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Review

# Chemical and biological characterisation of nutraceutical compounds of broccoli

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**Abstract**

People's diet offers a greater and more diverse group of plant bioactives than do drugs, and they often do not realise that many drugs are derived from the compounds originally discovered in plant foods.

Numerous epidemiological studies indicate that *Brassica* vegetables in general, and broccoli in particular, protect humans against cancer since they are rich sources of glucosinolates as well as possessing a high content of flavonoids, vitamins and mineral nutrients.

One unusual phytotherapic role of broccoli is for skin diseases—the juice of the leaves is used to treat warts. However, the main use of broccoli stems from its health-promoting properties. Some criteria have been proposed to evaluate the possibilities of developing new "functional foods" to reduce the risk of specific cancers; largely in broccoli, which is associated with cancer protection. Processing conditions, transport, domestic cooking, etc., affect the health-promoting properties of broccoli and these have been widely studied. This review makes an in-depth study of the chemical and biological characterization of the phytochemicals of broccoli and the effects on the bioactive composition of broccoli.
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*Keywords:* Broccoli; Phenolic compounds; Glucosinolates; Minerals; Chemical analysis; Bioavailability

## Contents



## 1. Importance of Brassicaceae on human health: broccoli

People ingest a vast diversity of pharmacologically active chemicals in the form of foods. Obtaining vegetables and fruits with enhanced nutritional and medicinal qualities will become a much larger component of private and public breeding programs [1].

Recognition of diet as a primary causative factor for cancer risk has directed much research attention towards the chemoprotective (i.e. reduction of cancer risk by specific chemical compounds) role of certain compounds in foods. Technological progress in manipulating plant metabolism and metabolites, combined with the explosive growth of the "functional food" industry has led to many attempts to enhance the concentration of these health-promoting compounds in specific plant-based foods [2]. "Functional foods" are foods that, by virtue of physiologically active components, provide a health benefit beyond basic nutrition" [3]. Functional/medicinal food is a category of botanical therapeutic available at the grocery store, which can

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be decrypted as "food engineered or supplemented to provide health benefits". Thus, the future of plant-based functional foods seems bright and, as a result, grocery and drug stores might eventually look very similar [4].

Optimisation of composition of fruits and vegetables would be a very cost-effective method of disease prevention, since diet-induced health improvements would not carry any added costs for the health sector. Recognised candidates as potentially health promoting compounds, and ones which are currently under investigation in many research projects include organosulphur compounds (glucosinolates and their degradation products) from *Brassica* species [5].

*Brassica* vegetables contain glucosinolates, the metabolic breakdown products of which are potent modulators of xenobiotic-metabolising enzymes that protect DNA from damage. This protective effect has been linked to the presence of glucosinolates in these vegetables [6]. A high intake of cruciferous vegetables is associated with a reduced risk of cancer, particularly lung and those of the gastrointestinal tract. The epidemiological literature provides modest support for the hypothesis that high intakes of *Brassica* vegetables reduce prostate cancer risk [7].

One unusual phytoterapic role of broccoli is for skin diseases, where the juices from leaves are used against warts [8].

In recent years, cancer prevention by natural products has received considerable attention. The potential protective role of cruciferous vegetables and active components present in these vegetables, such as isothiocyanates and indole-3-carbinol, has been extensively studied in experimental in vitro and in vivo carcinogenesis models. Results have consistently shown that the chemoprotective agents derived from this class of vegetables of the Cruciferae family influence carcinogenesis during initiation and promotion phases of cancer development. Similarly, reports from epidemiological studies and clinical trials support this notion. However, there are only few summarized reports of all the aspects of the association between cruciferous vegetables and cancer prevention [7,9,10]. Results clearly point towards a positive correlation between cancer prevention of many target organs and consumption of cruciferous vegetable or their active constituents. Crucifers contain many bioactive components including flavonoids (e.g. quercetin), minerals (e.g. selenium) and vitamins (e.g. Vitamin C) [11,12]. Among the most-studied bioactive compounds in crucifers associated with cancer protection are glucosinolates (GLS) [13]. More than 120 GLS have been characterized; although their function in the plant is unclear, their potent odour and taste suggests a role in herbivore and microbial defence [2,14].

