commercial resin were prepared as "R" and "C" enamels. "C" enamels contain zinc oxide to prevent sulfide staining and are used in cans packed with corn, peas, green beans, etc., while "R" enamels contain no zinc oxide and are formulated for use with acid-type foods, e.g., cherries, pumpkin and tomatoes. Pack tests of the "R" enamels (18 mg/4 sq in.) in water and cherries showed the commercial resin to be slightly superior. Both corn and dog food packs were prepared with "C" enamels coated at 21 mg/4 sq in. The commercial resin was again slightly better. Polymer films and the commercial resin were also baked for 10 min at 350F,375F,400F and 415F. Since both coatings exhibited equal preprocessing fabrication and water-pasteurization resistance at all bakes, a wide latitude of curing schedules is indicated.

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Mustard Seed Processing: Essential Oil Composition[1]

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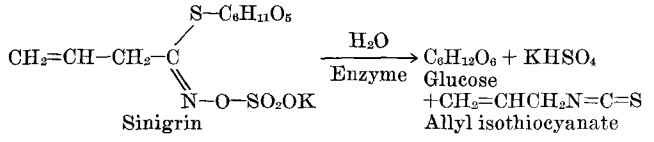
Abstract

Recent investigations into the processing of oriental mustard seed for production of livestock feed and of lipid-containing erucic acid have stimulated interest in the composition of the by-product essential oil. The development of a method for analysis of this oil by gas-liquid chromatography has led to the demonstration of an equilibrium reaction between the two main constituents. A similarity in composition and response to heat is shown between the natural essential oil and oils prepared synthetically. These observed similarities should lead to a better understanding of the enzymatic reaction responsible for release of this oil from the seed.

Introduction

ORIENTAL MUSTARD SEED, like other members of the Cruciferae family, contains thioglucosides, such as sinigrin, which are hydrolyzed to isothiocyanates (essential oils) by the action of enzymes naturally present in the seed. The following equation shows the main conversion products of sinigrin based on the thioglucoside structure of Ettlinger and Lundeen (3):



The composition of the essential oil derived from thioglucosides in oriental mustard seed (*Brassica juncea*) remains controversial. In their review article Vaughan and Hemingway (13) state that the oil is generally considered to be a mixture of allyl and crotonyl isothiocyanates. However, they also report the work of others who have concluded that allyl isothiocyanate is the only isothiocyanate present. Jensen et al. (6) of Denmark report allyl isothiocyanate as the sole volatile constituent of this seed.

Other compounds have been reported associated with allyl isothiocyanate derived from plant sources. Traces of allyl thiocyanate were reported by Schmidt (10) in allyl isothiocyanate recovered from an enzyme hydrolysis of sinigrin at 0C. This occurrence of organic thiocyanates seem to have been neglected until observed by Gmelin and Virtanen (4) in 1959. They reported allyl thiocyanate derived from sinigrin in *Thlaspi arnense* and benzyl thiocyanate derived from glucotrapaeolin in two *Lepidium* species. They suggested a dual enzyme system in some seeds to release either the isothiocyanate or the thiocyanate form from the thioglucoside.

In addition to the thiocyanate, allyl cyanide and carbon disulfide have also been reported with allyl isothiocyanate. Carbon disulfide is believed formed by chemical hydrolysis of the isothiocyanate (2).

Oriental mustard is being considered as a possible new crop in this country, and a process has been developed for removing the essential oil which otherwise contributes pungency to the meal (7,8). This oil, which has value for the pharmaceutical, flavoring, pesticide and plastics industries, can be recovered in a yield of 12 lb/ton of seed processed. If substantial quantities become available for marketing, more exact information regarding composition is needed.

Experimental

Materials and Equipment. Montana-grown oriental mustard seed, containing 4.5%moisture, 26% protein, 37% lipid and 0.7% isothiocyanate, was used throughout these studies. The commercial sample of allyl isothiocyanate was "practical grade" purchased from Distillation Products Industries (16).

Equipment for recovering essential oil from the seed was that described by Mustakas et al. (8) and consisted of a steam-jacketed stainless-steel vessel capable of processing 4,000 g seed and fitted with a steam-sparging coil, meshing-rod agitation system, condenser and spray nozzle. A Beckman Model GC-2A was used for gas-liquid chromatography (GLC) determinations. The column was 6-ft, 0.25-in. stainless steel packed with acid-washed celite 545 mesh size 80–100, coated with 20% Apiezon M. A Spinco MS amino acid analyzer was used for determination of the sulfur-containing amino acids, methionine and cystine. Synthetic oils were prepared in a 1,000-ml flask equipped with magnetic stirrer, condenser, receiver flask and vacuum pump.

