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Lipid Analysis of Maturing Crambe Seed

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LIPID ANALYSIS OF MATURING CRAMBE SEED

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

Head, Chemistry Department Date

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INTRODUCTION[1]

For several years, plants have been screened in the search for compounds that might have a potential use in industry and which presently have no other suitable source (1). During a screening study, Mikolajczak et al. (2) found that crambe (common name for Crambe abyssinica) contained a high percentage of erucic acid. Erucic acid has many potential industrial uses and is currently being obtained from imported rapeseed (3). Because there is presently no suitable domestic source of erucic acid, crambe is a potential new crop for the United States.

Field studies of crambe have shown that the seeds tend to shatter when mature and thus seed losses occur while the crop is being harvested (4). If the crambe seed could be harvested while immature, the shattering problem would be reduced. Early harvesting would not necessarily decrease the value of the crop if the erucic acid content of the seed reached its maximum before the seed matured. Sims (5) and McKillican (6) have studied the lipid changes in maturing crambe seeds, but their experiments were not conducted in controlled environments. Sims' results show that erucic acid continually increases to maturity. Similarly, McKillican states that "on a weight per seed basis, . . . erucic acid increases steadily to maturity in crambe." However, the data she presents appear to indicate that there is a relatively constant erucic acid content from 20 DAF (days after fertilization) to maturity.

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[1]The writing style of the journal Lipids was used as the guide in preparing this thesis.

Since there appears to be some variation in the above authors' results and because environmental conditions affect seed oil composition, the lipid changes in maturing crambe seed will be examined under greenhouse conditions.

In the original plans of this study, all lipid classes were to be analyzed and thus a general method of analysis was desired. Such a method has been described by Privett et al. (7). The method involves the use of thin-layer chromatography (TLC) and densitometry. TLC has the advantages that it is fast, it has good resolving power, and it allows the detection of small quantities (8). TLC also enables the completeness of the separation of the components to be observed. Besides being suitable for the analysis of all lipid classes, the densitometric procedure permits the quantitation of compounds without removing them from the plate.

Romans (9) used a whole-spot reflectance method for the quantitation of lipids on thin-layer chromatoplates. This method is similar to densitometry but does not require the expensive equipment nor does it require as much time to complete an analysis.

Because of the potential usefulness of Romans' method, plant lipids will be used to re-examine the precision and to determine the accuracy of the method.

LITERATURE REVIEW

This review consists of 3 sections which are areas of concern to this thesis. The first section will consider crambe as a potential new crop, the second will review lipid changes in oilseeds and the third will review quantitative lipid analysis.

Crambe, A Potential New Crop

In the search for new industrial oils, Earle et al. (10) studied some plants of the Cruciferae family. The Cruciferae family (mustard family) includes about 300 genera and 2,500 species. Some of the better known representatives of the family are radishes, mustard, rape and cabbage (2). The seeds of the Cruciferae were found to contain a high percentage of oil. The oil had a high iodine value and generally the oil contained erucic acid (10). Between the species of Cruciferae, the erucic acid content of the oil varied from 0-59% (2). The genus Crambe, containing about 20 species, was found to produce the highest percentage of erucic acid (2,11,12).

Crambe is a native of the Mediterranean region and it is also located in Western Europe and Central Asia (13). It is an annual herb, growing to a height of 61-91 cm depending on the environmental conditions. The inflorescence is of the racemic type and continues for 2-3 weeks. Only 1 seed, in a spherical pod, develops from each white flower. Crambe is a cool season crop, adaptable to many of the wheat growing areas of the United States (14). It was first used as a crop in the U.S.S.R. in 1933 (11), and in 1953 it was evaluated in Poland

as a source of vegetable oil (15). Crambe seed was introduced into the United States in the 1940's. In 1965, about 1,500 acres in the US were planted to crambe (14). Crambe was first grown in South Dakota in 1963 (16).

The recent interest in crambe is due to the potential uses of its oil. The yields of 5 Crambe species are listed in Table I. The species Crambe abyssinica has been found to be the highest erucic acid producing species of the Cruciferae family (2,17). Some of the properties of crambe oil are: $d^{20} = 0.9100$, solidification point = -8.5C, $n_D^{20} = 1.4718$, acid no. = 2.3, saponification no. = 169.4, iodine no. = 91.2 and fatty acid content = 94.97% (18). Crambe oil has proven to be a superior lubricant in the molds used for continuous casting of steel. It also has been used as a rubber additive (19). Erucic acid has been used to prepare nylon 1313, a tough nylon that can be used for molded plastic articles such as bearings or for heavy fibers like brush bristles (20).

Derivatives of erucic acid also have many potential industrial uses. For example, erucamide is used as an additive to certain types of plastic films. The erucamide allows the plastic films to slide over one another without sticking together (19). Brassylic acid, which is formed by ozonolysis of erucic acid, can be used as a plasticizer in polyvinyl chloride plastics (20). Brassylic acid can also be used in polyesters and lubricants (21). The other product of erucic acid ozonolysis is pelargonic acid. Pelargonic esters are used in jet lubricants, insect repellents and in synthetic flavorings and odors (22).

TABLE I

Yields of Five Crambe Species

Species	Seed weight gm/1000 seeds	Oil content %	Erucic acid content, %
Crambe cordifolin Stev.[a]	17.5	26	36
Crambe hispanica L.[a]	4.7	45	55
Crambe orientalis L.[a]	6.7	43	36
Crambe tatarica Jacq.[a]	15.5	33	27
Crambe abyssinica[b]	4.2	36	59

[a] Reference 12.
[b] Reference 2.

Crambe seed meal, a by-product of seed oil processing, could be used in areas where other seed meals are usually used such as in plywood adhesives (19). In the future, the seed meal may also be used as a feed supplement because it contains 40-50% protein and the protein has a favorable amino acid composition (11). However, the seed meal contains several sulfur compounds which make it toxic to rats (21). Processes are being developed to remove or destroy the toxic materials in the seed meal (23,24,25).

Crambe is a potential new crop for South Dakota. The results of a 1 year study (Table II) show the gross value of crambe per acre is higher than either wheat or flax, although it is not as high as corn (16). Brunn and Matchett (22) calculated that the chemical industry could pay 7.7 cents per pound for crambe oil and use it to make brassylic

TABLE II[a]

Gross Value of Several Crops

Crop	Average yield lbs/acre	Value per 100 lbs $	Gross value per acre $
Crambe	1058	4.85	51.31
Corn	4127	1.88	77.59
Flax	353	5.40	19.06
Wheat	672	3.30	22.18

[a] Reference 16.

and pelargonic acid. They estimated that crambe would produce a net return of $19 per acre.

Lipid Changes in Oilseeds

Changes in the lipid composition of oilseeds can be caused by various external factors such as temperature (26,27,28,29,30), moisture (27,31), photoperiod (28,29), nitrogen (32), time of flowering (33), and maturity. Of these factors, only maturity will be reviewed.

