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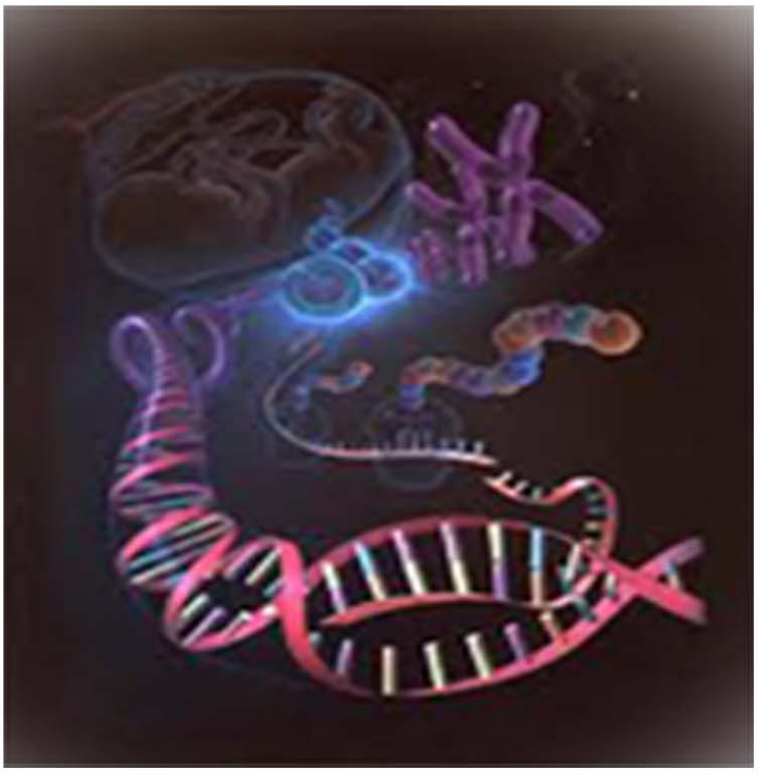

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Review Article

GLUCOSINOLATES: TRANSPOSING TRENDS OF IDENTIFICATION METHODS FROM PAPER CHROMATOGRAPHY TO MICROCHIP ANALYSIS

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Glucosinolates or β-thioglucoside N-hydroxysulfate are important class of secondary metabolites present in plants. This review is focused on providing a highlight over the advancements in the glucosinolate estimation methods. Initially, the estimation of the GSLs was dependent on the less specific and cheap methods of estimation using released glucose, test paper analysis, paper chromatography and thin layer chromatography. These methods were successfully superseded by HPLC, gas chromatography, mass spectrometry, etc., which have shown their accurate, reliable and reproducible results. But, some drawbacks are also associated with these methods like sophisticated operations and expensive instrumentation. Some light is also thrown over the latest methods, viz., strong ion exchange centrifugal partition chromatography and microchip analysis, having potential to be a successful alternate to the earlier methods Studies need to be conducted for improving non-HPLC methods of isolation for a rapid, accurate and a cheaper alternate.

Keywords: Glucosinolate, Chromatographic methods, Non-chromatographic methods, HPLC, Microchip

INTRODUCTION

Plants with potential therapeutic agents have been used throughout history. Modern science encompasses a range of approaches for the isolation, identification and elucidation of pharmacologically active principals (Sonderby, 2010). Glucosinolates (GSLs) constitute one such group of plant secondary metabolites (Figure 1), which have long been the focus of research pertaining to their antinutritional properties as well as their immense benefits. These natural products are known to be present in 500 different species and around 16 families of angiospermic plants (Fahey *et al.*, 2001; Troyer *et al.*, 2001; Toribio *et al.*, 2007).

The GSLs found in the plants are of diverse nature and are classified in a variety of ways, such as on the basis of variability in R chain structure, which includes aliphatic, ω-methylthioalkyl, aromatic, heterocyclic, olefins, hydroxyl or

