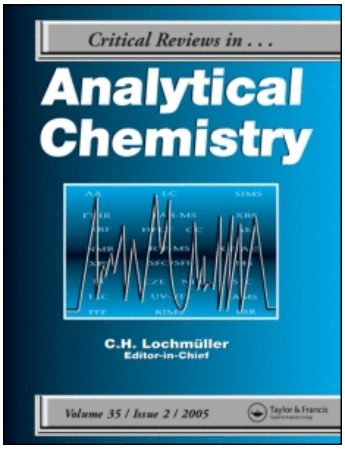

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Determination of Glucosinolates and Their Decomposition Products–Indoles and Isothiocyanates in Cruciferous Vegetables

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Several epidemiological studies have shown that a high intake of cruciferous vegetables, for example cabbage, broccoli or Brussel sprouts has beneficial influence on human health. Consumption of these vegetables is related to a lower risk of several cancers. Bioactive compounds such as glucosinolates and products of their decomposition—isothiocyanates and indoles—have an influence on anti-cancer properties. However, there is poor information about methods of detrmination of these compounds present in cruciferous vegetables. A significant analytical challenge for scientists is to gain reliable data about the presence and quantity of compounds with anti-cancer properties in samples of vegetable material. The purpose of this review is to present and introduce advantages and disadvantages of different methods of determination of glucosinolates, isotiocyanates and indoles present in cruciferous vegetables. High-performance liquid chromatography is general technique of analysis of glucosinolates, indoles and total isotiocyanates content. Identification of isothiocyanates from plant samples is accomplished by gas chromatography.

Keywords glucosinolates, indoles, isothiocyanates, analysis, cruciferous vegetables

INTRODUCTION

The of epidemiological research and of experiments performed in animal models suggest that a high level of consumption of vegetables from the *Brassicaceae* (*Cruciferae*) family, such as broccoli, Brussels sprouts and cabbage, may protect the human organism from the development of cancer (1–3). In this connection, these vegetables have engendered the interest of researchers as a potential source of substances that may find application in the prevention of oncological diseases. The antitumour properties of cruciferous edible plants are linked with the presence of glucosinolates which, under the influence of the enzyme myrosinase, are decomposed to isothiocyanates and indole compounds. Both glucosinolates and their decomposition products exhibit biological activities crucial from the point of view of chemoprevention of neoplasms (4). It is, therefore, important to develop optimal methods of identifying and quantitatively determining such compounds in vegetable tissues, as well as in food products and body fluids.

GLUCOSINOLATES AND THEIR DECOMPOSITION PRODUCTS CONTAINED IN CRUCIFEROUS PLANTS

Because of their chemical composition, more than 300 compounds already described in the literature (5) have been classified as glucosinolates (GLS). They are present mainly in plants from the crucifer family ($>$ 3000 species); the representatives of only a few genera are edible, however. All glucosinolates have a similar basic structure, which includes:

- A β-D-thioglucose group,
- A sulphonated oxime group,
- A side chain derived from one of seven protein amino acids (6) (Fig. 1).

Depending on the chemical structure of the side chain (R), glucosinolates can be divided into several groups:

- Aliphatic glucosinolates,
- ω-methylalkyl glucosinolates,

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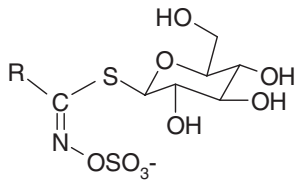

FIG. 1. Chemical structure of glucosinolates.

- Aromatic glucosinolates,
- Heterocyclic (indole) glucosinolates.

Chemically, glucosinolates are highly stable. Biologically active isothiocyanates (ITC) and indoles form as the result of the enzymatic hydrolysis of glucosinolates. The enzyme catalzsing this reaction is myrosinase (E.C. number 3.2.3.1, also known as β-thioglucosidase and β-thioglucoside glucohydrolase), which occurs in so-called myrosin cells (idioblasts). When vegetables are cut or chewed, myrosinase is liberated and hydrolysis of the glycosidic linkage ensues, as a result of which glucose and the unstable thiohydroximate-O-sulphate are formed; the latter converts spontaneously into the corresponding isothiocyanate. At pH 7–8, the final enzymatic degradation products of glucosinolates with a side chain derived from methionine or phenylalanine consist of ITC. On the other hand, the unstable β-hydroxyisothiocyanates undergo ring closure to oxazolidine-2-thiones. The indole isothiocyanates formed from glucosinolates with an indole side chain derived from tryptophan are converted to the corresponding alcohols (7). Indole-3-carbinol (I3C) is the most important of the unstable GLS decomposition products: it may undergo further transformation, yielding mainly such derivatives as 3,3′-diindolylmethane (DIM – product of condensation of two I3C molecules), ascorbigen (product of condensation with ascorbic acid), indole-3-acetic acid (IAA) and indole-3-acetonitrile.

Important qualitative and quantitative differences exist in the glucosinolate profile of individual plant species belonging to the *Brassicaceae* family. Information on the glucosinolates profile of such plants as broccoli, cabbage, cauliflower, kale or Brussel sprouts has been widely reported (e.g., 8–11). Quantities of glucosinolates in these vegetables range from 0.1 to 2.5 g/kg and vary depending on a region and cultivation conditions (12), the degree of advancement of the plant's development (seedlings, shoots, young plants, mature plants), the plant part, genetic factors and environmental factors (10, 13). Glucosinolates are found in the roots, seeds, leaves and stem of the plant; the youngest tissues contain the greatest amounts (14). Most plant species contain a specified number of glucosinolates (as a rule fewer than 12), although 36 different glucosinolates have been identified in *Arabidopsis thaliana* (15).

