were compared to verify that the peaks had been correctly identified.

The area under each peak was integrated with the Omega 2 software program licensed to Perkin-Elmer. The FFA of each peak was calculated from the following equations:

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$$\text{mass of IFFA} = (\text{Area IFFA}/\text{Area C-17}) \times \text{mass of C-17} \quad [1]$$

$$\text{IFFA\%} = \text{mass of IFFA}/\text{total oil} \quad [2]$$

$$\text{total oil} = \text{mass of sample extracted} \times \text{oil\%} \quad [3]$$

where IFFA was the individual free fatty acid, C-17 was a 17-carbon FFA added as an internal standard and Area IFFA and Area C-17 were the areas under the IFFA and C-17 peaks, respectively. The percentage of oil in the seed on a dry basis was measured with a Newport 4000 NMR analyzer (Oxford Analytical Instruments, London, United Kingdom) (7). The percentages represented by each peak were then added together to obtain the total FFA concentration for the sample.

Relationship between GLC and standard titration method. Two comparisons were conducted with two independent sets of seedlots. In the first set, a total of 20 seedlots representing two cultivars, Kristina and Global, were used. In the GLC method, one 10-mL sample was taken from each seedlot, and each sample was subjected to three separate extractions from 200-mg subsamples, with one injection per extraction. In the standard titration method, two separate samples were taken from each seedlot and ground. Two Soxhlet extractions, each with 4 g of ground canola, were conducted per sample. The resulting FFA concentration for each extraction was determined by titration (5). The average FFA concentrations were obtained for each of the two methods, and a regression of titration results on GLC results was determined with n = 20. In the second set, 26 seedlots representing several cultivars were used. Each seedlot was sampled twice, and one Soxhlet and one GLC extraction were conducted from each sample. The percentages of FFA from each sample were used to obtain a regression between the two methods with n = 26.

Variation due to extraction and machine procedures. To determine the amount of variation introduced by the extraction and injection steps of the method, a nested design was used to proportion the variation among seedlots and the extraction and injection steps of the method. Eleven seedlots from two cultivars, Global and Westar, were used. One 5-mL sample was taken from each of the 11 seedlots and ground. Two separate extractions were made from each seedlot, and three injections were made from each extraction.

Variation due to sampling within seedlots. To determine the variation introduced by sampling within seedlots, a nested design with the same 11 seedlots used in the previous experiment was used to determine the variation among seedlots, samples and the combination of the extraction and injection steps. Four 5-mL samples were selected per seedlot, and three separate extractions per sample were conducted with one injection per extraction.

Volume required for sampling. A preliminary investigation of sample size was conducted with one seedlot and a nested design to determine if the size of sample selected would affect the variability due to sampling. Two volumes, 2.5 mL and 10 mL, were used and five samples of each volume were taken from the seedlot. Each sample was extracted once with one injection per extraction.

Sample size was investigated in greater detail by using a factorial design with four different seedlots representing a range of FFA concentrations and four volumes, 1.2, 2.5, 5 and 10 mL. Four samples from each seedlot were selected for each volume tested. Each sample was subjected to one extraction with one injection per extraction.

Statistics. All statistical analyses were conducted with SAS version 6.06 (8). The SAS procedure used in the experiments shown in Tables 2, 3 and 4 was nested procedure (proc nested), and the means and standard errors in Tables 1 and 5 were generated using general linear model procedure (proc glm). Regression procedure (proc reg) was used to obtain the regression equations used to adjust the results from the two methods. In several experiments, the data obtained did not exhibit homogeneity of variance when tested by Levene's test of homogeneity of variance (9). The lack of homogeneity occurred because in some experiments the standard deviation increased in proportion to the mean. A natural log transformation restored homogeneity of variance to the data sets, but the transformation did not affect the level of significance for treatments. Therefore, the results for the analyses with untransformed data are shown.

RESULTS AND DISCUSSION

Peak identification. A sample chromatogram produced by the GLC method is shown in Figure 1. The distribution of individual FFA in a sample reflected the distribution of fatty acids in the oil, except that the percentage of the palmitic and stearic acids in FFA were about 50% higher than in canola oil from the same cultivar. Palmitic and stearic acids are primarily attached at the *sn*-1 and *sn*-3 positions of the triacylglycerol molecule (10). Therefore, the results suggest that the *sn*-1 and *sn*-3 positions are preferentially cleaved to produce FFA or that saturated fatty acid precursors accumulate because they are being synthesized in greater quantities than required for oil synthesis.

