Chapter - V

A NEW AND RAPID COLORIMETRIC METHOD FOR QUANTITATIVE ESTIMATION OF GLUCOSINOLATE IN SEEDS OF *Brassica campestris* L.cv.B - 54.

INTRODUCTION

Oleferous brassicas offer a potential source of plant proteins for utilisation as food, feed, manure, chemicals and pharmaceuticals. However, their use is limited on account of certain antinutritional factors present in the oil and meal. Out of several antinutritional factors present in the oil and meal, the class of glucosinolate is one of them. Thus it is very essential to know a suitable method for estimation of glucosinolate in any sample of *Brassica* for understanding their quantitative value.

Numerous methods have been reported for the quantitative analysis of glucosinolate (Lein 1970). Most of the quantitative methods are based upon the analysis of one or more of the enzyme hydrolytic products and therefore assays for both total and individual glucosinolates are available (Underhill, 1980).

Colorimetric (Johnston and Jones 1966) and titrimetric (Raghava *et. al,* 1971) assays of thiocyanate ion have been adapted for the estimation of the indole glucosinolates and p-hydroxybenzyl glucosinolate. But according to Underhill (1980) coloured material found in plant extracts often interfere with the assay. Sorensen (1985) suggested that it was impossible to obtaine100 percent pure glucosinolate and the exact actual purity of glucosinolates is essential in connection with quantitative estimation.

In addition to the methods mentioned earlier quantitative determinations of glucose released by myrosinase treatment (Heaney and Fenwick, 1981), and spectrophotometric determination of Pd-glucosinolate complexes (Moller et al 1985) have also been used. But in this connection Sorensen (1985) also pointed out that it was necessary to use all the methods based on different glucosinolates as none of them gives reliable results for all glucosinolates.

Because of the lack of purified glucosinolates McGregor (1985) proposed the use of the "thymol test for calibration of HPLC of glucosinolate. But while indirect determination of HPLC response to factor involving "thymol test" some glucosinolate were found partly at the cap of the loosely capped tubes. It was assumed that perhaps a small quantity could escape from the tubes. Possibly this "evaporation effect" affects different glucosinolate to different extract.

Methods based on the use of near-infrared reflectance spectroscopy (Starr

et. al, 1985) X-ray fluorescence spectroscopy (Evans et al, 1989) Gc - MS (Uda et al 1982) are also available.

Flash Chromatography (Peterka and Fenwick 1988) isotachophoresis (Klein 1981) High performance capillary gas spectrometry (Shaw et al, 1989) have been observed. But often laboratories are not provided with such facilities.

Thus an attempt has been made to standardise an easy and rapid colorimetric method for quantitative determinations of glucosinolate in seeds of *Brassica campestris* B-54 after purification by column chromatography, with the help of which a large number of samples derived from various physiological experiments quantitative determinations will be possible.

METERIALS AND METHODS

MATERIALS

Seeds of *Brassica campestris* B-54 were collected from the experimental plot for research, Centre for Life Sciences, North Bengal University.

METHODS

Extraction of glucosinolate

(Harborne 1973), 1 gm of freshly collected seeds was extracted with boiling 70% methonol in a homogenizer. It was concentrated to evaporate methanol.

Purification of glucosinolate

Concentrated extract was purified after column chromatography. Alumina the adsorbent was pretreated with dilute H_2SO_4 (1%) and packed in a glass column. The column was washed with distilled water for several times to free the column for H_2SO_4. The concentrated mass of extract was adsorbed on alumina and eluted directly with methonol-water. The methonol-water containing pure glucosinolate was made to volume upto 25 ml. with distiled water to serve as a stock solution of glucosinolate.

Preparation of Ammonical $AgNO_3$ Solution

To 100 ml of 1% $AgNo_3$ solution 5 drops of (4N) NH_4OH was added to make the solution alkaline.

Preparation of reaction mixture for quantitative estimation of glucosinolate

To 1 ml. of glucosinolate in water, 1 ml of ammonical $AgNo_3$ (1%) was added. The mixture was boiled for 1-2 minutes and kept at room temperature (27± 1°C) for 30 min. Characteristic colour was developed and optical density was determined colorimetrically.

