Table IV. Quantitative Comparison of High Molecular Weight Compounds from Selected Pyrolyses (Gram/Mole Pyrolyzed)

Pyrolysis	Phenan.–anthr.	Fluor-anthene	Py-rene	Chrysene (mixt.)
Leucine	0.25	0.09	0.11	0.05
Amino acid mixt.	0.26	0.05	0.12	0.06
Protein	0.22	0.04	0.11	0.06
Phe–Trp mixt.	0.5	0.06	0.11	0.06

of 2,5-piperazinedione, the proposed cyclic intermediate in the glycine pyrogenesis of hydrogen cyanide, produced over twice as much hydrogen cyanide (per molecule of N orginally present) as the pyrolysis of glycine itself.

A comparison was made between the amounts of the identified high molecular weight compounds obtained from the protein and amino acid mixture pyrolyses and previously published amounts of the same compounds from the pyrolysis of individual amino acids (*cf.* Patterson *et al.*, 1969, 1971; Smith *et al.*, 1973). The results of the protein pyrolysis and amino acid mixture, in respect to high molecular weight compounds, were very similar to those of leucine (Table IV). This is significant because almost all of the individually pyrolyzed amino acids gave much higher amounts of these substances per mole pyrolyzed than did leucine. If, in the case of high molecular weight compounds, the pyrolysis of a mixture of amino acids reflected an average of individually pyrolyzed amino acids, the amounts of high molecular weight compounds would have been higher.

Comparison was also made between the identified high molecular weight compounds from the protein and amino acid mixture pyrolyses and the published amounts of the same compounds from the pyrolysis of an equimolar mixture of phenylalanine and tryptophan (Table IV). The pyrolysis of the phenylalanine–tryptophan mixture represented the pyrolysis of a mixture of only aromatic amino acids. Yet, it is interesting to note that the phenylalanine–tryptophan results are similar to the protein and amino acid mixture results, in respect to the high molecular weight compounds.

Our pyrolytic studies at 850° show that pyrolyses of amino acids give the same results qualitatively and, with a few exceptions, quantitatively, as those obtained by pyrolyses of protein having the equivalent amino acid composition. These results give added significance to our previous studies on pyrolysis of individual amino acids and of simple mixtures and should be useful in the future in predicting pyrolytic products of other proteins.

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Glucosinolate Determination in Cruciferous Seeds and Meals by Measurement of Enzymatically Released Glucose

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Hydrolysis of glucosinolates by thioglucoside glucohydrolase EC 3.2.3.1 (thioglucosidase) releases β-D-glucose. The glucose was measured colorimetrically after specific enzymatic oxidations with readily available reagents containing a glucose oxidase, peroxidase, and chromogen. By this procedure glucosinolates were determined successfully in seed extracts after interfering substances were removed with charcoal. Samples containing glucosinolates equivalent to 0.01–0.15 mg of glucose were analyzed with either added or endogenous thioglucosidase. In the procedure involving addition of thioglucosidase, endogenous enzyme was first heat-inactivated and free glucose was determined. This modification has been used in assessing changes during seed storage and processing. Crambe seed analyses by the two methods agreed and were within 10% of results by an established independent method. Relative standard deviation by three tests was 3.3, 4.2, and 6.5%. Test paper impregnated with the enzymes and chromogen permitted screening of plant breeding samples for glucosinolate content.

The plant family Cruciferae includes rape (*Brassica campestris, B. napus*), mustard (*B. juncea, B. hirta*), and crambe (*Crambe abyssinica*). Seeds of all members of the family so far examined contain glucosinolates (thioglucosides) which are the source of organic compounds that contribute to the flavor of these plants but which also may be harmful when the defatted seed meal is consumed in large amounts by livestock. Reviews are available on the chemistry of these compounds (Ettlinger and Kjaer, 1968), problems related to the use of the plants in food or feed (VanEtten and Wolff, 1973), and removal of glucosinolates by processing or by plant breeding (Tallent, 1972).

