Extraction and Methanolysis of Oil from Whole or Crushed Rapeseed for Fatty Acid Analysis[1,2]

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ABSTRACT

Three methods are described for extraction of oil from rapeseed for routine determinations of fatty acid composition. In the "whole-seed method," ca. 50% of the total seed oil is extracted, without prior crushing of the seeds, by soaking the dried seeds in petroleum ether and benzene at room temperature for 2 days. For certain types of rapeseed with a "less permeable seed coat," a presoaking in water is required to rupture the seed coat. The extracted oil has practically the same fatty acid composition as the total seed oil, and can therefore be used as a representative sample for determination of the fatty acid composition of the total seed oil. In the "crushing method," the seeds are lightly crushed before the oil is extracted. In the "half-seed method," the outer cotyledon of a single seed is dissected from the embryo; the oil is extracted from this cotyledon for fatty acid analysis, while the remaining part of the embryo can be germinated and planted to produce the progeny of the seed. In all three methods the extracted oil is converted to fatty acid methyl esters by a rapid reaction with sodium in methanol at room temperature.

INTRODUCTION

Plant breeding experiments for improving the quality of rapeseed and other oilseeds often require that large numbers of seed samples be analyzed for fatty acid composition of the extracted oils. Conventional methods (1) for extracting the oil usually involve some initial crushing or grinding of the seed to destroy the cell structure, followed by extraction with a solvent. For subsequent determination of the fatty acid composition, the extracted oil is usually reacted with methanol and a catalyst such as sodium methoxide for interesterification (methanolysis) of the glycerides to give the methyl esters of the fatty acids. The grinding of rapeseed for analytical purposes can involve certain difficulties, because the oil tends to separate out and form a smear on the grinding equipment.

This paper describes a time saving method, the "whole-seed method," for extraction of oil from rapeseed and related seeds. The whole seeds are extracted without prior crushing or, alternatively, after steeping the seeds in water which effects rupture of the seed coat. The paper also describes two other rapid methods, the "crushing method" and the "half-seed method," for extraction of rapeseed oil; in these methods the seeds are lightly crushed or broken before the oil is extracted. In all three methods, methanolysis of the extracted oil is carried out with sodium in methanol at room temperature.

EXPERIMENTAL PROCEDURES

Materials

Three samples of rapeseed were used as testing material for developing the present methods: "Target-I" (oil content 46.8%, dry wt) and "Target-II," *Brassica napus* cultivar target, both with "more permeable seed coat," and "Yellow sarson," *B. campestris* var. *sarson* (oil content 43.8%), with "less permeable seed coat." The Target-II sample was required only to replace the similar Target-I sample when the stock of the latter had been exhausted. When required, seed samples were dried at 105 C for 6-16 hr.

Unless otherwise specified, the solvents used were petroleum ether of boiling range 36-57 C ("Skelly F," Skelly Oil Co., Kansas City, Mo.); benzene, crystallizable (Fisher "certified" reagent); and methanol, absolute (Baker "analyzed" reagent). All solvents were used as purchased without further purification.

Swedish Method for Oil Extraction

The method of Troëng (2), referred to as the "Swedish method" in many laboratories, is used with minor modifications. A sample of dried rapeseed (5 g or less) is placed in a steel extraction tube together with three steel balls. Petroleum ether (40 ml) is added with an automatic pipette. The tube is stoppered (solvent-resistant stoppers of fluorosilicone rubber) and shaken in its longitudinal direction (Eberbach laboratory shaker, stroke length 1.5 in., 200 double strokes per minute) for 1, 3 or 5-16 hr, for samples of 1, 3 or 5 g, respectively. The tube is placed vertically for sedimentation of the solids for 3-16 hr. Part of the clear petroleum-ether solution is transferred by pipette into a flask and evaporated to a smaller volume for subsequent preparation of the methyl esters of the fatty acids.

Conventional Methanolysis with Sodium

The method used is essentially as described by Luddy et al. (3). A sample of rapeseed oil (0.1 g or less) in 1-10 ml petroleum ether is refluxed for 10 min with 5 ml of a 0.02 N solution of sodium in methanol. The solution is cooled to room temperature and neutralized (approximately) by addition of 0.5 ml of a 0.2 N solution of acetic acid in petroleum ether. Water (5 ml or more) is added and the mixture swirled for washing of the petroleum ether layer. Part of the petroleum ether layer is decanted into a test tube and evaporated to a small volume of appropriate concentration for gas chromatographic analysis.