Maturity studies have been conducted with plants that are not in the Cruciferae family. Investigators (34,35) found that in castor plant seeds the percentages of palmitic, linoleic and linolenic acids decreased as maturity increased, while the percentage of oleic acid increased until 15 DAF and then decreased. Ricinoleic acid and stearic acid percentages increased to maturity. Simmons and Quackenbush (36) found that all fatty acids in soybean seeds increased throughout seed maturation but they increased at different rates. Linolenic acid

reached a constant percentage in the seeds before 30 DAF. Oleic acid and the saturated fatty acids reached a constant percentage about 40 DAF, whereas linoleic acid reached a constant percentage about 45 DAF. Maturity studies (37) of flaxseed have shown that the hydrocarbon, sterol ester, and triglyceride percentages increased as the seed matured, whereas the free sterol and phospholipid percentages decreased. The oil at 20 DAF resembled the oil of mature seeds (40 DAF). The oil of immature seeds contained a high percentage of palmitic acid and a low percentage of linolenic acid. The sterol esters of flaxseed contained more saturated fatty acids than the phospholipids and the phospholipids were more saturated than the triglycerides.

Zeman and Kratochvil (38) noted the lipid changes in winter rape oil. Erucic acid reached its maximum at maturity and the percentage of oleic acid decreased as the percentage of erucic acid increased. The inverse relationship of erucic acid and oleic acid could be expected since oleic acid is the precursor of erucic acid (39, 40). The oil of another member of the Cruciferae family, mustard, did not change in iodine value or saponification value during maturation (41).

Maturity studies of crambe have been reported by Sims (5) and McKillican (6). Neither of the reports included greenhouse or growth chamber studies. Sims analyzed the oil by gas-liquid chromatography (GLC). His values for erucic acid are given in Table III. No units for the values were given, but they are probably area percent or weight percent. Sims' data shows that erucic acid increases steadily to

TABLE III

Reported Changes in Maturing Crambe Abyssinica

	Days after fertilization							
	McKillican's data[a]				Sims' data[b]			
	10	20	30	Mature	7	14	21	42[c]
Erucic acid (%)	24.3	61.6	55.0	54.9	0.0[d]	26.0	43.5	60.4
Seed weight (mg/100 seeds)	356.4	600.0	728.0	654.0				
Oil (%)	11.8	32.5	33.2	32.2				
Triglyceride (%)	84.8	80.4	91.9	94.9				
Erucic acid[e] (mg/100 seeds)	8.7	96.6	122.2	109.7				

[a] Reference 6.
[b] Reference 5.
[c] Mature seed.
[d] No units were given but probably are area percent or weight percent.
[e] Calculated from McKillican's data using the formula erucic acid (mg/100 seeds) = seed weight X oil % X triglyceride % X erucic acid %.

maturity. McKillican did a more complete study of crambe oil by analyzing for all lipid classes and the fatty acid composition of each lipid class. The triglyceride fraction, seed weight, percent oil and percent triglyceride fraction reported by McKillican are also given in Table III. Her values indicate the percent erucic acid increases to 20 DAF and then remains relatively stable to maturity. However, in her article she states that "on a weight per seed basis . . . erucic acid increases steadily to maturity in crambe." This observation is not evident from the reported data. Using her values, the mg of erucic acid per 100 seeds was calculated and is given in Table III.

Various other studies have been conducted with crambe. Papathanasiou et al. (42) studied the number of primary branches, the number of pods, plant height, seed weight and the yield of crambe. Earle et al. (43) compared crambe samples from 17 states and found the oil content of the seed without pericarp varied from 36-54% with most being in the range of 42-48%. All samples, except 2 grown in Alaska, contained 51-60% erucic acid. In other studies (44), lipase in crambe seeds at 5-7% moisture was found to have no activity and even seeds at 10-15% moisture had low lipase activity.

Quantitative Lipid Analysis

Many methods of lipid analysis are specific for only one lipid class or one type of compound. An example of such a method is the Liebermann-Burchard test for Δ^5, 3-OH steroids (45). Triglycerides have been determined by measuring the glycerol content (46), while phospholipids have been measured by determining the phosphorus content of the lipid (47). Fatty acids have been determined colorimetrically (48,49,50) and by titration (51). Quite often fatty acids are also measured by GLC (6,30,34,37,52,53,54).

Several methods have been developed which are nonspecific. These methods are suitable for analyzing most lipid classes and compounds. Such a method has been reported by Amenta (55). The compounds were separated by TLC and then scraped from the plate into a test tube. A dichromate solution was added, the tube was heated and then the remaining amount of $Cr_2O_7^=$ was measured. Marsh and Weinstein (56) scraped the lipid from a thin-layer chromatoplate and charred it in a

test tube using sulfuric acid and heat. A linear relationship between the optical density (at 375 mμ) and the lipid concentration was obtained.

Methods which are nonspecific and which do not require the removal of the lipid from the thin-layer chromatoplates have an advantage over those requiring the extra steps of scraping the chromatoplates. For example, the fluorescent dyes such as fluorescein (57) and Rhodamine 6G (58) provide a method for the direct analysis of lipids on chromatoplates.

Privett et al. (7) used TLC and densitometry to determine the quantities on mono-, di-, and triglycerides. This procedure was later extended to include all lipid classes (59,60). The general procedure involves separating the components on a thin-layer chromatoplate, charring the compounds by spraying the plate with an oxidizing reagent and then heating. Privett and Blank (61) found that spraying the plates with a 70% (v/v) aqueous sulfuric acid solution saturated with potassium dichromate and heating them at 180C for 25 min resulted in the compounds having equal charring intensities. The compounds were compared on the basis of μg of carbon. Weaker oxidizing agents and higher temperatures allowed saturated compounds to evaporate before being charred, thereby causing the spots of the saturated compounds to be less dense. The densitometric method involves measuring the decrease in the light transmitted through the charred lipid spots.

Rouser et al. (62) analyzed polar lipids by TLC and densitometry. The authors pointed out that silicic acid is important in the charring

process. When magnesium silicate or aluminum oxide is used, little or no charring occurs. The authors also observed a direct relationship between charring intensity and R_f.

A method very similar to densitometry is the reflectance method (63). Instead of measuring the decrease in transmitted light, the decrease in the reflected light from the plate is measured.

The transmitted or reflected light can be measured by either scanning the spot using a narrow slit of light or by using a large area of light that covers the entire spot. The scanning method appears to be more popular.

Romans (9) used a whole-spot reflectance method for analyzing lipids. Using animal lipids, he obtained a precision of 1.9-7.3%. Other investigators have reported using reflectance for measuring compounds on thin-layer chromatoplates (63,64,65). They reported precisions of 5-12%.

This study will determine the accuracy of Romans' procedure and apply it to the analysis of lipid changes in maturing crambe seed.

EXPERIMENTAL PROCEDURES

Growth and Collection of Crambe Seed

Both greenhouse and field studies were conducted. The seed bed in the greenhouse studies consisted of vermiculite packed in 4-l crocks. A layer of sand, about 1.3 cm deep, was placed on top of the vermiculite. Crambe abyssinica seeds from the 1966 South Dakota State University (SDSU) field trials were obtained from the Agronomy Department. The seeds were planted in the sand which was then kept moist until after germination. Each crock was seeded with 10-30 seeds. The plants were thinned over a period of about a month. Light bulbs (300 watt) located 4 ft above the crocks were used to produce a 16-hr photoperiod. The plants were given mechanical support to prevent lodging. During the harvesting procedure, the pedicels were removed from the seeds. The pericarps, however, were left intact and all seeds were analyzed in this form.