Analytical methods include estimating the split products, such as mustard oils and goitrin, formed from each glucosinolate under specific conditions of hydrolysis by

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thioglucosidase glucohydrolase EC 3.2.3.1. Indirect methods for estimating specific glucosinolates include those described by Appelqvist and Josefsson (1967), Youngs and Wetter (1967), and Daxenbichler *et al.* (1970). Total glucosinolates can also be determined indirectly by measuring the bisulfate ion (McGhee *et al.*, 1965; Tookey, 1973; VanEtten *et al.*, 1965) or glucose (Lein and Schön, 1969), which apparently are always formed by the thioglucosidase regardless of hydrolysis conditions.

First Keston (1956) and then Teller (1956) reported determination of glucose in body fluids by the action of glucose oxidase on glucose to form hydrogen peroxide, which in the presence of peroxidase reacted with a chromogen to form a colored product. Lein and Schön (1969) assayed total glucosinolate based on estimation of enzymatically released glucose, but they employed a different series of reactions culminating in formation of NADPH, which was measured by ultraviolet (uv) spectrophotometry. Bjorkman (1972) applied the enzymes used by Keston and Teller to the analysis of enriched glucosinolate fractions and pointed out that the procedure was not suitable for crude solutions that contain colored or other interfering substances. We overcame these difficulties by treating hydrolyzed extracts of crambe and *Brassica* meals with charcoal. This modification permits commercially available reagents to be employed to determine total glucosinolates by a rapid simple procedure that is particularly useful in cruciferous plant-breeding programs. With the method one technician can estimate the glucosinolate content of 36 samples in three groups of 12 each per day. Variations of the procedure were applied to estimation of thioglucosidase activity, the extent of prior glucosinolate hydrolysis, and the amount of glucose (from glucosinolate hydrolysis) chemically bound during experimental seed processing and storage. Because of the simplicity and short time required, these procedures should be useful in controlling processing conditions. As demonstrated by Lein (1970), we confirmed that a test paper impregnated with the reagents can be utilized advantageously for preliminary screening.

EXPERIMENTAL SECTION

Materials. The reagent for the glucose determination (Glucostat X4) was purchased from Worthington Biochemical Corp., Freehold, N. J., and the test paper (Tes-Tape) for qualitative examination came from Eli Lilly & Co., Indianapolis, Ind. Glucostat X4 consists of β-D-glucose-oxygen oxidoreductase (EC 1.1.3.4) and donor-hydrogen peroxide oxidoreductase (EC 1.11.1.7) in a glucose reagent and *o*-dianisidine as the chromogen. In Tes-Tape, *o*-tolidine is the chromogen (Comer, 1956). Other purchased materials were sinigrin (allylglucosinolate, Aldrich Chemical Co., Inc., Milwaukee, Wis.), Darco G-60 Activated Carbon (Atlas Chemical Industries, Inc., Wilmington, Del.), and Celite analytical filter aid (Johns-Manville, Joliet, Ill.).

Crude thioglucosidase was prepared from yellow mustard seed (*Brassica hirta*) as described by Wrede (1941). The phosphate buffer pH 6.8 to 7.0, 0.05 *M*, was prepared according to Gomori (1955). For all analyses, cleaned *Brassica* or dehulled crambe seed was either ground in a hammer mill or flaked between smooth rolls and extracted with petroleum ether at room temperature. The defatted seed meal was air dried and reground, if required, to pass a 40-mesh screen. Optical density measurements were made with a Spectronic 20 Bausch & Lomb spectrophotometer with matched 0.5-in. o.d. Pyrex test tubes.

Calibration Curve. A 200-mg portion of glucose reagent in 10 ml of water was added to 20 mg of chromogen dissolved in 80 ml of water within 2 hr of use. The final volume was made to 100 ml. In the matched tubes, 1 ml of each glucose standard was mixed with 3 ml of the combined reagent solution. In accordance with well documented instructions provided with Glucostat X4, one drop of 6 *N* hydrochloric acid was added to stop color development after 10 min at room temperature. The stabilized color in the acid solution was read at 430 nm. Because the calibration curve varied from day to day, solutions containing 0, 0.05, 0.10, and 0.15 mg of glucose/ml were run with each group of determinations.