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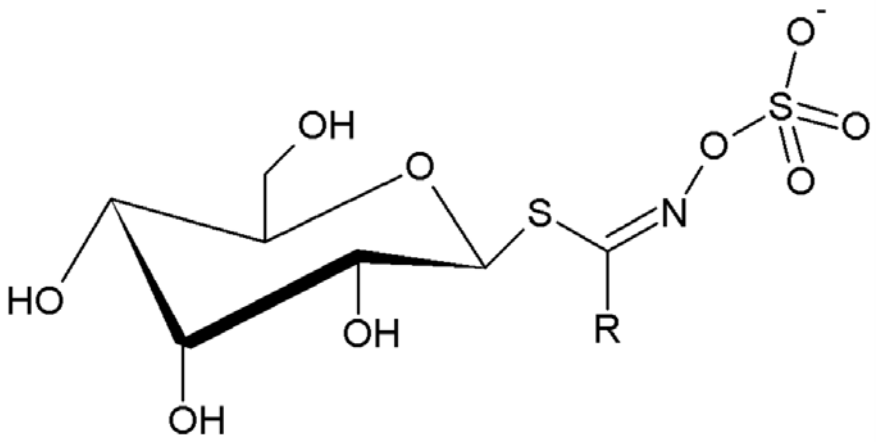
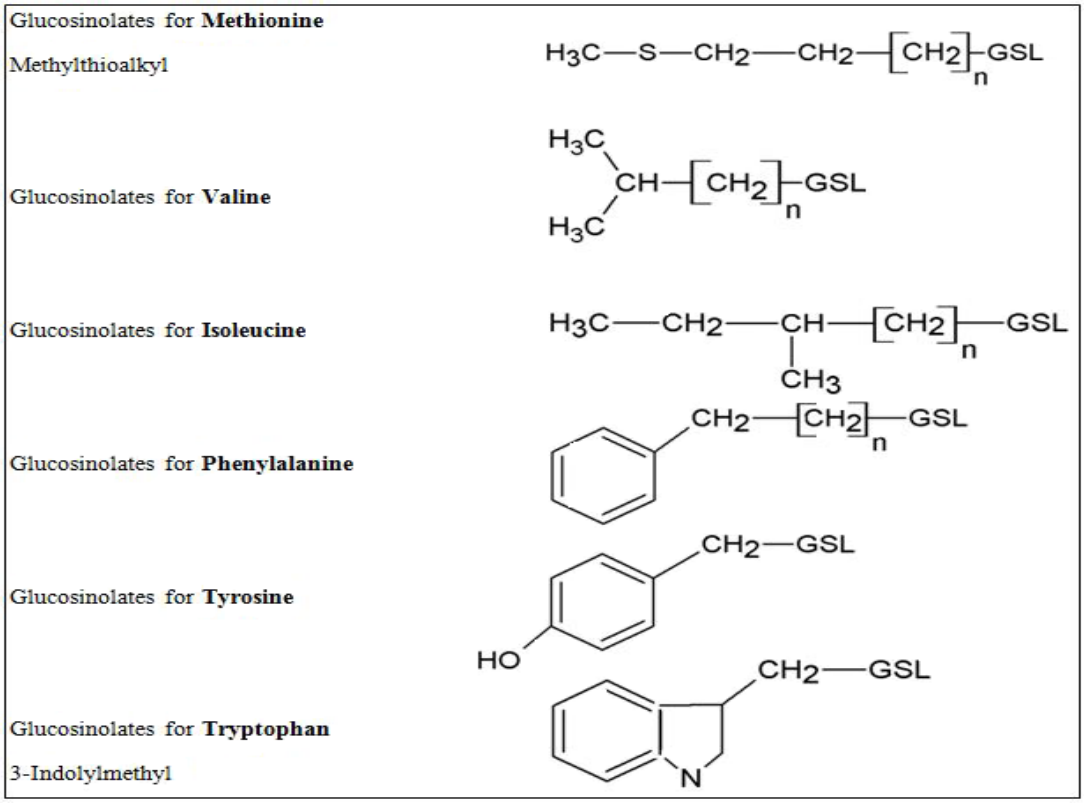
Figure 1: Structure of Glucosinolate with R Side Chain



carbonyl groups, etc. (Fahey *et al.*, 2001). But, the easiest way to classify is on the basis of the amino acid precursor like aliphatic glucosinolates derived from methionine, isoleucine, leucine or valine, aromatic glucosinolates derived from phenylalanine or tyrosine, and indole glucosinolates derived from tryptophan (Figure 2) (Redovnikovic *et al.*, 2008). The number of

Figure 2: R Side Chains of Glucosinolates Based Upon the Amino Acid Precursor



individual GSLs have been agreed to be 120 (Fahey *et al.*, 2001). Some new GSLs have been reported but are waiting to be proved using methods like NMR (Clarke, 2010). GSLs in addition also contain an enzyme called myrosinase (β-thioglucosidase) (Karcher *et al.*, 1999). GSLs and myrosinase both are accumulated in all the parts of the plant, i.e., root, shoot, stem, seed, etc. (Heaney *et al.*, 1987; Elfakir and Dreux, 1996; Brown *et al.*, 2003). GSLs have been widely studied mainly because of their immense advantages to humans. This plant metabolite has been found to be a potent bacteriocide and acts against pathogens like *Agrobacterium tumefaciens*, *Pseudomonas cichorii* and *Xanthomonas campestris* (Fahey *et al.*, 2002; Zukalova and Vasak, 2002; Aires *et al.*, 2009; Vig *et al.*, 2009). It also acts as an antioxidant, bioherbicide and fungicide (Brown and Morra, 1995; Manici *et al.*, 1997; Fahey and Talalay, 1999; Zukalova and Vasak, 2002; Vig *et al.*, 2009). But the most important property of GSLs has been the anti-carcinogenic or the anti-tumorigenic (Zhang and Talalay, 1994; Vang *et al.*, 1997; Fimognari and Hrelia, 2007; Vig *et al.*, 2009), thus anticipating their potential as an effective anticancer drug.