METHODS OF DETERMINING GLUCOSINOLATES AND THEIR DECOMPOSITION PRODUCTS IN CRUCIFEROUS VEGETABLES

In samples of vegetable matter in which the matrix is characterized by a complex composition and the compounds to be determined occur at low concentration levels, identification and quantitative determination of phytocompounds are possible when the sample is properly prepared for the final determination stage. The proper sample preparation, while maintaining optimal conditions that prevent degradation and loss of compounds, is indispensable for accurate determination.

In results most cases, the first step in sample preparation is lyophilization. The next step is the isolation and enrichment of analytes obtained from samples of cruciferous plants. Extraction techniques enable the compounds of interest to be transferred to a matrix with a composition simpler than that of the primary sample matrix. This makes the final assay easier and more accurate. Figure 2 shows the procedures applied in the analysis of plant matter samples for glucosinolates and their decomposition products—isothiocyanates and indoles.

SAMPLE PREPARATION

It is extremely important to prevent analyte loss from the plant matter. Cutting, chewing, boiling or fermentation destroys the cell structure of cruciferous vegetables; as a result, GLS compounds are hydrolyzed, or thermally degraded when heated to $>125^{\circ}$C, to their decomposition products (14, 16–18). Hence, when GLS are to be determined, vegetables should be stored in an uninjured state, as injury may reduce the levels of these compounds. Disintegrated lyophilized plants are kept at -20°C until analysis. In the case of large plants, however, to prevent injury and reduce myrosinase activity, the plant matter is frozen with liquid nitrogen (-196°C) or solid carbon dioxide (-80°C) (19). The juice obtained from plants is also used as a sample for determining GLS decomposition products, i.e., ITC and indole compounds.

Where ITC are to be determined, it is of great importance to choose the right conditions for extraction from plant matter samples; otherwise, not all GLS will be decomposed or other decomposition products may form, e.g., nitriles instead of ITC. Conditions such as pH $>$ 5–8, higher temperatures and increased hydration favor the formation of ITC as GLS decomposition products (8, 20). However, even more acidic conditions cause the enhanced formation of nitriles (21, 22). This should be borne in mind, as the use of an improperly chosen technique for extracting analytes from vegetable samples can lead to their loss or to the formation of other compounds (23, 24). Acidic hydrolysis (pH = 1.0) performed ahead of the extraction increases the yield of sulphoraphane released from glucoraphanin; this method has been indicated as being much faster than other hydrolysis procedures and giving a higher yield of sulforaphane (25). On the other hand, the hypothesis that glucoraphanin is totally decomposed into sulforaphane in cruciferous vegetable samples in a chemically inert environment under the influence of myrosinase has not yet been verified, nor that about the conversion of glucoraphanin into sulforaphane when pH = 1. It has been shown, however, that chemical degradation of silver-labelled glucosinolates at pH = 7 in the presence of iodide ions leads to the formation of isothiocyanate (26).

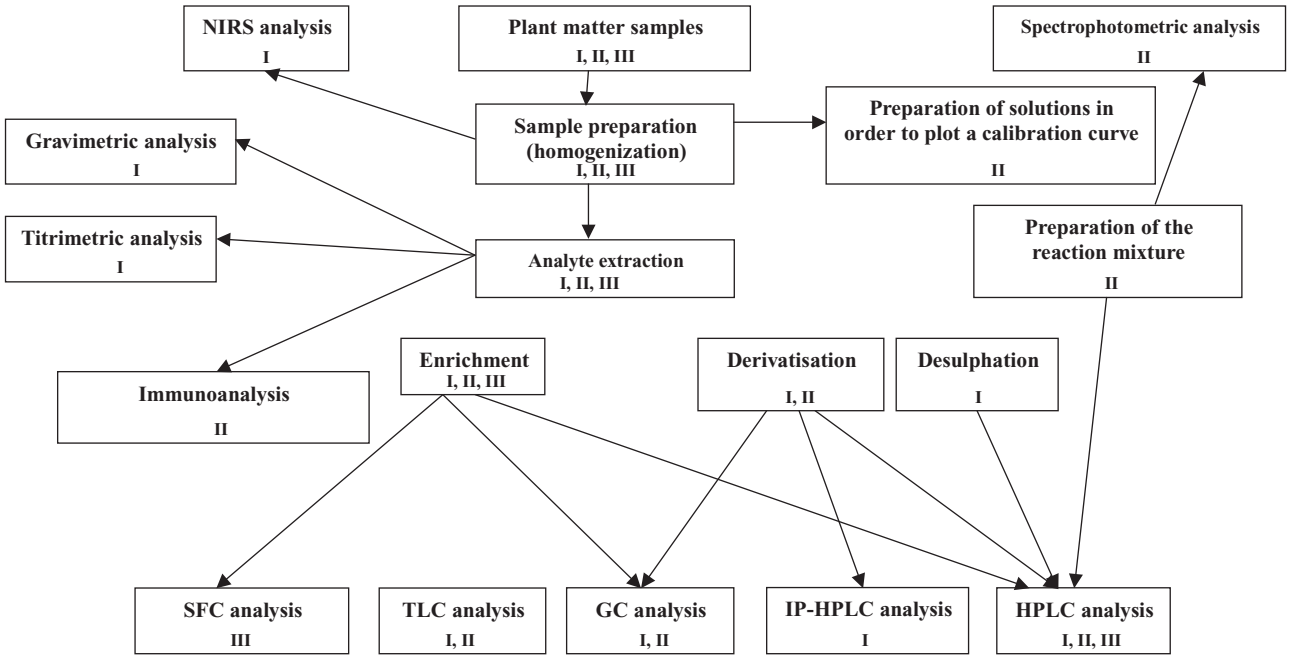

FIG. 2. The procedures applied in the analysis of plant matter samples for glucosinolates (yellow) and their decomposition products—isothiocyanates (blue) and indoles (pink).