Recovery of Natural Essential Oil. The seed was tempered by addition of water to 7% moisture and rolling in a sealed drum for 2 hr. Flakes of 0.005-in. average thickness were prepared either by cracking on corrugated rolls set at 0.002-in. clearance followed by rolling through smooth rolls or by a double pass through smooth rolls. About 4,000 g of the prepared

[1] Presented at AOCS Meeting in Atlanta, 1963.
[2] A laboratory of the No. Utiliz. Res. & Dev. Div., ARS, USDA.

flakes were charged to the converter-cooker and moistened to 30%. After mixing at room temp for 5 min, the charge was heated to 55C and held at this temp for 15 min to promote enzymatic hydrolysis of the thioglucoside to isothiocyanate. The myrosin enzyme system present naturally in the seed was sufficient to catalyze the reaction. Following this conversion period, live steam was admitted to the charge and the volatile essential oil was distilled at 100C. The essential oil was recovered from approx 2,500 ml condensate by centrifuging and removing as the lower layer. Ca. 80% of the total analyzable isothiocyanate in the seed was recovered by this technique; the remainder was discarded in the aqueous phase. When recovery was conducted under vacuum, a solid carbon dioxide trap served to condense the essential oil.

Preparation of Synthetic Esstential Oils. Synthetic essential oils were prepared via the reaction either of allyl amine and carbon disulfide or of potassium thiocyanate and allyl bromide according to the following procedures (9).

Approximately 25 g allyl amine were added dropwise to 50 g carbon disulfide with rapid agitation at 0C. (The reaction was highly exothermic and could not be controlled at room temp.) The dithiocarbamate intermediate after recovery by filtration and air-drying was dissolved in absolute ethanol. To this solution was added 200 ml of a 25% aqueous solution of lead nitrate at room temp. After 2 hr agitation, during which time the temp of the mixture rose to 33C, the charge was distilled. The volatile oil (ca. 5 g) was recovered by steam distillation at 100C as the lower phase. Attempts to recover the essential oil by vacuum distillation resulted in only traces of product.

In the second method, 121 g potassium thiocyanate in 200 ml water were added to 500 ml 95% ethanol. The solution was cooled to 0C in an ice-salt bath, and 100 g allyl bromide were added slowly while maintaining a constant temp. After holding for 1 hr, the crude mixture was distilled under vacuum (35C) to recover approx 20 g of the essential oil as a lower phase clarified by centrifuging. Essential oil was also prepared by reacting at room temp and distilling at atmospheric pressure (100C). The importance of the isolation temp upon essential oil composition will be discussed later.

Sulfur Balance Studies. The quantity of total sulfur in mustard was determined by the method of Shaw (11) in which 0.5 g defatted meal was digested in a nitric-perchloric acid mixture to convert all sulfur to sulfate. Sulfate was determined gravimetrically. Volatile sulfur was determined from total sulfur contents of the meal before and after removal of the essential oil.

Inorganic sulfate released by thioglucoside hydrolysis was determined by slurrying 10 g defatted meal in 250 ml distilled water and holding at 45–55C for 1 hr followed by filtration and precipitation with barium chloride. The filter cake was reslurried in 200 ml water at 100C, filtered and washed three times with 300 ml hot water; second extract and washings were combined with the first extract for precipitation. Barium sulfate was determined by ashing in the conventional manner.

Volatile isothiocyanate sulfur in the seed was determined by the method of Wetter (15). Volatile thiocyanate sulfur was taken as 10% of the volatile isothiocyanate sulfur (a relationship established by GLC studies).

TABLE I

Comparative GLC Analysis of Essential Mustard Oils, Both Synthetic and Natural, Recovered at 100C[a]

Essential oil	Avg composition, %	
	Allyl isothiocyanate	Allyl thiocyanate
Synthetic		
Commercial practical grade	91.5	8.5
From carbon disulfide and allyl amine	94.7	5.3
From allyl bromide and potassium thiocyanate	86.7	13.3
Natural		
From oriental mustard	90.5	9.5

[a] Analyses are reported free of carbon disulfide.