In experiment I, 96 crocks were used with each crock containing 6 plants. The plants were given Hoagland's nutrient solution (66) once a week and deionized water was supplied whenever the vermiculite appeared dry. A single row of 8 bulbs located above the center of the greenhouse table was used. After all of the plants had started blossoming, open flowers were tagged by tying a string (6.4 cm long) around them. Three thousand flowers were tagged in 1 day.

The experimental design for harvesting the seeds consisted of collecting seeds from each crock on each collection date. This design would help reduce the experimental variation caused by the genetic and

environmental factors. The number of tagged seeds in each crock was determined. This number was divided by 5 (the number of collection dates) to determine how many seeds would be collected from each crock on each collection date. The number of seeds collected per crock per collection date varied from 0-5. Seeds were harvested at 7 day intervals beginning with 15 DAF. The seeds were placed in a vial and stored under nitrogen at -15C. About 2 months later the seeds were freeze-dried, divided into replications, counted and weighed. The seed weight, in mg/100 seeds, was determined on 41-50 seeds.

Since the plants in experiment I appeared to be crowded, only 85 crocks with 5 plants per crock were used in experiment II. The plants were given Hoagland's solution every 2 weeks in the early and later stages of growth and once a week during the heavy growth stages. Additional deionized water was supplied whenever the plants showed signs of wilting. During the heavy growth stages, the plants were checked for lack of moisture 3-4 times each day. To provide more uniform lighting, the number of bulbs was increased to 16 and they were placed in 2 rows.

In experiment I, the flowers that blossomed during the early stages of the inflorescent period (early flowers) were observed to produce a larger percentage of seeds than those flowers which blossomed in the later stage of the inflorescent period (late flowers). Because of this, the blossoming period of all plants in experiment II was synchronized by cutting off the first flowers. After all plants had started blossoming, the flowers were tagged with thread which was 6.4 cm long. Thread was used instead of the heavy string employed in

experiment I to reduce the possibility of damaging the seed. Three thousand nine hundred flowers were tagged over a 2 day period.

A different procedure for harvesting the seeds was used so that all tagged seeds would be utilized and the process would be simplified. The greenhouse table was divided into 3 sections and the crocks within each section were randomized. This stratified randomization design should reduce the experimental variation caused by the genetic and environmental factors. All tagged seeds from 4 crocks in each section were harvested on the first 2 collection dates (10 and 15 DAF). On all other collection dates, the seeds were harvested from 3 crocks in each section. After harvesting, the seeds were immediately divided into 3 replications (116-146 seeds per replication), weighed and then freeze-dried for at least 10 hr. The seeds in each replication were then weighed again, counted, placed in a vial and stored under nitrogen at -15C.

The field study was conducted in the summer of 1966 in the Plant Pathology field plots. In the first tagging, the open flowers were tagged with a red string. Over a 2 day period, 380 flowers were tagged. The second tagging, also a 2 day period, consisted of 500 yellow strings. The second tagging was performed 13 days after the first tagging. All seeds were harvested "at random" at 7-day intervals beginning with 22 DAF for the red strings and 16 DAF for the yellow strings. After the seeds were harvested, they were stored under nitrogen at -15C. About 8 months later, the seeds were freeze-dried,

counted and weighed. The seed weight, in mg/100 seeds, was determined on 11-28 seeds.

Lipid Extraction

All solvents were redistilled in a completely glass system before being used. The oil was extracted from the seeds by homogenizing in hexane with a Virtis 45 homogenizing mill. Up to 150 seeds were extracted at 1 time. Each extraction was performed for a 2 min period, under nitrogen and in an ice bath. Two extractions, each consisting of 15 ml of hexane, were used. After the first extraction, the hexane was decanted and filtered. The second extract was filtered and the residue was washed 3 times with hexane. To remove nonlipid contaminants, the combined extracts were transferred to a separatory funnel and washed with 20 ml of 0.05% aqueous $CaCl_2$ solution (67). To remove any traces of water, the extract was passed through a column of anhydrous Na_2SO_4. The hexane was then removed using a rotary evaporator and a 28C water bath. The flask containing the oil was flushed with nitrogen and stored in the refrigerator until the end of the day. All samples extracted during the day were then transferred to tared vials. After the vials had been dried in vacuo for 16.5 hr, the weight of the oil was determined. The vials were flushed with nitrogen and stored at -15C.

This extraction procedure was compared to a continuous extraction method. Crushed seeds were placed in a Soxhlet extractor and extracted for 6 hr. The hexane was removed and the weight of the oil was determined. The percentage of oil extracted by the homogenizing procedure was

a little larger than the percentage of oil obtained by the continuous extraction procedure. This indicated an efficient lipid extraction was being obtained with the homogenizing method.

Quantitative Thin-Layer Chromatography

The triglyceride, free fatty acid and sterol ester classes were determined by the following quantitative TLC procedure. A slurry consisting of 30 gm of Silica Gel G and 60 ml of water was spread on 5 (20 x 20 cm) plates at a thickness of 250 μ. The plates were activated at 110C for 2 hr. The plates were stored over Dry-Rite and were used within 24 hr. A 50 μl Hamilton syringe was used to spot the plates and nitrogen was used to dry the spots.

Three concentrations of standard and 3 concentrations of crambe lipid were applied to each plate. All standard samples used throughout this study were obtained from Applied Science Labs., Inc., State College, Pa. In the maturity studies, 11.2, 25.2 and 39.1 μg of standard triolein were applied to the thin-layer chromatoplates. For the accuracy of the reflectance method, the following concentrations of standard triolein, oleic acid and cholesteryl oleate were applied: 11.2, 28.0, 41.9 μg; 8.9, 21.4, 39.2 μg and 12.0, 25.2, 38.4 μg, respectively.

The plates were developed in a solvent system of hexane-diethyl ether-acetic acid (85:20:1, v/v/v). After development, the plates were air dried for at least 2 min and then sprayed with a 70% (v/v) aqueous H_2SO_4 solution saturated with $K_2Cr_2O_7$ (61). The plates were placed on a hot plate (160C) and covered with a bottomless chloroform can (9).

After being heated for 30 min, the plates were cooled and the intensity of the charred lipid spots was determined by Romans' reflectance procedure (9). The Photovolt Model 610 Reflection Meter was modified by placing black tape on the sides of the light source. This decreased the field of light thereby increasing the sensitivity. The percent of reflected light (%R) was read from a galvanometer. The concentration of the standard lipids applied to the plates was plotted against 2-log %R. The lipid concentration in the 3 spots of crambe oil on the plate was then determined from the standard curve. The average of the 3 values was used as the observed concentration.

Preparative Thin-Layer Chromatography

This procedure was used to prepare the purified crambe lipid classes which were used in determining the accuracy of Romans' reflectance procedure. The plates were prepared and activated as previously described except that a layer thickness of 1 mm was used. The plates were heavily spotted with a crambe oil solution and then developed. A solvent system of hexane-diethyl ether (95:5, v/v) was used for the purification of the sterol esters while hexane-diethyl ether-acetic acid (75:35:1, v/v/v) was used for the free fatty acids and triglycerides. After the plates were air dried, they were divided into sections perpendicular to the direction of the lipid movement. The silica gel from 1 side of each section was not removed from the plate, whereas the rest of each section was scraped into a vial. The unremoved portion of the plate was then charred so that the compounds could be located. Diethyl ether was used to elute the compounds from

the silica gel into vials. The ether was removed and the lipid was stored under nitrogen at -15C. Since the free fatty acid content of the crambe oil was so small, a sample was prepared by saponification of the whole oil (45). The saponification mixture was acidified and the fatty acids were extracted with hexane. The fatty acids were then purified by TLC.