Treatment of Extracts with Charcoal. Inhibition of color development was assessed by analyses (without charcoal treatment) of test solutions that contained 0.1 mg of glucose plus heated aqueous extracts from 0 to 10 mg of crambe seed meal/ml. Glucose absorption was studied by mixing 0.1 to 2.0 g of charcoal in 20 ml of pH 7 buffer containing 0.1 mg of glucose/ml. After mixing, the solution was filtered and 1-ml samples were analyzed as described for the calibration curve.

Method 1. Glucose Released by Added Thioglucosidase. Samples of 50, 100, or 200 mg of seed meals were weighed into graduated 40-ml centrifuge tubes and heated in a boiling water bath for 2 min, after which 19 ml of the boiling pH 7 buffer was added. The tubes were heated for an additional 2 min in the bath and cooled to room temperature before 1 ml of aqueous thioglucosidase solution containing 5 mg/ml of the crude enzyme was added. After thorough mixing of contents, the tubes were held in a 35° constant temperature bath for 45 min. After hydrolysis the volume in each tube was adjusted to 20 ml of distilled water. About 0.2 g of charcoal estimated by volume was added. The content of each tube was thoroughly mixed by shaking and was filtered through 7-cm Whatman no. 2 filter paper. Cloudy filtrates were clarified by refiltration with vacuum through Celite on an 11-mm Hirsch funnel. Either 1 ml of the final filtrate or 0.5 ml of it and 0.5 ml of water were taken for analysis by treatment with 3 ml of the combined reagent, as described for the calibration curve.

To determine preformed free glucose, the same procedure was followed except that no thioglucosidase was added.

Method 2. Glucose Released by Endogenous Thioglucosidase. To each sample was added 20 ml of the buffer solution at room temperature. After thorough mixing, each sample was autolyzed at 35° for 45 min. Analyses were completed as in Method 1 after the autolyzed mixtures were heated to boiling to coagulate protein, cooled, and the volumes adjusted to 20 ml.

Calculations.

$$\% \text{ glucosinolate} = \frac{\text{glucose wt} \times \text{factor}}{\text{sample wt}} \times 100$$

where the glucose weight in milligrams was read from the calibration curve and appropriately corrected if preformed free glucose was determined. Sample weight was the milligrams of air-dried defatted seed meal represented by the aliquot analyzed. Though a mixture of glucosinolates is nearly always present in cruciferous seeds, for simplicity the glucose-glucosinolate conversion factor was based on the most predominant one. When this was (*R*)-2-hydroxy-3-butenylglucosinolate (progoitrin in *B. napus*) or its (*S*)-epimer (*epi*-progoitrin in *C. abyssinica*), the factor was calculated as follows.

$$\text{factor} = \frac{\text{mol wt of progoitrin}}{\text{mol wt of glucose}} = \frac{427.1}{180.2} = 2.37$$

Analogously calculated factors were 2.29 for 3-butenylglucosinolate (*B. campestris*), 2.20 for allylglucosinolate (*B. juncea*), and 2.27 for *p*-hydroxybenzylglucosinolate. Molecular weights for these glucosinolates were those of the potassium salts except for *p*-hydroxybenzylglucosinolate, which was for the anion.