Determination of absorption maxima (λ_{mas}) of glucosinolate

The reaction mixture for quantitative estimation of glucosinolate was prepared and kept for 30 minutes at room temperature 27 ± 1°C and O.d. value was determined at different wave length of light with the help of DU spetrophotometer, Beckman and absorption maxima (λ_{mas}) was determined.

Preparation of Standard curve for glucosinolate solution

1000 ppm, 100 ppm, 10 ppm, 1 ppm, 0.1 ppm, 0.01 ppm and 0.001 ppm of solutions were prepared from stock solution of glucosinolate solution after dilution with distilled water and standard curve was prepared after the preparation of reaction mixture with ammonical $AgNo_3$ solution.

Quantitative Determination of glucosinolate with the help of a colorimeter

Extraction and purification of glucosinolate were performed following the procedure mentioned earlier involving various seed samples. Reaction mixture was produced after combining with ammonical $AgNo_3$ and subsequent treatment for colour development and percentage of occurence of glocusinolate was determined with the help of a colorimeter using filter corresponding to 414 nm.

Estimation of glucosinolate in Seeds of *B.campestris* B-54. grown at different environmental conditions in West Bengal

Seeds were collected from the plants grown in the experimental plot. Dept of Botny NBU (Darjeeling district) and in Pulse and Oil Seeds Research Station. Berhampur (Murshidabad) in West Bengal and glucosinolate content in the seed was estimated, following the procedure metioned earlier.

RESULTS AND DISCUSSION

Numerous methods have been reported for the quantitative estimation of glucosinolates. These are based upon the analysis of one or more of the

enzymatic hydrolytic products and therefore assays for both total and invididual glucosinolates are not directly related to the undisturbed glucosinolate present within the tissue.

Glucosinolates co-occur with Myrosinase (Thioglucoside glucohydrolase E.C. 3.2.31) isoenzymes (Buchwaldt et al 1986) in all the Plant materials hitherto investigated.

It has been observed that a restricted number of plants have been shown to contain glucosinolates, mostly identified on the basis of degradation products of glucosinolates, some of these reports have been seriously questioned (Larson et al, 1988; B jerg *et. al*,1986).

Although the glucosinolate may be resolved by paper chromatography quantitative determinations of them are usually based on the products of enzymatic hydrolysis (Youngs and Welter 1967). The total glucosinolate content has been determined by some authors after enzymatic release of glucose or Sulfate (Van Etten et al, 1965). Determination of the amounts of the individual glucosinolate has been based on the analysis of the released mustard oils. Quantitative analysis of volatile isothiocyanates are generally based on indirect method. Conversion of isothiocyanates to thioureas gives material with a high UV absorption and provides a sensitive method of analysis (Kajeer *et.al*, 1973) But according to some authors separation of this individual thioureas by paper chromatography is not suitable for routine analysis of a large number of samples (Youngs and Welter,1967).

Spectrophotometric determination of Pd - glucosinolate complexes (Moller et al, 1985 b) has been used by some authors. A number of indirect methods have also been developed to estimate particular glucosinolate or groups of glucosinolates. Colorimetric (Johnstan and Jones, 1966) and tritimetric (Raghavan et al, 1971) assays of thiocynate ion have been adopted for the estimation of the indole glucosinolate and P-hydroxy benzyl glucosinolate. But often coloured materials found in plant extract interfere with the assay.

Gas liquid chromatography (GLC) has been used for identification of break down products (Youngs and elter 1967), intact glucosinolates (Underhill and Kirkland, 1971; Thies 1976) and desulphoglucosinolates (Theis 1977 1979; Heaney and Fenwick 1980). Similarly high performance liquid chromatography