Amino acid sulfur was determined from the analysis of methionine and cystine obtained by acid hydrolysis, followed by analysis on the Spinco analyzer (12).

Isothiocyanate content of the essential oil was obtained by diluting a 0.5-g sample to 500 ml with ethanol and analyzing a 10-ml aliquot. The method is similar, except for concn, to that reported by Guenther (5).

Column Preparation and Operation. The solid support was coated with Apiezon M by soaking it in a chloroform solution then air-drying. Prolonged conditioning of the column with helium at 115C produced a gradual improvement in the separation of thiocyanate from isothiocyanate. The column was operated at 115C with a helium rate of 60 cc/min. The thermal conductivity cell was operated at 400 ma; sample size was 1.0 μl with the attenuation control set high enough to eliminate base-line instability.

Results and Discussion

Essential Oil Composition. A typical chromatogram of the essential oil of oriental mustard indicates the following composition: carbon disulfide 1%, allyl thiocyanate 9%, allyl isothiocyanate 90%. Retention times relative to carbon disulfide were allyl thiocyanate 4.6 and allyl isothiocyanate 6.7. These components were identified by comparing retention times with known compounds. Allyl isothiocyanate was further confirmed by its reaction with ammoniacal silver nitrate and allyl thiocyanate by isomerization to allyl isothiocyanate as discussed later. The quantity of allyl isothiocyanate actually varied from 88–93% and agreed within 0.7% of corresponding values obtained by the chemical method.

The natural oil is very similar to commercial "practical grade" allyl isothiocyanate and to synthetic essential oils prepared from allyl bromide and potassuim thiocyanate or carbon disulfide and allyl amine as shown in Table I. Thus, the natural essential oil should be a satisfactory substitute in applications where the synthetic oil is now in use or where its use has been considered.

Equilibrium Reaction of Thiocyanate and Isothiocyanate. When allyl thiocyanate was prepared by the reaction of allyl bromide and potassium thiocyanate as suggested by Noller (9), only 10% of the product consisted of the thiocyanate. The remaining 90% was identified as the isothiocyanate isomer. When the steam-distillation recovery step was conducted under vacuum, GLC analysis indicated a much higher concn of allyl thiocyanate. Heating the oil on a steam bath for 2 hr resulted in an increased quantity of isothiocyanate. This increase is consistent with other reports which have shown that a rearrangement of certain organic thiocyanates to the corresponding isothiocyanates occurs with heat (1). According to Noller (9),

TABLE II

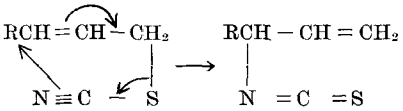
Comparative GLC Analysis of Essential Mustard Oils, Both Synthetic and Natural, Recovered at 35C and Equilibrated at 100C[a]

Essential oil	Avg composition, %	
	Allyl isothiocyanate	Allyl thiocyanate
Recovered at 35C from		
Allyl bromide and potassium thiocyanate	55.0	45.0
Allyl amine and carbon disulfide	94.7	5.3
Oriental mustard	96.0	4.0
Equilibrated at 100C from		
Allyl bromide and potassium thiocyanate	90.1	9.9
Allyl amine and carbon disulfide	92.3	7.7
Oriental mustard	92.4	7.6

[a] Analyses are reported free of carbon disulfide. Samples were equilibrated by heating in sealed bottles on a steam bath for 2 hr.

the reaction of allyl amine and carbon disulfide, followed by steam distillation from a mixture containing lead nitrate, should yield only allyl isothiocyanate. When essential oil was prepared by this method, allyl isothiocyanate was the major component comprising 94.7% of the total, the remainder being allyl thiocyanate (Table II). Heating this oil on a steam bath for 2 hr decreased the isothiocyanate content to 92.3%. The natural essential oil composition was affected similarly with application of heat.

Apparently heating any of the essential oils leads to an equilibrium mixture of allyl isothiocyanate and allyl thiocyanate. This equilibrium demonstration confirms a recent prediction that some of the reaction mixtures containing isothiocyanates and thiocyanates together may be equilibrium mixtures of the two forms (1). A suggested mechanism for rearrangement of thiocyanate is shown in the following equation where R is a methyl or ethyl radical (1):



Allyl thiocyanate is a special case of this mechanism in which the R group is hydrogen.