Rapid Methanolysis with Sodium

A sample of rapeseed oil (0.1 g or less) in 1 ml petroleum-ether solution, or in 0.5 ml petroleum ether and 0.5 ml benzene, is placed in a 10 ml volumetric flask with glass stopper and narrow neck (6-9 mm ID). For certain applications the meal or similar material may be included with the oil. One milliliter of 0.4 N solution of sodium in methanol is added. The petroleum ether and methanol reagent may be mixed in advance and added in one operation. For small samples (less than 30 mg oil) only half of the above volumes of petroleum ether, benzene and methanol reagent are used. The flask is allowed to stand at room temperature for 1-10 min for methanolysis to take place. Water is added, with swirling, until the entire petroleum ether and benzene layer has risen into the neck of the flask. If this layer is not clear, the flask is held for 1-3 hr for it to clarify; alternatively, if 1-2 drops of ethanol are added along the inner wall of the neck, the solvent layer clarifies within 10 min. Samples (usually 0.1-1 μl) can be

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withdrawn with a syringe directly from the clear solvent layer for gas chromatographic analysis.

Gas Chromatography of Fatty Acids

A Varian Aerograph instrument, model 1200, was used with a flame ionization detector. A polyester column (8 ft x 1/8 in. OD, copper) was packed with butanediol succinate on Anakrom ABS, 70-80 mesh, 1:10 w/w. With this column, the injection port, column and detector temperatures were 230, 170 and 250 C, respectively. A silicone column (5 ft x 1/8 in. OD, copper) was packed with 3% SE-30 on Varaport 30, 100-120 mesh. With this column, the injection port, column and detector temperatures were 250, 235 and 270 C, respectively. For both columns, the nitrogen flow rate was 22 ml/min; the injection volumes were usually 0.1-1 μl. Peak areas were measured with an electronic digital integrator (Infotronics model CRS-108). Fatty acid compositions were calculated as the relative peak areas for the methyl esters, without use of detector response factors.

Whole-Seed Method for Seed with More Permeable Seed Coats

A sample of whole seeds (0.25 g or less) in a 10 ml volumetric flask is dried. Petroleum ether (0.5 ml) and benzene (0.5 ml) are added, and the flask is held (stoppered) at room temperature for ca. 40 hr (minimum 24 hr, maximum 6 days) for extraction of oil. Finally, the "rapid methanolysis" procedure is applied to the mixture in the flask.

Whole-Seed Method for Seed with Less Permeable Seed Coats

A sample of whole seeds (0.25 g or less), in a 10 ml volumetric flask, is half covered with ca. 0.3 ml water and allowed to soak in the stoppered flask at room temperature for 22-24 hr (2-5 days, if more convenient). The steeped sample is dried (105 C, 16 hr). The remainder of the procedure is as described above for seed with the more permeable seed coats.

Crushing Method for Extraction and Methanolysis

A sample of seed (0.25 g or less), not necessarily dried, is placed in the test cylinder (1.13 in.) of a Carver laboratory press. Without using the hydraulic press, the sample is crushed lightly by moving the cylinder piston one to three times by hand. In this way, the seed is crushed so lightly that visibly no oil is expelled onto the piston or other parts of the test cylinder. The crushed seed is transferred with a spatula and funnel into a 10 ml volumetric flask. The test cylinder and spatula are wiped clean with a tissue paper or a brush before the next sample is treated. Finally, the rapid methanolysis procedure is applied directly to the crushed seed. A minimum reaction time of 10 sec, 1 min or 30 min is recommended for seed samples of 250, 50 or 10 mg, respectively.

Half-Seed Method for Extraction and Methanolysis

The seeds to be analyzed are kept on a moist filter paper in a Petri dish for 30-60 min to facilitate the later cutting of the seed. A single seed, held under a microscope with a pair of tweezers, is dissected with a scalpel into three parts: two outer parts consisting of mainly the outer cotyledon and a center part consisting of the remaining part of the embryo. The seed coat is not purposely removed and may for simplicity be included with the cotyledon in the analysis. The cotyledon pieces are transferred to a 10 ml volumetric flask; no further crushing of these is required. Petroleum ether (0.25 ml) is added, and the sample is held at room temperature for 1 hr (overnight, if more convenient). Finally, the rapid methanolysis procedure is applied to the mixture in the flask.