After the purified fractions of triglyceride, sterol ester and free fatty acid had been obtained, the purified lipids were transferred to tared vials and placed in vacuo until they reached constant weight. A Mettler microbalance was used for the weighings. Solutions of these purified lipid classes were used to determine the accuracy of Romans' procedure. The same method of analysis as described under the Quantitative Thin-Layer Chromatography section was used.

Gas-Liquid Chromatography

BF_3 (68) was used to esterify the crambe oil. One ml of the BF_3 reagent (25% BF_3-MeOH[1], 20% benzene, 55% MeOH, v/v/v) was used for each 6-14 mg of oil. The samples were placed in screw-capped tubes and heated for 40 min in a boiling water bath. The 40 min reaction time was selected after conducting a rate study. During the rate study, samples were removed at 10 min intervals and the reaction was quenched by cooling, adding water and extracting the esters with hexane. The hexane extracts were then subjected to TLC. The thin-layer chromatoplate used to determine the degree of esterification is shown in Figure 1. As can be seen,

[1]Applied Science Labs., Inc., 14% BF_3 (w/v).

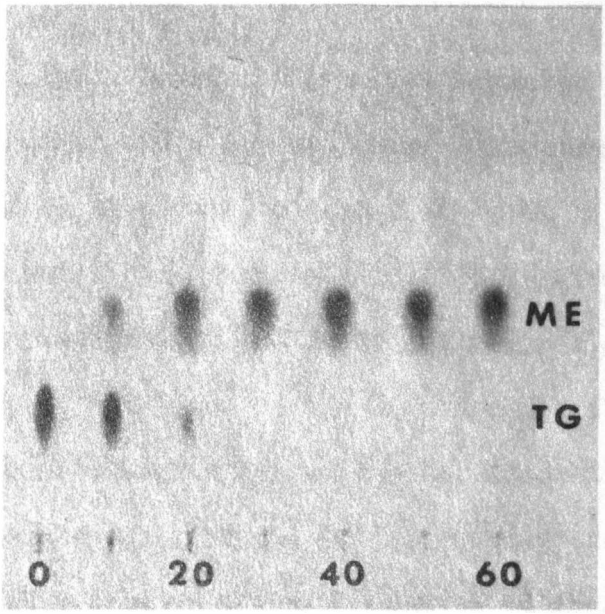

FIG. 1. Esterification rate study of crambe oil. The numbers represent the reaction time in minutes, the TG represents the triglyceride portion of crambe oil and ME represents the fatty acid methyl esters formed by the BF_3-MeOH esterification.

the esterification was almost complete at 30 min. To ensure complete esterification, the crambe samples were heated an additional 10 min. In processing the actual samples, the methyl esters were obtained by extracting the esterified samples 3 times with 2 ml portions of hexane. The extracts were placed in a vial and taken to dryness in vacuo. The methyl esters were diluted with a known volume of hexane and analyzed by GLC.

An Aerograph, HyFi, Model 600 gas chromatograph equipped with a flame ionization detector and a Sargent Model SR recorder was used. A DEGS (diethylene glycol succinate) column (1/8 in OD x 5 ft) was used. The conditions used were: temperature, 193-197C; hydrogen flow rate, 30 ml/min; nitrogen pressure, 13 lbs/sq in; nitrogen flow rate, 30 ml/min; attenuation, 8; run time, 25 min and recorder filter setting, 2. A standard mixture containing methyl palmitate, stearate, oleate, linoleate and linolenate was combined with a standard sample of methyl erucate. These 6 fatty acid esters were used to calibrate the gas chromatograph. In most cases, 3 μl injections were used. The area under the peaks was measured by triangulation and the area percent of each component was calculated. Correction factors for the weight percent of the 6 standard esters were determined each day and applied to the crambe samples.

The standard solution, injected 4 times during 1 day, was used to determine the precision of the GLC procedure. The percent standard deviation of the erucic acid values was 3.6%.

Quantitative Phosphorus Analysis

The phospholipid content of crambe oil was determined by using Bartlett's method of phosphorus analysis (69). The procedure had to be modified slightly since the amount of oil necessary for phosphorus measurements was too large for the digestion process. The phospholipids were first separated from the other lipids on a silicic acid column. The crambe oil sample was placed on the column with hexane. Most of the neutral lipids were removed with diethyl ether-hexane (1:1, v/v). The phospholipids were then eluted with methanol into 50-ml test tubes. The phospholipid and standard phosphorus samples were digested using the following sequence: 0.5 ml of 10 N H_2SO_4 was added to each tube and then the tubes were heated in an oven (160C) for 2 hr; 0.5 ml concentrated HNO_3 and 2 more hr of heating were then used; 10 drops of 30% H_2O_2 followed by 2 more hr of heating and finally 5 drops of H_2O_2 and 2 hr of heating finished the digestion. The color development was performed as described by Bartlett (69). After adding the reagents and developing the color, the samples were centrifuged to remove the silicic acid. The %T of the samples was obtained at 830 mμ using a Beckmann DU Spectrophotometer. The μg of phosphorus in the crambe samples were determined from the standard curve. The quantity of phospholipid in crambe oil was then calculated by assuming an average phosphorus content of 4%.

RESULTS AND DISCUSSION

Quantitative Lipid Analysis

Since the maturity studies of crambe depend upon the methods of lipid analysis, the quantitation of lipids will be discussed first.

The precision of Romans' reflectance method was measured by the repeated analysis of purified crambe triglyceride. The percent standard deviation obtained from 6 thin-layer chromatoplates was 8.3%. This deviation is within the range reported by other investigators (64,65). Romans obtained a lower percent standard deviation, but he used weight percent values, whereas μg/μl quantities were measured in this study.

Romans' method was checked for accuracy by determining the concentration of the gravimetrically prepared solutions of purified crambe lipid classes. The method was found to give a higher concentration than the gravimetrically determined concentration for the crambe triglyceride when triolein was used as the standard. The method also yielded high results for crambe free fatty acid and sterol ester classes when oleic acid and cholesteryl oleate were used as standards. To correct for these high results a correction factor (CF) was calculated by the formula $CF = \frac{\mu g \text{ lipid}/\mu l \text{ obtained gravimetrically}}{\mu g \text{ lipid}/\mu l \text{ obtained by reflectance}}$. The factors were: 0.81 for triglyceride, 0.90 for free fatty acid and 0.89 for sterol ester. These correction factors may have been due to the use of standards which were not typical of the material being analyzed. The correction factors were applied to all crambe oil samples in the maturity studies. Typical thin-layer chromatoplates used in this study are shown in Figure 2.

