

## A Method for Estimating Benzyl Isothiocyanate, Benzyl Thiocyanate, and Benzyl Nitrile in the Crushed, Moistened Seeds of *Lepidium sativum*

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Methods for the quantitative estimation of benzyl isothiocyanate, benzyl thiocyanate, and benzyl nitrile are described. Using these methods the enzymic formation of the compounds in question could be followed. Benzyl thiocyanate was shown to be formed from benzyl isothiocyanate, whereas benzyl nitrile as well as benzyl isothiocyanate were found to be primary products of the enzymic splitting of benzyl mustard oil glucoside.

When the natural enzymic cleavage of the thioglucoside, glucotropaeolin, in the seeds of *Lepidium sativum* was investigated, it was observed that besides the known fission product benzyl isothiocyanate (BITC) two other compounds are formed, namely benzyl nitrile (BCN) and benzyl thiocyanate (BTC). At a low temperature (+1 to +2°C) and within short reaction times (5 min or less) the glucoside is completely split, BITC and BCN being the primary reaction products, while BTC is formed secondarily from BITC by way of some isomerase mechanism<sup>1,2</sup> (Fig. 1 and 2). This concept of the formation of BCN presupposes that it is formed from the same glucoside as BITC.

Since the investigation was made on a microscale, a method suitable for determining minute amounts of the three components in the same reaction mixture had to be developed.

Whole *Lepidium* seeds were ground in a mill, defatted with petrol ether, air-dried, and ground once more to a very fine powder. About 2/3rds of the husk was removed in this operation.

250 mg of the seeds were placed in a Pozzi-Escot microdistillation apparatus<sup>3</sup>, 20 ml of water was added, and after an appropriate reaction time the mixture was steam-distilled for about 40 min so that the distillate amounted to 150 ml. The distillate was made up to 160 ml, of which 50 ml was taken for BCN estimation. 15 ml of saturated NaCl solution was added to the other part of the distil-

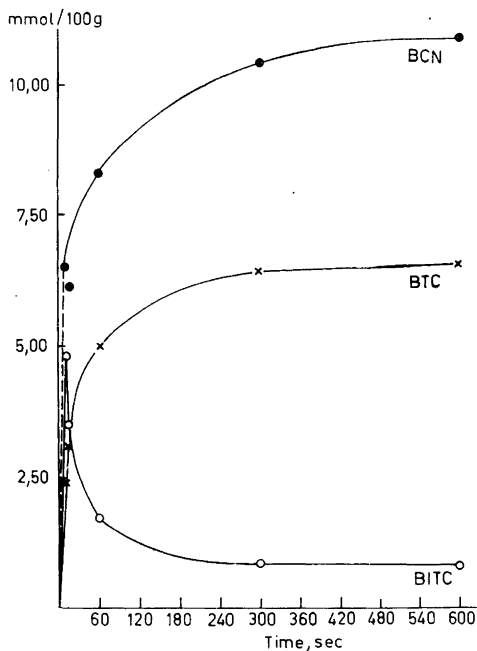


Fig. 1. Formation of benzyl nitrile (BCN), benzyl isothiocyanate (BITC), and benzyl thiocyanate (BTC) in finely ground *Lepidium sativum* seeds. Reaction temperature about +1°C.

