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Review

Current Methods for the Extraction and Analysis of Isothiocyanates and Indoles in Cruciferous Vegetables

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Abstract: Cruciferous vegetables are characterized by the presence of sulfur-containing secondary plant metabolites known as glucosinolates (GLS). The consumption of cruciferous vegetables such as broccoli, cabbage, rocket salad, and cauliflower has been related to the prevention of non-communicable diseases. Their beneficial effects are attributed to the enzymatic degradation products of GLS, e.g., isothiocyanates and indoles. Owing to these properties, there has been a shift in the last few years towards the research of these compounds and a wide range of methods for their extraction and analytical determination have been developed. The aim of this review is to present the sample preparation and extraction procedures of isothiocyanates and indoles from cruciferous vegetables and the analytical methods for their determination. The majority of the references that have been reviewed are from the last decade. Although efforts towards the application of eco-friendly non-conventional extraction methods have been made, the use of conventional solvent extraction is mainly applied. The major analytical techniques employed for the qualitative and quantitative analysis of isothiocyanates and indoles are high-performance liquid chromatography and gas chromatography coupled with or without mass spectrometry detection. Nevertheless, the analytical determination of isothiocyanates presents several problems due to their instability and the absence of chromophores, making the simultaneous determination of isothiocyanates and indoles a challenging task.

Keywords: *Brassicaceae*; sulforaphane; indole-3-carbinol; broccoli; extraction; HPLC; GC; glucosinolates; mass spectrometry

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1. Introduction

Cruciferous vegetables are members of the *Brassicaceae* (*Cruciferae*) family and include vegetables such as broccoli, cauliflower, Chinese cabbage, radish, arugula, cabbage, Brussels sprouts, turnip, watercress, kale, horseradish, garden cress, and wasabi. They are abundant in bioactive compounds, e.g., flavonoids, phenolic acids, carotenoids, terpenoids, phytosterols, and glucosinolates (GLS) [1]. Cruciferous vegetables can be considered functional foods, since their consumption can improve normal body function and can help prevent the development of cancer, cardiovascular disease, diabetes, and chronic inflammation [2–6]. The series of S- and N-secondary metabolites, known as GLS, underpin this beneficial effect. When GLS undergo enzymatic hydrolysis from the endogenous enzyme myrosinase, there is a release of aglucon products, which have a positive impact on human health [7]. Depending on the pH values, temperature, and other factors, the predominant aglucon products are isothiocyanates (ITCs) and indoles [8]. These compounds also contribute highly to the defense mechanism system of plants against herbivores and pathogens [9–11].

One of the most important ITCs is sulforaphane (SFN), which derives from the enzymatic hydrolysis of the glucosinolate glucoraphanin. It is highly effective in activating phase II detoxification enzymes in the human body [12] and suppresses histone deacetylase, and thus has attracted substantial interest [13]. Glucobrassicin is a notable glucosinolate as well, yielding indol-3-ylmethylisothiocyanate upon enzymatic hydrolysis. Nevertheless, this particular isothiocyanate (ITC) lacks stability, so indole-3-carbinol (I3C) is detected instead [14]. The latter may present chemopreventive activity, making it the focus of extensive research in recent times [15–17].

A range of analytical techniques have been devised to determine ITCs and indoles from cruciferous vegetables, owing to the high contribution of these metabolites to human health. Liquid chromatography (LC) is the main technique used to determine ITCs [18–24], while pre-column derivatization has been additionally employed [25]. Moreover, approaches based on gas chromatography (GC) have been reported [24,26–30]. However, in some cases, thermal degradation has been observed [31,32]. These compounds can be also quantified via Liquid Chromatography-Mass Spectrometry (LC-MS) methods, not only in *Brassica* [33,34] or broccoli products [35], but also in honey [36] and body fluids [37–41]. High Resolution Mass Spectrometry (HRMS) offers additional sensitivity, allowing a more precise and simultaneous determination of ITCs and indoles [42,43]. Moreover, total ITCs have been quantified through UV spectrophotometry [44] and infrared Fourier-Transform spectroscopy [45].

The aim of this review is to provide an overview of the methods that have been employed in the last decade for the conventional and non-conventional extraction of ITCs and indoles from cruciferous vegetables, as well as to present the analytical methods and techniques that have been developed for this purpose. The literature research was performed from March to July 2021, using databases such as Web of Science, Scopus, PubMed, and JSTOR by keywords such as cruciferous vegetables, *Brassicaceae*, analysis, extraction, and determination.

2. Cruciferous Vegetables

Cruciferous vegetables are members of the order *Brassicales* and the *Brassicaceae* botanical family (also called *Cruciferae*). The latter encompasses nearly 400 genera (e.g., *Brassica, Raphanus, Armoracia, Nasturtium, Wasabia, Alyssum, Arabidopsis*, etc.), and over 4000 species [46,47]. The majority of *Brassicaceae* species are not comestible, but there are several members of the family such as *Brassica oleracea* and *Brassica rapa* that are edible and economically significant (Table 1).

Table 1. Edible *Brassicaceae* crops.

Common Name	Genus	Species and Cultivar
Cauliflower	*Brassica*	*B. oleracea* (L.) var. *botrytis*
Cabbage		*B. oleracea* (L.) var. *capitata*
Brussels sprouts		*B. oleracea* (L.) var. *gemmifera*
Broccoli		*B. oleracea* (L.) var. *italica*
Savoy cabbage		*B. oleracea* (L.) var. *sabauda*
Kale		*B. oleracea* (L.)var. *acephala*
Bok choy		*B. rapa* var. (L.) *chinensis*
Turnip		*B. rapa* (L.) var. *rapa*
White mustard		*B. alba* (L.) Rabenh.
Indian mustard		*B. juncea* (L.) Czernj
Radish	*Raphanus*	*R. raphanistrum* subsp. *Sativus* (L.) Domin
Horseradish	*Armoracia*	*A.rusticana* (L.)
Watercress	*Nasturtium*	*N.officinale* W.T.Aiton
Garden cress	*Lepidium*	*L.sativum* (L.)
Rocket	*Eruca*	*E. sativa* Mill.
Wasabi	*Wasabia*	*W.japonica* (L.)

According to recent epidemiological research, certain types of cancer are less likely to develop when the dietary intake of cruciferous vegetables is high, providing support for the classification of these vegetables as functional foods. [48–52]. Consequently, the market has been inundated with dietary supplements that contain different extracts or compounds derived from cruciferous vegetables. GLS, and the products of their enzymatic hydrolysis are the primary source of the positive health effects of cruciferous vegetables [53], while polyphenols and triterpenes are beneficial to health as well [54,55]. The action against cancer, oxidants, inflammation, and cardiac disease that has been attributed to cruciferous vegetables can be also attributed to the synergistic activity of the above-mentioned phytochemicals [55–57].

3. The Chemistry of Glucosinolates, Isothiocyanates, and Indoles

Isothiocyanates and indoles are naturally occurring molecules that are produced from the enzymatic hydrolysis of GLS (Figure 1) performed by the enzyme β-thioglucoside glucohydrolase, EC 3.2.3.1 (myrosinase) [58,59]. Phytoalexins, phytoanticipins, and phytohormones also contain indole rings [14,60–62]. Glucosinolates display chemical stability and are found in members of the order *Brassicales* and the genus *Drypetes* (order *Malpighiales*, family *Putranjivaceae*) [59,63]. They are organic anions soluble in water that contain β-D-thioglucose and sulfonated oxime moieties. Three types of GLS are classified according to the amino acid precursor structure: (1) aliphatic GLS, derived from methionine, alanine, leucine, valine, or isoleucine, (2) arylaliphatic GLS, derived from phenylalanine or tyrosine, and (3) indole GLS, derived from tryptophan [59]. Cyanogenic glycosides that exist throughout the plant kingdom [64,65], are the basis of the evolution of GLS. The species *Carica papaya* (order *Brassicales*, family *Caricaceae*) is the only plant that contains both cyanogenic glycosides and GLS, and is capable of the concomitant production of cyanogenic glycosides and phenylalanine-derived GLS [66].

During the chewing, cutting, or wounding of cruciferous vegetables, endogenous myrosinase is released and catalyzes the hydrolysis of GLS to glucose with an intermediate lack of stability (Figure 1), which is immediately transformed into different compounds such as isothiocyanates, thiocyanates, nitriles, epithionitriles, and oxazolidine-2-thiones [67]. The ITCs are usually formed when enzymatic hydrolysis occurs at a neutral pH. The generated ITCs are cyclized to oxazolidine-2-thiones if the C-2 of the GLS side chain contains a hydroxyl group. Nitriles are formed in vitro when the pH level is low and Fe^{2+} ions are present [68]. The nitrile formation in vivo occurs when protein factors such as the epithiospecifier protein (ESP), thiocyanate-forming protein (TFP), and epithiospecifier modifier protein (EMP) are active [69,70]. It is likely that the epithiospecifier modifier (ESM1) interferes with ESP and directs the hydrolysis towards the production of ITCs [71]. Glucosinolates undergo enzymatic hydrolysis to epithionitriles when ESP and TFP are present. Thiocyanates are formed from the hydrolysis of benzyl-, allyl-, and 4-methylsulfinylbutyl GLS [72].