Large numbers of studies have been conducted on the isolation of GSLs (Bjorkqvist and Hase, 1988; Heeremans *et al.*, 1989; Shaw *et al.*, 1989; Prestera *et al.*, 1996; Kaushik and Agnihotri, 1999; Mellon *et al.*, 2002). Isolation of GSLs has been achieved both as intact molecules as well as desulfo-GSL. But, considering the factors like increased time requirement in case of the extraction of desulfo-GSL, due to sample processing and the desulfation process as well as the fact that not all GSLs can be isolated using this method, isolation of intact GSLs is more beneficial (Kaushik and Agnihotri, 1999; Mellon *et al.*, 2002). From the time of discovery, the isolation of GSLs has evolved considerably. Many methods have been employed for the isolation, such as Paper Chromatography (PC), Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Hydrophilic Interaction Liquid Chromatography (HILIC), Gas Chromatography (GC) and some of the very modern methods like strong ion exchange Centrifugal Partition Chromatography (SIXCPC) and Microchip method, etc. Many analytical techniques have been used to profile the GSL content of different plants. In this paper, an attempt has been made to review briefly the different methods for the estimation of GSLs, which can help the researchers to a large extent (Table 1).

ESTIMATION OF GLUCOSINOLATES

The Discovery and history of GSLs was well explained by Challenger (1959). Although, Gadamer (1897), gave the first structure of GSLs, but the correct structure was presented by Ettlinger and Lundeen (1956). With this discovery, there was a rush in finding its biological properties. GSLs have been found to be toxic to humans and animals in high concentrations (Smith and Dacombe, 2006), but in low concentration a wide number of beneficial effects of some of its hydrolytic products have been reported as stated above. The research on these bioactivities of GSLs has lead to a large number of methods being employed for their identification as has been discussed in this review.

The methods for the estimation of GSLs may

Table 1: Commonly Used Methods for the Quantitative and Qualitative Analysis of Glucosinolates

Type of Analysis	Methods Employed	References Cited
1. Chromatographic estimation	i. Paper chromatography	Kjaer and Rubinstein (1953); and Olsen and Sorenson (1980)
	ii. Thin layer chromatography	Elliott and Stowe (1971) and Russo and Reggiani (2012)
	iii. High performance liquid chromatography	Wade et al. (2007); Brown et al. (2003); Fahey et al. (2003) and Tolra et al. (2000)
	iv. Gas chromatography	Kjaer and Jar (1956); Vinjamoori et al. (2004) and Cools and Terry (2012)
	v. Centrifugal partition chromatography	Toribio et al. (2007); Hamzaoui et al. (2011) and Hamzaoui et al. (2012)
	vi. Mass spectroscopy	Bailey et al. (1961); Tolra et al (2000); Mellon et al. (2002); Gratacos-Cubarsi et al. (2010) and Maldini et al. (2012)
2. Spectroscopic estimation	i. X-ray spectroscopy	Schnug and Haneklaus (1987)
	ii. Nuclear magnetic resonance spectroscopy	Tapper and MacGibbon (1967) and Prestera et al. (1996)
	iii. Near Infrared Reflectance Spectroscopy	Tkachuk (1981) and Kumar et al. (2010)
3. Enzymatic estimation	i. Enzyme linked immunosorbent assay	Hassan et al. (1988) and van Doorn (1998)
	ii. Test paper method	Comer (1956); Bjorkman (1972) and Saini et al. (1987)
	iii. Released glucose method	Smith and Dacombe (2006); Ishida (2003) and Gardrat and Prevot (1987)
4. Other methods	i. Microchip analysis	Fouad et al. (2008)

be divided into 2 main types i.e. chromatographic methods and non-chromatographic methods. These methods are divided further into many subtypes (Flow Chart 1).