The present demonstration of an equilibrium reaction between the thiocyanate and the isothiocyanate forms accounts for their presence together in the essential oil of mustard. A dual enzyme system is not necessary, therefore, to account for the release of both compounds from sinigrin since the release of either form will give rise to the other by chemical rearrangement. This result is in contrast to that observed with benzyl isothiocyanate where the formation of the thiocyanate form is apprently enzymatic (14). Although the evidence is not conclusive, the increased isothiocyanate content of natural essential oil with reduced temp indicates that isothiocyanate is initially formed. This conclusion seems confirmed because this form is the one almost exclusively reported for members of the Cruciferae plant family.

Thioglucoside Content. All the compounds identified by GLC analysis of *Brassica juncea* essential oil have been reported at least in traces by other workers as hydrolysis compounds of sinigrin (2). To determine whether sinigrin is the only thioglucoside present in the seed, a sulfur balance was attempted from analysis of sulfur constituents in the meal (Table III). In column 1 of Table III, all figures are based on the assumption that sinigrin is the only thioglucoside present. Volatile sulfur was obtained from an analysis for allyl isothiocyanate and allyl thiocyanate released during enzymatic conversion and recovered by steam stripping. The inorganic sulfate value was taken as equivalent to the volatile sulfur since sinigrin

TABLE III

Evidence of Nonvolatile Isothiocyanate in Hydrolyzed Mustard Seed Meals by Sulfur Balance

Sulfur compound	Sulfur in g/100 g meal[a]	
	Column 1	Column 2
Volatile sulfur	0.44	0.40
Inorganic sulfate sulfur	0.44	0.69
Amino acid sulfur	0.24	0.24
Total	1.12	1.33
Total sulfur by oxidation		1.78

[a] Defatted, moisture-free basis.

hydrolysis releases a gram of inorganic sulfate for each gram of isothiocyanate or thiocyanate sulfur. The amino acid sulfur derived from quantitative amino acid analysis is also shown. The sum total of column 1 represents the total sulfur expected, under the assumed conditions.

In column 2, volatile sulfur was determined from total sulfur values obtained on the meal before and after enzyme conversion and steam stripping. This value, then, represents the actual sulfur in the form of volatile compounds released during conversion. Inorganic sulfate sulfur was determined quantitatively in the meal after hydrolysis by precipitation with barium chloride. The amino acid sulfur reported in column 1 is repeated in column 2. The total sulfur value in column 2 was derived by oxidation of all sulfur compounds to sulfate and, therefore, represents the actual sulfur in the unconverted meal. Since the volatile sulfur figures of the two columns compare favorably, it would seem that sinigrin is the only thioglucoside in the seed which yields volatile sulfur compounds during hydrolysis. However, a comparison of values indicates the presence of more inorganic sulfate sulfur and total sulfur than can be accounted for by the sinigrin present. These differences may indicate that other thioglucosides are present which yield nonvolatile isothiocyanates upon hydrolysis. Additional work is necessary before definite conclusions as to the total number of thioglucosides can be drawn.

Absence of Allyl Cyanide. There was no evidence of allyl cyanide in any of the essential oil chromatograms although its presence in such oils has been reported many times (2). Its absence here may result from the flake preparation used for enzyme reaction. Allyl cyanide reportedly varies in amount depending upon how thoroughly the seed has been macerated before conversion. Where the seed is thoroughly macerated, as in the present meals, little cyanide formation is found. Also the conversion conditions used here are different from those of previous workers in the amount of moisture present for conversion. These workers used water slurries of the meal, whereas the present conversions were conducted in a comparatively dry state.

Application of GLC. GLC should be a very useful tool for volatile isothiocyanate analysis. Sample analysis is rapid, only small quantities are required, and more important, the presence of organic thiocyanates can readily be determined. Although data reported here were developed from pilot-plant preparations, the general method should be applicable to an analytical technique employing smaller quantities of meal with conversion and isolation procedures similar to those of Wetter (15). In the past the thiocyanate form has generally been overlooked when the conventional method of conversion of isothiocyanate to thiourea, followed by paper chromatographic separation, has been used.

There is a possibility that some conversion of thio-

cyanate to isothiocyanate or isothiocyanate to thiocyanate occurs within the column so that GLC analysis may not represent the exact ratio of these two forms in a sample. Unfortunately, satisfactory resolution was not obtained when operating the column at a temp lower than 115C. This disadvantage is not serious when essential oils are recovered by the conventional atmospheric steam-distillation technique since they would be driven to an equilibrium mixture before analysis.