Unlike other GLS hydrolysis products, those with an indole ring are distinct because the ITCs formed at a neutral pH are unstable and ultimately undergo conversion to indol-methanols, ascorbic acid conjugates, and oligomeric mixtures [67,70,73]. When glucobrassicin is hydrolyzed (Figure 2), indole-3-acetonitrile and I3C are formed as a result of the fast reaction of the unstable isothiocyanate intermediate. The formation of indole-3-acetonitrile is promoted by the ESP protein in a way that is comparable to aliphatic GLS. Furthermore, the epithiospecifier modifier 1 gene (ESM1) [74,75] regulates indole-3-acetonitrile generation as well. Compared to indole-3-acetonitrile, I3C displays a greater chemical instability and yields products such as 3,3-diindindolylmethane, indole-3-carboxaldehyde, and ascorbigen (Figure 2) [14,73].

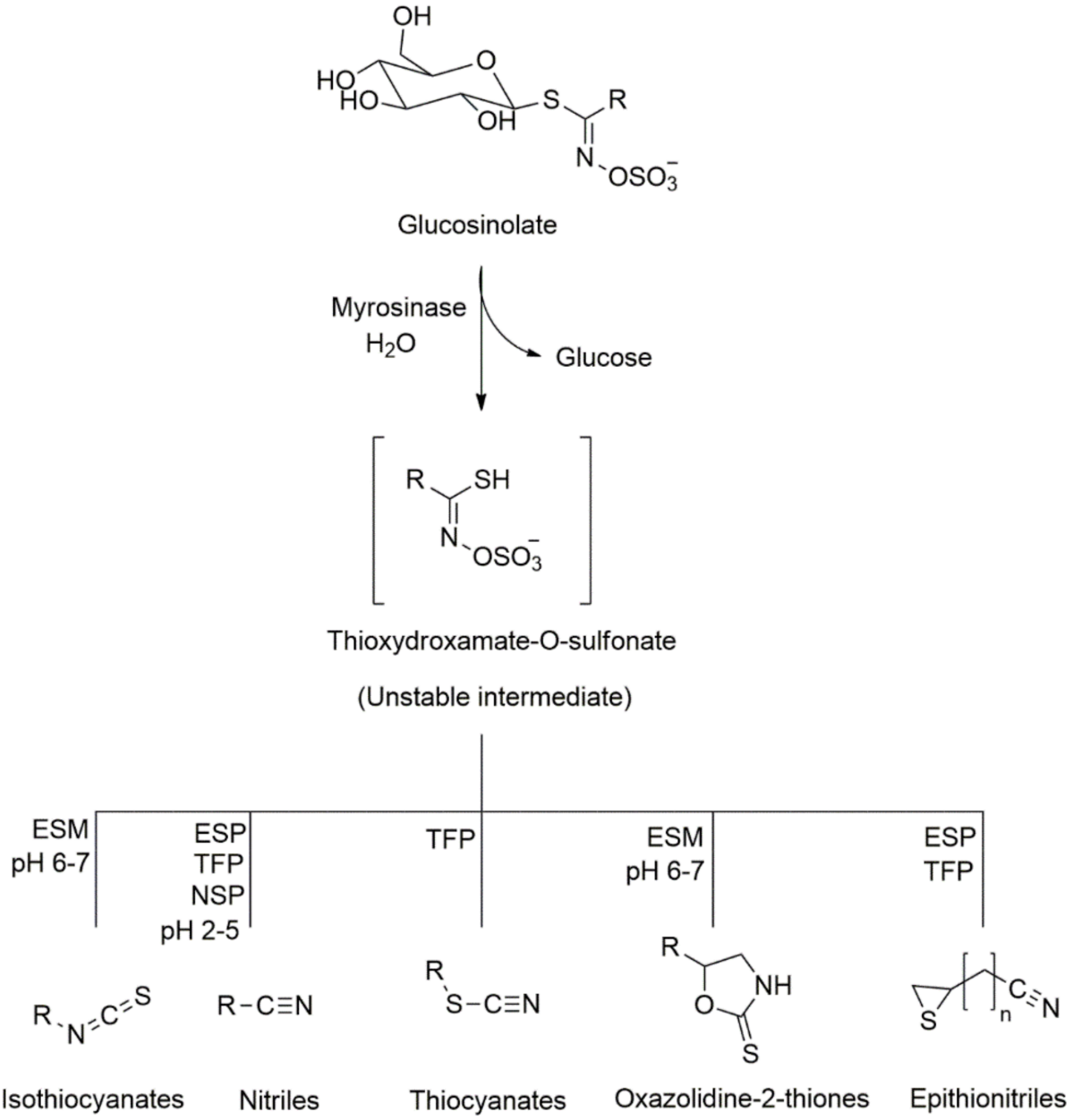


Figure 1. Chemical structures of aliphatic glucosinolates and their enzymatic hydrolysis products. ESP, epithiospecifier protein; NSP, nitrile-specifier proteins; TFP, thiocyanate-forming protein; ESM, epithiospecifier modifier protein.

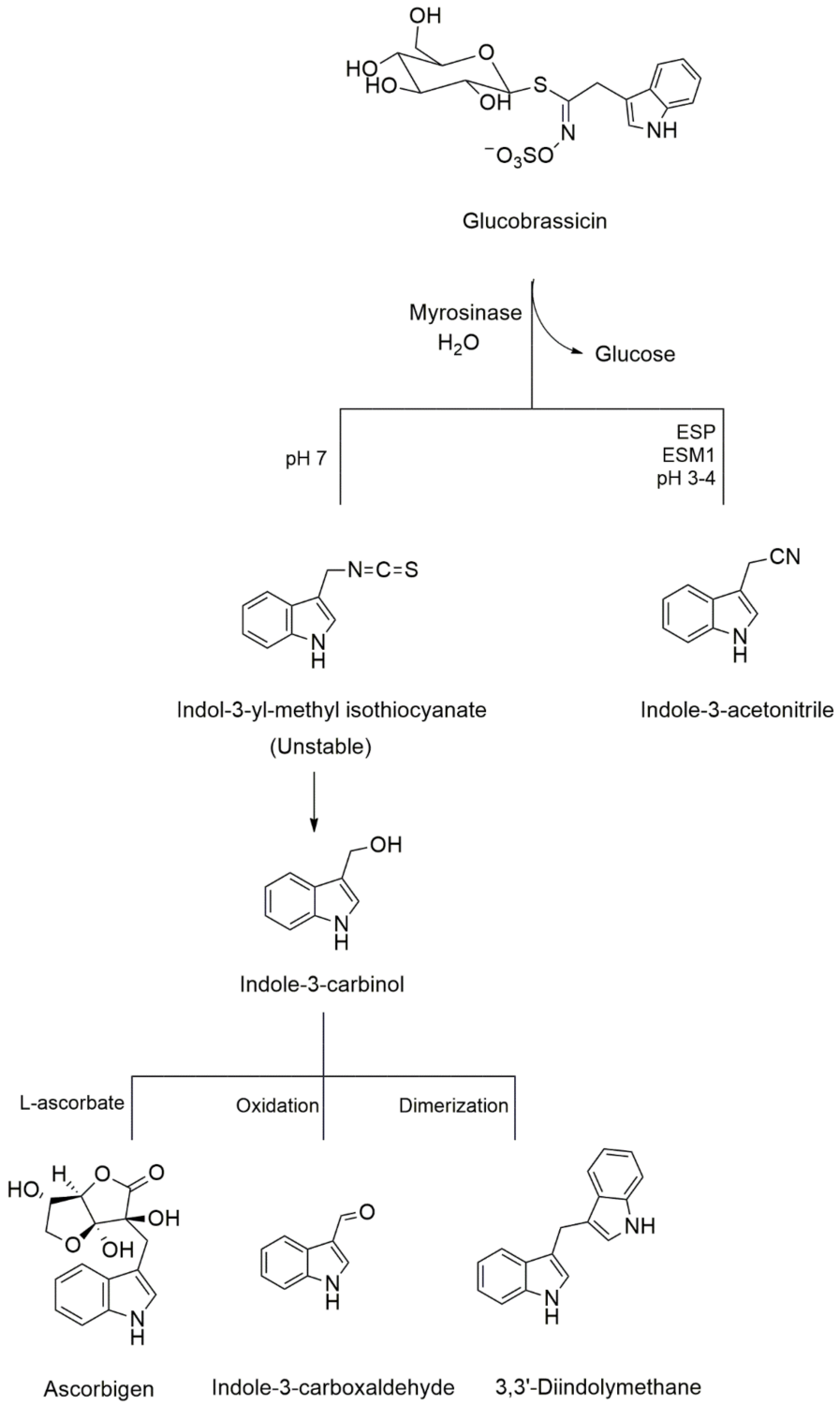
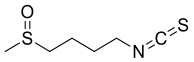
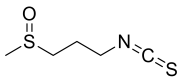
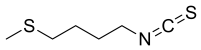
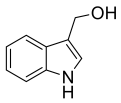
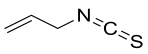
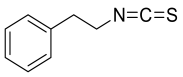
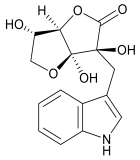
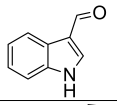
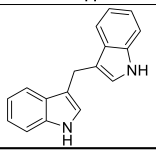


Figure 2. Chemical structure of glucobrassicin and its enzymatic hydrolysis products. ESP, epithiospecifier protein; ESM1, epithiospecifier modifier 1.