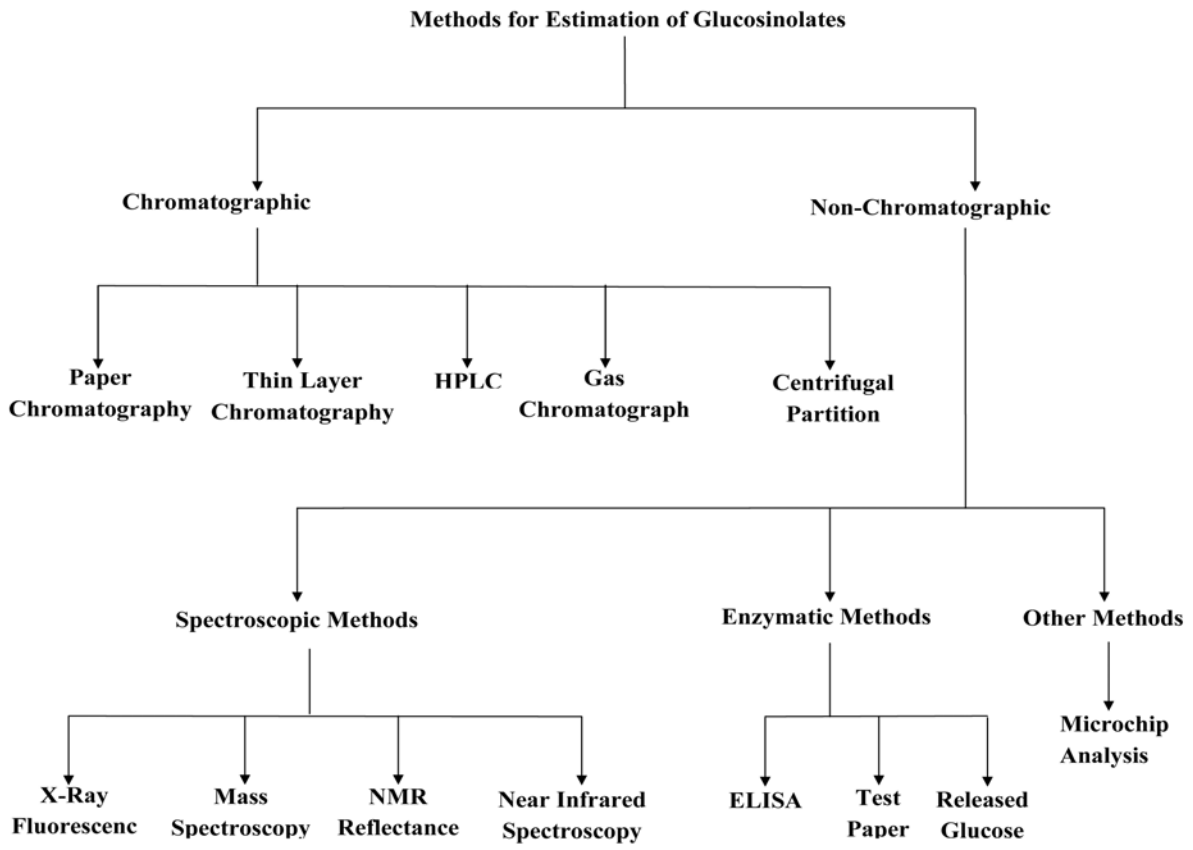
Chromatographic Methods

Chromatography is the most widely applied method for the analysis of GSLs. Among these, HPLC and GC have many applications.

Paper Chromatography (PC)

PC is a very simple and rapid method for separation of GSLs. It was widely used in 1900s for the estimation of GSLs by Kjaer and Rubinstein

Flow Chart 1: Methods of Estimation of Glucosinolates



(1953). PC was employed for the separation of thioureas, formed by the reaction between isothiocyanates and ammonia (Josefsson, 1967). Two different mobile phases are normally used: (a) water saturated chloroform for hydrophobic thioureas and (b) Butanol : toluene : water mixture for the hydrophilic thioureas. PC was used by many researchers (Kjaer and Rubinstein, 1953; Ettlinger and Lundeen, 1956; Kjaer and Gmelin, 1956; Kjaer and Thomson, 1963; Josefsson, 1967; Elliott and Stowe, 1971; Bjorkman, 1972; Olsen and Sorensen, 1980; Olsen and Sorensen, 1981; Smith and Dacombe, 2006) as an efficient method for estimation of intact GSLs.

This technique had disadvantage that different compounds showed same retention values in same solvent. Moreover, the reproducibility of the technique was dependent on the solvent temperature, chromatography paper, solvent purity and amount of substance. This led to the decrease in popularity and so the application of this technique.

Thin Layer Chromatography

TLC is a better, rapid and much reliable method. It has a lower detection limit than PC as claimed by Josefsson (1967). It was employed for the detection of thiourea formed by the reaction

between isothiocyanates and ammonia. The method was highly recommended by Elliott and Stowe (1971) due to its excellent separation results. TLC was used for the estimation of both intact GSLs and their hydrolytic products (Gadamer, 1897). Silica or cellulose gel plates formed a very efficient stationary phase. Due to unknown reasons, TLC was not much accepted method as compared to PC. There are only a few significant applications of TLC for the estimation of GSLs (Bjorkman, 1972; Barron *et al.*, 1988; Emam and El-Moaty, 2009; Devi and Thangam, 2010; Russo and Regiani, 2012).

But recently, Russo and Reggiani (2012), used this method for the estimation of GSLs. Hopefully, owing to many advantages of this method such as: rapid results, large number of samples estimated at the same time, reproducibility and very low cost, this method may resurface as a successful alternate to HPLC.

