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⁂Recent Advances in the Analysis of Glucosinolates

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ABSTRACT

Occurrence and biochemistry of glucosinolates are briefly discussed. The chemistry of intact glucosinolates and their degradation products is considered in relation to the methods used for their determination. Different methods have been used, including ion-exchange chromatography, paper chromatography, high-voltage electrophoresis, ^{1}H- and ^{13}C-NMR spectroscopy. The quantitative analysis of trimethylsilylated desulfoglucosinolates by gas chromatography and of intact glucosinolates by high performance liquid chromatography is discussed in relation to previously used methods based on the determination of glucosinolate degradation products.

INTRODUCTION

Glucosinolates are natural products which produce particularly characteristic properties of most of the plants belonging to the order Capparales (1). This seems to be due to the co-occurrence of glucosinolates and thioglucoside glucohydrolases (EC 3.2.3.1.) (myrosinases) in all parts of glucosinolate-containing plants, leading to many different hydrophilic and lipophilic autolysis products when the plants are crushed. The pungency, flavor and many undesirable toxic manifestations of different crucifer materials are associated with glucosinolates, affecting, e.g., the technically and economically important oils and proteins from these plants. Hence, vast analytical interest is associated with the glucosinolates. We are still faced with numerous problems within this field, but novel techniques have recently been developed and may conceivably become of great importance.

Glucosinolates encompass more than 80 different compounds, most of which seem to be biosynthetically derived from a few of the known α-amino acids in higher plants (2-9). Normally, only a few of the glucosinolates are present in appreciable amounts in a particular plant. However, new plant varieties obtained by plant breeding involve the possibility of dominating compounds other than those known from related species. Our potential to disclose the existence of novel glucosinolates is closely associated with our knowledge of the biosynthetic capacities of the plants, the chemistry of the glucosinolates, and especially the applied analytical techniques. We need to consider more closely the advantages and disadvantages of the different analytical methods.

Glucosinolate analysis was previously based solely on estimations of the products produced by myrosinase or acid-catalyzed hydrolysis, often of only partially purified preparations (10-14). Ultraviolet (UV) spectrophotometric, thiocyanate ion, and quantitative glucose determinations on hydrolysis products from crude or partially purified preparations of glucosinolates are often relatively fast, cheap, and easy to perform (7-9 and refs. cited therein). These methods estimate glucosinolates as a class and are adequate for some purposes, but are inadequate for specific glucosinolate compositional data. Some glucosinolates escape detection and other plant constituents may interfere.

Newly developed methods involve isolation of intact glucosinolates by ion-exchange chromatography or other chromatographic purification methods preceding high performance liquid chromatography (HPLC) of intact glucosinolates and/or gas liquid chromatography/mass spectroscopy (GLC-MS) of trimethylsilylated desulfoglucosinolates. These new methods have been of great importance in searching for efficient methods of isolation, separation, and quantitative determination of glucosinolates.

MATERIALS AND METHODS

Plant material, purity and preparation of reagents and glucosinolates are described elsewhere (10,15-17).

Paper chromatography (PC), ion-exchange chromatography, and high voltage electrophoresis (HVE) were performed as previously described (15-17) by use of the following solvent and buffer systems: (solvent 1) 1-butanol/acetic acid/water (12:3:5); (solvent 2) 1-butanol/pyridine/water (6:4:3); (solvent 3) 1-butanol/ethanol/water (4:1:4); (buffer pH 1.9) acetic acid/formic acid/water (4:1:45), for 2 hr at 3.2 kV and 90 mA; (buffer pH 3.6) pyridine/acetic acid/water (1:10:200), for 2 hr at 3 kV and 90 mA; (buffer pH 6.5) pyridine/acetic acid/water (25:1:500), for 50 min at 5 kV and 90 mA. HPLC was performed by reversed-phase ion-pair liquid chromatography as recently reported (18).

GLC was performed as previously described (16).

^{1}H- and ^{13}C-NMR spectra were determined in D_2O solutions as described elsewhere (15-17).

RESULTS AND DISCUSSION

Occurrence and Biochemistry of Glucosinolates

Glucosinolates are a well known group of natural products (2-9). They occur in appreciable amounts in plants of the order Capparales (1) and in a few other dicotylodonous plants outside this order (3). They apparently are always accompanied by myrosinases (3,5,19-22). High concentrations of these compounds are found especially in seeds (23,24) and in actively growing parts of the plants (8,15, 17). Generally, the concentrations found in green plant parts range from about 0.001 to 5 mg/g fr. wt. (8,10,16,17,25) corresponding to 10^{-4}-0.5%, whereas in seeds, up to about 100 mg/g dry wt., corresponding to 10%, may be found. The glucosinolate pattern varies considerably within varieties belonging to the same species (16) in different parts of the same plant (2,10,15,17,25) and during the development of the plant (26), and some glucosinolates in previously investigated plants may not have been detected (10,16,27).

The glucosinolate biosynthetically derived from glycine (R = H; Fig. 1) is unknown but is the basis for the semisystematic glucosinolate nomenclature (see next section). Methylglucosinolate, biosynthetically derived from alanine, has not yet been detected in Brassicaceae but is characteristic of Capparaceae. Glucosinolates that are biosynthetically derived from methionine (**2-13** and homologs thereof; Table I) are characteristic of cruciferous plants, e.g., rapeseed. This is also the case for some phenylalanine; tyrosine- and tryptophane-derived glucosinolates (**14-22**; Table I). Some of the other compounds (**17-21**) have been detected in only a few plant species (17), whereas **22** and derivatives thereof are found in many plant species (8 and refs. cited). The structure of **19** has not yet been published but **19** occurs as a dominating glucosinolate in some plants (28). Glucosinolates that are derived from valine, leucine and isoleucine—not included in Table I—are well known constituents of different plants, whereas glucosinolates derived from basic amino acids and glucosinolates with a free carboxylic acid group in the side chain are unknown.