4. Content of Isothiocyanates and Indoles in Cruciferous Vegetables

The content of isothiocyanates has been studied mainly in broccoli as it contains the highest concentration of total ITCs compared to other cruciferous vegetables, with the anticancer isothiocyanate SFN predominating. Sulforaphane, contrary to other ITCs, is present in a wide range of cruciferous vegetables [28,31,46,76–78]. The contents of various ITCs and indoles in cruciferous vegetables are summarized in Table 2.

Table 2. Content of isothiocyanates and indoles in cruciferous vegetables.

Glucosinolate	Products	Structure	Vegetable	Content	References
Glucoraphanin	Sulforaphane		Broccoli	0.14–370.3 mg/100 g FW [1]	[31,76]
			White cabbage	540 µg/g FW	
			Red cabbage	48 µg/g FW	[28]
			Turnip	60 µg/g FW	
			Cauliflower	2–190 µmol/100 g FW	[46]
			Arugula	5.90 µmol/g DW [2]	[77]
			Radish	111.94 µg/g FW	[78]
Glucoiberin	Iberin		Broccoli	2.40 mg/g DW	[79]
			White cabbage	5–280 µmol/100 g FW	[46]
			Cauliflower	0–330 µmol/100 g FW	
			Arugula	1.55 µmol/g DW	[77]
Glucoreucin	Erucin		Broccoli seeds	0.38–1.08 mg/g	[80]
Glucobrassicin	Indole-3-carbinol		Broccoli	24.6 µmol/100 g DW	[81]
			White cabbage	0.116 µmol/100 g FW	[23]
			Cauliflower	39.5 µmol/100 g DW	[81]
Sinigrin	Allyl-ITC		Broccoli	7.54 mg/g DW	[79]
			White cabbage	4–160 µmol/100 g FW	[46]
			Turnip	5 mg/100 g FW	[82]
			Arugula	1–160 µmol/100 g FW	[46]
Gluconasturtiin	Phenethyl-ITC		Broccoli	1.93 mg/g DW	[79]
			Turnip	8 mg/100 g FW	[82]
			Watercress	14–29.3 µmol/g DW	[83]
Glucobrassicin	Ascorbigen		Broccoli	236 µmol/100 g DW	[81]
			White cabbage	0.081 µmol/100 g FW	[23]
			Cauliflower	929 µmol/100 g DW	[81]
Glucobrassicin	Indole-3-carboxaldehyde		White cabbage	1.88 mg/100 g FW	[84]
			Cauliflower	10 µg/100 g FW	
Glucobrassicin	3,3′-Diindolylmethane		Broccoli	3.1 µmol/100 g DW	[81]
			White cabbage	0.00235 µmol/100 g FW	[23]
			Cauliflower	3.1 µmol/100 g DW	[81]

[1] FW: fresh weight; [2] DW: dry weight.

Broccoli, in addition to SFN, contains a variety of other ITCs and indole derivatives with significant biological activity such as allyl-ITC, phenethyl-ITC, iberin [79], erucin [80], ascorbigen, I3C, and 3,3′-diindolylmethane [81]. White cabbage and cauliflower are also important sources of ITCs and indoles such as SFN [28,46], iberin [46], allyl-ITC [46,82], ascorbigen, I3C, and 3,3′-diindolylmethane [23,81]. Turnips contain SFN [28] and phenethyl-ITC [82], while arugula (salad rocket) contains SFN and iberin [77]. Phenethyl-ITC is mainly present in watercress [83], while indole-3-carboxaldehyde has been detected in white cabbage and cauliflower [84].

The content of these compounds is indicative as it is influenced by numerous factors including variety, geography, season, and environmental factors, e.g., the infestation of pathogenic microorganisms, soil fertility, and plant growth regulators [85]. In addition, another important factor to note is the sample preparation and extraction conditions. The enzymatic hydrolysis of GLS must be complete and under appropriate conditions conducive to the production of ITCs over the corresponding nitriles. This explains the

discrepancies between studies regarding the SFN concentration in broccoli, which has been reported to be in the range of 0.14–370.3 mg/100 g fresh weight (Table 2). [84]

5. Extraction Procedures

5.1. Conventional Extraction

The optimum extraction conditions must be chosen carefully, given that ITCs and indoles are the products of GLS enzymatic hydrolysis. Glucosinolates have to undergo complete hydrolysis to ITCs rather than other compounds (e.g., nitriles) that exhibit a potent genotoxic risk [86]. In conventional extraction (Table 3), fresh or lyophilized samples are subjected to homogenization in a blender, with the addition of water or a buffer solution [25,87–89]. For Brassicaceae seeds, n-hexane is usually employed as the solvent for defatting purposes before the addition of water or a buffer. In this step, sonication for several minutes may be used [90]. To complete the hydrolysis of GLS, the mix is then allowed to autolyze at an ambient temperature or is subjected to incubation in a water bath at 35–45°C for 1–4 h. A buffer solution is typically added instead of deionized water because it affords a constant neutral pH that is required to ensure that GLS hydrolysis yields ITCs [25,87,91,92]. Nevertheless, it has been argued that for SFN, water with a pH of 3–6 should be added to attain the maximum amount of this compound when glucoraphanin is hydrolyzed [19,93]. Once hydrolysis is completed, a suitable solvent is used for the extraction of ITCs and indoles. At this step, derivatization may be also applied [25,87,94]. Table 3 lists the organic solvents necessary for standard ITC and indole extraction, with the chlorinated solvents dichloromethane (CH_2Cl_2) [42,43,78,93,95–97] and chloroform ($CHCl_3$) [90] being preferred. Nevertheless, other solvents have been also proposed such as ethyl acetate (AcOEt) [98] acetone [96,99], and methyl t-butyl ether (MTBE) [19,96]. The general procedure is briefly described in Figure 3.

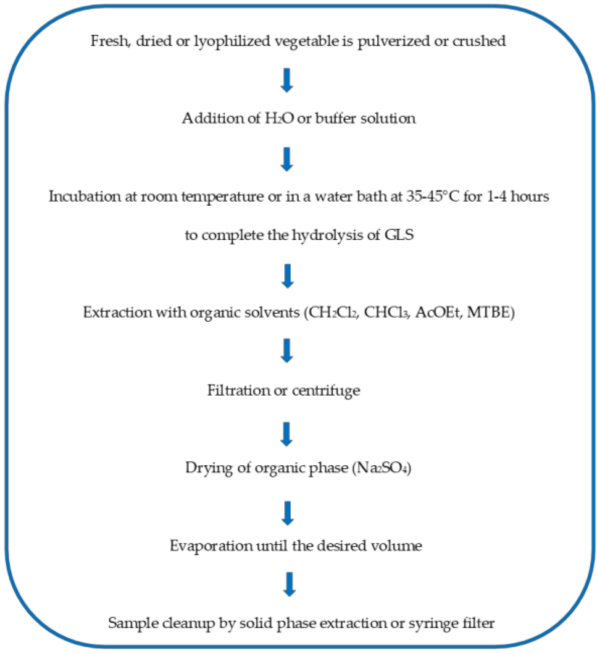


Figure 3. General procedure for the sample preparation and extraction of the hydrolysis products of glucosinolates from cruciferous vegetables.

In the case of analysis using gas chromatography with a flame ionization detector (GC-FID) or a mass spectrometer (GC-MS), CH_2Cl_2 is mainly used for the extraction of the hydrolysis products of GLS (Table 4) [100–106]. Additionally, Clevenger hydrodistillation has been also employed to extract the essential oil of cruciferous vegetables in which a great variety of ITCs, nitriles, epithionitriles, and oxazolidine-2-thiones have been identified [107,108].

Table 3. Sample preparation, conventional extraction, and determination of isothiocyanates and indoles using liquid chromatography/mass spectrometry.