High Performance Liquid Chromatography

HPLC is among the most preferred and most studied method for the analysis of GSLs (Mullin, 1978, Helboe *et al.*, 1980; Minchinton *et al.*, 1982; McGregor *et al.*, 1983; Sang *et al.*, 1984; Hogge *et al.*, 1988; Heeremans *et al.*, 1989; Kokkonen *et al.*, 1991; ISO, 1992; Betz and Fox, 1994, Matthaus and Fieblig, 1996; Prestera *et al.*, 1996; Arguello *et al.*, 1999; Kaushik and Agnihotri, 1999; Szmigielska and Schoenau, 2000; Kiddle *et al.*, 2001; Rangkadilok *et al.*, 2002; West *et al.*, 2002; Brown *et al.*, 2003; Fahey *et al.*, 2003; Mohn *et al.*, 2007; Wade *et al.*, 2007; Barbieri *et al.*, 2008; Troyer *et al.*, 2008; Berhow *et al.*, 2010; Maldini *et al.*, 2012; Russo and Regiani, 2012) due to its accurate and reproducible results, ability to detect minor quantities of compound and better speed of analysis than GC (Mullin, 1978). Interestingly, it was not given much importance earlier, as it was time consuming and the detection of the separated GSLs was difficult (McGregor et al., 1983). It was further added by Mullin (1978), that the mobile phase containing methanol reacts with isothiocyanates and thus resulted in the decreased use of this technique. But with the improvements and emergence of new detection techniques such as MS, this method gained credence. HPLC is being applied for both the qualitative and quantitative analysis.

The technique can be employed for the detection of both intact GSLs (50) and desulfo-GSLs (Mullin, 1978; Minchinton *et al.,* 1982). Analysis of intact GSLs is performed using reverse phase columns (Troyer *et al.*, 2001; Devi and Thangam, 2010) and desulfo-GSLs generally employ ion paired columns (Rangkadilok *et al.*, 2002). While HPLC analysis can be used for separation of all GSLs in less time for intact GSLs, the estimation of desulfo-GSLs is still preferred due to the simpler solvent requirement and the easy separation of GSLs after desulfation.

The earlier methods of HPLC detection mostly included the initial desulfation of GSLs, followed by separation on C_{18} column (ISO, 1992), but the GSLs isolated were biologically inactive and could not be hydrolyzed (Wade *et al.*, 2007). So new improvements have been introduced for solving the above problems. These include the use of isocratic paired ion or gradient HPLC in combination with photodiode array (UV detection) or mass spectrometer (MS) (Betz and Fox, 1994; Prestera *et al.*, 1996; Rangkadilok *et al.*, 2002; West *et al.*, 2002). These alternatives were very attractive, but faced the problems of time consuming desalting procedure for isocratic

paired ion and the requirement of lengthy gradients in case of gradient HPLC (Wade *et al.*, 2007). With the further advancements in HPLC, highly specific technique of HILIC was developed (Wade *et al.*, 2007). It solved much of the above faced problems.

New methods for the isolation of GSLs using HPLC are continually being developed. Many novel improvements such as use of graphitized carbon column (Brown *et al.*, 2003), anion exchange membrane extraction for HPLC analysis (Szmigielska and Schoenau, 2000), high speed counter-current chromatography (HSCCC) (Fahey *et al.*, 2003), HPLC atmospheric pressure chemical ionization mass spectrometry (Tolra *et al.*, 2000) and subsequently improving solvents have ensured the continual use and probably the most rapid, accurate and sensitive technique in the near future.

Gas Chromatography

Since the earliest report of the use of GC for the analysis of GSLs by Kjaer and Jart (1956), this technique has widely been explored. It has shown wonderful results even at low efficiency and has been successfully applied for the separation and quantitative and qualitative analysis of GSLs (Jart, 1961). The volatility of GSLs was assured by the trimethylsilylation of GSLs. The trimethylsilylation of intact GSLs causes loss of sulphate ions, which further interferes with silylation (Vig, 2000). This problem was overcome by the desulfation of GSLs and precipitation of sulfur by barium acetate or lead acetate prior to GSL analysis developed by Thies (1978).

With the increasing interest, many modifications were made, mostly in the types of column used. In order to increase the efficiency and accuracy of the method, it was mostly combined with mass spectrometry (Cole, 1975; Gil and McLeod, 1980; Hasapis *et al.*, 1981). Some of the common columns used were carbowax 20 M (Cole, 1975; Hasapis *et al.*, 1981), EGSS-X (VanEtten *et al.*, 1974), apielson L (VanEtten *et al.*, 1974; Cole, 1975), OV-17 and OV-1 (Olsen and Sorensen, 1980a). The earlier used stainless steel columns were replaced by glass columns since the trimethylsilyl derivatives were decomposed on metal columns (McGregor *et al.*, 1983). It is generally preferred to use more than 1 column with differential polarity, for better difference in the elution times (McGregor *et al.*, 1983). Helium has been the most preferred carrier gas, although nitrogen has been used instead in a few instances (Kjaer and Jart, 1956; Elliott and Stowe, 1971).