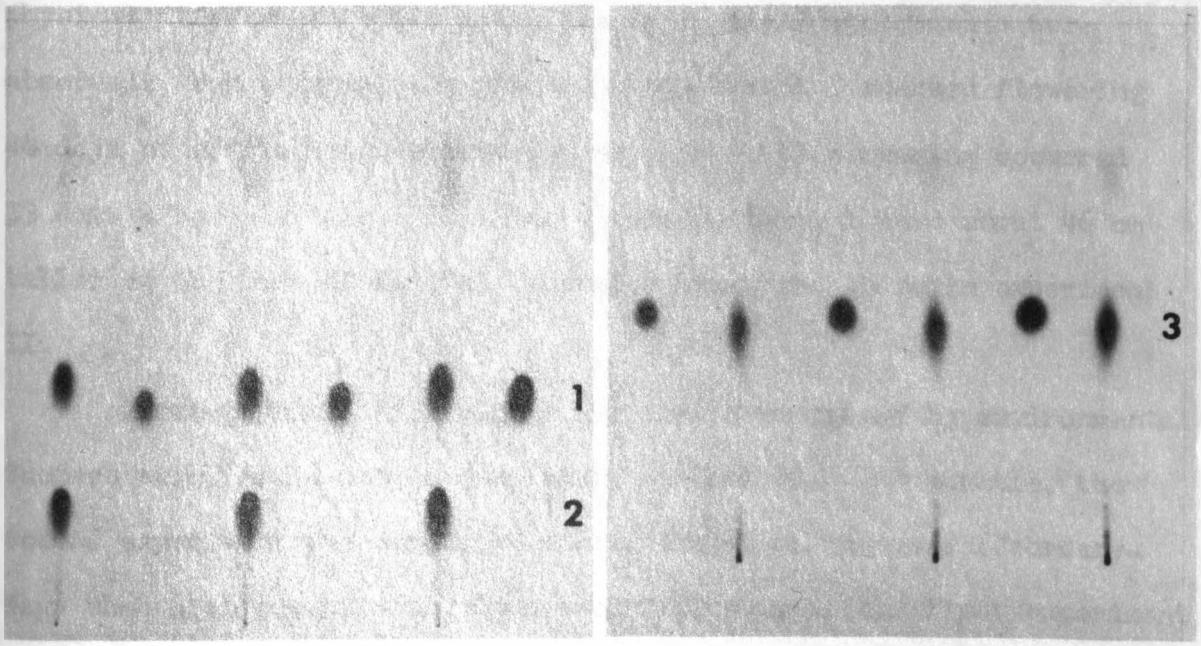

FIG. 2. Thin-layer chromatoplates of purified crambe lipid. The lipid classes are: 1 = triglyceride, 2 = free fatty acid and 3 = sterol ester. Those samples which have charred material at the origin are the crambe lipid classes, whereas the other samples are either standard triolein or cholesteryl oleate.

Changes in Maturing Crambe

In the maturity experiments, some general observations other than lipid changes were noted. These general observations will be discussed first, followed by the discussion of the specific lipid changes in maturing crambe.

General Observations. In the greenhouse studies, several physical differences between the plants in the 2 experiments were observed. For example, the plants in experiment I started flowering 44 days after planting, whereas in experiment II flowering occurred 33 days after planting. The plants in experiment I were about 46 cm taller at the time of initial flowering than were those in experiment II.

These physical differences may have been caused by environmental factors which could not be completely controlled. For example, the second experiment was conducted during a time of the year (February-May) when more bright sunny days occurred, whereas the first experiment was conducted during a season (October-January) having more cloudy days. Also, the plant population was not as large in experiment II as it was in experiment I. Not only was there 1 less plant per crock, but the number of crocks was decreased from 96-85. Another environmental difference between the experiments was that the plants in experiment I were watered more often and, in fact, probably received too much water. The roots of the plants may have become water logged. What effect this would have is not known.

The number of seeds formed and the seed weight also differed between experiments. A seed set of 31.9% was obtained from the tagged flowers in experiment I and the average seed weight of the mature seeds (42 DAF) was 416 mg/100 seeds. These values are contrasted with a 68.6% seed set and an average seed weight (40 DAF) of 893 mg/100 seeds in experiment II. The time of seed set may explain these differences. Since the flowers in experiment II were continually removed from the plants until the tagging period, early flowers were tagged. In experiment I, however, the plants were allowed to blossom freely until all of the plants were flowering. Thus, many of the plants were in the later stages of flowering and many of the tagged flowers were late ones. The results of the field study also support this hypothesis since the mature seeds from the early flowers (red strings) weigh more than the seeds from the late flowers (yellow strings). The average weights at 37 DAF were: red, 786 mg/100 seeds and yellow, 315 mg/100 seeds.

The experiments in this study were not designed to determine the differences due to light, moisture or time of seed set and therefore no definite conclusions can be given. These observations seemed to be of interest and are thus mentioned.

Stefansson and Hougen (70) reported the erucic acid content varied from 6-50% between individual plants of rapeseed. Because of this report, a preliminary study was conducted on plant to plant variations in crambe. No statistical differences at the 0.05 level were found for percent erucic acid, percent oil or seed weight. The description of the experiment and the data are given in the appendix.

Experiment I. The changes in crambe which were observed in greenhouse experiment I are given in Table IV. The values are the average of 3 replications. Dunnett's (71) one-sided test of significance at the 0.05 level was used to compare the highest value of each component with all other values for that particular component. The underlined values are those which are not significantly different from the largest value.

The seed weight reached a maximum at 28 DAF and then remained constant (not significantly different) throughout the remaining ripening period. The percent oil did not change during the maturation period which was studied; however, the actual amount of oil increased until 28 DAF because of the increase in seed weight. The percent triglyceride followed the same pattern as the seed weight in that it increased during the 15-28 DAF period and then leveled off during the 28-42 DAF period.

The percent phospholipid decreased as the seeds matured. These values were not statistically analyzed because a true picture of the phospholipid changes in the crambe seed could not be obtained. A preliminary study showed that chloroform extracts contained 20 times as much phosphorus as hexane extracts. However, hexane extracts were used in this study because the commercial process of oil extraction involves hexane.

Palmitic acid was at a maximum in the early (15 DAF) seeds. At all other seed ages the palmitic acid content was significantly lower. The palmitoleic acid content was not different at 15 DAF and

TABLE IV

Changes in Maturing Crambe: Greenhouse Study I

	Days after fertilization				
	15	21	28	35	42
Seed weight (mg/100 seeds)	358	395	459[a]	414	416
Oil content (%)	20.4	20.7	21.3	21.6	20.2
Triglyceride content (%)	42.9	67.7	98.2	101.7	92.1
Phospholipid content (%)	3.1	3.3	1.1	0.7	0.4
Fatty acid content (weight %)[b]					
16:0	3.8	2.3	2.1	2.2	1.9
16:1	0.7	0.4	0.4	0.3	0.5
18:0	1.0	0.9	0.6	0.5	0.7
18:1	17.3	15.6	15.3	14.0	14.1
18:2	15.3	12.8	13.0	12.3	13.3
20:0	0.8	0.9	0.0	0.7	0.3
18:3	14.3	10.8	9.9	9.8	10.0
Unknown	2.5	2.7	1.9	1.9	2.5
22:1	45.0	54.4	58.6	58.9	58.6

[a] All underlined values in a given row are not significantly different ($P < 0.05$) from the largest value in the row.

[b] Fatty acids are listed in the order of increasing retention time using a DEGS column.

42 DAF, but it was slightly lower at the in-between ages. Stearic acid was constant throughout the maturity study except for a slight decrease at 35 DAF. Arachidic acid and the unknown fatty acid remained constant from 15-42 DAF. The above changes, even though statistically

significant, are not very important in relation to the over-all lipid pattern. The total percentages of palmitic, palmitoleic, stearic, arachidic and unknown acids comprised less than 10% of the oil.