TABLE I

Yields of Extracted Oil from Whole Seeds[a]

Solvent	Extracted oil, % of total seed oil
Petroleum ether[b]-benzene	47
Petroleum ether-methanol-chloroform	45
Chloroform	41
Benzene	34
Petroleum ether	34
Petroleum ether (boiling range 63-69 C)	30
Petroleum ether-benzene-methanol	28
Benzene-methanol	26
Petroleum ether-methanol	11
Methanol	4

[a]Dried Target-I seeds (0.25 g) soaked in 5 ml solvent for 40 hr at room temperature.

[b]Boiling range 36-57 C. The solvent mixtures consisted of equal volumes of each solvent.

RESULTS AND DISCUSSION

The described whole-seed, crushing and half-seed methods for extraction and methanolysis of rapeseed oil were compared with conventional methods by analyzing standard seed samples. The gas chromatographic conditions for these analyses were chosen for rapidity, possibly at the expense of some accuracy and precision. The polyester column, used for separation according to chain length and double bonds, gave 8-min analyses of rapeseed oil. Stearic and oleic esters were not separated by this column; the results given as oleic acid thus include ca. 1% stearic acid which occurred in the oils. The silicone column, used for separation according to chain length only, gave 3-min analyses. This column is preferred whenever "double bond separation" is not required. Results from this column differ slightly from those of the polyester column unless detector response factors are used.

Whole-Seed Method

The method is based on the finding that more than half of the total oil contained in rapeseed can be extracted from the whole (unbroken) seeds by soaking the dried seeds in a solvent at room temperature. For certain types of rapeseed with a "less permeable seed coat" an initial steeping in water is required so as to rupture the seed coat. The extracted oil has practically the same fatty acid composition as the total oil of the seed. The extracted portion of the total oil can therefore be used as a representative sample for analysis of the fatty acid composition of the total seed oil. The methyl esters of the fatty acids are prepared for this purpose, at the end of the extraction period, with sodium methanoate in methanol at room temperature. The entire process takes place in a single flask,

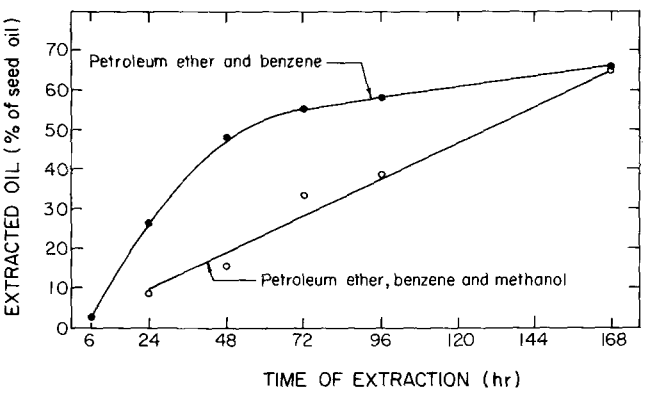

FIG. 1. Yields of oil extracted from whole rapeseeds (Target-I) by two solvent mixtures for 6-168 hr.

TABLE II

Fatty Acid Composition of Oils Extracted from Whole Seeds of Target-I, Conventional Methanolysis

Time of extraction, hr	Replicate analysis	Fatty acid, area %					
		16:0	18:1	18:2	18:3	20:1	22:1
6	2	4.3[a]	20.8[a]	11.5	6.0[a]	16.3[a]	41.3[b]
24	3	4.2[a]	21.4[b]	12.4	7.5[a]	15.3	39.4
48	2	3.8	21.0[b]	12.2	8.2	15.2	39.7
72	2	3.6	20.7[a]	12.6	8.4	15.0[b]	39.9
96	2	4.0[b]	21.2[b]	11.6	8.1	15.6	39.7
168	2	3.7	20.6[a]	11.6	8.0	15.6	40.7
Residual oil[c]	2	3.7	21.0[b]	12.8	8.6[b]	15.2	38.7
Total seed oil[c]	4	3.6	22.6	11.2	8.0	15.6	39.1

[a,b]Comparison with the "total seed oil" sample is significantly different at the 1 and 5% levels, respectively.

[c]Swedish extraction procedure.

which simplifies the routine handling and cleaning of glassware. Concurrently with this work, essentially the sampe principle of whole-seed extraction of oil seeds has been briefly reported by Yermanos and coworkers (4,5).