Much is known about the biosynthetic pathways of glucosinolates at the substrate level (4-6) but only little is known about their catabolism (15,17,29-31). No description is available in the literature concerning the site of their synthesis or the transport in plants, but it has been indicated that indol-3-ylmethylglucosinolates in *Isatis tinctoria* L. have a rapid turnover (32). Also, the physiological effect of different glucosinolates and products thereof is only insufficiently known (3,8,16 and refs. cited therein). However, it is known that autolysis may lead to different products (8,29,33), to some extent depending on the glucosinolates structure (8,29) but the type of products formed from one and the same glucosinolate (vide infra) is also affected by other plant constituents (3,8,15,17,29, 33-35). Therefore, the chemistry and the properties of intact glucosinolates and their degradation products and the reliability of the analyses used in the investigations of these compounds have to be studied further.

Structure and Properties of Glucosinolates

The structural formulas for the side chains of the glucosinolates discussed in this paper are shown in Table I.

The nomenclature of these compounds is thoroughly discussed in the authoritative review already mentioned (3) and needs little further comment. It is sufficient to mention

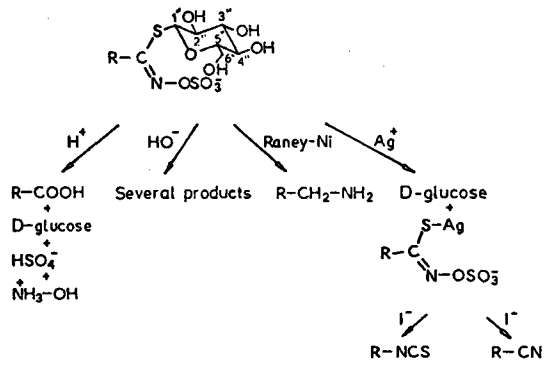


FIG. 1. Nonenzymatic degradation of glucosinolates.

that it is recommended to use the semisystematic nomenclature in which the anion (R = H; Fig. 1) is designated by the term glucosinolate and the chemical name of the side chain R (R ≠ H) is used as prefix. In accordance herewith, we assign the glucosinolate aglucone carbon atom no. 0 (Fig. 1), the first carbon atom in the side chain no. 1, and so on (Table I).

The glucosinolate structure (Fig. 1) has been confirmed by X-ray crystallographic investigation of the potassium salt of allylglucosinolate (sinigrin; **2**) (36) and by chemical investigations performed on both sinigrin, the sinapine salt of *p*-hydroxybenzylglucosinolate (sinalbin; **18**) and the tetramethylammonium salt of benzylglucosinolate (glucotropaeolin, **14**) (37,38). The glucosinolates have near planarity around the double bond, i.e., the oxygen atom, nitrogen atom, carbon atom no. 0, thioglucosidic sulfur atom, and carbon atom no. 1 are almost coplanar. The configuration around the double bond is Z as shown in the formula in Figure 1. This general glucosinolate structure has, furthermore, been confirmed for all of the intact glucosinolates investigated in our laboratory by use of HVE and ^{1}H- and ^{13}C-NMR spectroscopy (see following text).

The sulfate group imparts strongly acidic properties to the glucosinolates. The very low pK_a value of this group implies that glucosinolates occur in nature as anions and, because of the instability of glucosinolates in strong acidic solutions, it is also necessary to work with these compounds as salts in the laboratory. The sulfur-connected carbohydrate is an unsubstituted β-D-glucopyranosyl moiety in all of the glucosinolates investigated up to now and with a preferred conformation as shown in Figure 1. This is revealed from X-ray data obtained for sinigrin (36) and the NMR and HVE data obtained for this compound and other glucosinolates in water as shown later.

The sulfate group and the thioglucose moiety impart nonvolatile and hydrophilic properties to all glucosinolates. This is important when proper methods for analysis of intact glucosinolates have to be considered. The R group, although perhaps always derived from amino acids, varies in properties from lipophilic to marked hydrophilic (Table I) (2-9,15). This is important when proper analysis methods based on determination of glucosinolate degradation products are to be considered (8,16).

Nonenzymatic Degradation of Glucosinolates

Figure 1 shows some important nonenzymatic degradation possibilities used in investigations of glucosinolates.

The glucosinolates are relatively easily decomposed in both strongly acidic and strongly basic solutions (37-39). In acidic solutions, the hydrolysis products are the corresponding carboxylic acids, D-glucose, hydroxylammonium ion, and hydrogen sulfate (37,38). The carboxylic acids

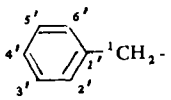
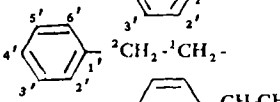
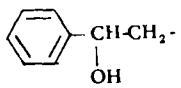
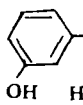
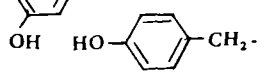
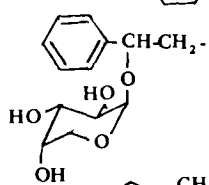
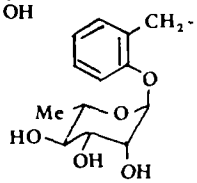
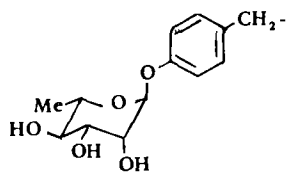
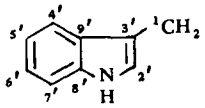
and D-glucose are easily determined by GLC-MS of the trimethyl silylated products (15,17); for D-glucose, enzymatic determination is efficient, as well. The identity of the product may also be established by PC and HVE (15,17). However, it is difficult to transform the glucosinolates quantitatively into carboxylic acids (39) and some glucosinolate side chains, e.g., indolyl derivatives, are unstable in acidic solutions. Therefore, this method is not to be recommended as a quantitative method for glucosinolate analysis. Acid-catalyzed hydrolysis is of value in the identification of new glucosinolates (15,17); otherwise, it is necessary to avoid acidic conditions during glucosinolate isolation (10,16).