For the analysis of indole compounds, the procedure at the sample preparation stage is similar with both SFC and HPLC; it is most often based on homogenization of the vegetable matter with water and acetone. After evaporation of acetone the residue is extracted with ethyl acetate. When the extract has been evaporated to dryness, the residue is dissolved in the mobile phase.

ANALYTICAL TECHNIQUES USED TO DETERMINE GLUCOSINOLATES AND THEIR DECOMPOSITION PRODUCTS

Methods Based on the Measurement of Glucose or Sulphates Released Following Treatment of Glucosinolates with Myrosinase

The thymol test used to determine saccharides gives the total GLS content in food samples. The action of strong acids on sugars causes dehydration and ring closure leading to the formation of furaldehydes. The red-colored product is the result of the condensation reaction between furaldehyde derivatives and thymol. This assay gives a positive result with both free and bound sugars (6, 27, 28).

Glucosinolates can be determined also using methods based on the measurement of the glucose (29–31) or sulphate ions (32, 33) that form as a result of GLS decomposition by myrosinase (Fig. 3). Belongs to this type of total GLS determination. Biosensing with enzyme—immobilized eggshell membranes (34)

Glucose tests are employed to determine the glucose released. However, when this method is used, the possible presence of endogenic glucose should be taken into consideration. The effect of endogenic glucose on GLS determinations can be eliminated through the prior purification and elimination of free glucose and other interfering compounds (4).

Apart from the above methods, several gravimetric and titrimetric methods for determining sulphate ions formed after the hydrolysis of GLS by myrosinase have been described (6). One of them is based on the precipitation of sulphate with barium chloride and testing the barium sulphate crystals by photoemissive spectroscopy (X-ray emissive spectroscopy—XES) (35).

Enzyme-Linked Immunosorbent Assay (ELISA)

Sinigrin and progoitrin in plant extracts have been determined using the ELISA technique. A double-bond test has been used (ELISA in a sandwich configuration) based on the fact that an antigen is linked between two "layers" of specific polyclonal antibodies. The antigen is formed by the synthesized conjugates sinigrin-BSA or sinigrin-ovalbumin and progoitrin-BSA or progoitrin-ovalbumin. Specific antibodies are useful in the quantitative determination of glucosinolates in samples extracted with phosphoric acid (36). It is obviously possible to synthesize conjugates of other GLS and generate antibodies to be used in ELISA.

Spectrophotometric Methods

Indole glucosinolates can be determined spectrophotometrically after prior reaction with diazotized sulfanilic acid in the presence of o-phosphoric acid. The red-colored coupling product has an absorption maximum at 510 nm (37).

Determination of the total ITC content in samples of juice obtained from *Brassicaceae* plants or vegetable extracts is possible

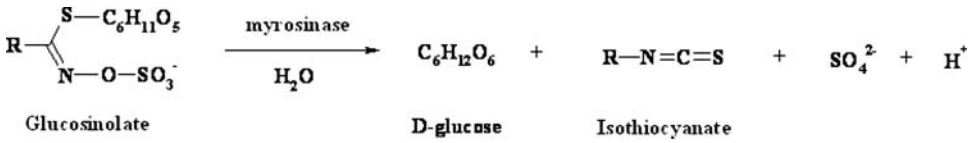

FIG. 3. Decomposition of glucosinolates in the presence of myrosinase

by means of a spectrophotometric technique based on the ability of ITC to react with commercially available 1,2-benzenedithiol (Fig. 4).

Thiocyanates, isocyanates and cyanates do not interfere during the reaction. As a result of cyclocondensation, a non-volatile, colored product is formed, 1,3-benzodithiole-2-thione, exhibiting maximum absorption at 365 nm, due to which a quantitative measurement by means of spectrophotometric techniques is possible. The measurement sensitivity is of the order of 1 nmole when using this method (38). The choice of 1,2-benzenedithiol for the cyclocondensation reaction provides two important advantages since:

(1) It is easily available and reacts quickly and quantitatively with almost all isothiocyanates;
(2) It forms a stable condensation product with compounds from the ITC-group (39).

Near-Infrared Spectroscopy (NIRS)

The use of near-infrared spectroscopy allows the determination of a wide range of GLS, whereas glucose tests permit only the elimination of genotypes containing GLS above a certain amount (28). With this technique, sample preparation merely means placing the vegetable matter directly in the NIRS measurement cell.

Chromatographic Techniques

Chromatographic techniques are most often applied in the determination of glucosinolates and their decomposition products in cruciferous plant samples.

Thin-Layer Chromatography (TLC) and Paper Chromatography (PC)

The simplest techniques for determining GLS compounds are based on paper chromatography or thin-layer chromatography (40).

Paper chromatography was also historically the first technique used for the determination of ITC (41, 42). Conversion of ITC into thiocarbamide derivatives enabled the identification of four of them: allyl-, 3-methylthiopropyl-, 3-butenyl- and 3-methylsulphonylpropylthiourea. At the same time the conversion of allylthiocarbamide into a crystalline derivative S-methyl-N-allylisothiuronium picrate enabled the derivative of allyl isothiocyanate to be determined by X-ray diffraction analysis (XRD) (41).