GC has been an excellent technique for identification of GSLs, but has a few drawbacks such as: it is time consuming and due to the high temperature, the GSLs get denatured and it is difficult to obtain pure samples (Mullin, 1978). GC takes longer time for the analysis and the capacity of the instrument is low as compared to HPLC, but still the method has always been of prime interest. This simple procedure and accurate results have attracted researchers for adopting this technique for GSL analysis and this has been continued till date (Kjaer and Jart, 1956; Jart, 1961; Kjaer and Friis, 1962; Elliott and Stowe, 1971; Underhill and Kirkland, 1971; Bjorkman, 1972; VanEtten *et al.*, 1974; Cole, 1975; Mullin, 1978; Thies, 1978; Daxenbichler *et al.*, 1979; Olsen and Sorensen, 1979; Gil and MacLeod, 1980; Olsen and Sorensen, 1980a; Hasapis *et al.*, 1981; McGregor *et al.*, 1983; Shaw *et al.*, 1989; Vig,

2000; Vinjamoori *et al.*, 2004; Kamel and El-Gengaihi, 2008; Chaudhary *et al.*, 2012; Cools and Terry, 2012).

Strong Ion-exchange Centrifugal Partition Chromatography (SIXCPC)

SIXCPC technique developed by Toribio *et al.* (Toribio *et al.*, 2007), is another important technique developed for the GSL estimation. The equipment was fitted with aliquat 336, a strong anion exchanger for generating lipophilic entities (Toribio *et al.*, 2007). A unique biphasic solvent system has been used for CPC. The compounds with strong affinity with anion exchanger competitively remove those with low affinity. This method is capable of providing 2.4 g of pure sinalbin from 12 g of mustard crude extract in 170 min, thus a productivity of 3.3 g/h/L_{VC}. Due to the size of the instrument, a flow rate of only 2 ml/min could be achieved (Hamzaoui *et al.*, 2012).

Another method with extractor in place of chromatograph has also been developed (Hamzaoui *et al.*, 2011). SIXCPE instrument on the other hand has less number of partition cells with larger columns. This method allows a flow rate of 30 ml/min, thus resulting in an 8.5 fold increase in the productivity, i.e., 28.3 g/h/L_{VC} (Hamzaoui *et al.*, 2012).

Both these techniques promise a better GSL estimation method. These methods may be employed for the industrial scale due to high purity. Expectantly, these techniques will be highly applicable in the near future.

Non-Chromatographic Methods

There are many methods apart from chromatography. Among these many methods have wide applications, and are usually used in combination with the chromatographic methods.

Spectroscopic Methods

Estimation of GSLs has been performed by many methods, but almost every method is followed by a spectroscopic analysis for the better identification and estimation of the compounds. Four types of spectroscopic methods are generally followed: X-ray spectroscopy, MS, Nuclear Magnetic Resonance (NMR) spectroscopy and Near Infrared Reflectance Spectroscopy (NIRS). Among these, MS has been the most commonly used method.

X-ray Spectroscopy

GSL analysis with the help of X-ray fluorescence was first reported by Schnug and Haneklaus (1987). It is the most simple and rapid analytical method based on the principle of nondestructive assay of total sulfur content in the sample (Vinjamoori *et al.*, 2004). The GSL content is then determined by comparing the results with the values of the reference samples (Vinjamoori *et al.*, 2004).

The method had been thoroughly improved by Schnug and Haneklaus (1987), who also reported the basic factors which might affect the accuracy. Among these, most important are the reduction of moisture content below 10 % and controlled heating of the seeds before crushing, in order to prevent the GSL breakdown (66). Although, not much importance has been given to this method (Schnug and Haneklaus, 1987; Vig, 2000; Vinjamoori *et al.*, 2004), still it is expected to obtain more applications in the near future.

Mass Spectrometry

MS has always been a method of interest for GSL

estimation among researchers. Ever since its first application reported by Bailey *et al.* (1961), which was further confirmed by Kjaer and Thomson (1963) and Kjaer (1963), it is continually being modified and improved. MS analysis could be performed directly using mass spectrometer (Helboe *et al.*, 1980; Mohn *et al.*, 2007), or it may be coupled with GC (Gil and MacLeod, 1980; Hasapis *et al.*, 1981; Olsen and Sorensen, 1981a), HPLC (Hogge *et al.*, 1988; Kokkonen *et al.*, 1991a) or Ultra Performance Liquid Chromatography (UPLC) (Gratacos-Cubarsi *et al.*, 2010; Ikeura *et al.*, 2010). But generally, due to the presence of GSLs in complex mixture, the combination of sensitive and specific method of MS with the highly efficient chromatographic technique is preferred for better information of structures (Heeremans *et al.*, 1989).