Glucosinolates are easily transformed into several products in basic solutions (39,40). Compounds containing activated hydrogen at C-1, i.e., allyl- and benzylglucosinolates, form the corresponding α-amino acids and thioglucose in a reaction proposed to involve a Neber-type rearrangement (40). Some R-groups, e.g., phenols are also unstable in basic solutions (15,17).

The stabilities of but-3-enylglucosinolate (gluconapin; 3) and 2-hydroxybut-3-enylglucosinolate (progoitrin; 5)

TABLE I

Selected Glucosinolates[a] Representing the Requirements to Methods of Glucosinolate Analaysis

No.	Structure of R group[a]	Semisystematic names
1	$^{1}CH_3$-	Methylglucosinolate
2	$^{3}CH_2$=^{2}CH-$^{1}CH_2$-	Allylglucosinolate
3	$^{4}CH_2$=^{3}CH-$^{2}CH_2$-$^{1}CH_2$-	But-3-enylglucosinolate
4	CH_2=CH-CH_2-CH_2-CH_2-	Pent-4-enylglucosinolate
5	CH_2-CH(OH)-CH_2-	2-Hydroxybut-3-enylglucosinolate
6	CH_2=CH-CH_2-CH(OH)-CH_2	2-Hydroxypent-4-enylglucosinolate
7	CH_3-S-CH_2-CH_2-CH_2-	3-Methylthiopropylglucosinolate
8	CH_3-S-CH_2-CH_2-CH_2-CH_2-	4-Methylthiobutylglucosinolate
9	$^{4}CH_3$-SO-$^{3}CH_2$-$^{2}CH_2$-$^{1}CH_2$-	3-Methylsulfinylpropylglucosinolate
10	CH_3-SO-CH_2-CH_2-CH_2-CH_2-	4-Methylsulfinylbutylglucosinolate
11	CH_3-SO-CH_2-CH_2-CH_2-CH_2-CH_2-	5-Methylsulfinylpentylglucosinolate
12	CH_3-SO_2-CH_2-CH_2-CH_2-	3-Methylsulfonylpropylglucosinolate
13	CH_3-SO_2-CH_2-CH_2-CH_2-CH_2-	4-Methylsulfonylbutylglucosinolate
14		Benzylglucosinolate
15		Phenethylglucosinolate
16		2-Hydroxy-2-phenylethylglucosinolate
17		*m*-Hydroxybenzylglucosinolate
18		*p*-Hydroxybenzylglucosinolate
19		2-(β-L-Arabinopyranosyloxy)-2-phenylethylglucosinolate
20		*o*-(α-L-Rhamnopyranosyloxy)benzylglucosinolate
21		*p*-(α-L-Rhamnopyranosyloxy)benzylglucosinolate
22		Indol-3-ylmethylglucosinolate

[a]For the glucosinolate structure, see Fig. 1.

have been examined at different pH values and temperatures (41). The results indicate that these glucosinolates are unstable in both acidic and basic solutions, and they are especially unstable in borax solutions. They show the greatest stability in neutral solutions, but it is evident that glucosinolates are not very stable in aqueous solutions. It is obvious that a more systematic investigation of these properties is required.

Aqueous solutions of glucosinolates containing mercury and/or silver ions or, very likely, other metal ions capable of forming nearly insoluble sulfides, result in the formation of D-glucose and the metal derivative of the aglucone (Fig. 1). The metal derivative can be further degraded under controlled conditions to the corresponding nitrile or isothiocyanate, depending on pH of the solution (17,42). Furthermore, some isothiocyanates rearrange to the corresponding thiocyanates and/or decompose to other products (8,16,29,31) because of the great reactivity of these compounds.

Glucosinolates can be reduced to primary amines using Raney-Nickel catalyses in aqueous solution at room temperature (37-39). The position of the amino group is at the carbon atom corresponding to C-0 in the glucosinolates; these amines are thus different from the amines proposed to be catabolic products of glucosinolates (15,17,30,31). The amino group in these compounds is at the carbon atom corresponding to C-1 in the glucosinolates.

Enzymatic Degradation of Glucosinolates

Glucosinolate catabolism is a subject of controversy. The known enzyme-induced glucosinolate degradation products (Fig. 2) have been discussed elsewhere (2-9), and only a few points related to glucosinolate analysis will be discussed briefly.

Desulfoglucosinolates (Fig. 2) are known intermediates in the biosynthesis of glucosinolates (9) and they are formed in a sulfatase-catalyzed hydrolysis used for analytical purposes (43). The sulfatases have not been described as constituents of plants but the enzymes have been isolated from other sources (43).