Analyte	Vegetable	Sample Preparation	Extraction Solvent	Purification	Analytical Method	Conditions	Reference
Sulforaphane	Broccoli	Fresh or lyophilized sample, addition of acidic water (pH 6), incubation at 45 ± 2 °C for 2.5 h	CH_2Cl_2	Filtration with Whatman no. 41 paper, purification with Bakerbond SPE silica gel 3 mL,	HPLC-UV [1]	Wavelength: 202 nm Column: SS Exil ODS C18/25 × 0.46 cm, 5 µm Mobile phase: MeCN and H_2O	[95]
Ascorbigen indole-3-carbinol, indole-3-acetonitrile, 3,3′-diindolylmethane	Fermented cabbage	Sample homogenized with distilled water and NaCl	Acetone-ethyl acetate (for ascorbigen), Methyl-t-butyl ether (for indole-3-carbinol), CH_2Cl_2 (for indole-3-acetonitrile, 3,3′-diindolylmethane)	filtration	HPLC-UV/fluorescence	(for ascorbigen) Wavelength: 280 nm Column: RP-18 Mobile phase: 10% MeCN in 0.1 M NH_4OAc buffer pH 5.7 and 80% MeCN in 0.1 M NH_4OAc buffer pH 5.7. (for the other indoles) Fluorescence detection: ex.285 nm, em.340 nm Column: RP-18 Mobile phase: 10% MeCN and 80% MeCN	[96]
Sulforaphane	Broccoli	Addition of acidic water (pH 3), incubation at 35 °C for 4 h (optimum conditions)	CH_2Cl_2	Filtration with 0.22 µm membrane filter and purification with silica SPE cartridge	HPLC-UV	Wavelength: 205 nm Column: OptimaPak C_{18}/250 mm × 4.6 mm, 5 µm Mobile phase: MeCN and H_2O	[93]
Total ITCs	Broccoli Cabbage Cauliflower Brussels sprout Kale Collard green Mustard green Turnip green	Homogenization with deionized water and centrifuge, supernatant mixed with 100 mmol/L potassium phosphate buffer pH 8.5 and 10 mmol/L 1,2-benzenedithiol in MeOH for cyclocondensation reaction	-	Centrifuge at low speed	HPLC	Wavelength: 365 nm Column: Whatman Partisil ODS-2 RP-C_{18}/250 mm × 4.5 mm, 10µm Mobile phase: MeOH and H_2O	[25]

Table 3. *Cont.*

Analyte	Vegetable	Sample Preparation	Extraction Solvent	Purification	Analytical Method	Conditions	Reference
Sulforaphane	Broccoli florets, stems, and leaves	Dried sample at 40 °C, addition of acidic water pH 5.0 or 6.0 using 0.1 M hydrochloric acid, incubation at 35–45 °C for 1–4 h	Methyl-t-butyl ether	Filtration with Albet 140 paper and purification with SPE with silica cartridges	HPLC-DAD [2]	Wavelength: 196 nm Column: Synergi^{TM} Hydro-RP C_{18}/150 mm × 4.6 mm, 4 µm Mobile phase: 20 mM NH_4HCO_2 in H_2O and MeCN (55:45, *v*/*v*)	[19]
Sulforaphane, sulforaphene	*Raphanus sativus* L. var. *caudatus* Alef	Homogenization with deionized water for 30 min and autolyze at r.t. for 2 h.	CH_2Cl_2	-	HPLC-DAD	Wavelength: 254 nm Column: HiQsil RP-C_{18}/250 mm × 4.6 mm, 5 µm Mobile phase: THF and H_2O	[78]
Total ITCs	Fresh and cooked green cauliflower, purple cauliflower, rutabaga	Lyophilized samples mixed with 0.01 M sodium phosphate buffer pH 7.4, incubation at 37 °C for 3 h, centrifuge and purification by SPE. Retained ITCs submitted to a cyclocondensation reaction	-	Supernatant passed through Bakerbond SPE C18 500 mg cartridge. Elution of ITCs and indoles with MeOH	HPLC-DAD	Wavelength: 365 nm Zorbax Eclipse XDB-C_8/150 mm × 4.6 mm, 3.5 µm Mobile phase: 1% (*v*/*v*) formic acid in H_2O and MeOH	[87]
Indole-3-carbinol Indole-3-acetic acid Indole-3-acetonitrile Diindolylmethane Total indoles		Lyophilized samples mixed with 0.01 M sodium phosphate buffer pH 7.4, incubation at 37 °C for 3 h and centrifuge	-		HPLC-DAD-FLD [3]	Fluorescence detection: ex.280 nm, em.360 nm Column: Zorbax Eclipse XDB-C_8/150 mm × 4.6 mm, 3.5 µm Mobile phase: MeCN and H_2O	
Indole-3-carbinol	Broccoli, cabbage	Freeze-dried samples homogenized with sodium dihydrogen phosphate and citric acid buffer for 1.5 h	Ethyl acetate	Centrifuge for 10 min at 5500× *g* and filtration with Agela No. 0.22-µm (D) nylon filter paper	HPLC-UV	Wavelength: 279 nm Column: C_{18}/250 × 4.6 mm, 5 µm Mobile phase: H_2O and MeCN	[92]

Table 3. *Cont.*

Analyte	Vegetable	Sample Preparation	Extraction Solvent	Purification	Analytical Method	Conditions	Reference
Alyll-ITC	Mustard	Samples homogenized with LC-grade water and ACN at 450 rpm for 10 min and sonicated for 30 min	-	Centrifuge for 10 min at 1300× *g* at 7 °C and filtration with Phenomenex RC 0.45 µm membrane filter	HPLC-PDA-UV [4]	Wavelength: 242 nm Column: ReproSil-Pur 120 C_{18}-AQ/250 × 4.6 mm, 5 µm Mobile phase: H_2O with 0.5% formic acid and MeCN with 0.5% formic acid	[88]
Sulforaphane	Broccoli	Fresh sample homogenized	CH_2Cl_2	Filtration	HPLC-DAD	Wavelength: 254 nm Column: Agilent C_{18}/250 × 4.6 mm, 5 µm Mobile phase: H_2O and MeCN	[97]
Ascorbigen, indole-3-carbinol, indole-3-acetonitrile, 3,3′-diindolylmethane	Fermented cabbage	Sample homogenized with water	Acetone (for ascorbigen), Methyl-t-butyl ether (for indole-3-carbinol), CH_2Cl_2 (for indole-3-acetonitrile, 3,3′-diindolylmethane)	Filtration with PTFE filter 0.22 µm (for ascorbigen, indole-3-acetonitrile, 3,3′-diindolylmethane), filtration with filter paper Munktell, grade: 390 (for indole-3-carbinol)	HPLC-UV	(for ascorbigen) Wavelength: 280 nm Column: LiChrospher® 100 RP-18/250 × 4 mm, 5 µm Mobile phase: 10% MeCN in 0.1 M NH_4OAc buffer pH 5.7 and 80% MeCN in 0.1 M NH_4OAc buffer pH 5.7. (for the other indoles) Fluorescence detection: ex.285 nm, em.340 nm Column: LiChrospher® 100 RP-18/250 × 4 mm, 5 µm Mobile phase: 10% MeCN and 80% MeCN	[99]
Sulforaphane, iberin	Broccolini, kale	Freeze-dried samples extracted with MilliQ-H_2O for 24 h at room temperature	H_2O	Centrifuge for 5 min at 17,500× *g*	UHPLC-QqQ-MS/MS[5]	Column: ZORBAX Eclipse Plus C_{18}/50 × 2.1 mm, 1.8 µm Mobile phase: H_2O/ammonium acetate 13 mM (pH 4) with acetic acid 99.99:0.01 *v/v* and acetonitrile/acetic acid; 99.99:0.1, *v/v*	[89]