Many important improvements in the regular MS detection have already been applied for the estimation of GSLs such as thermospray liquid chromatography-MS (Mellon *et al.*, 1987; Hogge *et al.*, 1988), thermospray liquid chromatography-MS-MS (Heeremans *et al.*, 1989), negative ion electrospray MS (Zrybko *et al.*, 1997), fast atom bombardment liquid chromatography/MS (Kokkonen *et al.*, 1991a; Kokkonen *et al.*, 1991b) and the recent development of atmospheric pressure chemical ionization (APCI) MS (Tolra *et al.*, 2000), and programmed cone voltage electropray LC/MS (Mellon *et al.*, 2002). The new MS methods are superseding the earlier ones due to the improved performance, minimal sample requirement and better sensitivity.

MS is evolving continuously and getting more accurate, simpler, sensitive and automated with minimal sample preparation. It has always been among the most attractive method for the estimation of GSLs (Bailey *et al.*, 1961; Kjaer, 1963; Cole, 1975; Gil and MacLeod, 1980; Hasapis *et al.*, 1981; Olsen and Sorensen, 1980a; Mellon, 1987; Burke and Cominos, 1988; Hogge *et al.*, 1988; Heeremans *et al.*, 1989; Kokkonen *et al.*, 1991a; Kokkonen *et al.*, 1991b; Zrybko *et al.*, 1997; Tolra *et al.*, 2000; Kiddle *et al.*, 2001; Mellon *et al.*, 2002; Barbieri *et al.*, 2008; Millan *et al.*, 2009; Gratacos-Cubarsi *et al.*, 2010; Ikeura *et al.*, 2010; Maldini *et al.*, 2012). These methods have been applied for the isolation of both intact and desulfate GSLs and have continued to be in use till date (Maldini *et al.*, 2012).

Nuclear Magnetic Resonance (NMR) Spectroscopy

The use of NMR for the analysis of GSLSs was successfully reported by Tapper and MacGibbon (1967). This technique exploits the magnetic properties of the different GSLs to give the various physical and chemical characteristics of the compound. It is applicable for only those compounds which have spin.

NMR is a very useful, non-destructive method which provides information about structural confirmation. It has also proved to be very efficient for both qualitative and quantitative analysis and is highly applicable in identification of some GSLs which are not easily identified by other methods (Prestera *et al.*, 1996).

In spite of the above stated advantages, this technique is not preferred by many researchers due to its major drawbacks, viz., high cost, time consumption and the difficulty in interpretation of the spectra. From the time of the first application of this technique for GSL analysis, it has not been applied for much estimation (Tapper and

MacGibbon, 1967; Elliott and Stowe, 1971; Bjorkman, 1972; Olsen and Sorensen, 1979; Olsen and Sorensen, 1980a; Olsen and Sorensen, 1980b; Cox *et al.*, 1984; Arguello *et al.*, 1999; Kiddle *et al.*, 2001; Berhow *et al.*, 2010).

Near Infrared Reflectance Spectroscopy

NIRS is a rapid, reliable, reproducible and cost effective screening technique. It is a non destructive method with high accuracy (Velasco *et al.*, 1997). The different types of GSL are mainly determined by irradiating the compound and measuring the reflected light. This reflected light gives the information about the type of bonds present. Three types of bonds are mainly detected, viz., C-H, N-H and O-H (Foley *et al.*, 1998; Bala and Singh, 2013). This technique has been successfully employed for evaluating the GSL content of rapeseed (Mika *et al.*, 1997).

The basic requirement for analyzing the data of NIRS is the development of the calibration equation for converting the spectral information into chemical information (100). Since its first application by Tkachuk (1981) for GSL analysis, this method has not received much attention. But with the increasing demand for a non destructive method of analysis of GSLs attention has been provided to this method recently (Tkachuk, 1981; Mika *et al.*, 1997; Velasco *et al.*, 1997; Foley *et al.*, 1998; Kumar *et al.*, 2010; Bala and Singh, 2013).

Enzymatic Methods

The enzymatic methods of estimation of GSL are based on the use of an enzyme like in ELISA or by measuring the enzymatic degradation of the GSLs by myrosinase as in Test paper analysis and estimation using released glucose.

Enzyme Linked Immunosorbent Assay

ELISA is a highly specific method for GSL estimation developed by Hassan *et al.* (1988). Due to the small size of the GSL, it is required to be conjugated with high molecular weight immunogenic carrier such as proteins for invoking the immune response. This purpose was solved by conjugating sinigrin or progoitrin with either Bovine Serum Albumin (BSA) or ovalbumin (Hassan *et al.*, 1988; Van Doorn *et al.*, 1998). The antisera raised against the conjugate showed great specificity towards sinigrin, progoitrin and glyconapin, but had little cross reactivity with GSLs having aromatic side chain or oxidized thiogroup (Vig, 2000).