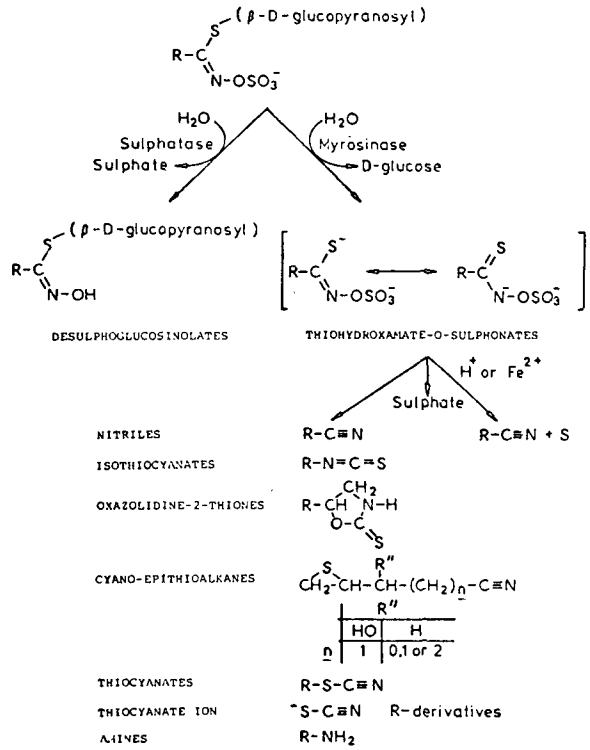


FIG. 2. Enzymatic degradation of glucosinolates.

Myrosinases catalyze the hydrolytic cleavage of the thioglucosidic bond (Fig. 2) by which glucosinolates produce D-glucose and thiohydroxamate-O-sulfonates. The thiohydroxamate-O-sulfonates collapse under release of sulfate either to the corresponding isothiocyanates or nitriles and elementary sulfur, depending on the concentration of H^+ or other constituents, e.g., Fe^{2+} (17,39,42, 44). In a study of the degradation of sinigrin (42), the ratio of nitrile to isothiocyanate was found to be directly proportional to the hydrogen ion concentration in the pH range 2-5. Metal ions likewise promote nitrile formation and it is well known that the myrosinases have different cofactor requirements, e.g., vitamin C (17,45,46). Myrosinase-catalyzed reactions often produce end products other than isothiocyanates and nitriles (47,48) from the glucosinolate side chain. There is no description of glucosinolate-containing plants which do not contain myrosinases.

Autolysis, i.e., degradation of endogenous glucosinolates in aqueous suspensions of disrupted plant material due to catalysis of endogenous myrosinases and maybe other plant constituents, often leads to many different products. Some glucosinolates, e.g., 2,8,14 (Table I) form thiocyanates as autolysis products in some plants, although the same glucosinolates in other plant species are transformed mainly into isothiocyanates (29).

All methods of quantitative glucosinolate analysis require that uncontrolled glucosinolate degradations are avoided in the initial steps of homogenization and preparation of the crude glucosinolate extracts. Interfering substances must be removed, and often, concentrations of the glucosinolate fractions are required for reliable quantitative methods of analysis. Furthermore, if the methods of quantitative analysis of individual glucosinolates are based on products from myrosinase-catalyzed hydrolysis, it is not possible to obtain well defined aglucone products from all glucosinolates, some of the products just mentioned are difficult to detect quantitatively, and some isothiocyanates are very unstable (see following text).

Isolation of Glucosinoaltes by Ion-Exchange Chromatography

As revealed from the discussion just given, isolation of intact glucosinolates requires that some metals and strongly acidic and basic conditions are avoided. This is achieved by the newly described technique (16,17) in which a strongly acidic ion-exchange column is connected in series to a weakly basic Ecteola anion-exchange column. The latter has tertiary amino groups (pK_a ca. 7.5) as functional groups, which permits the elution of the total pool of glucosinolates by removal of the positive charges on the Ecteola column using M pyridine as eluant. This eluant is volatile, and the glucosinolates are easily isolated as pyridinium salts under gentle conditions (16 and refs. cited therein).

Previously, enzyme solutions have been used to release measurable products of the glucosinolates from other types of anion-exchange columns; desulfoglucosinolates released by sulfatase (43,49,50) and aglucone products and D-glucose released by myrosinase (13) (Fig. 2).

Paper Chromatography (PC) and High Voltage Electrophoresis (HVE)

It is recommended to use PC instead of TLC when examining glucosinolates. This is a general experience in chromatographic analysis of salts, hydrophilic compounds, and/or impure preparations of natural products. However, TLC is recommended for investigations of thiourea derivatives and

TABLE II

R_B-Values[a] from PC, Ionic Mobilities from HVE, and k'-Values from Reversed Phase Ion-Pair HPLC[b] of Some Glucosinolates

Glucosinolate Compound no. (Table I)	MW	R_B-value from PC in solvent system[b]			Distance in cm by HVE in buffer system[b]			k'-value from HPLC[b]
		1	2	3	pH 1.9	pH 3.6	pH 6.5	
1	331	0.46	0.54	0.38	23.6	21.3	15.0	3.3
2	357	0.65	0.62	0.50	22.8	20.3	14.2	4.2
3	371	0.98	0.81	0.84	22.5	19.8	13.7	–
4	385	1.16	0.91	1.18	22.0	19.6	13.2	5.0
5	387	0.68	0.63	0.50	21.8	19.6	13.2	3.7
9	421	0.35	0.22	0.31	20.4	18.8	12.5	2.1
14	407	1.00	1.00	1.00	21.4	18.9	12.7	6.7
15	421	1.27	0.98	1.30	21.2	18.5	12.5	9.8
16	437	1.02	0.93	0.91	20.8	18.1	12.2	6.2
17	423	0.79	0.81	0.74	20.8	18.3	12.1	6.4
18	423	0.73	0.81	0.68	21.1	18.8	12.0	5.0
19	577	0.55	–	0.45	17.8	15.0	9.5	–
20	579	0.69	0.60	0.67	18.2	15.2	9.8	–
22	446	0.96	0.89	0.97	20.3	17.8	11.8	9.8

[a]The R_B-values are mobilities relative to benzylglucosinolate.