The unknown fatty acid had a retention time similar to 22:0 (behenic acid) (72). Since retention time was the only property used for identifying the fatty acid, no positive identification could be made. McKillican (6) did not report the presence of behenic acid. She did report the presence of gadoleic (20:1) but this fatty acid was not detected in the present study. Other investigators (2) have reported the presence of both fatty acids in crambe.

The 4 major fatty acids were oleic, linoleic, linolenic and erucic. Oleic acid remained constant until 28 DAF and then decreased. The decrease in oleic acid during maturation has been observed by several authors (6,38,40). Linoleic and linolenic acid reached a maximum in the 15 DAF seeds. Both were significantly lower at all other ages. Erucic acid increased to 21 DAF and then did not significantly change.

Experiment II. The greenhouse study was repeated because of the poor seed set encountered in experiment I. Table V contains the results of experiment II. The values are the average of 3 replications and Dunnett's test was again used to analyze the data.

The seed weight increased to 25 DAF, remained constant to 30 DAF and then decreased slightly at 35 and 40 DAF. The percent oil increased to 25 DAF, remained constant at 30 and 40 DAF but it dropped slightly at 35 DAF. This differs from experiment I where the percent oil was

TABLE V

Changes in Maturing Crambe: Greenhouse Study II

	Days after fertilization						
	10	15	20	25	30	35	40
Seed weight (mg/100 seeds)	479	672	857	946[a]	931	894	893
Oil (%)	1.8	12.8	22.7	24.9	24.4	23.7	24.2
Triglyceride (%)	58.1	92.8	93.2	102.3	104.9	96.2	96.1
Phospholipid (%)	6.4	1.7	0.2	0.2	0.1	0.2	0.2
Fatty acid (weight %)[b]							
16:0	7.1	3.1	2.0	1.8	1.9	1.8	2.3
16:1	3.3	1.1	0.4	0.3	0.3	0.3	0.3
18:0	2.8	1.2	0.8	0.7	0.8	0.9	0.9
18:1	25.0	21.3	18.8	18.3	18.1	18.6	19.0
18:2	24.8	12.4	9.7	9.9	9.8	9.0	9.4
20:0	0.0	1.7	0.9	1.0	0.9	0.9	0.8
18:3	24.3	18.8	12.8	11.6	11.8	11.4	11.4
Unknown	0.0	0.9	2.1	1.7	2.1	2.3	2.2
22:1	5.6	38.2	53.8	55.8	54.7	55.6	54.4
Moisture (%)	83.3	77.3	65.7	52.9	8.0	4.2	3.7

[a] All underlined values in a given row are not significantly different ($P < 0.05$) from the largest value in the row.

[b] Fatty acids are listed in the order of increasing retention time using a DEGS column.

constant throughout the entire maturing period. The percent triglyceride increased to 15 DAF and then did not change. As in experiment I, the percent phospholipid decreased during seed maturation.

In terms of significant changes, all fatty acids except arachidic, erucic and the unknown were at a maximum at 10 DAF and then significantly lower at all other ages. The percentages of arachidic and the unknown acid were stable during most of the ripening period. They were not detected, however, in the very young seeds. As in experiment I, the percent erucic acid increased to 20 DAF and then did not significantly change. Even though the percent erucic acid did not change beyond 20 DAF, the actual amount of erucic acid increased until 25 DAF because of the increase in the seed weight.

Experiment III. The maturity studies conducted in the field could not be statistically analyzed because replications of each sample were not obtained. Many of the strings used for tagging the flowers were blown off and thus the number of seeds collected was too small to be divided into replications. In general, the trends observed in the field study (Table VI) were similar to those observed in the 2 greenhouse studies. The erucic acid content of the seeds appeared to change very little beyond 22 DAF.

The differences in the bulk harvested seed[1] (Table VI) obtained in 1965 and 1966 were statistically analyzed. The seed weight and percent oil differences were found to be insignificant, but the difference in percent erucic acid was significant ($P < 0.05$).

The observed lipid changes in the maturity experiments were very similar. The trends which were observed in this study appear to

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[1]The seed was furnished by the Agronomy Department, SDSU.

TABLE VI

Field Studies of Crambe

	Changes in maturing crambe									Bulk harvest[b]	
	Days after fertilization									Years	
	Early flowers[a]					Late flowers					
	22	29	36	43	50	16	23	30	37	1965	1966
Seed weight (mg/100 seeds)	810	687	786	734	769	357	471	440	315	732	540[c]
Oil (%)	25.8	24.7	29.1	28.7	29.8	19.5	25.0	25.8	20.3	29.4	28.4[c]
Triglyceride (%)	42.2	93.1	95.6	92.0	89.6	38.2	24.4	91.4	101.2		
Phospholipid (%)	0.8	0.4	1.3	2.5	0.6	2.5	0.9	0.5	1.2		
Fatty acid (weight %)[d]											
16:0	2.2	1.9	1.5	1.6	1.9	3.6	2.0	1.9	2.6	2.2	1.8
16:1	0.3	0.3	0.2	0.1	0.3	0.5	0.3	0.2	0.3	0.2	0.2
18:0	0.8	0.5	0.4	0.6	0.5	1.2	0.7	0.6	0.9	1.2	0.4
18:1	17.4	18.8	17.0	17.4	19.2	17.6	16.7	15.4	16.9	18.6	16.7
18:2	9.0	10.3	8.8	9.4	11.0	11.9	9.6	10.0	11.9	11.2	9.4
20:0	0.0	1.1	0.4	0.8	0.8	0.9	0.7	0.9	0.6	1.2	1.2
18:3	10.5	13.9	11.8	12.7	12.2	19.4	13.4	14.0	14.0	21.0	10.8
Unknown	2.6	2.0	2.3	1.6	1.6	1.5	2.3	1.4	1.9	1.4	2.3
22:1	58.1	52.7	58.9	57.1	53.4	45.4	55.5	57.1	52.5	45.4	58.2*[c]

[a] Early flowers were tagged 7/14/66; late flowers were tagged on 7/27/66.

[b] Mature seeds harvested by combine.

[c] These values were analyzed for significant differences ($P < 0.05$); those with * were found significant.

[d] Fatty acids are listed in the order of increasing retention time using a DEGS column.

agree with McKillican's data (6). Since the seed weight, percent oil and percent erucic acid reached their maximum about 25 DAF, impetus is given to the idea of harvesting crambe earlier than 40 DAF. Although the early harvesting of crambe may not decrease the potential value of the crop, there are other problems related to early harvesting which may affect its value. For example, the moisture content of the seeds may be a problem. Even though seeds 30 DAF and older have a moisture content (Table V) low enough for storage (44), drying may be necessary if too many seeds younger than 30 DAF are present during harvesting. If drying would be needed, the effect it may have on the quality of the oil is not known. Because problems such as this one may affect the net value of crambe, further studies will be required before definite recommendations can be made.

SUMMARY

Crambe abyssinica (crambe) is a potential new crop for South Dakota. The oil of crambe is valuable because it contains a high percentage (50-60%) of erucic acid. Erucic acid has many potential industrial uses and is presently being obtained from imported rapeseed.