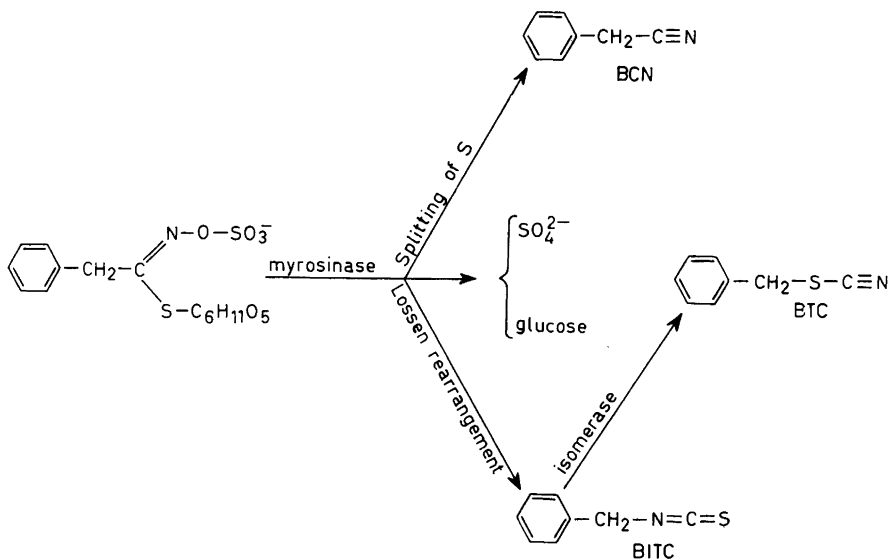


Fig. 2. Enzymic splitting of benzyl mustard oil glucoside in finely ground *Lepidium sativum* seeds.

late, the volatile compounds were extracted four times with about 25 ml of ether, and the ether was divided into two 50 ml portions from which BTC and BITC estimations were made.

#### Estimation of benzyl isothiocyanate

BITC was converted to thiourea in the usual manner with ammonia. 15 ml of ethanol and 10 ml of concentrated ammonia were added to one of the 50 ml portions of ether. The mixture was kept overnight at room temperature, distilled to dryness under a vacuum (motor pump), and the remaining benzyl thiourea (BTU) was taken up in 50 to 250 ml of methanol. The maximum absorption at 243  $m\mu$  was measured in a Beckman Spectrophotometer DK 2. The amount of BITC in the sample was calculated from a standard curve prepared from pure BTU.

#### Estimation of benzyl thiocyanate

The method is based on the usual method of estimating inorganic thiocyanate as a coloured ferric complex. In this case the usual method did not give reproducible values.

1 ml of 1%  $\text{Na}_2\text{S}$  in water and 15 ml of 96% ethanol (to ensure that the  $\text{Na}_2\text{S}$  is evenly dispersed throughout the ether solution) were added to the 50 ml of ether. Subsequently the bottle was kept in a water-bath until completely dry. During the ether evaporation the water-bath was kept at about 60–80°C, after which it was allowed to boil. 10 ml of acetone, 5 ml of 10% hyamine\* in ethanol, and 5 ml of ferric reagent\*\* were added to the dry residue. The bottle was made up to 25 ml with acetone and the red colour measured in a Klett-Summerson colorimeter, with a green filter (No. 50). The amount of BTC was calculated by comparison with a standard curve prepared from pure BTC in the same manner. The error in concentrations ranging from 0.2 to 1.5 mg BTC/25 ml was about  $\pm 5\%$ . It made no difference whether the standard was made directly from pure BTC or was treated in exactly the same manner as the sample. It is very important that the solutions to be added (acetone, Hyamine, and ferric reagent) after the water-bath treatment are added in the right proportions, because otherwise the final solution is cloudy and unsuited for colorimetric measurement.

#### Estimation of benzyl nitrile

Attempts to estimate BCN have presented some difficulties because of the fact that all three components, BITC, BTC, and BCN, were present in the test solutions and the estimation of BCN was disturbed in one way or another by the other two. However, it was observed that sodium 1,2-naphthoquinone-4-sulphonate (NQS), a usual reagent for reactive  $\text{NH}_2$  and  $\text{CH}_2$  groups, gives a brown-red colour with BCN, whereas BTC and BITC do not. The colour is proportional to the concentration of BCN within certain limits. On this basis a colorimetric micromethod for estimating BCN was developed. Although the NQS is by no means a specific reagent for BCN, it was concluded that under our reaction conditions – and in the absence of other nitriles – it is responsible for the colour developed. The method is as follows: 10 to 100  $\mu\text{l}$  of standard solution (200 mg of BCN/10 ml ethanol) and 20 ml of water or 20 ml of the test solution, which has to be neutral, were placed into a 50 ml test-tube (190  $\times$  18 mm, glass stopper), to which exactly 2 ml of 0.3% NQS in water was then added. The tubes were placed in a smoothly boiling water-bath and kept there for exactly 4 min. Precisely 2 ml of 0.5 M NaOH solution in water was added, and the tubes were left to stand for 20 min at room temperature, and finally put to cool in an ice-bath. The contents were poured into 25 ml calibrated flasks and filled with water up to the mark. A solution prepared in the same way with NQS and NaOH served as a blank. The brown-red colour was measured in a Klett-