Glucosinolates are chemically defined compounds; all characterized GLS share a similar basic structure consisting of a β-D-thioglucose group, a sulphonated oxime group and a side chain derived from methionine, phenylalanine, tryptophane or branched-chain amino acids (Fig. 1):

The sulphate group of a GSL molecule is strongly acidic and plants accumulate GSL by sequestering them as potassium salts in plant vacuoles [15]. GLSs are not bioactive in the animal that consumes them until they have been enzymatically hydrolysed to an associated isothiocyanate [16] by the endogenous myrosinase enzyme that is released by disruption of the plant cell through harvesting, processing, or mastication [2].

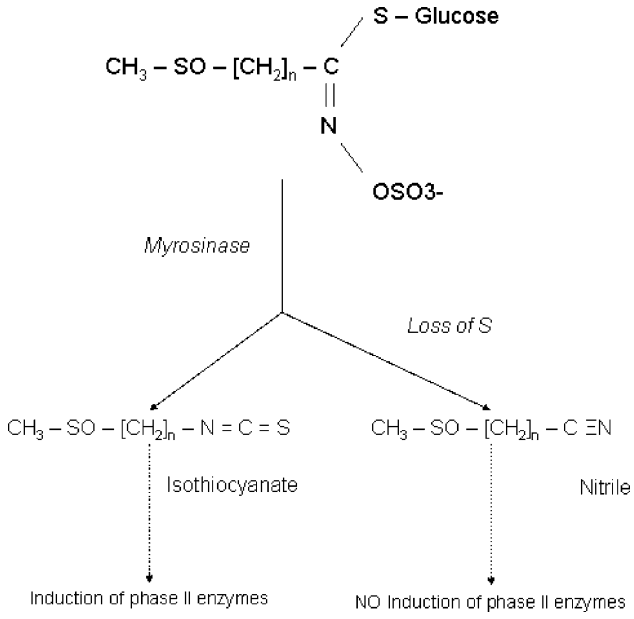



Fig. 1. General accepted hydrolysis of glucosinolates.

In vitro and in vivo studies have reported that isothiocyanates affect many steps of cancer development, including modulation of phases I and II detoxification enzymes. They function as a direct antioxidant or as an indirect antioxidant by phase II enzyme induction, modulating cell signalling, induction of apoptosis, control of the cell cycle, and reduction of *Helicobacter* infections. The most characterized GLS compounds are sulphoraphane, phenethyl isothiocyanate, allyl isothiocyanate and indole-3-carbinol, but many other isothiocyanates that are present in lower quantities may also contribute to the anti-carcinogenic properties of crucifers [17,18 and references therein].

A great deal of research on functional foods like anticarcinogens has focused on broccoli and on a single bioactive component within broccoli, sulphoraphane [2,7,19]. Some researchers have concluded that the evidence for health benefits from sulpharaphane is strong enough to warrant product development [20,21], and broccoli sprouts with a uniformly high concentration of sulphoraphane are a patented, commercially available product [22,23].

Indole-3-carbinol, benzyl isothiocyanate and phentyl isothiocyanate, natural bioactives in broccoli, GLS breakdown products, may be responsible for selective induction of apoptosis in cancer cells, supporting the potential preventive and/or therapeutic benefit of the GLS hydrolisis products against different type of cancers [24,25].

Brassicaceae family (Cruciferae) includes vegetables that are commonly grown and include broccoli, Brussels sprouts, cabbage, collards, kale, mustard, rape, etc. Broccoli was derived from a species of wild cabbage, *Brassica oleracea* [26]. Broccoli consumption is widespread in Europe (Eurostat) [27]. Broccoli consumption or supplementation and cancer prevention

study reports appear with ever greater frequency in the scientific literature, with multiple references to different type of cancers and the complexity affecting the study of gene–diet interactions and cancer risk in humans [28–33]. Examples of the wide range of studied effects of dietary anticancer bioactives from broccoli can be found elsewhere: antiproliferative effects of sulphoraphane in human breast cancer [19,34,35]; reduced risk of cancer via decreased damage to DNA [36,37]; effects on the regulation of intestinal cell growth and death in colon cancer [38], as well as the cancer-protective effect of high-selenium broccoli [28]; or the exertion of a protective effect in prostatic tumours [23,39–41], such as sulphoraphane-induced apoptosis in prostate cancer cells, is initiated by reactive oxygen species generation, and the fact that both intrinsic and extrinsic caspase cascades contribute to the cell death caused by this highly promising cancer chemopreventive agent [42]. Additional effects of bioactives (isothiocyanates) from broccoli on bladder carcinoma cells [43,44], on antioxidant capacity and on cellular oxidative stress [45], as well as cholesterol-lowering effects [46] and protective effects on cardiovascular disease [47] and *Helicobacter pylori* infections [20], supporting the fact that the dose level of bioactives may be effective through human consumption of *Brassica* vegetables could contribute to the lower incidence of different types of cancer and diseases in individuals who regularly consume such vegetables.