Reference Methods. For comparison of results by Methods 1 and 2 with published procedures, samples were

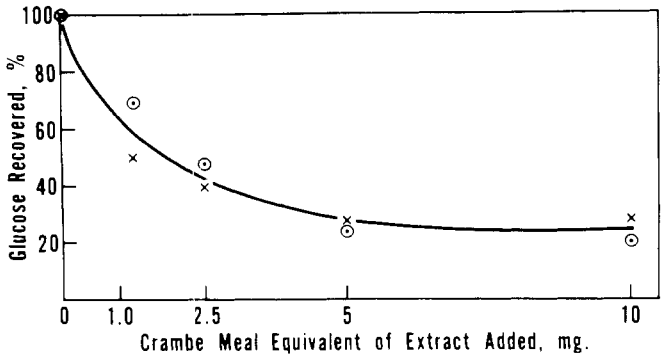

Figure 1. Interference by water-soluble components in two different crambe meal preparations (⊙ and X). Each point represents the average of duplicate analyses (without charcoal treatment) of solutions containing meal extract and 0.1 mg of glucose.

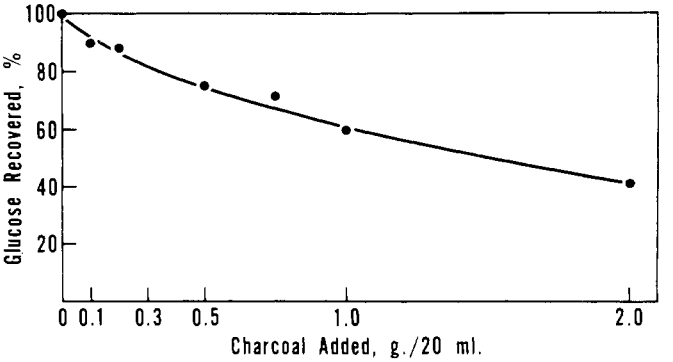

Figure 2. Charcoal adsorption of glucose from solutions containing 2 mg/20 ml. Each point represents the average of four or more determinations.

analyzed by slight modifications of the spectrophotometric procedure of Appelqvist and Josefsson (1967) and of the titrimetric procedure of Tookey (1973). Modifications in the spectrophotometric procedure included using 100 mg of defatted meal, omitting ascorbic acid during thioglucosidase hydrolysis, extracting goitrin with dichloromethane, and measuring uv absorption in ethanol instead of ethyl ether. In the titrimetric procedure, 0.5-g samples of seed meals were mixed with 20 ml of water and 10 mg of crude thioglucosidase. Acid released by hydrolysis of the glucosinolates was titrated with standard NaOH dispensed by a radiometer titrimeter set to maintain a constant pH of 5.3.

Estimating Glucosinolate with Test Paper. For each test, about 20 mg of seed meal was transferred to the well of a white test plate and 0.1 ml of water was added. After autolysis for 15 min, one end of a ½ in. strip of test paper was dipped into the meal slurry. After 1 min, the color developed on the strip wetted by capillary action was compared either with the color developed from seed meals of known glucosinolate content treated similarly or with the four shades of green related to glucose concentration on the Tes-Tape container.

Seed Meal Treatments. Relative thioglucosidase activity of treated meals was determined by autolysis of 100-mg samples in 1 ml of water for 5, 15, 30, and 60 min. Thioglucosidase activity was stopped by adding 19 ml of boiling buffer. After cooling to room temperature and adjusting to 20 ml, the solutions were analyzed for free glucose as described for Method 1 without added thioglucosidase.

The relationship of temperature and moisture treatment to the amount of free glucose from glucosinolate hydrolysis was studied in a reference crambe meal prepared as described for meal 5 by VanEtten *et al.* (1969). It contained no glucosinolate, 3.2% free glucose, and 8.8% moisture. Samples (100 mg) of this meal were weighed into 15 × 45 mm vials with screw caps. After adding various amounts of water, the vials were tightly capped and heated at 95–98° for 15, 30, 60, and 120 min. After treatment, the meal was made to 20-ml volume with pH 7 buffer and analyzed for free glucose as described for Method 1 without added thioglucosidase.