Presently not much work has been done in this direction, but it is expected that this method might gain more acceptance in the near future especially for the rapid analysis of a large number of samples for breeding purpose (Van Doorn *et al.*, 1998).

Test Paper

Test paper method or Glucose test paper method has been used for many years for the indirect determination of GSLs. It was used particularly due to its rapid and simple nature and the low cost. The whole experiment could be completed within 5 minutes. This method was developed by Comer (1956) for the determination of glucose in urine. The technique was soon adopted by many researchers for the determination of GSLs (Comer, 1956; Lein, 1970; Bjorkman, 1972; VanEtten *et al.*, 1974; McGregor and Downey, 1975; Saini *et al.*, 1987; Vig, 2000).

The glucose required for the estimation was released by GSLs after the reaction with myrosinase enzyme. The glucose thus released

reacts with glucose oxidase impregnated on the paper to form gluconic acid and hydrogen peroxide. This hydrogen peroxide further reacts with peroxidase, which changes the color of the chromagen impregnated on the paper (Lein, 1970; Saini *et al.*, 1987; Vig, 2000).

The method seems to be very easy, but has the limitations that the peroxidase is inhibited by the phenolic compounds of the extract and the method gives only an approximate quantity of GSLs. Though, some improvements were made such as removal of phenolics by ion exchange chromatography (Bjorkman, 1972), activated carbon (McGregor and Downey, 1975) and charcoal (VanEtten *et al.*, 1974), but technique was still not considered very effective and was not preferred by most of the researchers.

Estimation Using Released Glucose

Estimation of GSLs using enzymatically released glucose is an indirect method for the determination of GSLs. It is a simple technique based on the principle that glucose released by the hydrolysis of GSLs reacts with glucose oxidase to release hydrogen peroxide. The hydrogen peroxide formed by the reaction reacts with chromagen in presence of peroxidase to yield a color change. This color is determined by UV spectrophotometer and compared with calibration curve to give the concentration of GSLs in the sample (Miwa *et al.*, 1972; Ishida *et al.*, 2003). Glucose from GSLs may be obtained by using the endogenous myrosinase or the enzyme may be supplemented from outside.

Since, this technique provides an approximate concentration of GSLs, so it may be used mostly for the screening purposes and may not be employed for the technical purposes. This method may be followed for the initial screening and separation of plants with low GSLs from plants having high GSL content. Due to the rapid nature and the low cost, this technique was promoted by few researchers (Bjorkman, 1972; Miwa *et al.*, 1972; VanEtten *et al.*, 1974; Carlson *et al.*, 1981 Smith *et al.*, 1985; Gardrat and Prevot, 1987; Heaney *et al.*, 1988; Ishida *et al.*, 2003; Vinjamoori *et al.*, 2004; Smith and Dacomber, 2006).

This may be due to the few disadvantages: it gives an approximate result, it is difficult to make sure that all GSLs have been hydrolyzed and this technique is not suitable for the colored extracts (Bjorkman, 1972). It also faces the problem of inhibition of peroxidase by phenolic compounds, which was solved using the same alternate as in case of estimation using test paper.

Other Methods

Apart from the above mentioned methods, there are some other methods which recently been employed for the estimation of GSLs. Among these, microchip analysis is the latest technique and offers a potential alternate to the above mentioned processes.

Microchip Analysis

The method for the analysis of GSLs by microchips was developed by Fouad *et al.* (2008). This technique is based on the Capillary Electrophoresis on Microchips (u-CE). It is a rapid, automated and high throughput method requiring low quantity of samples. This nondestructive method performs the qualitative determination by exploiting the abilities of GSLs to form charge transfer complex with xanthene dyes. Both qualitative and quantitative determination of GSLs can be performed rapidly at the same time (Fouad *et al.*, 2008).

Due to its rapid procedure and the low reagent consumption, it may become a successful alternate to the earlier methods.

CONCLUSION

GSLs have always been a topic of interest among scientists due to its many beneficial properties. Various methods have been formulated to estimate this natural product with the main aim of developing an easy and rapid method. All the procedures had their own advantages and disadvantages, but one should always adapt according to convenience. While the earlier procedures like test paper method, PC and TLC were rapid, but lacked specificity. On the other hand, methods like GC, HPLC, ELISA, etc. provided separation with specificity. Many modern methods like microchip analysis, ion-exchange centrifugal partition chromatography, hydrophilic interaction liquid chromatography and centrifugal partition chromatography are providing hope for a potential alternate to the earlier methods.

However, research must be conducted on developing a rapid GSL estimation using only a few reagents and for small sample size. Moreover, a problem of limited availability of the standards needs to be addressed.

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