Table 3. *Cont.*

Analyte	Vegetable	Sample Preparation	Extraction Solvent	Purification	Analytical Method	Conditions	Reference
3-(methylsulfinyl)propyl-ITC, 4-(methylsulfinyl)butyl-ITC, 6-(methylsulfinyl)hexyl-ITC, 9-(methylsulfinyl)nonyl-ITC, 4-(methylsulfinyl)-3-Butenyl-ITC, 3-(methylsulfonyl)propyl-ITC, 3-(methylthio)propyl-ITC, 4-(methylthio)butyl-ITC, 5-(methylthio)pentyl-ITC, propyl-ITC, allyl-ITC, 3-butenyl-ITC, 4-pentenyl-ITC, benzyl-ITC, phenethyl-ITC	Seeds of *Sinapis alba* (yellow mustard) *Brassica napus* *Brassica juncea* var. *rugosa* *rugosa* (Chinese mustard)	Extraction of GLS with MeOH at 65 °C. Extract evaporated, resolubilized in tert-butanol and freeze-dried. Addition of myrosinase and derivatization with N-acetyl-L-cysteine	-	-	RP-UHPLC–ESI-MS[6]	Column: Acquity UPLC-BEH shield RP18/150 mm × 2.1 mm, 1.7 µm Mobile phase: 0.1% (v/v) FA in H_2O and 0.1% (*v*/*v*) FA in MeCN	[94]
Sulforaphane, indole-3-carbinol	Broccoli	Fresh samples homogenized for 5 min, addition of distilled water, incubation at 45 ± 3 °C for 3 h	CH_2Cl_2	Filtration with Whatman filter paper grade 1	UPLC–HRMS/MS [7]	Column: Merck Chromolith RP/100 × 4.6 mm Mobile phase: MeOH and H_2O	[42]
Indole-3-carbinol, indole-3-carboxaldehyde, ascorbigen, indole-3-acetic acid	White cauliflower, red cabbage, white cabbage, green broccoli, purple broccoli, radish, turnip	Lyophilized samples mixed with distilled water, incubation at 45 ± 3 °C for 3 h	CH_2Cl_2	Filtration with Whatman filter paper grade 1	LC-Q-TOF-MS [8]	Column: Agilent Zorbax C18/50 × 2.1 mm, 1.8 µm Mobile phase: MeOH and H_2O	[43]

[1] HPLC-UV: High Performance Liquid Chromatography-Ultraviolet Detector; [2] HPLC-DAD: High Performance Liquid Chromatography-Diode-Array Detection; [3] HPLC-DAD-FLD: High Performance Liquid Chromatography-Diode-Array Detection-Fluorescence Detector; [4] HPLC-PDA-UV: High Performance Liquid Chromatography-Photodiode-Array Detection-Ultraviolet Detector; [5] UHPLC-QqQ-MS/MS: Ultrahigh Performance Liquid Chromatography-Triple Quadruple-Mass Spectrometry/Mass Spectrometry; [6] RP-UHPLC-ESI-MS: Reverse Phase-Ultrahigh Performance Liquid Chromatography-Electronspray Ionization-Mass Spectrometry; [7] UPLC-HRMS/MS: Ultraperformance Liquid Chromatography-High Resolution Mass Spectrometry/Mass Spectrometry; [8] LC-Q-TOF-MS: Liquid Chromatography-Quadruple-Time of Flight-Mass Spectrometry.

Table 4. Sample preparation, conventional extraction and determination of isothiocyanates and indoles analysis using gas chromatography/mass spectrometry.

Analyte	Vegetable	Sample Preparation	Extraction Solvent	Purification	Analytical Method	Conditions	Reference
4-methylthio-3-butenyl-ITC	Chinese radish roots	Samples cut into 1 cm cubic pieces, blended and ground. Filtration with two layers of cotton gauze. Centrifuge of the filtrate with CH_2Cl_2 at 3× *g* for 1.5 h at 37 °C	CH_2Cl_2	Centrifuge for 15 min at 756× *g* Filtration with 0.45 µm membrane filter	GC-FID [1]	Column: HP-5/30 m × 0.32 mm × 0.5 µm, splitless mode, T injector = 250 °C T detector = 280 °C	[100]
4-methylthio-3-butenyl-ITC	Chinese white radish	Cooked samples were homogenized and filtered through two layers of cotton gauze. Ultrasonic extraction for 10 min	CH_2Cl_2	Centrifuge for 10 min at 3000 rpm	GC-FID	Column: HP-5/30 m × 0.25 mm × 0.25 µm, splitless mode, T injector = 200 °C T detector = 200 °C	[101]
2-propenyl-ITC, 3-(methylthio)propyl-ITC, propyl ITC, 3-butenyl-ITC, 3-butenenitrile, 4-pentenenitrile, 4-(methylsulfinyl)butyl-ITC, 5-(methylsulfinyl)-pentanenitrile, 3,4-epithiobutanenitrile	Broccoli seeds	Seeds suspended in water and ground in a mortar. Washed with water and incubated at 25 °C for 2 h.	CH_2Cl_2	Centrifuge	GC-FID	Column: HP5/30 m × 0.25 mm × 0.25 µm, splitless mode, T injector = 200 °C	[102]
sec-butyl-ITC, allyl-ITC, benzyl-ITC 3-(methylthio)propyl-ITC, 2-phenylethyl-ITC	Horseradish roots	Samples were peeled and ground. Addition of CH_2Cl_2 and vigorous stirring for 30 min.	CH_2Cl_2	Filtration and solvent-assisted flavor evaporation at 50 °C	HRGC-O/FID [2]	Column: DB-FFAP/30 mm × 0.32 mm × 0.25µm or DB5/30 m × 0.32 mm × 0.25µm, split ratio (1:1), T injection = 40 °C (cold on-column technique)	[103]
4-(methylthio)butyl-ITC, 2-phenylethyl-ITC, 4-(methylthio)-3-butenyl-ITC, 4-methylpentyl-ITC, benzyl-ITC, 5-(methylthio)-4-pentenenitrile, benzenepropanenitrile	Radish	Clevenger hydrodistillation	H_2O	-	GC-FID and GC-MS [3]	For GC-FID: Column: HP-101/25 m × 0.2 mm × 0.2 µm split ratio 1:50 T injector = 250 °C T detector = 300 °C For GC-MS: Column: HP-20 M/50 m × 0.2 mm × 0.2 µm, split ratio 1:50, T injector = 250 °C	[108]

Table 4. *Cont.*

Analyte	Vegetable	Sample Preparation	Extraction Solvent	Purification	Analytical Method	Conditions	Reference
2-butyl-ITC, phenylethyl-ITC	Indian mustard	Dried and defatted seeds and leaves mixed with deionized water and incubated at 37 °C for 2 h. Addition of myrosinase and L-ascorbic acid. Incubation for 3 h at 37 °C.	CH_2Cl_2	Centrifuge for 15 min at 4000 rpm.	GC-MS	Column: HP-5 m/0.25 mm × 30 m × 0.25 µm, split ratio 1:50, T injector = 280 °C	[104]
sec-butyl-ITC, 3-butenyl-ITC, 4-pentenyl-ITC, 4-(methylsulfanyl)butyl-ITC, 5-(methylsulfanyl)pentyl-ITC, 4-(methylsulfinyl)butyl-ITC, 2-phenylethyl-ITC, 1-methoxyindole-3-carbinol, 3-methylpentanenitrile, 4-pentenenitrile, 3-hydroxypentenenitrile, 5-hexenenitrile, 5-(methylsulfanyl)pentanenitrile, 6-(methylsulfanyl)hexanenitrile, 5-(methylsulfinyl)-pentanenitrile, 3-phenylpropanenitrile, indole-3-acetonitrile, 1-methoxyindole-3-acetonitrile, 4,5-epithiopentanenitrile, 3-hydroxy-4,5-epithiopentanenitrile, 5,6-epithiohexanenitrile, 3-hydroxy-5,6-epithiohexanenitrile, 5-vinyl-1,3-oxazolidine-2-thione	*Brassica rapa* leaves	Fresh samples homogenized with deionized water	CH_2Cl_2	Centrifuge	GC-MS	Column: SGE BP5MS/30 m × 0.25 mm × 0.25 µm, splitless mode, T injector = 190 °C	[105]

Table 4. *Cont.*

Analyte	Vegetable	Sample Preparation	Extraction Solvent	Purification	Analytical Method	Conditions	Reference
3-butenyl nitrile, 4-pentenenitrile, 2-propenyl-ITC, 3-butenyl-ITC, 3,4-epithiobutyl nitrile, 4-(methylsulfanyl)butenyl nitrile, 4,5-epithio-pentanenitrile, 5-(methylsulfanyl)-pentanenitrile, 3-(methylsulfanyl)propyl-ITC, 4-(methylsulfanyl)-butyl-ITC, 2-phenylethyl-ITC, 5-vinyl-1,3-oxazolidine-2-thione, 3-hydroxy-4,5-epithiopentane-nitrile, 5-(methylsulfinyl)pentanenitrile, 3-(methylsulphinyl)propyl-ITC, 4-(methylsulfinyl)-butyl-ITC	Chinese kale sprouts	Samples homogenized with ddH_2O and incubated at 25 °C for 1 h	CH_2Cl_2	Centrifuge for 10 min at 4000 g at room temperature	GC-MS	HP-5ms/30 m × 0.25 mm × 0.25-µm, splitless injection, T injector = 250 °C	[106]
allyl-ITC, 2-butyl ITC, 3-butenyl-ITC, 4-pentenyl-ITC, 4-methylpentyl-ITC, hexyl-ITC, heptyl ITC, benzyl-ITC, erucin, phenethyl-ITC	Broccoli, daikon, mustard, rocket salad, watercress	Clevenger hydrodistillation	H_2O	-	GC/EI-MS [4]	Column: HP-5MS/30 m × 0.25 mm × 0.25 µm, T injector = 220 °C	[107]

[1] GC-FID: Gas Chromatography-Flame Ionization Detection; [2] HRGC-O/FID: High Resolution Gas Chromatography-Olfactometry/Flame Ionization Detector; [3] GC-MS: Gas Chromatography-Mass Spectrometry; [4] GC/EI-MS: Gas Chromatography/Electron Impact-Mass Spectrometry.