Qualitative purity of GLS hydrolysis products from Lesquerella and broccoli seed was determined by TLC on silica gel plates using either 95:5 chloroform/methanol or 100% acetone as the mobile phase, followed by development of the plates in iodine vapor (43).

Gas Chromatography (GC)

Because of their ionic nature, GLS cannot be directly determined by gas chromatography, so pre-column derivatization or conversion to volatile desulpho-GLS derivatives has to be carried out. However, in some laboratories GC analysis of sililated desulfo-GLS is preferred for routine application due to shorter analysis time and lower running costs.

The determination of the content of volatile compounds such as ITC, as well as their identification, are possible through the use of gas chromatography (43). The instability of these compounds at high temperatures may cause difficulties in qualitative and quantitative analysis. For istance, sulforaphane has been shown to decompose in 80% sulforaphane to 3-butenyl isothiocyanate (44). The procedure for the GC determination of GLS and ITC is shown in Table 1.

The resolution of glucosinolates in complex mixtures can be accomplished using partition chromatography (gas chromatography with a liquid stationary phase) (6). Partition chromatography (GLC) of GLS derivatives is a method of identifying and quantitatively determining individual GLS compounds.

Prior to the final determinations, the analytes are desulphated and derivatized with trimethylsilane (45). Partition chromatography has also been used in combination with mass spectrometry (46).

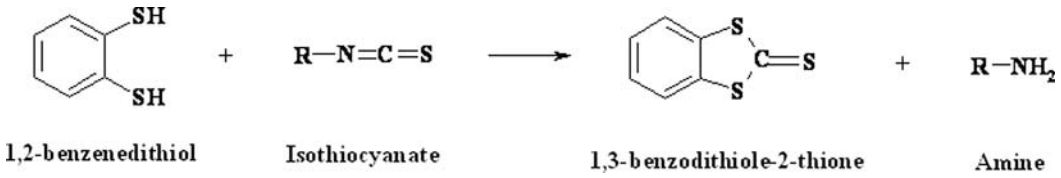

FIG. 4. The reaction of ITC with 1,2-benzenedithiol

TABLE 1
Procedure for the determination of isothiocyanates in plant extracts using gas chromatography

Step	GLS	ITC
Preparation of sample	– Weihting of seeds (0.3 g) to the scintillation vial;	– Cutting the material into small particles and thorough comminution; – Lyophilization; – Heating the lyophilized material (5 g) with the addition of hot ethanol, in hot water (15 min.) – Homogenization after cooling and filtering
Extraction and enrichment	– Addition of methyl alcohol (2 mL), internal standard — glucotropaeolin (0.5 mL) and barium acetate (0.5 mL) – shaking 1 hour	– Double extraction with a hot ethanol-water mixture (80/20 mL) – Concentration of combined extracts to a final volume of 25 mL at 40°C; – Centrifugation (4–5°C, 15 min., 3000 rpm) and filtration of the supernatant (dissolved in 50 mL of water) – Pouring the supernatant (20 mL) into an anion exchange column and flushing with water until loss of reaction with Molisch's reagent – Stirring the resin with chloromethane (5 mL), myrosinase (50 mg), ascorbic acid (1 mL, 10 mM) and phosphate buffer (5 mL, 0.1 M) – Stirring for 18 h and centrifugation of the enzyme – Desiccation of the methyl chloride layer with Na_2SO_4
Desulphation and silylation	– Preparing columns – Washing by distilled water (2 × 1.8 mL) – Transferring of extract to the Sephadex column – Washing column of methanol (67%, 1.6 mL), water (2 × 1.6 mL), pyridine acetate (0.02 M, 1 mL) – Addition to the column of sulphatase (0.075 mL) – Leaving overnight in room temperature – Elution of desulpho-GLS with methanol (67%, 2 × 1.8 mL) – Removing the methanol by heating in 60°C – Addition of sililating mixture (300 μL)	
GC	– Injector temp. 300°C; – Detector temp. 300°C; – Oven temp. 271°C;	– Capillary column with OV-7 phase - 1 m × 3.17 mm – Mobile phase flow rate – 1.0 mL/min. – FID detector
GC/MS		– Capillary column with O.D. phase; 230 × 0.25 nm, film thickness 0.25 μm – Eluent gas – helium – Mobile phase flow rate – 1.0 mL/min. – Identification of the substance with the aid of a spectrum library

Supercritical Fluid Chromatography (SFC)

The decomposition products of GLS from the indole group can be determined with mobile phase supercritical fluid chromatography (SFC) (47) combined with UV detection (6). Supercritical fluid chromatography (SFC) is used both for analytical separations and preparative purification of a wide range of glucosinolate degradation products, especially those formed during degradation of sinalbin and the indol-3-ylmethylglucosinolates (48).

High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography is commonly used to determine glucosinolates and their decomposition products. Analytical procedures based on this technique are employed in the analysis of samples of appropriately prepared extracts from vegetable matter or limpid juices, without the need to convert them into volatile derivatives. Some glucosinolates, in particular the indole GLS, are thermally unstable, so HPLC is the preferred technique for the determination of these compounds.

The technique most widely used for the qualitative and quantitative determination of glucosinolates in cruciferous vegetables is HPLC with an inverted phase system, described in ISO standard 9167-1 (49). The introduction of the enzymatic desulphation step with the use of sulphohydrolase (sulphatase) meant that sulphate could be eliminated. But there are difficulties with this approach, the most important of them being:

- the influence of pH on enzymatic activity;
- defining the duration of the reaction and the concentration of the enzyme during desulphation (9).