Comparison of the two methods. The GLC method was precise, but consistently underestimated the values obtained with the titration method. A significant, positive linear relationship occurred between the GLC method and the standard titration method ($r^2 = +0.98$). The relationship between the two methods was described by the following equation:

$$Y_{\text{titration method}} = 1.090\ X_{\text{GLC method}} + 0.095 \quad [4]$$

The means and standard errors of the FFA measured by each method on 20 different seedlots are shown in Table 1. The standard errors with both methods were small and similar. A second experiment to compare the methods was conducted with a different set of 26 seedlots, ranging from 0.18 to 3.9% FFA. The significant linear relationship ($r^2 = +0.99$) between the two methods was described by the following equation:

$$Y_{\text{titration method}} = 1.092\ X_{\text{GLC method}} + 0.097 \quad [5]$$

These results indicate that values obtained by the GLC method should be adjusted to agree with the values obtained from the standard titration method. Means obtained from the GLC method were consistently slightly lower than the means obtained from the standard method. One possible explanation for this difference could be that the naturally-occurring FFA were less completely extracted than the internal C-17 standard during the cold solvent extraction step in the GLC method.

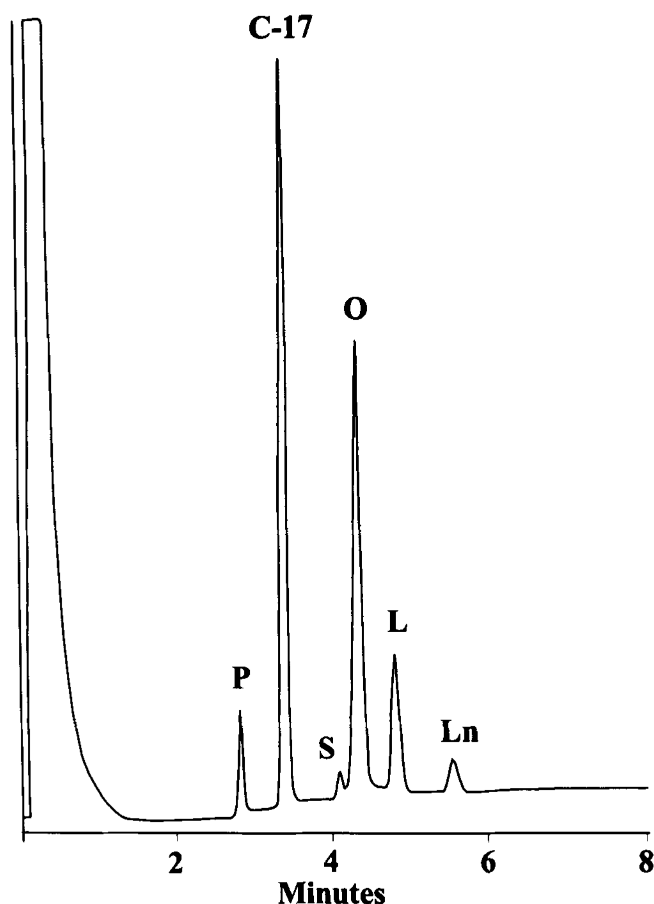

FIG. 1. Gas-liquid chromatogram of free fatty acids (FFA) from a sample of canola seed. The peaks in the chromatogram are palmitic (P), C-17, stearic (S), oleic (O), linoleic (L) and linolenic (Ln) acids. The percent FFA of the oil for this sample was 0.71%.

Variation caused by the GLC method. Two nested experiments were conducted to determine the variability caused by various steps in the method. In the first experiment, the variation due to the extraction and injection procedures was measured over a range of FFA levels (Table 2). Under the conditions of this experiment, the variation in FFA content accounted for by extraction and injection were 0.11 and 0.018%, respectively, of the total variation. The variation attributed to injection would also include the variation caused by other steps in the method that occurred after the injection of the sample on the column, including peak integration. The low proportion of variation attributed to the extraction and injection steps indicated that the GLC method was precise and repeatable, and differences among seedlots for FFA levels should be statistically distinguishable. The standard errors of the means of the different seedlots were small, but there was a small increase in $s_{\bar{x}}$ as the mean increased.