The assumption underlying "functional foods" is that the bioactive components (in the food) are efficacious for the improvement of health; the available evidence should be rigorously scrutinized to ascertain this. To allow health claims on food products, the US Food and Drug Administration (FDA) has developed an extensive set of such criteria that are used to decide whether there is "significant scientific agreement" or "emerging evidence" regarding biological functionality of food components [48,49]. Yet we are still far from understanding completely the effects of combinations of chemopreventive phytochemicals present in these cruciferous vegetables or their overall action mechanism(s) in providing protective effects [10,50]. Many laboratories are studying the action mechanisms of individual bioactive food components; but, relatively few studies are concerned with the interaction of the many components found in a single food or with a comparison between effects of the putative major bioactive component and the whole food [51].

The cancer-protective properties of *Brassica* (i.e. broccoli) consumption are most likely mediated through "bioactive compounds" that induce a variety of physiological functions including acting as direct or indirect antioxidants, regulating enzymes and controlling apoptosis and the cell cycle. The "functional food" industry has produced and marketed foods enriched with bioactive compounds, but there are no universally accepted criteria for judging efficacy of the compounds or enriched foods. The lack of understanding bioactive compounds and their health benefits should not serve to reduce research interest but should, instead, encourage plant and nutritional scientists to work together to develop strategies for improvement of health through food [2].

## 2. Phytochemical health-promoting compounds in broccoli and their analysis

Cruciferous vegetables are an excellent dietary source of phytochemicals including glucosinolates (and glucosinolate-breakdown products), phenolics and other antioxidants like vitamins (C, K1, etc.), as well as dietary essential minerals (Ca, Mg, Na, K, Fe, Zn, etc.). Dietary antioxidants (i.e. vitamins, flavonoids) present in broccoli may decrease the risk of certain cancers [11,12]. Since the content for these broccoli components varies significantly, it may not be easy to advise the general public on how much vegetable to include in their diet. An examination of 50 broccoli varieties showed that β-carotene levels varied over six-fold [52]. α-Tocopherol and ascorbic acid also varied, but not in concert with the β-carotene.

One of the new natural functional foods are sprouts, which is a result of one of the leading ways to increase the use of different seeds in human nutrition by the popularisation of their consumption in a germinated form, and Cruciferae seeds and ready-to-eat sprouts are a good source of Vitamin $B_1$ and $B_2$, dietary fibre and minerals [53]. Vitamin K (phylloquinone) is a fat-soluble vitamin that functions as a coenzyme and is involved in blood clotting and bone metabolism, and broccoli contain >100 μg phylloquinone/100 g vegetable, either raw or cooked [54].

Broccoli is a good source of health promoting compounds since it also contains polyphenolics. The flavonoid composition of broccoli inflorescences has been studied by liquid chromatography-UV diode-array detection-electrospray ionisation mass spectrometry [55], and a large number of hydroxycinnamic acid esters of kaempferol and quercetin glucosides were characterised and the structures of the flavonoid glycosides were analysed after alkaline hydrolysis, and identified as 3-sophoroside/sophorotrioside-7-glucoside/sophoroside of kaempferol, quercetin and traces of isorhamnetin.

Research on *Brassica* vegetables has been focused on the edible parts. However, scarce information is available regarding the corresponding by-products, which are in fact a good source of phenolic compounds [56], of this unusual food product (i.e. cauliflower leaf as by-product) with possible uses as a dietary or food antioxidant. Flavonoids from roots, shoots and roots exudates of *B. alba* have also been identified from their UV, IR, $^1$H NMR, $^{13}$C NMR, EIMS and HREIMS spectra [57].