Research aimed at improving the effectiveness of this method is in progress, particularly with regard to the sample preparation procedure, enzymatic desulphation, peak identification and evaluation of the response factor (RF) of the individual compounds. The possible optimization of the method has been investigated by a team of Italian scientists, who have put forward definite solutions for the individual stages of the procedure (19).

There is also another approach to the determination of glucosinolates: this avoids the process of desulphation, thus eliminating the problem of effective desulphation, which crops up in the determination of desulpho-GLS (50). The use of IP-HPLC enables electrically charged glucosinolates to be determined (51), and LC-MS permits their identification.

The determination of isothiocyanates using HPLC can be performed following the condensation reaction with 1,2-benzenedithiol. Using HPLC instead of a spectrophotometric technique has led to lower LODs being achieved, reaching the level of 10 pmoles. Such a sensitivity can easily be increased by injecting a larger volume sample (of the order of 500 μL) into the column.

Also, indole decomposition products can be determined by HPLC (39). Chromatographic analysis is carried out in an inverted phase system with gradient elution. In most cases, the mobile phase is a mixture of acetonitrile with an ammonium or phosphate buffer of pH 5.7–7.0. The procedure for the HPLC determination of desulphoglucosinolates, isothiocyanates and indoles is shown in Table 2.

Other Chromatographic Techniques and Related Techniques

In the case of glucosinolate analysis, supplementary chromatographic techniques can be applied:

- Based on reversed-phase ion-pair chromatography (IPC) for the determination of non-polar GLS;
- Normal-phase hydrophilic interaction chromatography (HILIC) for the determination of polar GLS (9, 52, 53).

A new approach consists of the use of one column in which residual -OH-silica groups are removed for separation in the reversed-phase system; the mobile phase gradient consists of 50 mM ammonium acetate-methanol for the determination of both non-polar and polar GLS present in isolates obtained during extraction with boiling water (54).

High-Speed Countercurrent Chromatography (HSCCC)

High-speed countercurrent chromatography (HSCCC) permits the determination of ITC compounds on a larger scale with simultaneous simplification of the sample preparation procedure. With this advanced technique both a normal and an inverted phase system can be employed; it is favored because:

(1) Total recovery of analytes is possible,
(3) It is simple (pressure-free option),
(3) It is relatively inexpensive (low-purity solvents can be used) (55).

In order to produce large amounts of pure sulforaphane for research purposes, a novel method using high-speed countercurrent chromatography (HSCCC) was developed. Without any initial cleanup steps, sulforaphane was successfully purified from the ethyl acetate extract of the broccoli seed meal by HSCCC. The separation was performed with two-phase solvent systems: *n*-hexane/ethyl acetate/methanol/water (1:5:1:5, v/v/v/v) (56).

Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) has been used to determine the total content of GLS and their decomposition products in vegetable extracts. In the case of GLS, this method is based on the enzymatic liberation of glucose from GLS as a result of reaction with myrosinase and the further conversion of this glucose into glucuronic acid in the presence of glucooxidase. The glucuronic acid (GA) thus formed is labelled fluorescently with 7-aminonaphthalene-1,3-disulphonic acid (ANDSA). The GA-ANDSA derivative has a short migration time, and when CE is linked with laser-induced fluorescence (LIF) detection an electropherogram of GA-ANDSA is obtained that is unequivocal and free from impurities (57).

TABLE 2
The procedure for determining desulphoglucosinolates, isothiocyanates and indoles in vegetable extracts or juices using HPLC

Stage	Glucosinolates	Indoles	Isothiocyanates
Sample preparation	– Lyophilization – Disintegration of material – Storage at −20°C	– Disintegration of material – Storage at −20°C	– Lyophilization – Disintegration of material – Atorage at −20°
Extraction and enrichment	– Addition of an extraction mixture (4 mL): ethanol/water (50:50) or methanol/water (70:30) to the sample (100 mg) – Mixing the solution at 75°C for 10 min. – Addition of an internal reference: sinigrin or glucotropaeolin after 2 min. – Centrifugation – Solution made up to 5 mL with water	– Extraction of juice (10 mL) with ethyl acetate (3 × 5 mL) – Combining the extracts and evaporation with a rotating evaporator – Dissolving the dry residue in a mix of acetonitrile – Phosphate buffer (1:1, 1 mL, pH = 7)	
Desulphation	– Conditioning the column filled with DEAE Sephadex gel (0.5–1.0 mL) with acetate buffer (20 mM; pH = 4.0 or 5.60) – Dosing of extract to the column – Flushing the column with acetate buffer – Dosing refined sulphatase to the column (0.1 mL; 0.28 U/MI) – Incubation for 12 h at room temp. – Elution of desulpho-GLS with water (3 mL)		Preparation of solutions to plot a calibration curve – Synthesis of 1,3-benzodithiole-2-thione: preparation of a mixture containing equal volumes of methanol with potassium phosphate (100 nM, pH=8.5) and reference ITC substances and 1,2-benzenedithiol – Incubation at 25°C, extraction of solution by dichloromethane, crystallisation of the product – Preparation of a series of solutions of the product in the concentration range 2.5–10 μM
Chromatographic analysis	– Column with octyl or octadecyl packing (4 mm to 4.6 mm). temp. 30°C – Mobile phase consisting of a water/acetonitrile mixture – UV or DAD detector, $\lambda = 229$ nm	– Column with octyl or octadecyl packing, temp. 30°C – Mobile phase consisting of a mixture of phosphate buffer (5 mM, pH = 7) and acetonitrile in phosphate buffer (5 mM, pH = 7) – UV or DAD detector, $\lambda = 280$ nm	– Column RP-C18; 4.5 × 250 mm – Isocratic elution programme – Mobile phase consisting of 80% methanol – Volume of proportioned sample 200 μL – Detection at $\lambda = 365$ nm
Quantitative and qualitative analysis	– Establishing the retention sequence of individual compounds – Comparison of UV spectra – Assessing the concentration of individual compounds [μmole/g d.m.] – Assessing the total GLS content by summing the number of individual compounds	– Establishing the retention sequence of reference indoles – Assessing the concentration of individual compounds [μmole/mL] on the basis of an analytical curve – Assessing the total indole content by summing the number of individual compounds	– Assessing the total ITC content on the basis of an analytical curve