\* "Hyamine", Rohm & Haas Co., Philadelphia, Pennsylvania, 50% solution in water, active ingredients: methyl dodecyl benzyl trimethyl ammonium hydroxide and methyl dodecyl xylene bis (trimethyl ammonium hydroxide).

\*\* Ferric nitrate 9  $\text{H}_2\text{O}$     5.0 g  
Nitric acid conc.        2.5 ml  
Aq. dest. to              100.0 ml

Summerson colorimeter, with a green filter (No. 50). The blank itself was quite dark and was always measured against distilled water first. If the Klett-Summerson values of the blank were higher than 370, it was impossible to adjust the blank to zero. The amount of BCN was calculated by comparison with a standard curve. The error in concentrations ranging from 0.1 to 1.0 mg of BCN/25 ml was at most  $\pm 6\%$ .

When the colour formed in the reaction was more brown than red, it was reasonable to assume that it might have been formed with some compound other than BCN. In such a case an additional test for nitriles was made. 3 ml of 0.5 M NaOH and 10 ml of ethylmethylketone were added to the reaction mixture. The bottle was shaken. When the layers separated, the upper layer had a clear violet colour if nitriles were present. The upper layer with BTC, BITC, and some other compounds tested, was pale yellow in colour.

#### DISCUSSION

The methods for estimating BITC and BTC need little comment. In the case of BTC the most important thing is to evaporate the solution to complete dryness, otherwise the moisture causes cloudiness when the reagents are added. When estimating BITC as thiourea, some other volatile compounds may cause error. Kjær *et al.*<sup>4</sup> have presented a method for correcting such error. However, in our experiments Kjær's correction factor for BITC was not used because the main foreign compounds were known and probably different from those in his experiments. We therefore treated the three compounds alone and in different relative concentrations together with ammonia and measured the absorbances. BCN caused no disturbance even at reasonably high concentrations, whereas if the concentration of BTC was very high in regard to BITC the curve could sometimes be flattened and lifted. However, this happened very seldom in our work, and the results were then discarded.

When estimating BCN, the sources of error were greater. In the method the point of importance was the heating time and the temperature. The tubes were kept for exactly 4 min in a water-bath, the temperature of which was 97–98°C. If the water-bath was boiling vigorously, the blank became too dark and dilution did not give true values. The other source of error could be caused by volatile compounds present in the test solution. In our experiments the compounds probably present in the distillate were some sulphides or mercaptans, ammonia, hydrogen sulphide, carbon disulphide, steamvolatile amines or amines produced as decomposition products after boiling BITC and BCN with alkali. Some of these compounds gave a faint brown colour with NQS and alkali, but when treated with more alkali and ethylmethylketone they gave no violet colour, and hence uncertain cases were easy to detect.

As a control, 300 g of crushed *Lepidium* seeds in water were steam-distilled when the enzymic reactions were completed. The oil recovered was separated into fractions in a counter-current apparatus (methanol:H<sub>2</sub>O:petrol ether 8:2:5). One of these fractions gave an infrared spectrum completely identical with pure commercial BCN.

It ought, perhaps, to be pointed out that the handling of the fine seed-powder of *Lepidium*, which in aqueous solution forms a very tough slime, needs some experience before reproducible values can be achieved.

Some of our results concerning the enzymic reaction products of crushed *Lepidium sativum* seeds and factors influencing these reactions have been published in preliminary reports<sup>1,2</sup>. The analytical methods described in the present

paper were not included in these reports. A more detailed paper will be published in the near future.

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