The antioxidant activity and total phenolic content of broccoli extracts have been evaluated by using a model system consisting of β-carotene and linoleic acid [58]. The total phenolic of the extracts was determined spectrophotometrically according to the Folin–Ciocalteau procedure. Broccoli found to have a high anti-oxidant activity correlated significantly and positively with total phenolics. Recently, other authors [45] have employed the phytochemical content of 22 broccoli genotypes to determine correlations among chemical composition (carotenoids, tocopherol and polyphenolics), chemical antioxidant activity (ORAC) and measures of cellular antioxidation (prevention of DNA oxidative damage and of oxidation of dichlorofluorescein in HepG2 cells) using hydrophilic and lipophilic extracts of broccoli.

Sulphur-containing phytochemicals of two different kinds are present in all *B. oleracea* (Cruciferae) vegetables. These are glucosinolates (GLSs, previously called thioglucosides) and *S*-methyl cysteine sulphoxide (SMCSO). The two types of organosulphur phytochemicals found in all *B. oleracea* vegetables, GLS and SMCSO, or, more specifically, many of their metabolites, show anticarcinogenic action that could be useful as cancer chemopreventive agents in humans. These phytochemicals, perhaps in concert with other constituents such as vitamins that are also present in Brassicas, could be the major efficacious agents [59,60].

At least 120 different GLSs, the precursors of isothiocyanates (ITC), have been identified in edible plants. Glucosinolates and/or their breakdown products have long been known for their fungicidal, bactericidal, nematocidal and allelopathic properties and have recently attracted intense research interest because of their cancer chemoprotective attributes. Numerous reviews have addressed the occurrence of glucosinolates in vegetables, primarily the family Brassicaceae (sync. Cruciferae; including *Brassica* spp. and *Raphanus* spp.) [17,18]. On the other hand, GLSs have traditionally been condemned because of their goitrogenic and growth retardation activities [61]. Glucosinolate breakdown products (oxazolidine-2-thiones) found in several oil meals may induce morphological and histological abnormalities of internal organs in animals [61,62], as exemplified in increased thyroid weight in pigs and poultry, as well as depressed growth, goiters, poor egg production, and liver damage.

The abundance and structural variety of the glucosinolates (Table 1 ), and the fact that each one produces different breakdown products makes their analysis very complicated [63]. The analytical methods available have been extensively reviewed by McGregor et al. [64] and more recently by Verkerk et al. [65] (Table 2). In the present review the predominant members of these biologically active and chemically diverse compounds found in Brassicas (i.e. broccoli; Table 3) and the analytical methods for their isolation and identification would be addressed. The most extensively studied glucosinolates are the aliphatic, ω-methylthioalkyl, aromatic and heterocyclic (e.g. indole) glucosinolates, typified by those found in the *Brassica* vegetables (Tables 1 and 3). The largest single group (one-third of all glucosinolates) contain a sulphur atom in various states of oxidation. Another small group of benzyl glucosinolates have an additional sugar moiety, rhamnose or arabinose, in glycosidic linkage to the aromatic ring [17,18,66].

There is a wealth of knowledge on methods for the efficient isolation and identification of glucosinolates in Brassicas and more specifically in broccoli [67–69]. Most early identifications relied on paper or thin-layer chromatography of the glucosinolates or on their presumptive hydrolysis products. Numerous techniques were used for the quantification of "total" glucosinolates [17]. For example, McGregor [70] performed GLS separations by gas liquid chromatography of trimethylsilylated derivatives of glucosinolates from which the sulphate group had been removed, and this technique was subsequently coupled with mass spectrometry [71]. Enzymatic removal of the sulphate prior to derivatisation led to multiple products [72,73]. This method was used as recently as 15 years ago by Daxenbichler et al. [74], who undertook an extensive survey of the glucosinolate composition of seeds from about 300 wild plant species using gas chromatographic detection of desulphoglucosinolate hydrolysis products.

Because glucosinolates coexist with myrosinase in the plant, fresh plant material processing in the presence of water (grinding, cutting) will initiate a rapid hydrolysis of the parent compounds, and this adds complexity to the problem. In general the analytical approach can be divided into methods for total glucosinolates, individual glucosinolates and the breakdown products. Inhibition of myrosinase activity is essential for analysis of intact glucosinolates. Before disruption of the material, samples should be completely dried by freeze-drying or frozen in liquid nitrogen. The use of aqueous methanol for extraction in combination with high temperatures, also inhibits myrosinase [63,75].