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Activation and Specificity of *Crambe abyssinica* Seed Lipase

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Abstract

The lipase of *Crambe abyssinica* seed is not active in crushed seeds stored at 5–7% moisture at room temp. Lipase activity is very low even at 10–15% moisture: free acids in the crambe oil increased 1.6% to a total of 4% in 6 weeks. At higher moisture levels the lipase is active, hydrolyzing the oil in 5–7 weeks. Oil in whole seed is resistant to lipolysis when stored air-dry. These results indicate good stability of the oil during seed storage and the usual steps in seed processing.

Crambe lipase hydrolyzes triglycerides in a nearly random fashion. The hydrolysis pattern indicates a small preference for the shorter chain acids (C_{16} and C_{18}), but no specificity for position within the triglyceride is apparent.

Introduction

RECENT INTEREST IN *Crambe abyssinica* Hochst. ex. R. E. Fries as a potential economic plant depends upon the high erucic acid content of its oil-bearing seed (1,2). For the seed to be economically harvested and stored, the oil must be relatively stable to autolysis while still in the seed. Common oilseeds are stable to storage: the oils in air-dry seeds of flax, mustard and rape are stable for 18 months when stored with normal ventilation (3); the oil in castor beans for 5 years (4).

Oil may be stable in whole seeds, but not in cracked or broken seeds. Broken castor beans contain more than 5% free acid in the oil after 5 years of storage compared to 1% free acid in oil from whole beans (4). A more startling example is provided by the seed of the Indian ironweed (*Vernonia anthelmintica*), which contains an easily activated lipase. Whole seeds survive 1–3 years of storage at 6–7% moisture with no significant change in free acid content of the oil (5,6), whereas the oil from coarsely ground seed, stored air-dry for 26 days, contains approx 40% free acid, or more than double its initial free-acid content (6). These figures were obtained by the usual Soxhlet extraction techniques following a 2- and a 26-day storage period after grinding. More recently an initial fatty acid content of 0.5% has been obtained by rapid extraction of freshly ground seed (7).

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Oil in seed at 7–8% moisture of *Dimorphotheca* and *Lesquerella* is stable for many months at room temp. As in ironweed, the oil in crushed seed is hydrolyzed rapidly: oil in crushed *Dimorphotheca* seed contains nearly 10% free acid after 21 days; that in *Lesquerella* seed ca. 15% after only 7 days (8).

Stability of the oil in *Crambe abyssinica* seed was investigated in both crushed and intact seed. Studies of crushed seed held at various moisture levels were undertaken to determine the limits of oil stability during seed processing. Oil in stored seed was analyzed to provide preliminary data on oil stability expected in intact seed from harvest to time of processing.

Several types of positional specificity of lipases are possible: a) cleavage of the triglycerides may be random, as with the lipase of castor beans (9); b) the 2-position may be preferentially split, as is suggested by the 1,3-divernolin present in partially hydrolyzed vernonia oil (6); or c) the 1- and 3-positions may be more easily cleaved, as shown by pancreatic lipase (10). If crambe lipase resembled pancreatic lipase and produced 2-monoglycerides plus the free acids from the 1- and 3-positions, the enzyme might be used to prepare purified erucic acid since it is known that, in the crambe triglycerides, erucic acid occurs exclusively in the 1- and 3-positions (11). However, it was found that crambe lipase catalyzes a nearly random hydrolysis of triglycerides.

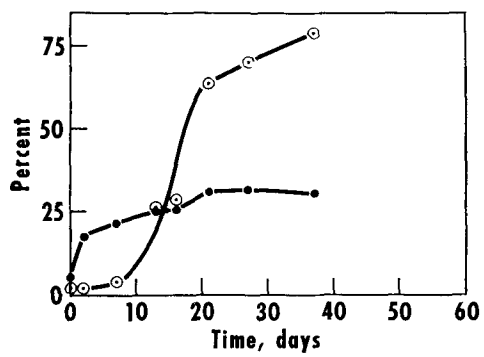

FIG. 1. Lipase activity in flaked crambe seed stored at high-moisture content. ●—● per cent moisture in flakes; ⊙—⊙ per cent hydrolysis of oil.