[b]For experimental conditions, solvent and buffer systems, see Material and Methods.

5-substituted oxazolidine-2-thiones (7 and refs. cited therein). Only qualitative investigations can be performed by PC and TLC and with simultaneous use of reference compounds of related structures. If we meet these conditions, important information can be obtained by these methods.

PC of crude extracts has previously been done (7 and refs. cited therein). The moderate success of this method is partly due to lack of specific reagents for detection of glucosinolates. More or less specific reagents for the glucosinolates and their different R groups are available (7,17 and refs. cited therein). Another reason for the moderate success of PC is that impure methanol/water extracts are only poorly separated. This is no longer an obstacle because it is now a fast and easy routine to isolate the total glucosinolates by ion-exchange chromatography as already described.

Table II presents some selected PC data which illustrate the obtainable information concerning the hydrophilic or lipophilic properties of the R groups. A definitive identification of glucosinolates by PC is not possible, but the results are easy to obtain and are valuable in connection with identification of new compounds (15,17) and in the screening of known compounds. The results obtained from PC combined with those from HVE and HPLC of intact glucosinolates and GLC of trimethylsilylated desulfoglucosinolates are very informative.

The information obtainable from HVE also is shown in Table II. The HVE mobilities are a function of ℓn molecular weight (MW) or, more correctly, molecular size of the

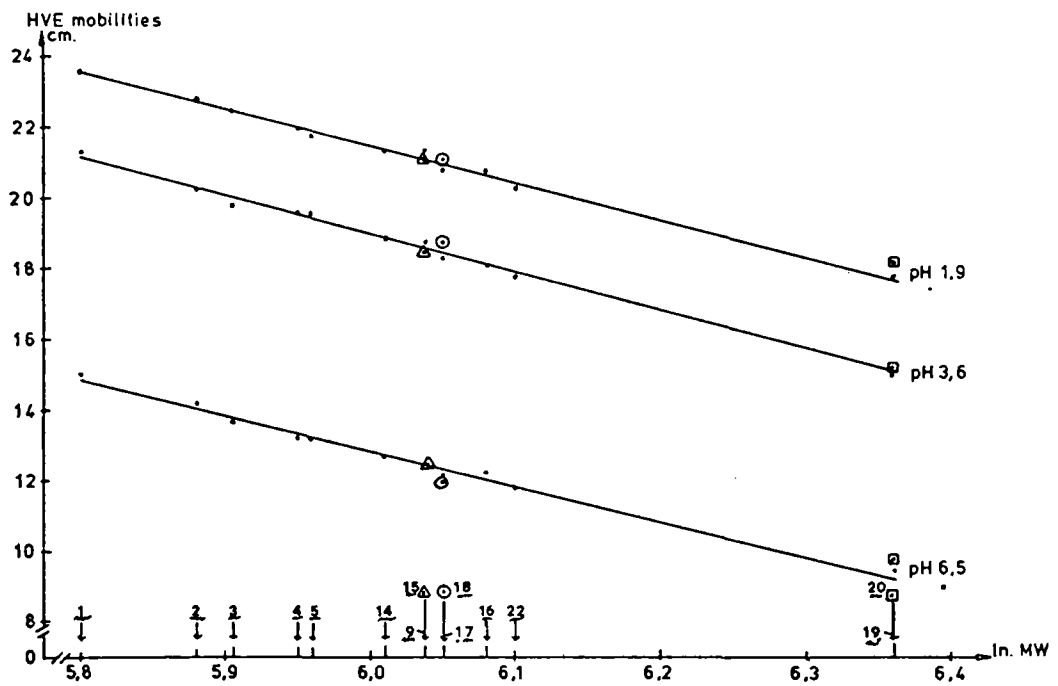


FIG. 3. Relationship between the natural logarithm of molecular weight (MW) and mobilities found by HVE (in cm) at different pH values for some glucosinolates. The points at the 3 lines correspond to the mobilities for the compounds shown by the no. (Table I) vertically below the points along the lnMW axis.

glucosinolates (Fig. 3). The mobilities are very sensitive to differences in the experimental conditions as can be seen from the mobilities in different buffer systems and, therefore, internal references are necessary. Nearly the same slope is found for the HVE mobilities at three different pH-values, and it is well documented that the HVE mobilities are directly proportional to the net charge of the compounds. The different glucosinolates thus have the same net charge in all three buffer systems, even at pH 1.9, reflecting the low pK_a-value of the sulfate group. HVE mobilities easily reveal the presence of more than one carbohydrate part (19-21; Tables I and II) (15). It will also be possible to reveal additional protolytic-active groups in the side chains, e.g., (N-sulfoindol-3-yl)methylglucosinolate or glucosinolates with side chains derived from acidic or basic amino acids. Preparative HVE at pH 1.9 provides an efficient separation of glucosinolates from other plant constituents, including carboxylic acids (51).

Spectroscopic Properties of Glucosinolates

Intact glucosinolates have a UV absorption maximum at 235 nm due to the glucosinolate group, which can be used for the detection of these compounds by HPLC (18). Furthermore, some glucosinolates may have UV absorptions at other wavelengths due to the R groups.