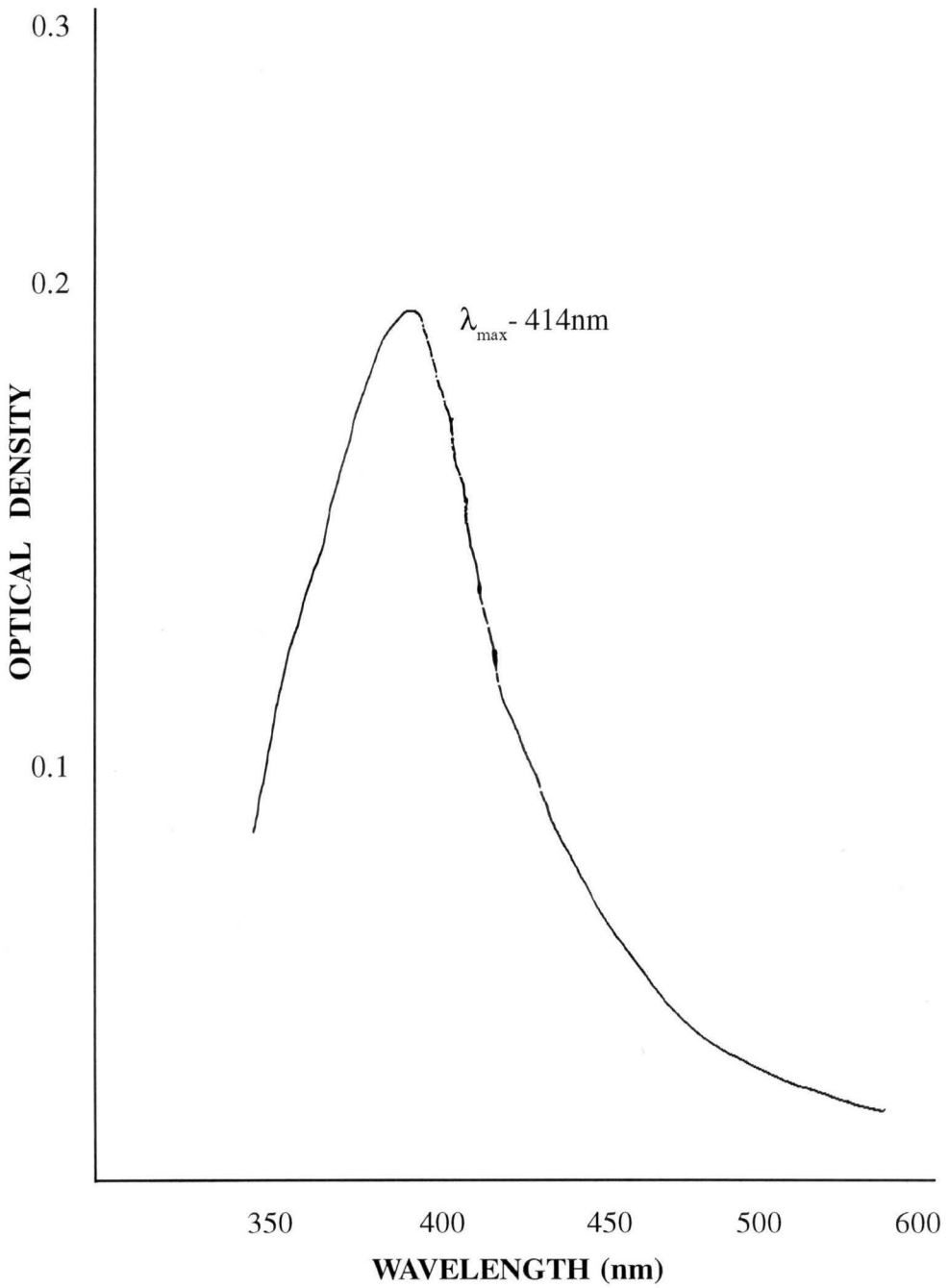

FIG. 26 ABSORPTION SPECTRUM OF GLUCOSINOLATE IN AMMONICAL SILVER NITRATE SOLUTION

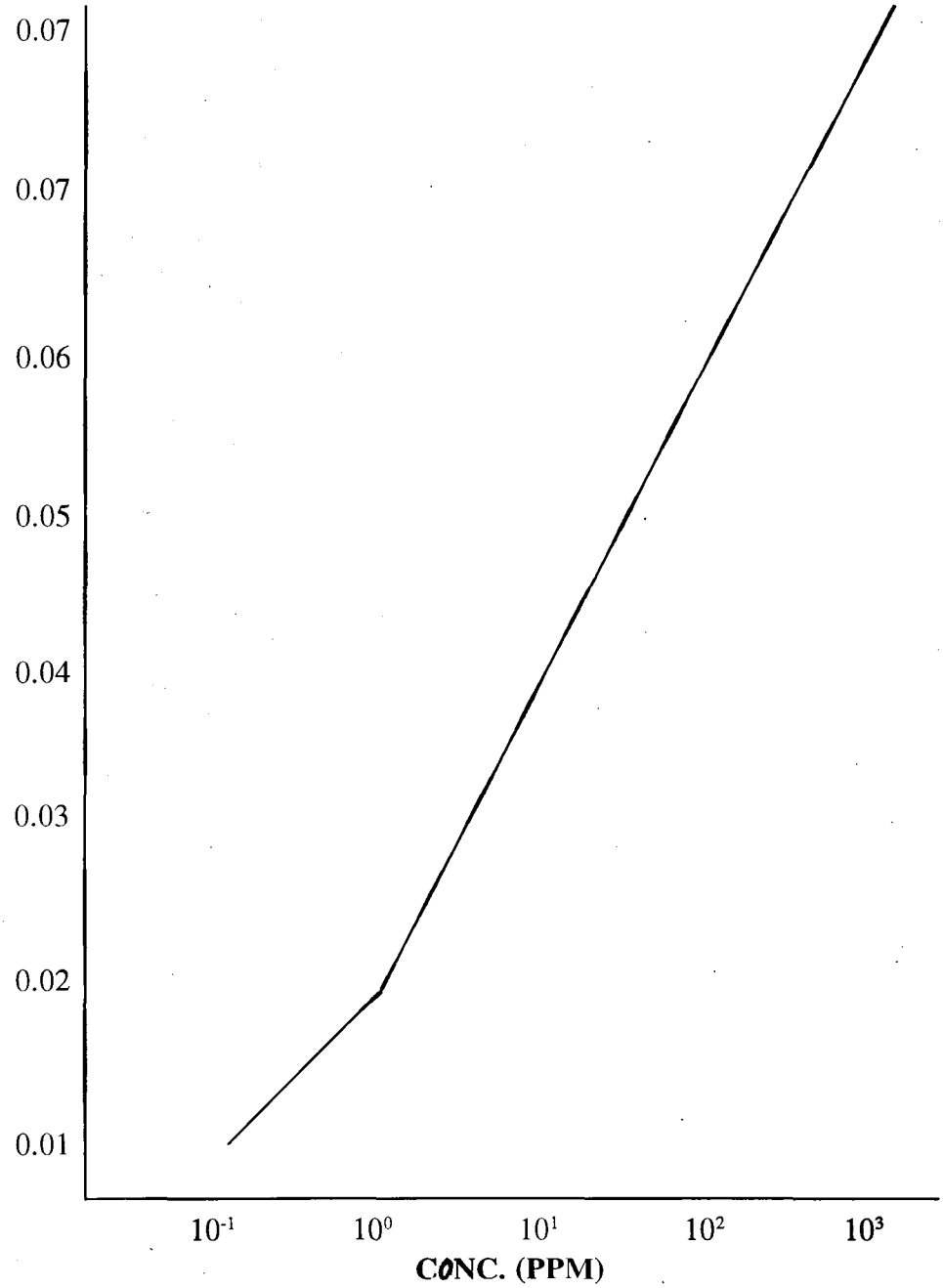

FIG. 27 STANDARD CURVE OF GLUCOSINOLATE IN AMMONICAL SILVER NITRATE SOLUTION

(HPLC) has been used for identification of break down products (Maheshwari et al, 1979) or intact glucosinolates (Holboe et al, 1980; Moller et al, 1984). But it has been the experience that those procedure involves freeze drying step and some amount of glycosinolate could escape from the tubes. This "evaporation effect" affects yield of different glucosinolates to different extent.

Methods based on the use of near infrared reflectance spectroscopy have been published (Starr et al, 1985); and the potential of X-ray fluorescence spectroscopy has been examined (Evans et al, 1989) GC-MS (Uda et al, 1982); flash chromatography (Peterka and Fenwick, 1988), thermospray liquid chromatography mass spectrometry (Hogg et al, 1988), elisa (Hassan et al, 1988), Isotachophoresis (Klein 1981), and high performance capillary gas spectrometry (Shaw et al, 1989) have been used din connection with quantitative estimation of glucosinolate.