Determination of GLS in vegetables with high-performance capillary electrophoresis (HPCE) is prevented for lack of trustworthy modified GLS references. Besides, the relevant electrophoretic systems and detection circuits should be so adapted that the opportunities offered by HPCE can be fully exploited in GLS determination (6). In this respect, only a few publications describe methods based on CE for the qualitative and quantitative determination of GLS or their decomposition products in vegetable samples (6, 57).

DETECTION OF GLUCOSINOLATES AND THEIR DECOMPOSITION PRODUCTS USING COMBINED TECHNIQUES

Identification of individual GLS compounds and their decomposition products is possible when the right method of detection is employed (58). Usually, however, there are limitations due to low analyte concentrations in the sample; hence, the adaptation of many detection systems to desired limits of detection.

In the case of HPLC, compounds are most often determined using diode array (DAD) and UV detectors. Desulpho-GLS are determined at the nmole level with HPLC coupled to a DAD detector (6). Quick ITC determination (time < 10 min) is possible using HPLC coupled to an evaporative light-scattering detector (ELSD (24).

Flame ionization (FID) and flame photometric (FPD) detectors are used in combination with GC equipment. An extremely useful set of techniques consists of HPLC-MS, GC-MS and NMR (59–61). They yield information on the molecular mass of an unknown substance, the chemical group it belongs to and its structure. Currently used methods like LC-MS frequently include analyzers with numerous quadrupole systems, and less often, time-of-flight spectrometers (TOF-MS) or such instruments operating on the ion-trap principle. Mass spectrometry has been found an invaluable tool for identifying and explaining the structures of GLS and their decomposition products.

Discovery of the structures of various desulpho-GLS is aided by the various ionization methods used in mass spectrometry, such as:

- Electron-impact ionization (EI),
- Chemical ionization (CI),
- Ionization through fast-atom bombardment (FAB) ,
- Chemical ionization under atmospheric pressure (APCI),
- Matrix-assisted laser desorption ionization (MALDI),
- Electrospray ionization (ESI) (4).

However, of all these ionization techniques in the specific case where both LC-MS and CE-MS are used to determine GLS, electrospray ionization is the one most often used, owing to its greater sensitivity in the determination of these compounds (59, 62–64). LC-APCI-APCI-MS has been used to identify desulpho-GLS (60); unfortunately, however, this technique is not sensitive enough in the case of GLS and ITC. On the other hand, mass spectrometry with ionization through fast-atom bombardment (FAB) has been found particularly useful in the analysis of unrefined vegetable extracts (65–67).

LC-MALDI-TOF-MS equipment has been used in an attempt to improve resolving power and thus to avoid overestimated values in the determination of compounds, but this is not a preferred technique because of its low LOD. MALDI-TOF-MS is very useful for GLS detection (68), and CE-ESI-TOF-MS can also be very effective in this respect (69); in addition, up to 20 GLS could be identified using LC-ESI-ITMS (67, 70). To determine ITC in vegetable samples, liquid chromatography with tandem mass spectrometry with ionization through electrospraying (LC-ESI(PI)-MS/MS) can be used, although the sensitivity of this method is not good enough to draw reliable conclusions. If, however, ITC are converted to the corresponding thiourea derivatives detection sensitivity increases considerably (71), and detection of ITC in samples of vegetable matter is then easy (66). Nonetheless, this technique is very time-consuming and, moreover, it is not possible to analyze a large number of samples without complicated sample preparation procedures.

DETERMINATION OF SYNTHETIC ISOTHIOCYANATES

Glucosinolates decompose in the presence of myrosinase to isothiocyanates, among other compounds, which are regarded as phytocompounds with the highest chemopreventive potential. Consequently, appropriate methods are necessary to determine them qualitatively and quantitatively. Aside from that, the isolation and purification of the hydrolysis products of GLS is also of interest in view of their potential application in organic analysis. However, finding an effective method to determine such compounds in vegetable matter has been a problem. Where synthetic isothiocyanates are concerned, gas chromatography (GC) is commonly employed (20).

DETERMINATION OF ISOTHIOCYANATES IN BODY FLUIDS (PLASMA AND URINE) FOLLOWING CONSUMPTION OF CRUCIFEROUS VEGETABLES

The isothiocyanates contained in cruciferous vegetables are metabolized in the body via mercapturic acid to *N*-acetylcysteine conjugates of isothiocyanates (NAC-ITC), which are excreted in the urine (72). These conjugates are formed as a result of the spontaneous reaction of the N=C=S group contained in the ITC structure with glutathione (GSH) and subsequent enzymatic modification in the presence of glutathione *S*-transferase (GST, E.C. 2.5.1.18) (73).

In connection with this, the search has started for analytical methods enabling isothiocyanates and their metabolites to be determined in urine samples. Determination of the total ITC content in urine is an excellent biological marker of the body's response to the action of isothiocyanates. These methods may also yield data on the biological availability of isothiocyanates from cruciferous vegetables (74).