Total glucosinolates yield equimolar amounts of glucose upon hydrolysis with myrosinase, and methods based on the measurement of enzymatically released glucose proved to be relatively rapid and simple to apply [76]. The total glucosinolate content of a food sample can be measured by determining the quantity of glucose released after treatment with the enzyme, but account must be taken of any endogenous glucose. To achieve this, extraction of glucosinolates can be performed, followed by selective cleanup to eliminate free glucose and other interfering compounds, after which controlled enzymatic release of bound glucose is possible [63].

Several titrimetric and gravimetric methods have been described for the quantification of the bisulphate ion (unstable aglycone, which after a Lossen rearrangement produces and equimolar quantity of bisulphate) generated after hydrolysis of glucosinolates by myrosinase. Schnug [77] has described a method in which the bisulphate liberated after sulphation is precipitated with barium chloride, and residual barium is measured by X-ray emission spectroscopy.

Gas liquid chromatography (GLC) of derivatised glucosinolates is the traditional method for the identification and quantification of the individual glucosinolates, extracted with boiling water, derivatised and separated by isothermal chromatography improved with the use of ion exchange purification steps to remove carbohydrates and other impurities before derivatisation [78,79].

West et al. [80] found a single column approach with reversed-phase separations using hydrophilic endcapped $C_{18}$-bonded silica and a 50 mM ammonium acetate-methanol gradient mobile phase to resolve both non-polar and polar glucosinolates present in isolates obtained using boiling water extraction, procedures which are extremely useful and valuable to other researchers studying broccoli GLSs as well.

Another major breakthrough in glucosinolate analysis has been achieved through the introduction of enzymatic on-column desulphation using aryl sulphatase. A desulphation step was introduced before derivatisation to eliminate sulphate that interfered with GC analysis. Desulphation was elegantly carried out on the ion exchange column, using a commercially available sulphatase isolated from an edible snail (*Helix pomatia*). Free sulphate in the glucosinolate extract, which could inhibit the

Table 1
Listing of predominant glucosinolates identified in *Brassica* spp.

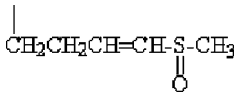
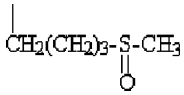
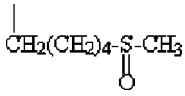
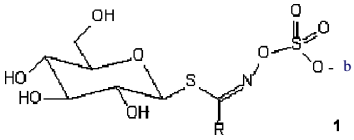
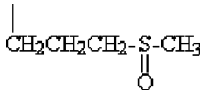
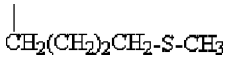
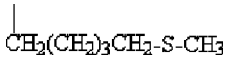
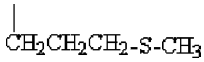
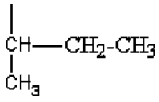
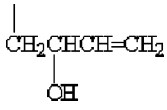
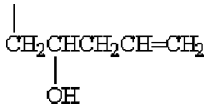
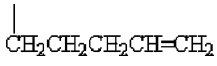
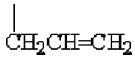
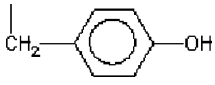
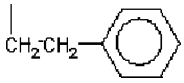
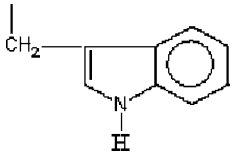
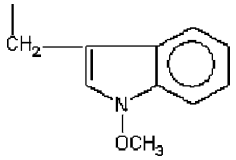
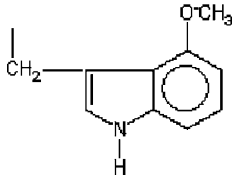
| Chemical class | Chemical name | Trivial name | Radical |
|---|---|---|---|
| Sulphur-containing GLS | 4-Mehylsulphinyl-3-butenyl | Glucoraphenin |  |
| | 4-(Methylsulphinyl)butyl | Glucoraphanin[a] |  |
| | 5-(Methylsulphinyl)pentyl | Glucoalyssin[a] |  |
|  1 | 3-(Methylsulphinyl)propyl | Glucoiberin[a] |  |
| | 4-(Methylthio)butyl | Glucoerucin |  |
| | 5-(Methylthio)pentyl | Glucoberteroin |  |
| | 3-(Methylthio)propyl | Glucoiberverin |  |
| Branched aliphatic GLS | 1-Methylethyl | Glucoputranjivin, isopropyl |  |
| | 2-Methylethyl | | |
| | 1-Methylpropyl | Glucochlearin, glucojiabutin, *sec*-butyl, 2-butyl |  |
| Aliphatic olefins | 3-Butenyl | Gluconapin[a] |  |
| | 2(*R*)-2-Hydroxy-3-butenyl | Progoitrin[a] |  |
| | 2-Hydroxy-4-pentenyl | [Gluco]napoleiferin |  |
| | 4-Pentenyl | Glucobrassicanapin[a] |  |
| | 1-Pentenyl | | |
| | 2-Propenyl | Allyl, sinigrin |  |
| Aromatic aryl GLS | 4-Hydroxybenzyl | [Gluco]sinalbin |  |
| | 2-Phenylethyl | Gluconasturtiin[a], phenethyl |  |
| Aromatic indole GLS | Indol-3-ylmethyl | Glucobrassicin[a] |  |
| | 4-Hydroxy-3-indolylmethyl | 4-Hydroxyglucobrassicin[a] | |