^{1}H- and ^{13}C-NMR spectra of intact glucosinolates are very informative with respect to the identification of intact glucosinolates (15,17).

A collection of NMR data obtained from different glucosinolates isolated as pyridinium salts by the procedure now described is presented in Table III.

Some of the spectral data have previously been used for the identification of a few of these compounds (15,17). They are included here as references, and for the purpose of obtaining a more general picture of the structure of glucosinolates in water solution. It appears that the anomer carbon atom in these thioglucosides has a ^{13}C-chemical shift very different from that of the anomer carbon atom in O-glucosides. This shift and the shift value for C-0 are especially characteristic of intact glucosinolates.

By correlation to the structure for sinigrin (2), which has been proved by X-ray crystallography, the structure for the other glucosinolates can be deduced from the NMR data. All of the investigated compounds have nearly the same ^{13}C-chemical shifts for C-0 and the glucose carbon atoms and, therefore, a configuration at the double bond and a β-thio-D-glucopyranosyl structure as shown in Figure 1 are present. The β-thio-D-glucopyranosyl configuration is also revealed from the ^{1}H-NMR spectra, but the data obtained therefrom (with respect to the glucose part) are difficult to give an exact interpretation. It is possible to identify the structures of the aglucone parts from NMR data as it is known from other natural products, e.g., the amino acid precursors.

Gas Liquid Chromatography (GLC) of Trimethylsilylated Desulfoglucosinolates and GLC-MS of These Compounds

Trimethylsilylation of glucosinolates isolated as pyridinium salts by ion-exchange chromatography results in derivatives of desulfoglucosinolates (Fig. 2) which are easily separated and determined quantitatively by GLC (16). This method allows quantitative analysis of even small amounts of glucosinolates in leaves or other plant parts (17). Furthermore, rather small amounts of specific glucosinolates can be detected in mixtures containing high concentrations of others. This is illustrated in Figure 4, which shows two chromatograms of different amounts of the same trimethylsilylated glucosinolate sample isolated from leaves of *Reseda media* Lag. The concentrations found for the identified glucosinolates (compound no. [Table I], mg/g fr. wt.) are: (14, 2.1), (15, 0.2), (17, 12.7), and (22, <0.01).

From a quantitative point of view, GLC of trimethylsilylated desulfoglucosinolates is much more reliable than methods based on glucosinolate degradation products. This is especially true when glucosinolates are involved that have β-hydroxylated, hydrophilic and aromatic R-groups (5,6, 16-22; Table I). However, some problems still exist concerning the quantitative GLC analysis of trimethylsilylated desulfoglucosinolates of the structural type 7-13 (Table I),

TABLE III

^{13}C Chemical Shifts (δ) for Pyridinium Salts of Glucosinolates in D_2O Solution; Carbon No. as Fig. 1 and Table I

	Glucosinolate no. (Table I)											
Atom no.	1	2	3	5	9	14	15	16	17	18	20[a]	22
Glucose moiety												
1"	82.5	82.4	82.5	82.7	82.4	82.2	82.5	82.6	82.2	82.2	82.4	82.2
2"	72.8	72.9	72.8	72.8	72.7	72.7	72.8	72.7	72.8	72.8	72.7	72.9
3"	78.0	78.0	77.8	77.8	77.8	77.9	77.9	77.9	77.3	77.9	77.8	77.9
4"	70.1	70.1	69.9	69.9	69.9	69.7	69.3	69.9	69.5	70.1	69.5	69.7
5"	80.8	80.7	80.7	80.7	80.9	80.6	80.9	81.1	80.6	81.1	80.6	80.8
6"	61.6	61.6	61.4	61.4	61.4	61.2	61.4	61.5	61.2	61.8	61.1	61.3
Aliphatic moiety												
0	162.7	163.6	161.1	161.5	163.3	163.3	161.6	161.0	163.4	163.7	163.7	162.9
1	19.0	36.9	33.2	40.0	31.6	39.0	34.7	42.1	38.9	38.4	34.7	30.1
2		132.9	31.5	70.3	20.5		44.1	71.9				
3		119.1	137.3	139.4	52.2							
4			116.8	117.1	37.4							
Aromatic moiety												
1'						136.0	141.4	143.0	137.6	127.6	120.8	
2'						130.1	129.6	129.8	115.3	130.4	154.2	124.7
3'						128.9	129.5	127.0	156.8	116.8	115.2	108.9
4'						128.4	127.4	129.2	115.8	155.7	131.2	119.5
5'						128.9	129.5	127.0	131.4	116.8	123.5	120.3
6'						130.1	129.6	129.8	121.1	130.4	130.0	122.9
7'												112.8
8'												136.8
9'												127.1

[a]For the rhamnose moiety, see ref. 15.