In this study, the lipid changes in maturing crambe were determined. The lipids were analyzed by using Romans' whole-spot reflectance procedure. This procedure was found to be precise, but a correction factor was needed to determine the actual amount of lipid in crambe oil. It is postulated that the correction factor was needed because atypical standards were used.

The lipid changes in maturing crambe were studied under greenhouse and field conditions. The minor fatty acids, palmitic, palmitoleic, stearic, arachidic and an unknown, varied during seed maturation, but their total percentage was always less than 10%. The unknown acid had a retention time similar to behenic acid but it was not positively identified. The major fatty acids were oleic, linoleic, linolenic and erucic. Generally, the C_{18} acids decreased as the seeds matured. Erucic acid increased to 20 DAF and then did not significantly change ($P < 0.05$). The percent oil and seed weight reached their maximum about 25 DAF. Since the actual amount of erucic acid reached its maximum before maturity, the possibility exists that crambe can be harvested before it is mature. The early harvesting would reduce seed losses due to shattering but other factors, such as moisture, will need further study before definite recommendations can be made.

REFERENCES

1. Earle, F. R., E. H. Melvin, L. H. Mason, C. H. VanEtten, I. A. Wolff and Q. Jones, Search for new industrial oils. I. Selected oils from 24 plant families, J. Am. Oil Chemists' Soc. 36, 304-307 (1959).

2. Mikolajczak, K. L., T. K. Miwa, F. R. Earle, I. A. Wolff and Quentin Jones, Search for new industrial oils. V. Oils of Cruciferae, Ibid. 38, 678-681 (1961).

3. English, S. S., Editor, Introducing crambe, Agr. Research 11, 6 (1962).

4. White, George A., and J. J. Higgins, Culture of crambe . . . A new industrial oilseed crop, Agricultural Research Service, U. S. Dept. Agr., Production Research Report No. 95, 1-20 (1966).

5. Sims, R. P. A., Changes in the fatty acid composition of the seeds of three oil-bearing species during increasing seed maturity, Can. J. Plant Sci. 44, 217-218 (1964).

6. McKillican, Mary E., Lipid changes in mature oil-bearing plants. IV. Changes in lipid classes in rape and crambe oils, J. Am. Oil Chemists' Soc. 43, 461-465 (1966).

7. Privett, O. S., M. C. Blank and W. O. Lundberg, Determination of mono-, di-, and triglycerides by molecular distillation and thin-layer chromatography, Ibid. 38, 312-316 (1961).

8. Stahl, Egon, "Thin-Layer Chromatography," Academic Press, Inc., New York, 1965, p. 179.

9. Romans, John R., "Lipid Mobilization in Beef and Rabbits as Affected by Marketing-Induced Stress or ACTH-Induced Stress," Ph.D. Thesis, South Dakota State University, Brookings, S. Dak., 1967, p. 34-43.

10. Earle, F. R., T. A. McGuire, Jean Mallan, M. O. Bagby and I. A. Wolff, Search for new industrial oils. II. Oils with high iodine value, J. Am. Oil Chemists' Soc. 37, 48-50 (1960).

11. Papathanasiou, G. A., and K. J. Lessman, Crambe, Purdue University Exp. Station, Research Bulletin No. 819, 1-8 (1966).

12. Miller, Roger Wayne, F. R. Earle, I. A. Wolff and Quentin Jones, Search for new industrial oils. XIII. Oils from 102 species of Cruciferae, J. Am. Oil Chemists' Soc. 42, 817-821 (1965).

13. Robison, Laren R., and James H. Williams, Crambe, potential oilseed crop, Nebraska Agr. Exp. Station, Farm, Ranch and Home Quarterly, Fall, 507 (1966).

14. White, George A., What we know about growing crambe, Crops and Soils 18, 10-12 (1966).

15. Moldenhawer, K., New oil plants, Przemyst Spozywczy 7, 58-62 (1953). Chem. Abstr. 51, 8455a (1957).

16. Kingsley, Q. S., and D. W. Beatty, Crambe: stranger on the prairie, S. Dak. Farm and Home Res. 15, 11-12 (1964).

17. Goering, K. J., Robert Eslick and D. L. Brelsford, Search for high-erucic acid-containing oils in the Cruciferae, Econ. Botany 19, 251-256 (1965).

18. Niewiadomski, H., B. Drozdowski and W. Zwierzykowski, Application of molecular distillation for the determination of the fatty acid composition of Crambe abyssinica seed oil, Fette, Seifen, Anstrichmittel 61, 897-899 (1959). Chem. Abstr. 54, 20247b (1960).

19. Wolff, Ivan A., The many uses of crambe products, Crops and Soils 18, 10-12 (1966).

20. Wolff, Ivan A., and George A. White, Crambe--a progress report, Crops and Soils 19, 16 (1967).

21. Agricultural Research Service, Crambe, U. S. Dept. Agr., Crops Research Bulletin No. 34-42, 1-9 (1962).

22. Bruun, Johannes H., and John R. Matchett, Utilization Potential of Crambe abyssinica, J. Am. Oil Chemists' Soc. 40, 1-5 (1963).

23. VanEtten, C. H., M. E. Daxenbichler, J. E. Peters and I. A. Wolff, Seed meal from Crambe abyssinica, J. Agr. Food Chem. 13, 24-27 (1965).

24. Kirk, L. D., G. C. Mustakas and E. L. Griffin, Jr., Crambe seed processing - improved feed meal by ammoniation, J. Am. Oil Chemists' Soc. 43, 550-555 (1966).

25. Tookey, H. L., C. H. VanEtten, J. E. Peters and I. A. Wolff, Evaluation of enzyme-modified solvent-extracted crambe seed meal by chemical analysis and rat feeding, Cereal Chem. 42, 507-514 (1965).

26. Dybing, C. Dean, and Don C. Zimmerman, Temperature effects on flax (Linum usitatissimum L.) growth, seed production, and oil quality in controlled environments, Crop Sci. 5, 184-187 (1965).

27. Dillman, A. C., Effect of climate on the yield and oil content of flaxseed and on the iodine number of linseed oil, U. S. Dept. Agr. Tech. Bull. No. 844, 1-69 (1943).

28. Sosulski, F. W., and R. F. Gore, The effect of photoperiod and temperature on the characteristics of flaxseed oil, Can. J. Plant Sci. 44, 381-382 (1964).

29. Howell, Robert W., and F. I. Collins, Factors affecting linolenic and linoleic acid content of soybean oil, Agron. J. 49, 593-597 (1957).

30. Canvin, David T., The effect of temperature on the oil content and fatty acid composition of the oils from several oilseed crops, Can. J. Botany 43, 63-69 (1965).

31. Sallans, H. R., Factors affecting the composition of Canadian oilseeds, J. Am. Oil Chemists' Soc. 41, 215-218 (1964).

32. Dybing, C. Dean, Influence of nitrogen level on flax growth and oil production in varied environments, Crop Sci. 4, 491-494 (1964).

33. Ford, J. H., and D. C. Zimmerman, Influence of time of flowering on oil content and oil quality of flaxseed, Ibid. 4, 653-656 (1964).

34. Canvin, David T., Formation of oil in the seed of Ricinus communis L., Can. J. Biochem. Physiol. 41, 1879-1885 (1963).

35. Chandra, K. S., Studies on the changes in fatty acid composition in developing seeds. I. Recinus communis (sic), J. Am. Oil Chemists' Soc. 41, 251-254 (1964).