RESULTS AND DISCUSSION

Glucose Release and Quantitative Determination. Without charcoal treatment, recovery of glucose added to seed extracts ranged from 20 to 70%, depending on the amount of crambe seed meal from which the extract was made (Figure 1). Even after the extract at room temperature was treated with powdered cellulose, silica gel, acidic alumina, Celite, Sephadex G-10, or an insoluble poly(vinylpyrrolidone) powder sold commercially for removing tannins, the interference remained. According to Salomon and Johnson (1959), charcoal removes interferences from extracts of natural products but also adsorbs glucose. We found that the amount of glucose adsorbed varied with the amount of charcoal added, as illustrated in Figure 2. When glucose concentrations ranged from 0.01 to 0.15 mg/ml, 0.2 g of charcoal/20 ml (as used in Methods 1 and 2) adsorbed 5 to 15% (average 12%) glucose. This amount of charcoal was added in the routine analytical procedures because sometimes this much was required to adsorb all the color from the extract. Except for the most accurate work, a correction was not made for this loss. A small correction from the blank in Method 1 was also not applied. This omission contributed a positive error equal to 0.1 to 0.4% glucosinolates from seed meals that had received no treatment other than grinding of the seed and extraction of the oil at room temperature with petroleum ether. The negative error due to glucose adsorption on charcoal and the positive blank approximately offset each other.

In preliminary tests, the thioglucosidase hydrolysis in Method 1 and the autolysis with native thioglucosidase in Method 2 required 30 min in a 35° bath for completion. To be certain of complete hydrolysis, 45 min was allowed. Ascorbic acid, a known thioglucosidase activator, was not added because it is an inhibitor of the peroxidase in the reagent (Comer, 1956).

Analyses (12 replicates) of a crambe meal by Method 2 gave an average of 9.41% *epi*-progoitrin, which makes up 95% of the glucosinolates in this species (Tallent, 1972). Precision is indicated by a standard deviation of 0.56 and a relative standard deviation (RSD) of 6.5%. Another measure of precision was provided by results from analyses of 30 *B. napus* accessions by Method 2. The standard deviation calculated from the difference between duplicates was 0.18, and RSD was 4.2%. A third determination of RSD (3.3% for combined data, from Methods 1 and 2) is described in the next section.

In eight analyses run on 50-mg samples of *B. napus* by Method 2, glucose was added to four of them equal to 0.1 mg/ml. The average glucose from hydrolysis of the glucosinolates in each sample containing no added glucose was 0.046 mg/ml. This amount was subtracted from the glucose found in the samples to which glucose had been added. The average recovery calculated from the remaining glucose was 86%; range was from 82 to 88%. If the net added glucose recovered was corrected for 12% loss on the charcoal, recovery was 97%. This experiment indicates that to obtain maximum accuracy, corrections should be made for both glucose in the sample before hydrolysis and glucose adsorbed by charcoal.

Glucosinolate Content of Meals by Different Methods. On the basis of significance at the 95% confidence level, the following observations are made. Applied to crambe seed meals, Methods 1 and 2 gave means for 15 accessions that did not differ significantly from each other but were significantly higher (10%) than the mean arrived

Table I. Glucosinolate Content of Crambe Seed Meals Determined by Four Methods[a]

Description	Mean, %	RSD,[b] %
Glucose determination (Method 1)	9.34	5.4
Glucose determination (Method 2)	9.39	6.6
Spectrophotometric procedure[c]	8.49	9.1
Titrimetric procedure[d]	10.76	14.5

[a] Computed on dry basis as *epi*-progoitrin from single analyses of 15 accessions (samples from individual plots or fields) of defatted seed meals randomly selected from a plant-breeding program. [b] RSD = relative standard deviation. [c] Slightly modified method of Appelqvist and Josefsson (1967). [d] Slightly modified method of Tookey (1973).

at by spectrophotometry and lower (13%) than the mean by titrimetry (Table I).

The mean from each of the five *Brassica* taxons was slightly lower by Method 2 than by Method 1 (Table II). The means by the spectrophotometric procedure were significantly lower, ranging from 74 to 84% of the means by Method 1 and 78 to 89% of the means by Method 2. The *p*-hydroxybenzylglucosinolate found in *B. hirta* agreed with the results of Josefsson (1970).