Ten pure or mixed solvents were tested for efficiency in extracting oil from whole seeds (Table I). The highest yield of extraction, 47% of the total oil, was obtained with a mixture of petroleum ether and benzene; this solvent mixture was preferentially used in the further work. To obtain reasonably high yields of extracted oil, the seeds were dried before extraction. Seed samples of ca. 7% moisture content, when extracted as above with petroleum ether, yielded only 6-15% of the total oil.

The influence of extraction time on the yield of oil obtained by the whole-seed procedure was tested with two solvent mixtures: petroleum ether-benzene 1:1, which had proved the most efficient for extraction (Table I), and petroleum ether-benzene-methanol 1:1:2, which was of interest for possible simultaneous extraction and methanolysis. The yield of oil increased with extraction from 6 to 168 hr (Fig. 1). To ensure a reasonably high yield of oil, 40 or 48 hr extraction was used in the further work. It should be feasible, however, to reduce the extraction time to 24 or 16 hr.

The fatty acid compositions were practically the same for the above oil fractions obtained by 6-168 hr extraction with petroleum ether and benzene as for the residual oil in the 168 hr extracted seed and for the total oil from replicate samples of seed (Table II). All samples in Table II were compared with the total seed oil. Analysis of variance and determination of the "least significant difference between means" showed that some of the fatty acid values, as footnoted in the table, differed significantly from those of the total seed oil. These differences, however, are not sufficiently large to be of practical concern to the plant breeder. Therefore each of the extracted samples, with the possible exception of the 6 hr sample, may be regarded as a representative sample for determining the fatty acid composition of the total seed oil.

For simplicity, methanolysis at room temperature was preferred. Rapid methanolysis at room temperature has been reported with sodium in methanol (6) and with HCl in methanol (7). Acetyl chloride in methanol has been used under refluxing temperature (8). Also, simultaneous extraction and methanolysis at room temperature has been reported for the oil in dry-milled corn, using HCl and dimethoxypropane in methanol and benzene (9).

The reagents sodium, HCl and acetyl chloride, all in methanol solution, were tested for simultaneous extraction and methanolysis of the seed oil at room temperature. Essentially, the described whole-seed method was followed, except that the three methanolysis reagents (0.4 N Na, 0.8 N HCl [gas] and 1 N acetyl chloride solutions) were added to the seed samples at the beginning of the 40 hr extraction period. The sodium-methanol reagent was furthermore tested for effecting rapid methanolysis subsequent to the extraction, as described in the whole-seed method. The HCl and acetyl chloride reagents resulted in incorrect fatty acid analyses and were not further considered.

The sodium-methanol reagent resulted in satisfactory fatty acid analyses (Table III), both when the reagent was added at the beginning and at the end of the extraction

TABLE III

Fatty Acid Composition of Target-II by Whole-Seed Method with Methanolysis during or after Extraction and by Crushing Method

Method	Replicate analysis	Fatty acid, area %					
		16:0	18:1	18:2	18:3	20:1	22:1
Whole-seed method							
Methanolysis during extraction	6	3.6 ± 0.08	20.7 ± 0.64	12.7 ± 1.20	8.4[a] ± 0.14	14.6 ± 0.23	40.0[a] ± 0.66
Methanolysis after extraction	6	3.5 ± 0.12	19.5 ± 0.67	12.7 ± 0.68	8.4[a] ± 0.06	14.7 ± 0.24	41.2 ± 0.32
Crushing method	10	3.5 ± 0.20	21.1 ± 1.11	13.1 ± 0.23	7.0[a] ± 0.29	14.2[a] ± 0.39	41.2 ± 1.09
Total seed oil[b]	10	3.5 ± 0.13	20.3 ± 0.90	12.5 ± 0.85	8.0 ± 0.17	14.8 ± 0.23	40.9 ± 0.96

[a]Comparison with total seed oil is significantly different at the 1% level.

[b]Swedish extraction procedure; conventional methanolysis.