36. Simmons, R. O., and F. W. Quackenbush, Comparative rates of formation of fatty acids in the soybean seed during its development, Ibid. 31, 601-603 (1954).

37. McKillican, Mary E., and R. P. A. Sims, Lipid changes in maturing oil-bearing plants. III. Changes in lipid classes in flax and safflower oils, Ibid. 40, 108-113 (1963).

38. Zeman, Ivo, and Vladimir Kratochvil, Changes in the composition of winter rape oil during seed maturation, Biologia Plantarum 9, 1-14 (1967).

39. Canvin, David T., Incorporation of acetate- 1- C^{14} into long chain fatty acids of some oilseed crops, Can. J. Botany 43, 71-74 (1965).

40. Downey, R. K., and B. M. Craig, Genetic control of fatty acid biosynthesis in rapeseed (Brassica napus L.), J. Am. Oil Chemists' Soc. 41, 475-478 (1964).

41. Kartha, A. R. S., and R. Narayanan, The biosynthesis of oil in ripening mustard seeds, J. Sci. Ind. Research 18c, 41-48 (1959). Chem. Abstr. 54, 1661h (1960).

42. Papathanasiou, G. A., K. J. Lessman and W. E. Nyquist, Evaluation of eleven introductions of crambe, Crambe abyssinica Hochst, Agron. J. 58, 587-589 (1966).

43. Earle, F. R., J. E. Peters, I. A. Wolff and G. A. White, Compositional differences among crambe samples and between seed components, J. Am. Oil Chemists' Soc. 43, 330-333 (1966).

44. Tookey, H. L., and I. A. Wolff, Activation and specificity of Crambe abyssinica seed lipase, J. Am. Oil Chemists' Soc. 41, 602-604 (1964).

45. Clark, John M., Jr., "Experimental Biochemistry," W. H. Freeman and Co., San Francisco, 1964, p. 52 and 61.

46. Van Handel, E., and D. B. Zilversmit, Micromethod for the direct determination of serum triglycerides, J. Lab. Clin. Med. 50, 152-157 (1957).

47. Rouser, George, A. N. Siakotos and Sidney Fleischer, Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots, Lipids 1, 85-86 (1966).

48. Duncombe, W. G., The colorimetric micro-determination of long-chain fatty acids, Biochem. J. 88, 7-10 (1963).

49. Itaya, Koichi, and Michio Ui, Colorimetric determination of free fatty acids in biological fluids, J. Lipid Res. 6, 16-20 (1965).

50. Novak, Milan, Colorimetric ultramicro method for determination of free fatty acids, Ibid. 6, 431-433 (1965).

51. Trout, David L., E. Harvey Estes, Jr. and Samuel J. Friedberg, Titration of free fatty acids of plasma: a study of current methods and a new modification, Ibid. 1, 199-202 (1960).

52. Horning, E. C., E. H. Ahrens, Jr., S. R. Lipsky, F. H. Mattson, J. F. Mead, D. A. Turner and W. H. Goldwater, Quantitative analysis of fatty acids by gas-liquid chromatography, Ibid. 5, 20-27 (1964).

53. Craig, B. M., and N. L. Murty, Quantitative fatty acid analysis of vegetable oils by gas-liquid chromatography, J. Am. Oil Chemists' Soc. 36, 549-552 (1959).

54. Craig, B. M., Varietal and environmental effects on rapeseed. III. Fatty acid composition of 1958 varietal tests, Can. J. Plant Sci. 41, 204-210 (1961).

55. Amenta, J. S., A rapid chemical method for quantification of lipids separated by thin-layer chromatography, J. Lipid Res. 5, 270-272 (1964).

56. Marsh, Julian B., and David B. Weinstein, Simple charring method for determination of lipids, Ibid. 7, 574-576 (1966).

57. Skipski, Vladimir P., Anson F. Smolowe, R. Clark Sullivan and Marion Barclay, Separation of lipid classes by thin-layer chromatography, Biochim. Biophys. Acta 106, 386-396 (1965).

58. Vroman, Hugh E., and Graeme L. Baker, Limits of detection of some lipids in thin-layer chromatography, J. Chromatog. 18, 190-191 (1965).

59. Blank, M. L., J. A. Schmit and O. S. Privett, Quantitative analysis of lipids by thin-layer chromatography, J. Am. Oil Chemists' Soc. 41, 371-376 (1964).

60. Privett, O. S., M. L. Blank, D. W. Codding and E. C. Nickell, Lipid analysis by quantitative thin-layer chromatography, Ibid. 42, 381-393 (1965).

61. Privett, O. S., and M. L. Blank, Charring conditions for the quantitative analysis of mono-, di-, and triglyceride by thin-layer chromatography, Ibid. 39, 520 (1962).

62. Rouser, George, Gene Kritchevsky, Claudio Galli and Dorothy Heller, Determination of polar lipids: quantitative column and thin-layer chromatography, Ibid. 42, 215-227 (1965).

63. Lines, J. G., Quantitative aspects of thin-layer chromatography applied to the lipids of biological materials, In "Biochemical Problems of Lipids," edited by A. C. Frazer, Vol. 1, Elsevier Publishing Co., New York, 1963, p. 17-23.

64. Frodyma, Michael M., Roland W. Frei and Donald J. Williams, The application of spectral reflectance to thin-layer chromatography, J. Chromatog. 13, 61-68 (1964).

65. Frodyma, Michael M., and Roland W. Frei, The determination by reflectance spectrophotometry of amino acids resolved on thin-layer plates, Ibid. 15, 501-509 (1964).

66. Hoagland, D. R., and D. I. Arnon, The water-culture method for growing plants without soil, California Agr. Exp. Station, Circular No. 347, 1-32 (1950).

67. Folch, Jordi, M. Lees and G. H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226, 497-509 (1957).

68. Morrison, William R., and Lloyd M. Smith, Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol, J. Lipid Res. 5, 600-608 (1964).

69. Bartlett, Grant R., Phosphorus assay in column chromatography, J. Biol. Chem. 234, 466-468 (1959).

70. Stefansson, B. R., and F. W. Hougen, Selection of rape plants with oil practically free from erucic acid, Can. J. Plant Sci. 44, 359-364 (1964).

71. Steel, Robert G. D., and James H. Torrie, "Principles and Procedures of Statistics," McGraw-Hill Book Company, Inc., New York, 1960, p. 111.

72. Greichus, Yvonne A., Personal communication.

APPENDIX

Plant to Plant Variation in Crambe

A preliminary study was conducted on plant to plant variations of seed weight, percent oil and percent erucic acid. After all seeds were harvested for the experiment II maturity studies, mature seeds were collected from individual plants. A paired data experimental design was used. This design enabled crock to crock variation to be separated from plant to plant variation. A total of 4 crocks were used and 49-52 seeds were collected from 2 individual plants within each crock. The seeds were analyzed as described in Experimental Procedures and the data are given below. No significant difference at the 0.05 level was found among any of the factors.

Pairs	Seed weight (mg/100 seeds)	Oil (%)	Erucic acid (%)
1	923	25.4	57.4
	947	20.7	50.9
2	830	23.9	57.6
	914	22.8	57.1
3	937	26.2	57.5
	928	25.6	56.7
4	800	20.7	57.1
	913	21.0	52.4