Glucosinolates can be subjected to enzymatic hydrolysis indirectly by an initial extraction of intact GLS. In this approach, the plant material is pulverized and the myrosinase enzyme is deactivated with liquid nitrogen or boiling water. Methanol or aqueous methanol is then employed to extract the intact GLS, which are desulfated on a diethylaminoethyl (DEAE) Sephadex column, with the addition of an artificial or natural myrosinase enzyme. This technique is advantageous primarily because of the possibility to adjust the column conditions, including pH (acidic, basic, or neutral) and temperature (low, room temperature, or high) depending on the type of hydrolysis product that is targeted to be obtained [59].

5.2. Non-Conventional Extraction

The conventional extraction methods are simple, especially for lab-scale processes; however, due to the large quantities of solvents and the long extraction times required, they may be replaced or enhanced by non-conventional extraction techniques such as High-Pressure Processing (HPP), High Voltage Electrical Discharges (HVED), Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), Supercritical Fluid Extraction (SFE), and Pressurized Fluid Extraction (PFE) (Table 5). Non-conventional extraction may be preferable, as it manages to reduce the extraction temperature and time, and therefore the consumption of the solvent, but at the same time it can achieve a higher efficiency and an improvement in the quality of the extracted compounds compared to the conventional methods. However, in the case of ITCs and indoles, the research for non-conventional extraction procedures is still ongoing and more studies are required for the establishment of these techniques as an alternative to conventional extraction.

The technique of HPP, (also known as High Hydrostatic Pressure (HHP)), is a non-thermal technology that utilizes liquids for a pressure transmission medium. Compared to thermal processing, HPP is more effective in the preservation of the flavor, texture, nutrients, and appearance of food products [109]. Applications of HPP in foods include the inactivation of microorganisms and enzymes and the increase in the shelf life of the products. Furthermore, the use of chemical preservatives is reduced [110]. After HPP on broccoli sprouts with 600 MPa, 85% of GLS were converted to ITCs [111] which suggested the inactivation of the ESP protein. Moreover, the glucosinolate–myrosinase system in broccoli sprouts was not negatively affected. Two studies [110,112] have reported that the application of HHP at 400 MPa in red cabbage and broccoli generated the highest amounts of ITCs. Moreover, it was found that the activity of myrosinase increased after processing, indicating that the increase in ITCs content is related to the stimulation of myrosinase activity [110].

Table 5. Non-conventional techniques applied for the extraction of isothiocyanates from cruciferous vegetables.

Vegetable	Compounds Studied	Technology	References
Red cabbage	Allyl-ITC	HHP [1]	[112]
Broccoli	Sulforaphane, erucin	HHP	[110]
Broccoli sprouts	Iberin, sulforaphane, erucin	HP [2]	[111]
Cauliflower by-products	Isothiocyanates	UAE [3]	[113]
Rapeseed, rapeseed press-cake	Isothiocyanates	HVED [4]	[114]
White cabbage	Sulforaphane	MAE [5]	[115]
Wasabi	Allyl-ITC	SFE-CO_2 [6]	[116]
Horseradish	Allyl-ITC	SFE-CO_2/Hydrodistillation/H_2O	[117]
Watercress	Isothiocyanates	PFE [7]	[83]
Garden cress	Benzyl-ITC	UAE/SFE-CO_2/ASE [8]	[118]

[1] HHP: High Hydrostatic Pressure [2] HP: High Pressure [3] UAE: Ultrasound-Assisted Extraction; [4] HVED: High voltage electrical discharges; [5] MAE: Microwave-Assisted Extraction; [6] SFE-CO_2: Supercritical Fluid Extraction-Carbon Dioxide; [7] PFE: Pressurized Fluid Extraction; [8] ASE: Accelerated Solvent Extraction.

High voltage electrical discharges are a non-thermal technique based on the electrical breakdown in water, while the air bubbles in water may accelerate the process. The HVED process initiates from the avalanche of electrons that is caused by the intense electrical field and constitutes the starting point of streamer propagation. The application of HVED improves the extraction yield of compounds through the destruction of the cellular structure and the enhancement of the mass transfer [119,120]. Barba et al. [114] employed HVED technology for the extraction of protein, polyphenols, and ITCs from rapeseeds and rapeseed press-cake. Different energy inputs (0–400 kJ/kg) were tested, and the researchers concluded that the optimal treatment energy input was 80 or 240 kJ/kg, while when this was exceeded, the yield decreased. It is important to note that the nature of the sample influences the treatment efficiency to a great extent; thus, for each starting material, an optimization of the HVED process is required.

Ultrasound-assisted extraction (UAE) facilitates cell disruption and solvent penetration in the samples. This technique manages to enhance the extraction yield of compounds owing to the cavitation phenomena that are created by the ultrasound pressure waves in the solvent used for the extraction. Although the use of a solvent is required, the amount of solvent and energy consumption are reduced [121]. Besides, eco-friendly solvents such as natural deep eutectic solvents may be used with UAE to obtain high-quality extracts with increased yields [122]. Diatuo et al. [118] applied the UAE at a frequency of 24 kHz in order to obtain extracts from cauliflower by-products that are rich in ITCs. The researchers developed a powder from the plant material, which was subjected to UAE. From the three solvents (distilled water, 70% methanol, and 80% acetonitrile) that were tested under UAE, the distilled water was the most efficient for the extraction of ITCs. The MAE is an alternative extraction procedure that has the potential to reduce the extraction time and the amount of solvent consumption while simultaneously increasing the extraction yield. Microwaves are electromagnetic waves with a frequency of 300 MHz to 300 GHz. The process is based on the ionic conduction and dipole movement which are responsible for the warming of substances. The choice of the appropriate solvent in the MAE process is crucial. Solvents transparent to microwaves such as hexane do not heat up when subjected to the microwaves, while microwave absorbing solvents, such as ethanol or water, are more suitable for the MAE extraction process [123]. Tanongkankit et al. [115] studied the MAE of SFN from white cabbage in comparison to the conventional solvent extraction method. Different solvent types (CH_2Cl_2 or water), microwave power, and extraction time were tested. MAE led to a higher extraction yield of SFN with a reduced extraction time compared to the classic solvent extraction method while both solvents, CH_2Cl_2, and water, provided similar yields.

In supercritical extraction, a supercritical fluid solvent, such as carbon dioxide, is used. The advantage of this technique is the avoidance of the use of organic solvents, which eventually reduces environmental concerns. The surface tension is not present in supercritical fluids and thus they have the potential to penetrate into small pores which are inaccessible to liquid solvents. This technique is very effective for the extraction of volatile compounds [124]. Li et al. [116] applied SFE for the extraction of allyl ITC from wasabi. The researchers tested different ranges of pressure (15–25 MPa) and temperature (35–55 °C) and found that the extraction yield was increased with the increase in pressure and the decrease in temperature. The highest extraction yield was obtained at 25 MPa and 35 °C, while the most significant parameters for the SFE process were pressure, temperature, and moisture content. Wu et al. [117] extracted allyl-ITC from horseradish using water extraction, hydrodistillation, and supercritical fluid extraction with carbon dioxide (SFE-CO_2). Hydrodistillation and SFE-CO_2 provided similar extraction yields, whereas the conventional water extraction yield was significantly lower. Rafińska et al. [118] used UAE, SFE, and Accelerated Solvent Extraction (ASE) for the extraction of bioactive compounds from garden cress. It was found that the SFE method applied in dried garden cress sprouts was more efficient in the extraction of ITCs compared to ASE and UAE. The main ITC was found to be benzyl-ITC.