No interaction was evident between taxons and methods on the basis of plotted results for one method *vs.* another and on analysis of variance. Any of the four methods in Tables I and II would be consistent for selection of accessions that are low in glucosinolates even though the glucosinolate content of a given sample varies with the method applied. Accordingly, for a plant-breeding program, Method 2 may be chosen because it is the simplest, is rapid, and requires only small amounts of seed meal.

The RSD's given in Table II include contributions from the methodology and differences between accessions. Mathematical removal of variation from accession differences and pooling of residual values gives RSD 3.3%.

The high RSD of the 14 accessions of *B. napus* reveals a marked variability within the group and favors selection for further plant breeding. Such variability is not so evident in the other *Brassica* taxons and crambe in Tables I and II.

By the titrimetric references method, crystalline sinigrin was 90% allylglucosinolate. In eight analyses made by Method 1 (except that the charcoal treatment was omitted since no interfering substances were present), an average allylglucosinolate composition of 89.5% was obtained. The range was 86 to 96%. No further attempt was made to determine the relative accuracy of the methods. In developing the spectrophotometric method, Appelqvist and Josefsson (1967) show incomplete recovery of allylisothiocyanate, sometimes as low as 83 to 86%. This loss of mustard oil may explain only in part differences among methods reported here for rapeseed and mustard species that contain large amounts of mustard oil-forming glucosinolates. Methods 1 and 2 may give high results because any glucose from sources other than the glucosinolates contributes to the measurement. Similarly, acidity from any other source than that from the glucosinolates generated during thioglucosidase treatment would introduce a positive error in the titrimetric procedure.

Rapid Screening by Test Paper. The rapid test paper procedure was evaluated with 200 rapeseed samples from a breeding program. As checked by quantitative Method 2, 13 accessions out of 15 were correctly grouped as containing 4.5% or less glucosinolate. However, 21 of 93 accessions containing 4.5 to 6.5% glucosinolates were also placed in the group containing 4.5% or less. None of the 92 accessions containing 6.5 to 9.1% were placed in the group of 4.5% or less. On the basis of these data, all high-glucosinolate seed meals can be rapidly eliminated in a search for low glucosinolate-containing *Brassica* seed.

Determination of Processing Effects. By selection of an appropriate procedure for glucose, a treated seed or meal may be analyzed for thioglucosidase activity, for extent of hydrolysis of glucosinolates to give free glucose, and for loss of free glucose (from glucosinolate hydrolysis) through chemical reaction with other constituents.

Processing *Brassica* or crambe oilseeds by prepress solvent extraction and heating gives a better quality feed from the meal if processing conditions are controlled so that no thioglucosidase activity remains in the meal and so that hydrolysis of the glucosinolates is held to a minimum (Youngs, 1965). Results of testing for such crambe meals are given in Figure 3. Prepress solvent extraction given to meal B reduced thioglucosidase activity markedly compared to the activity in meal A. However, in meal B, hydrolysis was more than 80% complete in 1 hr. Heating meal B as described to give meal C removed all thioglucosidase activity.

Loss of reducing sugars, as related to processed mustard seed meal color and nutritional quality of the protein, has been demonstrated by McGhee *et al.* (1964). The same phenomenon with crambe seed appears to be related to glucose from glucosinolate hydrolysis. The sensitivity of free glucose in crambe seed meals to heat treatments (95–98°) at low moisture contents is shown in Figure 4 (meals A and B). In contrast, the glucose in meal C is stable when given the same heat treatment in the presence of larger amounts of water.