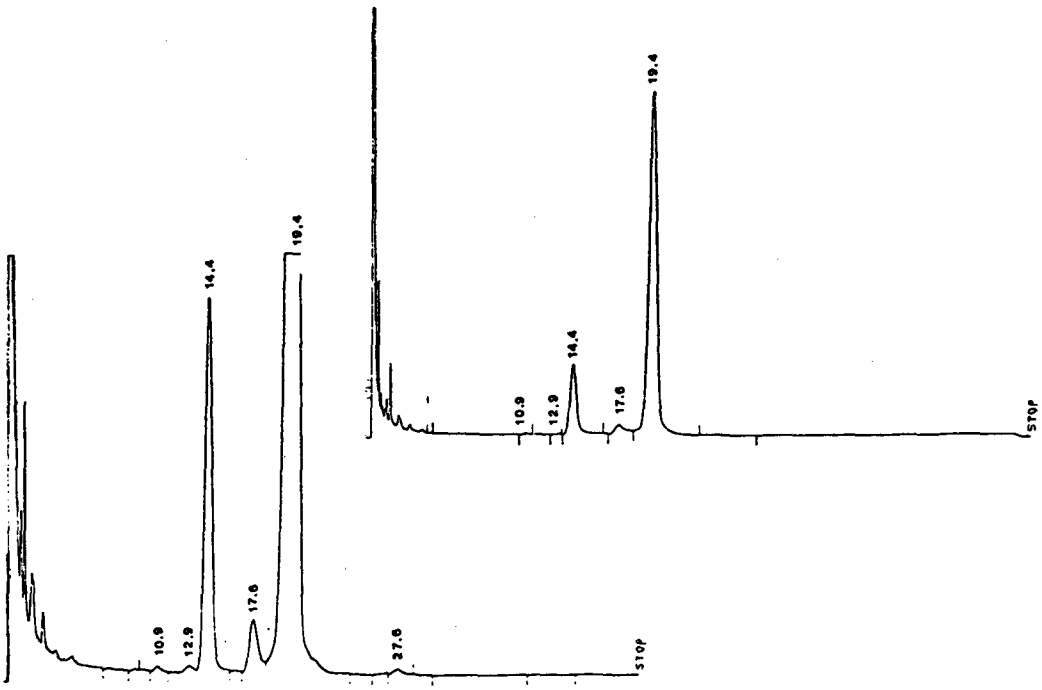


FIG. 4. GLC of trimethylsilylated glucosinolates isolated from leaves of *Reseda media* Lag. obtained by injection of 1 μL and 5 μL, respectively, of the silylated mixture. The peaks with retention time (t_R) (min) correspond to: t_R = 14.4: benzylglucosinolate (14); t_R = 17.6: phenethylglucosinolate (15); t_R = 19.4: *m*-hydroxybenzylglucosinolate (17); t_R = 27.6: indol-3-ylmethylglucosinolate (22); t_R = 10.9 and 12.9 are as yet unidentified.

possibly the same types of problems as described elsewhere for the quantitative GLC analysis of isothiocyanates produced from these compounds (11,12). This has not yet been satisfactorily investigated, but promising results are obtained by recently performed GLC-MS of trimethylsilylated desulfoglucosinolates (52), and HPLC can be applied in the study of these compounds.

Reversed-Phase Ion-Pair High Performance Liquid Chromatography (HPLC)

Glucosinolates isolated by the ion-exchange chromatographic method already described are efficiently separated by reversed-phase ion-pair HPLC as described elsewhere (18). Methanol is used as a modifier and tetraalkylammonium ions are used as counterion, the capacity factor (k') = retention time (t_R)/retention time of nonsorbed substance (t_o) is 1.0 for naphthalene. The obtained k' values are compared to the R_B values from PC (Table II). The results show that small R_B values in PC correspond to small k' values in this type of HPLC. This HPLC method has a very high resolution capacity compared to PC. HPLC is an efficient alternative and/or supplement to other methods used in the study of intact glucosinolates, e.g., PC and HVE, and GLC of trimethylsilylated desulfoglucosinolates. Furthermore, HPLC can be used for quantitative analysis of intact glucosinolates, and it is the only method described up to now which allows simultaneous determination of common types of glucosinolates.

New and Traditionally Applied Methods of Glucosinolate Analysis

Previously, the structural identification of glucosinolates was exclusively based on identification of their degradation products. The same methods adapted to quantitative glucosinolate analysis have lead to serious drawbacks. These are especially due to the analyst's ignorance of the many different hydrophilic and lipophilic enzymic hydrolysis products which may be found, especially during autolysis. If an inactivation of the endogenous myrosinases is performed in the homogenization steps and myrosinases isolated from another source are used instead, then it is possible to transform some glucosinolates into lipophilic isothiocyanates, oxazolidine-2-thiones or nitriles, but not always quantitatively. These compounds form the basis for most of the previously used methods of glucosinolate analysis (Fig. 5).

Isothiocyanates are fairly reactive compounds toward nucleophilic reagents, but some of them are sufficiently stable to permit isolation. With ammonia and amines, they produce substituted thioureas, and this property is often used for identification and analytical purposes. Thiourea derivatives are stable, often well crystallizing compounds having a characteristic UV-absorption at about 243 nm (53,54). Isothiocyanates are easily transformed into thiocarbamates by reaction with alcohols. Thus, the 2-hydroxy-substituted glucosinolates (Table I; 5,6,16) on myrosinase-catalyzed hydrolysis form isothiocyanates that spontaneously cyclize to 5-substituted oxazolidine-2-thiones. Correspondingly, tetrahydro-1,3-oxazine-2-thiones are produced from 3-hydroxysubstituted isothiocyanates. The thiocarbamate structure has UV-absorption at about 244 nm, which is used for analytical purposes (54,55). Some of the glucosinolates containing aromatic R-groups (Table I; 14-22) produce unstable isothiocyanates (16 and refs. cited therein) and, therefore, other stable end products may be formed, e.g., alcohols from solvolysis together with the thiocyanate ion. Furthermore, many glucosinolates produce only hydrophilic (nonvolatile) degradation products, as revealed from the structures shown in Table I.