The second experiment (Table 3) examined, over a range of FFA levels, the variation due to sampling within a seedlot and the combination of all other steps (extraction, injection and machine errors) in the method. The variation attributed to sampling and to the other procedures in the method was 2.21 and 1.09%, respectively, of the total variation. Therefore, about twice the amount of variation was attributed to sampling within seedlot than to the combination of the extraction and injection procedures. The amount of variation attributed to sampling and the increased $s_{\bar{x}}$ observed as the mean increased suggest that

TABLE 1

Means and Their Standard Errors for Free Fatty Acid Levels in 20 Different Seedlots, Measured by Gas-Liquid Chromatography and by the Standard Titration Method

Seedlot	% Free fatty acids: Gas-liquid chromatography method[a]	% Free fatty acids: Standard titration method[b]
1	0.168 ± 0.006	0.253 ± 0.014
2	0.173 ± 0.009	0.223 ± 0.007
3	0.254 ± 0.007	0.400 ± 0.010
4	0.403 ± 0.015	0.456 ± 0.033
5	0.434 ± 0.007	0.596 ± 0.033
6	0.453 ± 0.011	0.588 ± 0.032
7	0.657 ± 0.044	0.735 ± 0.034
8	0.865 ± 0.022	1.20 ± 0.02
9	1.03 ± 0.03	1.26 ± 0.03
10	1.17 ± 0.04	1.43 ± 0.08
11	1.18 ± 0.04	1.35 ± 0.03
12	1.37 ± 0.04	1.48 ± 0.04
13	1.38 ± 0.01	1.92 ± 0.08
14	1.46 ± 0.08	1.62 ± 0.04
15	1.64 ± 0.01	1.78 ± 0.07
16	1.70 ± 0.01	1.77 ± 0.01
17	1.90 ± 0.06	2.31 ± 0.04
18	2.26 ± 0.02	2.52 ± 0.06
19	2.46 ± 0.06	2.65 ± 0.14
20	2.51 ± 0.10	2.93 ± 0.04

[a]Values shown are means ± their SE for the gas-liquid chromatography method calculated from three observations. Each seedlot was sampled once, and each sample was subjected to three separate extractions with one injection per extraction.
[b]Values shown are means ± their SE for the standard titration method calculated from four observations. Each seedlot was sampled twice, and each sample was subjected to two separate Soxhlet extractions with one titration per Soxhlet extraction.

TABLE 2

Results of a Nested Experiment, Using Gas-Liquid Chromatography, to Determine Levels of Free Fatty Acids in 11 Different Spring Canola Seedlots, and Variation Associated with Extraction Nested Within Seedlots and with Injection Nested Within Extraction[a]

Seedlot	Free fatty acids (%)[b]	Source	DF	MS	% of Total variation
1	0.646 ± 0.004	Seedlots	10	14.27**[c]	99.87
2	0.697 ± 0.005	Extraction	11	0.0086**	0.11
3	0.723 ± 0.008	Injection	44	0.0004	0.018
4	0.817 ± 0.003	Total	65	2.20	100
5	1.06 ± 0.004				
6	1.53 ± 0.02				
7	1.85 ± 0.02				
8	2.51 ± 0.01				
9	2.94 ± 0.02				
10	4.65 ± 0.05				
11	4.82 ± 0.02				

[a]Abbreviations: DF, degrees of freedom; MS, mean squares.
[b]Values shown are means ± their SE calculated from six observations per seedlot. Each seedlot was sampled once, and each sample was subjected to two separate extractions with three injections per sample.
[c]Significant at $P \leq 0.01$.

TABLE 3

Results of a Nested Experiment, Using Gas-Liquid Chromatography, to Determine Levels of Free Fatty Acids in 11 Different Spring Canola Seedlots, and Variation Associated with Samples Nested Within Seedlots and with the Combination of Extraction and Injection Steps Nested Within Samples

Seedlot	Free fatty acids (%)[a]	Source	DF	MS	% of Total variation
1	0.486 ± 0.018	Seedlots	10	24.44**[b]	96.70
2	0.592 ± 0.021	Samples	33	0.16**	2.21
3	0.753 ± 0.031	Ex and Inj[c]	88	0.023	1.09
4	0.768 ± 0.040	Total	131	1.92	100
5	1.00 ± 0.022				
6	1.53 ± 0.036				
7	1.57 ± 0.056				
8	2.24 ± 0.086				
9	3.09 ± 0.072				
10	3.95 ± 0.11				
11	4.60 ± 0.15				

[a]Values shown are means ± their SE calculated from 12 observations per seedlot. Each seedlot was sampled four times, and each sample was subjected to three separate extractions with one injection per extraction.
[b]Significant at $P \leqslant 0.01$.
[c]Extraction and injection steps of the method. See Table 2 footnote for other abbreviations.

TABLE 4

Results of a Preliminary Nested Experiment, Using Gas-Liquid Chromatography and one Spring Canola Seedlot, to Determine the Variation Associated with Volume of Sample Taken from the Seedlot, with Samples Nested Within Volume and with the Combination of Extraction and Injection Nested Within Samples

Volume (mL)	Free fatty acids (%)[a]	Source	DF	MS	% of Total variation
2.5	0.749 ± 0.036	Volume	1	0.0004	0.00
10.0	0.742 ± 0.011	Sample	8	0.036**[b]	97.3
		Extraction and injection	20	0.0003	2.72
		Total	29	0.010	100

[a]Values shown are means ± their SE calculated from 15 observations per seedlot. Two volumes were used to sample one seedlot. Five samples were taken for each volume, and each sample was subjected to three separate extractions with one injection per extraction.
[b]Significant at $P \leqslant 0.01$. See Table 2 footnote for abbreviations for DF and MS.

sampling as part of the method required further investigation.