But according to Sorensen (1985) it is very difficult to obtain pure glycosinolates an identifications of the glycosinolates by GLC, HPLC, H^1 and C^{13} NMR, (GLC-MS etc do not give any information on the content of water, inorganic salts and some other impurities. Moreover, Sorensen (1985) pointed out that it was necessary to use all the methods based on different glucosinoles as none of them gave reliable result for all glucosinolates.

Another draw back for estimation of glycosinolate is that it is impossible to obtain 100% pure glycosinolate but purity of glycosinolate is very much required for the purpose (Sorensen, 1985).

Here in this part of work the procedure, established for quantitative estimation of glucosinolate isolated from seeds of *Brassica Campestris* B - 54 is very much related to the glucosinolate in intact form. As the isolated product is soluble in water, it can be purified with the help of Column Chromatography. The alumina has been pretreated with dilute H_2SO_4 (Harborne, 1973).

The method is basically based on the principle that the isolated product of glucosinolate in pure form produces characteristic colour in presence of ammonical $AgNo_3$ solution (Harborne, 1973). The method proposed in this part of work is a new one as no method utilising ammonical $AgNo_3$ solution has yet been published in connection with quantitative determination of glucosinolate. The reaction mixture is produced after mixing glucosinolate solution in water

with the same volume of ammonical $AgNo_3$ (1%). The characteristic colour develops after heating the reaction mixture for 1-2 minutes at 100^0 C and keeping at room temperature 27 $\pm$ 1^0C for 30 min. The absorption mixima (λ_{mas}) has been worked out to be at 414 nm (Fig.26). It is advantageous brcause the colour remains stable for 8 hours and quantitative estimation can be done with the help of a colorimeter which is available at any laboratory. The standard curve has been observed to follow. Beer's law for the concentration in the range of 10^0ppm to 10^3 ppm (Fig.27). Again with the help of this method only 500 mg of plant material has been observed to be sufficient for the estimation of glucosinolate. This is also less time consuming and large number of samples can be examined within a short time. Following the procedure the glucosinlate content in the seed sample of *B.campestris* B-54 grown in Darjeeling and Mushidabad district in West bengal were estimated to contain 1.80 and 1.38 percent respectively on dry weight basis. (Table-22).

Table - 22

Variation in glucosinolate content in seeds of *B. Campestris* B- 54 grown in the experimental plot Department of Botany NBU (Darjeeling district) and Pulse & Oil Seed Research Station, Berhampur (Murshidabad) in West Bengal).

Place of origin of seed sample	Glucosinolate Content (as percentage on dry Weight basis)
Research Plot, Dept of Botany. NBU, Darjeeling District West Bengal.	1.80
Research Plot. Pulseand Oil Seed Research Station, Berhampur, Murshidabad District, West Bengal.	1.38

SUMMARY

An easy method for quantitative estimation of glucosinolate has been established colorimetrically.

The glucosinolate has been purified by column chromatography taking alumina as adsorbent which has been pretreated with dilute H_2SO_4 and diluted with methanol-water.

The colour complex of glucosinolate solution with ammonical $AgNo_3$ (1%) after heating at 100°C for 1-2 minutes shows absorption mzxima (λmax) at 414 nm. and remains stable after 30 mins. to 8 hours.

Glucosinolate at the range of concentration 10^0 ppm to 10^3 ppm obeys Beer's Law.

The proposed method is supported to be advantageous because of the fact that glucosinolate can be determined from low concentration. Moreover only 500 mg of plant material is required for the estimation of glucosinole and it takes very small duration of time to estimate the chemical in large number of samples produced during experimentation.

During estimation of the glycosinolate no enzyme hydrolysis is required.

Following the procedures, 1.80 and 1.38 percent of glucosinolate, have been estimated, in the seed samples of *B. campestris* B-54 grown in Northern (Darjeeling District) and Southern (Murshidabad District) part respectively of West Bengal.