All types of glucosinolates present in plant materials must, however, be considered important when methods of their analysis are discussed. From the discussions in this paper and the recently reported data (12) we conclude that, no matter how sophisticated the GLC analysis of myrosinase-catalyzed glucosinolate degradation products will be,

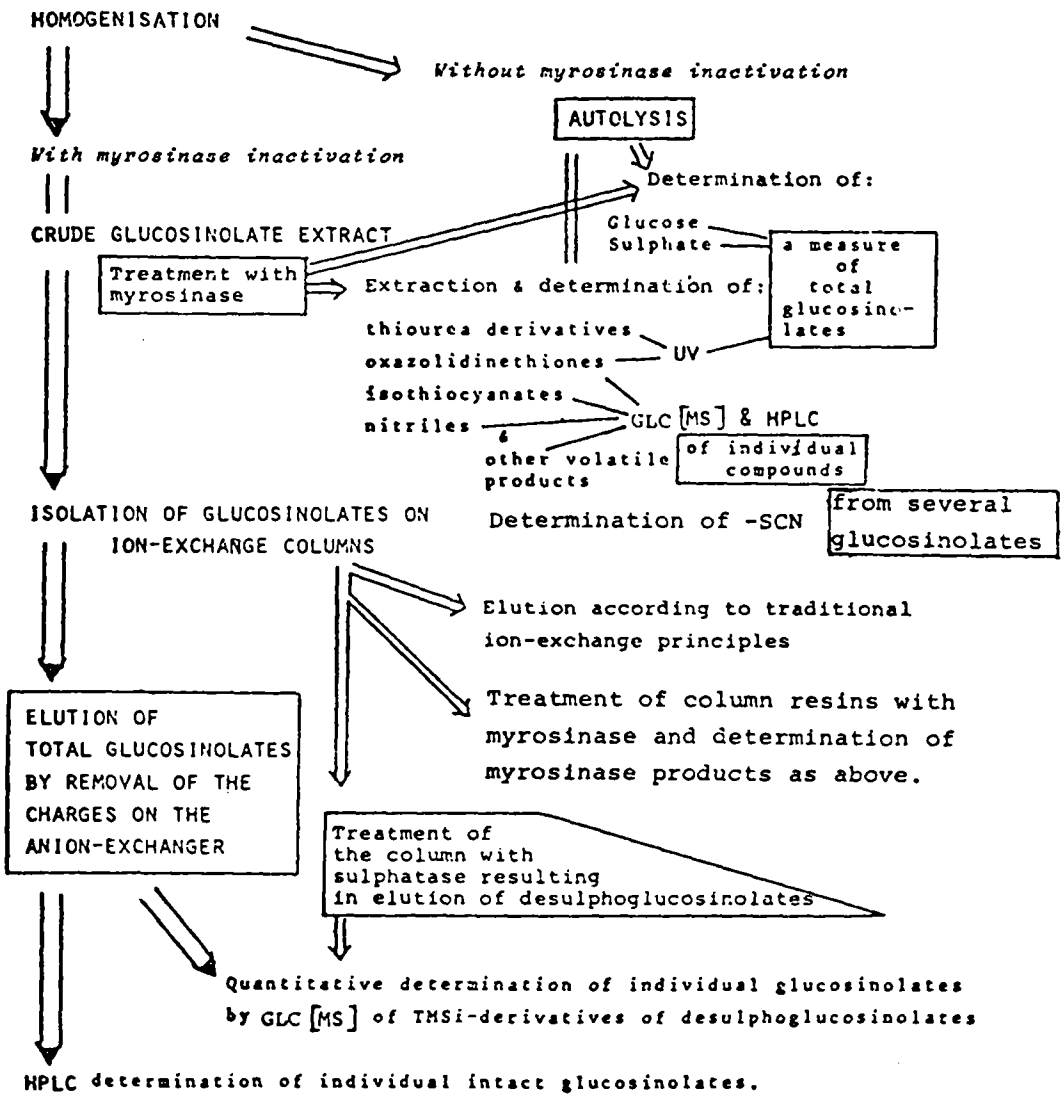


FIG. 5. Comparison of experimental steps involved in glucosinolate studies using the traditionally applied methods based on investigations of glucosinolate degradation products and new methods based on estimation of individual, intact glucosinolates.

it can only afford an approximate picture of the glucosinolates in the plants. Therefore, our efforts have been directed toward the determination of the intact glucosinolates.

Figure 5 shows a comparison of the experimental steps involved in the glucosinolate studies using different methods of analysis. Traditionally applied methods based on determination of enzyme-induced degradation products—UV-, glucose-, sulfate-, and thiocyanate ion—have been described elsewhere (7,8 and refs. cited therein). These methods allow only an estimation of glucosinolates without distinction, some escape detection and other plant constituents may interfere. However, the enzymatic glucose determination is elaborated as a fast, cheap and easy method, e.g., useful for different plant breeding purposes. The methods based alone on determination of glucosinolate R-groups after myrosinase treatment (7-9), e.g., GLC-MS (33,35,56) and HPLC (57) are efficient in structural identification of some glucosinolates but unacceptable for quantitative purposes as revealed from this discussion, especially if the methods are based on autolysis products (12,58).

Today, however, the access to powerful chromatographic methods, especially the ion-exchange technique (15,17), reversed phase ion-pair HPLC (18) and GLC of trimethylsilylated desulfoglucosinolates (15,17) combined with MS (52) and other spectroscopic methods (NMR, UV) (17) has made the recognition of known and new glucosinolates almost a matter of routine. PC and HVE are important tools in qualitative glucosinolate studies and for some preparative purposes (51). The methods now described involving ion-exchange chromatography followed by HPLC and/or GLC of trimethylsilylated desulfoglucosinolates allow separation and quantitative determination of microgram amounts of intact individual glucosinolates at gentle conditions in an easy, cheap, and fast procedure.

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