The total ITC content in urine samples was determined using HPLC and the method of measuring dithiocarbamates — derived from the reaction of isothiocyanates with glutathione and synthetic PEITC-NA conjugates (phenethyl isothiocyanate-N-acetyl conjugate). Synthesized PEITC-NA conjugates have been utilized to plot the calibration curve (75). NAC conjugates of other isothiocyanates such as allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC) and sulphoraphane (SFN) have also been determined in urine samples, following the consumption of mustard, radish or Brussels sprouts (76, 77). With solid-phase extraction (SPE) followed by HPLC-MS-MS, the individual NAC-ITC conjugates can be determined in urine samples over a range of low concentrations ($\sim$1 μM) (74).

However, determination of NAC-ITC conjugates will not provide an accurate quantitative evaluation of the human body's susceptibility to the action of individual isothiocyanates, because NAC-ITC conjugates are unstable and tend to decompose back to isothiocyanates (78). Apart from this, a number of NAC-ITC conjugates may form as a result of the reaction of an ITC side chain, as in the case of sulphoraphane (74). The total content of isothiocyanates and their metabolites in body fluids (blood and urine) can be determined if cyclocondensation with 1,2-benzenedithiol is applied. Using a reference NAC-ITC, it can be determined whether all isothiocyanate metabolites undergo cyclocondensation (72, 79). Neither the cyclocondensation product of 1,3-benzenedithiol-2-thion nor the NAC-ITC conjugate is commercially available, but methods of their synthesis have been described (38) in which problems with the final purification of products have been eliminated (72).

HPLC-MS-MS has been used to determine phenylethyl isothiocyanate (PEITC) in human plasma and urine at the 2 nM level. Because of the volatility of PEITC and other isothiocyanates and their low ionization capability, hexane has been employed for their extraction, after which the ITC were converted into phenylethylthiocarbamide derivatives reacting with ammonia (71, 74).

SUMMARY

To obtain results that are to be a source of credible and reliable information on the presence and amount of compounds in vegetable matter is a serious analytical challenge. This is because:

- The concentrations of the compounds of interest in samples with a complex and variable matrix composition are very low;
- There is a danger of interference from compounds with similar physical and chemical properties (thus difficult to separate) which may appear in the investigated samples, even at considerably higher levels than the analytes;
- The sample preparation step is labor-intensive and time-consuming;
- Reference materials are not always available, which causes serious problems in the analytical procedure and the calibration of measuring equipment.

Table 3 summarizes data from the literature regarding the analytical techniques used for samples of cruciferous vegetables to determine the content of GLS and their decomposition products, including those of the greatest public interest due to cancer chemopreventive properties.

The information presented in Tables 3 and 4 on the analytical techniques in use show that there is no ideal technique for the determination of glucosinolates and their decomposition products. Each of the techniques has its good points and shortcomings; most of them require much time and a considerable financial outlay, mainly due to the required procedures preceding the analysis. Because of their ionic nature, glucosinolates cannot be investigated directly by GC. Conversion of GLS into their volatile desulpho-GLS derivatives requires pre-column derivatization. For this reason, HPLC and capillary electrophoresis (CE) are more suitable for GLS analysis.

The most commonly used technique for determining glucosinolates is still high-performance liquid chromatography. Diode array detectors (DAD) are mostly employed, but they do not determine unknown structures. GLS identification requires the application of reference substances. More information on the structure of an investigated compound can be obtained by using a liquid chromatograph in tandem with a mass spectrometer or a nuclear magnetic resonance spectrometer (100). The introduction of various ionization methods, for instance, ion sources operating under atmospheric pressure and ionization by electro-spraying, has made LC-MS the most appropriate one for the determination of these compounds. On the other hand, near-infrared spectroscopy (NIRS) facilitates the quick determination of GLS in that the time-consuming sample preparation stage can be eliminated. Even so, many vegetable GLS cannot be identified through the use of modern analytical and spectroscopic methods such as HPLC, NMR, MS or supercritical fluid chromatography (SFC) with scattered-light detection (LSD) (9, 82).

Attempts have been made to modify and improve analytical methods for determining the glucosinolate content in vegetable extracts, based on the use of LC-MS at the mixture separation and individual constituent identification stages (84). These improvements consist of:

(1) The introduction of reverse-phase paired-ion chromatography (PIC) of alkylammonium salts (e.g., tetra-, octyl- or tetradecylammonium bromide) used together with hydrolysis by myrosinase and ITC determination by cyclocondensation with vicinal dithiols (9, 38, 39);
(2) A novel method for replacing the PIC counterions by ammonium ions, permitting direct bioassay mass and ^{1}H NMR spectrometry;
(3) Using mass spectroscopy with ionisation techniques with fast atom bombardment and chemical ionization;
(4) The use of high-resolution ^{1}H NMR spectroscopy to provide final confirmation and identification of intact glucosinolates (84).