TABLE IV

Fatty Acid Composition and Yield of Extracted Oil from Water-Steeped Whole Seeds of Yellow Sarson

Steeping time, hr	Extraction time, hr	Oil yield, % of total seed oil	Replicate analysis	Fatty acid, area %[a] 16:0	18:1	18:2	18:3	20:1	22:1
0	48	0							
6	48	1.8	2	7.7[b]	15.9[b]	13.7	8.2[c]	10.0[b]	44.9[b]
19	24	5.3	1	6.9	16.2	18.6	10.2	10.3	37.9
19	72	16.7	1	2.8	13.5	14.8	8.7	8.0	52.3
24	24	50.2	2	2.9	14.2	12.7	8.1[c]	8.6[c]	53.6
24	72	56.4	2	2.7	14.1	12.8	8.3	8.3	54.0
48	48	61.9	2	2.9	15.1[b]	12.6	8.4	7.5	53.6
72	48	53.7	1	2.9	13.5	14.1	8.5	7.5	53.4
144	48	54.0	1	2.8	13.6	13.2	8.9	7.5	54.1
Total seed oil[d]			3	2.2	13.9	12.6	8.7	7.8	54.8

[a]Conventional methanolysis. All fatty acid values, except those obtained by single determinations, are compared statistically with corresponding values for total seed oil.
[b,c]Significant differences at the 1 and 5% levels, respectively.
[d]Swedish extraction procedure.

period. Three fatty acid values differed significantly from those obtained by conventional analysis of the total seed oil. These differences, however, are too small to be of practical concern for the plant breeder. The results in Table III show that the fatty acid composition for rapeseed samples can be satisfactorily determined with oil extracted from whole seeds and interesterified by sodium-methanol at room temperature. The esterification reagent may be added to the sample at the end of the extraction period, or together with the extraction solvent at the beginning of the extraction period. Repeated experiments have indicated, in agreement with the results in Table III, that slightly lower erucic acid values (not statistically significant) tend to result when the esterification reagent is added at the beginning of the extraction period; the practice in our laboratory, therefore, has been to add the reagent at the end of the extraction period. The precision of analysis is indicated by the standard deviations for replicate analyses (Table III).

"Unusual" samples of rapeseed were encountered which gave low yields of oil by the whole-seed extraction procedure, apparently because the seed coat was less permeable to the solvents. Seeds of this type, when extracted with the usual petroleum ether and benzene mixture for 2 days, gave only 0-3% yields of oil. Longer extraction times, up to 35 days, increased these yields to 2-9%. To further increase the yield of extraction, the seeds with the less permeable seed coat were steeped in water and again dried, before being extracted by the usual soaking in the solvent mixture. The steeping in water resulted in a slight degree of germination of the seed, sufficient to rupture the seed coat and thus to facilitate the subsequent extraction of oil. With steeping in water for 24 hr or longer, the yields of extracted oil (Table IV) were more than 50% of the total oil. The fatty acid compositions of these extracted oils were all practically the same as the composition of the total oil of the seed (Table IV). With steeping in water for 19 hr or less, the yields of extracted oils were low; the fatty acid compositions of these oils tended to differ from the composition of the total oil, possibly because the samples available for gas chromatographic analysis might have been too small for reliable analysis. Steeping times of 22-24 hr were used in the further work. Although a longer time may occasionally be used for convenience, it is not practical to steep for more than 2-3 days because the germinating seeds then form an intertwined mat of rootlets which is cumbersome to clean out of the flask.

The rapid methanolysis procedure was assessed with water-steeping included in the extraction procedure as described in the whole-seed method. As no more Yellow sarson seeds were available, Target-II was used in this experiment. The fatty acid compositions of the interesterified oils were practically the same as for a control sample of total seed oil (Table V). The whole-seed method for extraction and methanolysis thus gives satisfactory results also with water-steeping included in the procedure. The precision of analysis with this version of the method is indicated by the standard deviations for replicate analyses (Table V).

Some recent experiments with soybeans by Singh et al. (10) tend to corroborate some of the above findings. These authors report that oil sampled from any of five different portions of mature, ungerminated soybean seed showed no significant differences in fatty acid composition except with regard to the stearic acid values. The slight differences for this acid, however, were not large enough "to constitute a serious problem for the plant breeder." This is in agreement with our findings with rapeseed—namely, that the smaller portions of oil obtained by extraction of whole seeds all have practically the same fatty acid composition as the total seed oil.