Rodrigues et al. [83] reported the use of Pressurized Fluid Extraction (PFE) with supercritical CO_2 for the recovery of ITCs and phenolic compounds from watercress. Pressurized fluid extraction is also an advantageous alternative method to the traditional extraction processes as it requires less solvent and a shorter extraction time. The results showed that the application of the PFE process was highly selective for the isolation of ITCs from watercress and the main isothiocyanate that was recovered was phenethyl-ITC. Moreover, an alternative extraction method for ITCs has been proposed using lactic acid bacteria (LAB). Jaiswal et al. [125] performed a fermentation-assisted extraction of ITCs from York cabbage using LAB. The researchers concluded that the employment of LAB facilitated the hydrolysis of GLS to the formation of ITCs. The factors with the highest influence in obtaining a high yield of ITCs were the fermentation time, the solid-to-liquid ratio, and the agitation rate. Yuan et al. [126] developed an ultrasound-assisted dispersive-filter extraction technique based on poly(deep eutectic solvent)-graphene oxide (PDES-GO) adsorbent for the isolation of I3C from broccoli. For the preparation of the PDES-GO, the researchers used a mixture of choline chloride and methacrylic acid in a 1:2 molar ratio in order to modify the graphene oxide surface. The specific method proved to be rapid, accurate, and low-cost. The efficiency of aqueous micellar systems (AMSs) was explored by Coscueta et al. [127] for the extraction of phenylethyl-ITC from watercress by-products. For this purpose, the surfactants Genapol X-080 (Clariant, Louisville, KY, USA) and Tergitol 15-S-7 (Sigma-Aldrich, Burlington, MA, USA) were utilized as it is known that they are effective in interacting with the low polar molecules of plant tissues. Liquid-liquid extraction was also performed using n-hexane and MeCN/$CHCl_3$. The highest content of phenylethyl-ITC was obtained using n-hexane; however, no statistically significant differences were observed between the extraction systems with Tergitol 15-S-7 (Sigma-Aldrich, Burlington, MA, USA) and MeCN/$CHCl_3$ compared to n-hexane, while the use of Genapol X-080 (Clariant, Louisville, KY, USA) afforded a slightly lower yield of phenylethyl-ITC.

6. Methods of Analysis

The analysis of ITCs and indoles has been performed using a great variety of chromatographic, spectrophotometric, and other techniques. However, the techniques that have been commonly employed are LC and GC, with or without the use of mass spectrometry detection (Tables 3 and 4). Furthermore, prior to the analysis of ITCs, derivatization may be applied owing to the high volatility and instability of these compounds and the absence of chromophore groups.

6.1. Spectroscopic Techniques

6.1.1. UV–Vis Spectrophotometry

Spectrophotometry is defined as the measurement of the absorption or reflectance properties of a substance as a function of the wavelength. The key advantages of the UV–Vis spectrophotometry methods are the low analysis time and the reduced labor consumption, while they simultaneously exhibit a high sensitivity and precision [128]. A UV–Vis spectrophotometric method for the determination of total ITCs content has been developed [44,129]. This method is indirect, and it is based on the quantitative reaction of ITCs with an excess of 1,2-benzenedithiol (Figure 4). The product of the cyclocondensation reaction, 1,3-benzodithiole-2-thione, is determined spectrophotometrically at 365 nm.

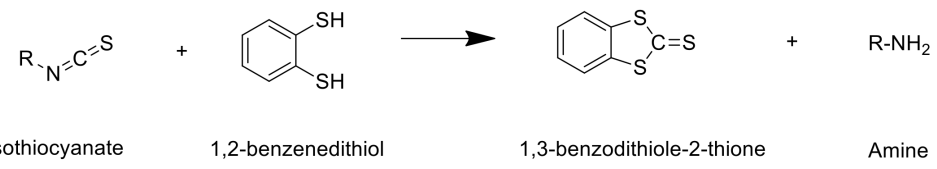


Figure 4. Cyclocondensation reaction of isothiocyanates with 1,2-benzenedithiol.

The reaction of 1,2-benzenedithiol with ITCs is highly selective; thus, other enzymatic hydrolysis products of GLS, such as thiocyanates, do not react. Although the sensitivity of the method is very low (1 nmol), it can be considered a time-consuming method of analysis due to the pre-required stage of derivatization. However, this cyclocondensation reaction has been utilized in several studies for the determination of total ITCs with High-Performance Liquid Chromatography (HPLC) [21,130,131] or GC-MS [132].

6.1.2. Fourier-Transform Infrared Spectroscopy (FT-IR)

Another method that has been reported in the literature for the determination of the total ITCs in cruciferous vegetables is the Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy (ATR-FTIR) method in combination with the partial least-squares (PLS) algorithm [45]. The sampling technique of ATR is advantageous as it enables the direct examination of samples without the requirement of a derivatization step [133]. The spectral range 2150–2020 cm^{-1} was used for the quantification of total ITCs in broccoli and the results showed that it is an equivalent method in terms of reproducibility and accuracy to the UV–Vis spectrophotometric method [44]. This method seems to be simple and rapid and constitutes a useful alternative for the determination of total ITCs in cruciferous vegetables.

6.2. Chromatographic Techniques

6.2.1. Paper Chromatography (PC)

Paper chromatography is the oldest method used to analyze GLS hydrolysis products [134,135]. Ammonia reacts with ITCs and produces thiourea-type derivatives which are separated by PC. The components that are hydrophobic are analyzed using water saturated $CHCl_3$, while the hydrophilic substances are analyzed with a mixture of water, butanol, and toluene as the developing solvent system. However, PC exhibits low reproducibility for the analysis of ITCs; thus, alternative chromatographic methods have been developed for their analysis [136].

6.2.2. Thin Layer Chromatography (TLC)

Thin layer chromatography has been used to determine the compounds 1-isothiocyanato-4-(methylsulfinyl)butane, 1-isothiocyanato-3-(methylsulfinyl)propane, 5-(methylsulfinyl)pentanenitrile, and 4-(methylsulfinyl)butanenitrile, from broccoli and *Lesquerella fendleri* [137].

6.2.3. Gas Chromatography (GC)

Gas chromatography is an excellent separation technique for the identification and determination of volatile organic substances. This technique in combination with a Flame Ionization Detector (FID) has been used for the determination of SFN [29,138–140] and other ITCs [100–103,108] in a great variety of cruciferous vegetables. However, the thermal degradation of SFN to 3-butenyl ITC has been observed under split/splitless conditions [31]. Besides, the thermal degradation studies that have been performed for ITCs show that these compounds are thermolabile [32,141,142]. Therefore, LC methods are often preferred for their analysis.

6.2.4. High-Performance Liquid Chromatography (HPLC)

For the analysis of ITCs and certain indoles, HPLC coupled with a UV detector has been proposed by various researchers [19,25,78,88,92,93,95–97,99]. For the determination of I3C, indole-3-acetonitrile, and 3,3′-diindolylmethane, HPLC with a Fluorescence Detector (FLD) is usually employed [87,99,143]. The determination is performed at excitation at 280–285 nm, and emission at 340 nm. In the case of SFN, the UV detection has been performed at 202 nm [95], 205 nm [93], and 196 nm [19]. However, the lack of strong UV chromophores in several ITCs, and especially SFN, creates analytical problems. For this reason, Nakagawa et al. [144] and Liang et al. [20] proposed the use of an Evaporative Light-

Scattering Detector (ELSD) for analyzing SFN, achieving a higher sensitivity compared to a UV detector. Another problem that also arises in the HPLC analysis of ITCs is their precipitation when aqueous mobile phases are used. This impacts the accuracy of determinations and leads to operational problems [22].

6.2.5. High Speed Counter Current Chromatography (HSCCC)

High speed counter current chromatography is a simple and low-cost method which is based on the distribution of substances between two immiscible liquids. This technique enables the recovery and purification of samples; thus, it can be used for the isolation of natural products from plant extracts [145,146]. Liang et al., applied HSCCC in broccoli seed meal in order to purify and recover SFN [147]. The two-phase liquid system used in this study was n-hexane/ethyl acetate/methanol/water (1:5:1:5, *v*/*v*/*v*/*v*) which was selected based on the partition coefficient of SFN. The results of HSCCC were compared with the preparative HPLC method. Sulforaphane was obtained with a slightly higher purity (97%) via HSCCC compared to preparative HPLC (95%), while the recovery obtained from HSCCC was higher (98.5%) than the preparative HPLC method (87.4%). High speed counter current chromatography has been also employed for the purification of sulforaphene from radish seeds [148]. The two-phase solvent system used in this study was n-hexane-ethyl acetate-methanol-water (35:100:35:100, *v*/*v*/*v*/*v*). High purity sulforaphene was obtained (96.9%), while the recovery of the compound was 95.2%.

6.2.6. Supercritical Fluid Chromatography (SFC)

Supercritical fluid chromatography constitutes a variation of HPLC. The difference between HPLC and SFC lies in the fact that in SFC, a supercritical fluid is used instead of the liquid mobile phase. This technique is considered a good alternative to HPLC, as the properties of the supercritical fluid used in the mobile phase is between a liquid and a gas. Supercritical CO_2 is commonly employed owing to its low cost, the fact that it is safe to use in the food industry, and also because it has no negative impact on the environment [149]. Supercritical fluid chromatography has been used for the analysis of the hydrolysis products of indole GLS. Buskov et al. [150] used SFC for the determination of ascorbigens in the autolysates of broccoli, white cabbage, red cabbage, cauliflower, Brussels sprouts, and various Portuguese cabbages, while Buskov et al. [151] studied the degradation products of 4-hydroxybenzylglucosinolate. The study of the hydrolysis products of indol-3-ylmethyl-glucosinolates in broccoli heads by SFC has been also reported [152].