TABLE 3
Basic information gleaned from the literature on the techniques employed to determine the glucosinolate content in cruciferous vegetable samples

Analytical technique	Application	Advantages	Drawbacks	References
		GLUCOSINOLATES		
Glucose test	Determination of total analyte content by determination of glucose liberated as a result of GLS decomposition	Simple and quick; inexpensive	Analyte identification not possible	(29–31)
Gravimetry and titration	Determination of total analyte content by determination of sulphate ions as a result of GLS decomposition	Simple and quick; inexpensive	Analyte identification not possible	(32, 33)
ELISA	Determination of total analyte content	Inexpensive; quick	Differences in determination results in comparison with HPLC; analyte identification not possible	(36)
Near-infrared spectroscopy (NIRS)	Simultaneous determination of total analyte, protein and fat content	Quick and inexpensive determination of analytes; wide application range; does not require sample preparation; hazardous substances not needed	High cost of equipment; calibration problems	(28, 80–83)
High-performance liquid chromatography with inverted-phase system (HPLC)	Quantitative and qualitative determination of desulpho-GLS	Identification and quantitative determination of analytes	Sample preparation step labor-intensive and time-consuming; determination of desulpho-GLS instead of GLS; analytical procedure expensive; utilization of desulpho-GLS as substrates for myrosinase not possible	(11, 25, 27, 47, 48, 64, 67, 82, 84–88)
High-performance liquid chromatography with inverted-phase system (HPLC)	Quantitative and qualitative determination of analytes, also thermally unstable indole compounds	Simplified method of preparing samples for analysis, higher analyte content yield	The risk of formation of GLS degradation products	(51, 59, 66, 89–92)
Gas chromatography (GC)	Determination of individual GLS	Determination of volatile compounds	Sample preparation step complicated and time-consuming; GLS must be converted into volatile derivatives	(20, 66)
High-performance capillary electrophoresis (HPCE)	Qualitative determination of analytes	Simple and quick (10–20 min); inexpensive; no advanced skills or complicated equipment required; limited consumption of solvents; small sample volume (2–4 nL)	Lack of reference compounds	(6, 57)

TABLE 4
Basic information gleaned from the literature on the techniques employed to determine decomposition products in cruciferous vegetable samples

Analytical technique	Application	Advantages	Drawbacks	References
		GLUCOSINOLATE DECOMPOSITION PRODUCTS		
X-ray fluorescence analysis (XRF)	Analysis of chemical composition and identification of ITC	Quick	Labor-intensive and costly	(93, 94, 95)
HPLC with the use of 1,2-benzenedithiol	Determination of total ITC content	Higher sensitivity in comparison with spectrophotometric methods	Analyte identification not possible	(16, 23, 39, 72, 74)
HPLC	Determination of indoles and ITC	Identification of individual indoles and ITC	Sample preparation step labor-intensive and time-consuming in the case of ITC	(20, 23, 24, 25, 96, 97, 98)
GC	Determination of individual ITC	Identifies volatile compounds; connection to a universal MS detector possible; affords excellent separation	Sample preparation step labor-intensive and time-consuming; long analysis time (>30 min); ITC may decompose at the temperatures used	(13, 20, 43, 44, 47, 61, 89, 99)
Spectrophotometric method with the use of 1,2-benzenedithiol	Determination of total ITC content	Inexpensive and quick; separation at low temperatures; can be combined with specific and universal detectors	Poor sensitivity	(38)

The introduction of these improvements offers a powerful method for identifying and determining quantities of GLS.

Decidedly less information is available on the determination and identification of indole compounds. As in the case of isothiocyanates, knowledge of their determination is scanty. This is because of the difficulties in determining this type of compounds, owing to their volatility and lack of stability. Applying the referential technique that HPLC is considered to be, it is possible to determine only the total isothiocyanate content, after prior condensation with 1,2-benzenedithiol. Quantitative determination of ITC is possible with Zhang's spectrophotometric method. On the other hand, identification of indole compounds is feasible only with GC-MS. Sample preparation and the use of HPLC for the simultaneous detection of ITC are also described.

As the problems with the use of various analytical techniques outlined above show, analysis of samples with a complex composition requires the use of combined techniques.

ABBREVIATION

AITC	Allyl isothiocyanate
ANDSA	7-aminonaphthalene-1,3-disulphonic acid
APCI	Atmospheric pressure chemical ionization
BITC	Benzyl isothiocyanate
CE	Capillary electrophoresis
CI	Chemical ionization
DAD	Diode array detector
DIM	3,3'-diindolylmethane
EI	Electron ionisation
ELISA	Enzyme-linkd immunosorbent assay
ELSD	Evaporative light-scattering detector
ESI	Electrospray ionization
FAB	Fast-atom bombardment
FID	Flame ionisation detector
FPD	Flame photometric detector
GA	Glucuronic acid
GC	Gas chromatography
GLC	Gas-liquid chromatography
GLS	Glucosinolates
GSH	Glutathione
GST	Glutathione *S*-transferase
HILIC	Hydrophilic interaction liquid chromatography
HPCE	High-performance capillary electrophoresis
HPLC	High-performance liquid chromatography
HSCCC	High-speed countercurrent chromatography
HS-SDE	High-speed single-drop extraction

I3C	Indole-3-carbinole
IPC	Ion-pair chromatography
IP-HPLC	Ion-pair high-performance liquid chromatography
ITC	Isothiocyanates
ITMS	Ion trap mass spectrometry
LC-ESI(NI)-MS/MS	Liquid chromatography-electrospray Ionisation (negative Ion)-tandem mass spectrometry
LC-ESI(PI)-MS/MS	Liquid chromatography-electrospray ionisation (positive ion)-tandem mass spectrometry
LIF	Laser-induced fluorescence
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption ionization
MECC	Micellar electrokinetic capillary chromatography
MS	Mass spectrometry
NIRS	Nuclear magnetic resonance spectroscopy
NMR	Nuclear infrared spectroscopy
NAC	N-acetylcysteine
PAD	Photodiode array detector
PEITC	Phenethyl isothiocyanate
PIC	Paired-ion chromatography
RP-HPLC	Reversed-phase high-performance liquid chromatography
SDE	Single-drop extraction
SFC	Supercritical fluid chromatography
SPME	Solid phase microextraction
XES	X-ray electron spectroscopy
XRD	X-ray diffraction analysis
XRF	X-ray fluorescence analysis

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