Singh et al. reported furthermore that practically no significant changes in fatty acid composition took place during germination of soybean seed for up to 6 days. Again this is in agreement with our findings with rapeseed—namely, that the oil extracted from whole seeds which have been steeped in water (germinated) for up to 6 days has practically the same fatty acid composition as the oil of the ungerminated seed. It thus appears, as Singh et al. suggest, that the utilization of the fatty acids in the seed during

TABLE V

Fatty Acid Composition by Whole-Seed Method with Water-Steeping of Seeds of Target-II

Method	Fatty acid, area %[a] C16[b]	C18	C20	C22
Whole-seed method	5.2[c] ± 0.10	37.9 ± 1.10	14.8 ± 0.49	42.0 ± 0.83
Total seed oil[d]	3.8 ± 0.06	39.5 ± 0.26	14.4 ± 0.12	42.5 ± 0.26

[a]Means of three replicate analyses.
[b]Fatty acids with 16 carbon atoms.
[c]Comparison with the total seed oil is significantly different at the 1% level.
[d]Conventional methanolysis.

germination is random. Singh et al. concluded that a seed sample "could have been taken any time during the germination period of 6 days to determine the fatty acid proportions of the mature, ungerminated seed"; this, in effect, is the principle employed in the present method of steeping whole rapeseed in water before extracting the oil for fatty acid analysis.

The whole-seed method of extraction and methanolysis is well suited to routine analyses of large numbers of samples. It may be difficult to predict whether a group of samples for analysis is of the type with the more or the less permeable seed coat. The method with water-steeping may then be used, as it is applicable to both types of seed. As a guide to the choice of method, however, it appears that the method with water steeping usually is required for yellow or light colored seed, while the slightly simpler method without water steeping may be used for brown or dark colored seed. The whole-seed method has given promising results also with soybean, sunflower and flax seeds.

Crushing Method

This method for extraction and methanolysis of rapeseed oil was developed from an earlier "press method" (F.W. Hougen, unpublished), in which the seeds were crushed under high pressure in a laboratory hydraulic press. In the present method the seeds are only lightly crushed and then soaked for a short time at room temperature in a mixture of petroleum ether and sodium-methanol reagent for simultaneous extraction and methanolysis of the oil.

The seed oil is not completely extracted by this method. A soaking time of 10 sec, or 1, 10 and 30 min, gave oil yields of ca. 65, 70, 85 and 95% of the total seed oil. The longer extraction times are used for small samples in order to obtain sufficient material for gas chromatographic analysis; the shorter extraction times may be used for larger samples.

The extracted fractions of oil may be regarded as representative samples for determination of the fatty acid composition of the total seed oil. This may be inferred from the above study of the whole-seed method, where smaller fractions of oil extracted from whole seeds proved to have practically the same fatty acid composition as that of the total seed oil (Tables II and IV). The methanolysis procedure, furthermore, is essentially the same as for the whole-seed method and has thus been proved satisfactory (Tables III and V). Analyses of replicate seed samples by the crushing method resulted in practically the same fatty acid composition as obtained by conventional analysis of the total seed oil (Table III). Two fatty acid values by the crushing method differed significantly from the corresponding values for the total seed oil; these differences are too small, however, to be of practical concern for the plant breeder.

The crushing method is well suited for routine analyses, particularly when the results are required without delay. The entire preparation of a seed sample for gas chromatographic analysis takes place in a single flask and is completed in 2-3 min. The precision of the method is indicated by the standard deviations for replicate analyses (Table III).

Half-Seed Method

As reported by Downey and Harvey (11) and recently by Thies (12), there can be certain time saving advantages in a plant breeding program if a single rapeseed is dissected so that the outer cotyledon may be analyzed for fatty acid composition while the remaining part of the embryo is left for subsequent germination and planting to produce the progeny of the seed. Although the morphological parts of rapeseed have been reported to differ in fatty acid composition (13), Thies (12) has shown that the fatty acid composition for the outer, larger, cotyledon is nearly the same as for the total seed. The oil of the outer cotyledon may therefore be regarded as a representative sample for determining the fatty acid composition of the total seed oil.

We have adopted the technique of Downey and Harvey (11) with certain modifications which include the method of rapid methanolysis with sodium at room temperature. The method is well suited to routine analyses. A trained person can dissect 40-50 seeds in an hour and also complete the extraction, methanolysis and gas chromatographic analysis of these samples in the same day.

Rapid Methanolysis

A procedure for methanolysis with sodium and methanol at room temperature was developed and incorporated as part of the whole-seed, crushing, and half-seed methods. This procedure gave the same results in fatty acid analyses as the conventional procedure adapted from Luddy et al. (Table III). These authors reported that the methanolysis of glycerides was substantially complete by their method (3). The present method is well suited to routine analyses and to methanolysis of fats and oils in general. Free fatty acids are not esterified by the sodium-methanol reagent.

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