6.3. Hyphenated and Other Techniques

6.3.1. Liquid Chromatography/Mass Spectrometry

The combination of mass spectrometry with liquid chromatography has been successfully applied for the determination of ITCs and indoles. Baenas et al. [89] used the Ultrahigh Performance Liquid Chromatography method coupled with Triple Quadrupole Mass Spectrometry/Mass Spectrometry (UHPLC-QqQ-MS/MS) for the determination of SFN and iberin in cooked broccolini and kale. Andini et al. [94] and Pilipczuk [153] analyzed ITCs with Ultra-High-Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS), applying a derivatization method using N-acetyl-L-cysteine (NAC) (Figure 5), achieving very low detection limits (0.9–2.6 μM) [94].

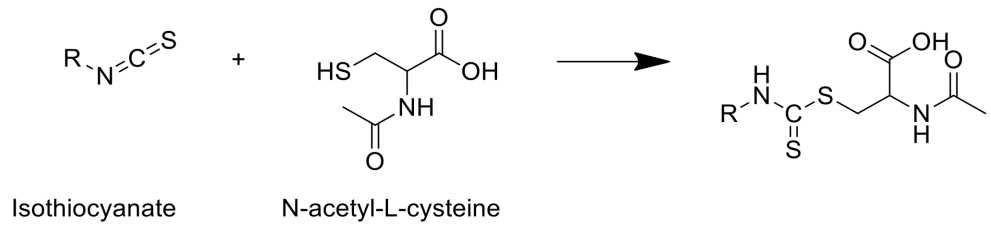


Figure 5. Derivatization reaction of isothiocyanates with N-acetyl-L-cysteine.

High resolution mass spectrometry has been used for the qualitative and quantitative determination of ITCs and indoles. A mass spectrometry study of SFN and indole-3-carbinol has been performed by Kokotou et al. [42] using Ultraperformance Liquid Chromatography-High Resolution Mass Spectrometry/Mass Spectrometry (UPLC–HRMS/MS) avoiding a derivatization step. The researchers also developed a method for the simultaneous determination of compounds in broccoli extracts.

The simultaneous determination of I3C, indole-3-carboxaldehyde, ascorbigen, and indole-3-acetic acid by Liquid Chromatography-Quadrupole-Time of Flight-Mass Spectrometry (LC-Q-TOF-MS) in a broad range of cruciferous vegetables has been also reported [43].

6.3.2. Gas Chromatography/Mass Spectrometry

The determination of ITCs with GC-MS has been employed [104–107,154–156]. For the analysis of volatile ITCs, the eco-friendly technique of Solid Phase Microextraction (SPME) in conjunction with GC-MS has been also proposed [157–161]. A method employing GC-MS with a pre-column derivatization step has been also developed by Latxague et al. [26].

6.3.3. Other Techniques

Gonda et al., reported the development of a capillary electrophoresis (CE) method for the simultaneous quantification of sinigrin, gluconasturtiin, and allyl-ITC [162]. The ITCs are determined after the myrosinase hydrolysis of GLS and derivatization of ITCs in-vial, utilizing mercaptoacetic acid (Figure 6). The analysis was performed in extracts of cruciferous vegetables such as Brussels sprouts, radish, horseradish, and watercress. This method has the advantage that it can be used to determine both GLS and ITCs, and it also requires a minimum sample size.

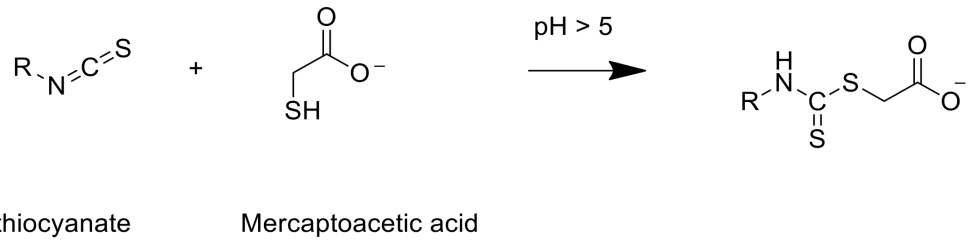


Figure 6. Derivatization of isothiocyanates with mercaptoacetic acid.

7. Conclusions

Since ITCs and indoles are products of enzymatic hydrolysis, the sample preparation procedure is considered crucial for their determination. The hydrolysis of glucosinolates by myrosinase should be complete and the conditions must be in favor of producing ITCs and indoles, rather than other compounds with an increased toxicity. Following the sample preparation, the extraction solvents that are commonly used are chlorinated. The toxicity of the extraction solvents poses limitations in the use of extracts in the development of food products enriched with these health-promoting compounds. Considering the solvent toxicity, future research should be focused on the employment of eco-friendly solvents, such as natural deep eutectic solvents, which are still unexplored in this field. These efforts may be enhanced by the use of non-conventional techniques, such as ultrasound and microwave-assisted extraction. Further research on the development of non-conventional techniques is required towards the control and optimization of the processing conditions, as the treatment efficiency may be affected by the nature of the raw materials. The analytical determination of ITCs and indoles is commonly performed by liquid or gas chromatography. Nevertheless, the determination of ITCs presents analytical problems such as the precipitation in the liquid chromatography column and the weak DAD signal, while in gas chromatography certain ITCs can be degraded. These problems are attributed to the high volatility, instability, and the lack of chromophores which can be addressed by the use of derivatization reagents

and the employment of hyphenated mass spectrometry methods. Due to the differences in the chemical properties between ITCs and indoles, the analytical methods for the simultaneous determination of these compounds in cruciferous vegetables are scarce, and therefore additional analytical methods must be developed for this purpose.

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Abbreviations

GLS	Glucosinolates
ITCs	Isothiocyanates
SFN	Sulforaphane
I3C	Indole-3-carbinol
ITC	Isothiocyanate
LC	Liquid Chromatography
GC	Gas Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
HRMS	High Resolution Mass Spectrometry
ESP	Epithiospecifier protein
TFP	Thiocyanate-forming protein
EMP	Epithiospecifier modifier protein
ESM1	Epithiospecifier modifier 1 gene
NSP	Nitrile-specifier proteins
AcOEt	Ethyl acetate
MTBE	Methyl t-butyl ether
GC-FID	Gas Chromatography-Flame Ionization Detection
GC-MS	Gas Chromatography-Mass Spectrometry
DEAE	Diethylaminoethyl
SPE	Solid Phase Extraction
UAE	Ultrasound-Assisted Extraction
HVED	High Voltage Electrical Discharges
HPP	High-Pressure Processing
SFE	Supercritical Fluid Extraction
PEF	Pulsed Electric Fields
HHP	High Hydrostatic Pressure
SFE-CO_2	Supercritical Fluid Extraction with Carbon Dioxide
ASE	Accelerated Solvent Extraction
LAB	Lactic Acid Bacteria
PDES-GO	Poly(Deep Eutectic Solvent)-Graphene Oxide
PC	Paper Chromatography
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
HSCC	High Speed Counter Current Chromatography
SFC	Supercritical Fluid Chromatography
FTIR	Fourier-Transform Infrared Spectroscopy
ATR-FTIR	Attenuated Total Reflectance-Fourier-Transform Infrared Spectroscopy
MS	Mass Spectrometry
CE	Capillary Electrophoresis
MAE	Microwave-Assisted Extraction

HRGC-O/FID	High Resolution Gas Chromatography-Olfactometry/Flame Ionization Detector
GC/EI-MS	Gas Chromatography/Electron Impact-Mass Spectrometry
UHPLC-QqQ-MS/MS	Ultrahigh Performance Liquid Chromatography-Triple Quadruple-Mass Spectrometry/Mass Spectrometry
UHPLC-MS	Ultra-High-Performance Liquid Chromatography-Mass Spectrometry
NAC	N-acetyl-L-cysteine
UPLC–HRMS/MS	Ultraperformance Liquid Chromatography-High Resolution Mass Spectrometry/Mass Spectrometry
SPME	Solid Phase Microextraction
PLS	Partial Least-Squares
FLD	Fluorescence Detector
ELSD	Evaporative Light-Scattering Detector
HPLC-UV	High Performance Liquid Chromatography-Ultraviolet Detector
HPLC-DAD-FLD	High Performance Liquid Chromatography-Diode-Array Detection-Fluorescence Detector
HPLC-DAD	High Performance Liquid Chromatography-Diode-Array Detection
HPLC-PDA-UV	High Performance Liquid Chromatography-Photodiode-Array Detection-Ultraviolet Detector
RP-UHPLC-ESI-MS	Reverse Phase-Ultrahigh Performance Liquid Chromatography-Electronspray Ionization-Mass Spectrometry
LC-Q-TOF-MS	Liquid Chromatography-Quadruple-Time of Flight-Mass Spectrometry

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