Size of samples within seedlot. An experiment (Table 4) was conducted on one seedlot to determine the appropriate volume of the sample. Because one seedlot was used, all the variation was proportioned among the different steps of the method. The variation due to volume was not significant and the variation attributed to sampling was highly significant. The variation attributed to the extraction and injection steps of the method was 2.7%, indicating that these steps have a high degree of precision and reproducibility. The differences among samples within one seedlot accounted for 97.3% of the variation in the method, indicating that most of the variability occurred among samples within the one seedlot.

The same seedlots were used to obtain the results in Tables 2 and 3, but the means in the two tables for each individual seedlot were slightly different. As the means were derived from one 5-mL sample per seedlot in Table 2 and from three 5-mL samples per seedlot in Table 3, the difference between the means of individual seedlots in Table 2, compared to the means in Table 3 for the same seedlot, was apparently caused by variation in the sampling step of the procedure. The individual seedlots had higher $s_{\bar{x}}$ values in Table 3 than in Table 2. The $s_{\bar{x}}$ in both tables includes variation contributed by the injection and extraction steps of the method, but the $s_{\bar{x}}$ in Table 3 also includes variation contributed by the sampling step of the procedure. Hence, the increased size of $s_{\bar{x}}$ from Table 2 to Table 3 was attributed to variation caused by sampling within one seedlot.

The large variation among samples within a seedlot can be explained by the results of other research (May, W.E., and D.J. Hume, unpublished data) in which the position of the seed on the raceme of the canola plant affected the FFA level found in the seed. Therefore, the level of FFA varied from seed to seed, and seed-to-seed variability increased with higher levels of FFA.

The variation attributed to volume (Table 4) was calculated from the differences between the means of each volume, 0.749% and 0.742%, for the one seedlot. Because the two means represent the FFA level for the same bag of seed, it was not surprising that the variation due to volume was not significant. However, the $s_{\bar{x}}$ of these means were different. Conducting an F test, as suggested by Bartlett (10), showed that the $s_{\bar{x}}$ for the 2.5-mL sample size was significantly ($P \leqslant 0.05$) higher than $s_{\bar{x}}$ for the 10-mL sample size. Therefore, larger sample volumes should reduce the variation introduced by the sampling step of this method.

The last experiment involved a more detailed investigation of the effects of sample size. Four seedlots with different levels of FFA and four sampling volumes were used. In this experiment (Table 5) the effect of volume of seed used was significant. For the 1.2-, 2.5- and 5-mL volumes, as the mean increased, the $s_{\bar{x}}$ increased. For the 10-mL volume, $s_{\bar{x}}$ did not increase with the mean. This indicated that a minimum sample volume of 10 mL should be used. It also demonstrated that the volume of the sample to be ground became more important as the level of the FFA in the seedlot increased.

The GLC method provided precise measurements ($r^2 = +0.98$) of the FFA level in individual seedlots, but the accuracy was improved by using a linear regression equation to convert results to those obtained with the standard method. The variation due to the new GLC method itself was small, but the variation among samples within the seedlot was larger than desired, particularly at high FFA levels. To reduce this problem, the sample size needed to be at least 10 mL. The GLC method has additional advantages. It can be used with an automatic sampler on the gas chromatograph, allowing samples to be run overnight. The results also indicate which fatty acids are being cleaved from the oil.

TABLE 5

Means and Standard Errors for Free Fatty Acids Levels for Four Seedlots and Four Sample Volumes

	Amount of seed ground (mL)			
Seedlot	1.25[a]	2.5	5	10
	(% free fatty acid in oil)			
A	0.719 ± 0.074	0.927 ± 0.071	0.980 ± 0.067	0.888 ± 0.076
B	1.21 ± 0.11	1.17 ± 0.03	1.27 ± 0.08	1.31 ± 0.04
C	1.96 ± 0.08	2.13 ± 0.13	2.05 ± 0.13	2.19 ± 0.08
D	3.10 ± 0.16	3.54 ± 0.16	4.02 ± 0.19	3.88 ± 0.03

[a]Values shown are the means ± their SE calculated from four observations per seedlot. Four samples were taken per seedlot, and each sample was subjected to one extraction with one injection per extraction.

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