Binding of free glucose also accompanies loss of protein efficiency. A crambe meal that had been extracted with aqueous acetone to remove all glucosinolates and derived harmful products gave a protein efficiency ratio (PER) of 2.55. After a heat treatment of this meal (95–98° for 30

Table II. Glucosinolate Content of *Brassica* Seed Meals by Three Methods[a]

Brassica taxon	Number of accessions	Method 1		Method 2		Spectrophotometric[b]	
		Mean, %	RSD, %	Mean, %	RSD, %	Mean, %	RSD, %
campestris var. *dichotoma*	6	6.56	6.7	6.10	5.5	5.14	7.7
campestris var. *sarson*	5	6.99	9.0	6.84	5.4	5.85	9.8
campestris var. *toria*	6	6.84	4.4	6.33	6.6	5.55	12.6
juncea L.	7	7.01	13.0	6.56	16.7	5.54	16.1
napus	14	5.48	22.5	5.26	21.7	4.08	29.5
hirta (*Sinapis alba*)	4			9.16[c]	5.0		

[a] Calculated as percentage in dry defatted meals of 3-butenylglucosinolate for *B. campestris;* allylglucosinolate for *B. juncea*, 2-hydroxy-3-butenylglucosinolate for *B. napus*, and *p*-hydroxybenzylglucosinolate for *B. hirta* for Methods 1 and 2. For the spectrophotometric method, the volatile mustard oil-forming glucosinolates were calculated as 3-butenylglucosinolates and the progoitrin as 2-hydroxy-3-butenylglucosinolate. The two were added for the total. The mean values by the three methods may be compared because the difference in calculation introduces only a minor error; *e.g.*, 0.1% for *B. napus* by spectrophotometry. [b] Slightly modified method of Appelqvist and Josefsson (1967). [c] Josefsson (1970) found an average of 8.83% glucosinolates, dry basis, in defatted seed from five accessions of *B. hirta* by a method to determine *p*-hydroxybenzylglucosinolate in this plant.

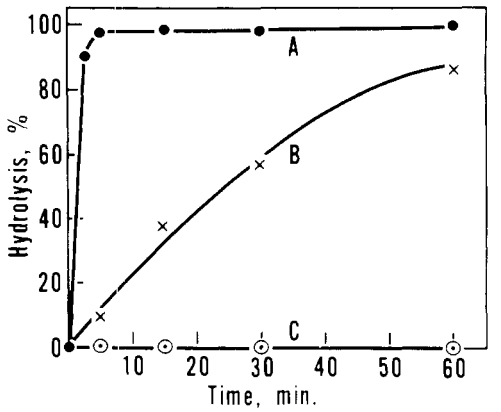

Figure 3. Glucose release as a measure of thioglucosidase activity. Meal A (—●—) from dehulled flaked crambe seed extracted with petroleum ether at room temperature. Meal B (—X—) crambe meal prepared by prepressing and solvent extraction. Meal C (—⊙—) meal B after heating to 85° and then treating with steam to 20% moisture content at 95° for 10 min.

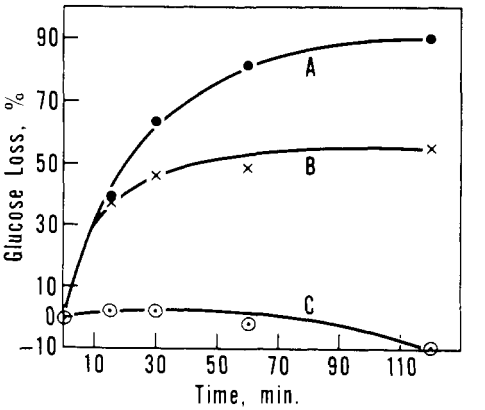

Figure 4. Loss of glucose vs. moisture in meals heated at 95–98°. Curve A (—●—), no water added to vials before heating; meal contained 8.8% moisture. Curve B (—X—), one drop of water added to give a meal containing 27 to 34% moisture. Curve C (—⊙—), 0.5 ml of water added to give a slurry of 1 part meal to 5 parts water, pH 5.3.

min) the PER dropped to 1.51 (VanEtten *et al.*, 1969). Recent analyses of these meals, which had been stored under refrigeration, by Method 2 showed that the extracted meal contained 1.0% free glucose before and 0.2% after the heat treatment.

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