Table 1 (*Continued*)

| Chemical class | Chemical name | Trivial name | Radical |
|---|---|---|---|
| | 1-Methoxyindol-3-ylmethyl | Neoglucobrassicin[a] |  |
| | 4-Methoxyindol-3-ylmethyl | 4-Methoxyglucobrassicin[a] |  |

Information extracted from Fahey et al. [17,18], Daxenbichler et al. [74], and references therein.
[a] General accepted structural formula for glucosinolates in plants.
[b] Glucosinolates isolated in broccoli varieties.

Table 2
Listing of some of the commonly used methods for the analysis of glucosinolates and their breakdown products

| Compound | Method | Reference |
|---|---|---|
| Total glucosinolates | Palladium chloride and thymol assays | [19,20,63,75,76,89,103] |
| | Glucose- and sulphate-release enzyme assays | [63,67,68,74–79,89] |
| | ELISA | [63,92,93] |
| | Near infra-red reflectance (NIR) spectroscopy; alkaline degradation and thioglucose detection | [63,67,68,73,78,79] |
| | High resolution nuclear magnetic resonance (NMR) spectroscopy | [84–89] |
| Individual intact glucosinolates | Reverse phase HPLC | [63,75,78,79,89–91] |
| | Thermospray LC with tandem MS; high performance capillary electrophoresis; capillary GC–MS, GC–MS, GC–MS–MS | [89,90,91,94] |
| Desulpho-glucosinolates | Reverse phase HPLC | [55,63,71,80–83,89,90,93] |
| Degradation products | X-ray fluorescence spectroscopy (XRF); GC or GC–MS; HPLC | [19,63,74,88,89,95,97] |

sulphatase, was precipitated by addition of barium acetate and removed by centrifugation before addition of the extract to the ion exchange column [55,63].

Some glucosinolates, particularly the indoles, are thermally unstable and HPLC is therefore the preferred method. High performance liquid chromatography (HPLC) has the advantage of directly determining of glucosinolates [81]. Glucosinolates were purified and desulphated on-column, and then separated by ion-exchange chromatography [82] or reverse phase ion-pairing chromatography using a $C_{18}$ nucleosil column with gradient elu-

Table 3
Listing of some of the cruciferous plants and tissue sources of glucosinolates

| *Brassica* species | Plant organ source | Glucosinolate class |
|---|---|---|
| *Arabis hirsuta* | Hairy rock cress young leaves | Aromatic |
| *Barbarea praecox* | Land cress young leaves | Aromatic |
| *Barbarea vulgaris* | Bitter winter cress young leaves | Aromatic |
| *Brassica campestris* | Chinese cabbage seeds | Indolyl |
| *B. juncea* | Brown mustard young leaves | Alkenyl |
| *B. napus* | Oilseed rape young leaves | Mixed |
| *B. nigra* | Black mustard young leaves | Alkenyl |
| *B. oleracea* var. *botrytis* subvar. *Cymosa* | Calabrese broccoli florets | Mixed |
| *Conringia orientalis* | Hares ear cress young leaves | Alkyl |
| *Isatis tinctoria* | Woad young leaves | Indolyl |
| *Lepidium sativum* | Garden cress young leaves and roots | Aromatic |
| *Nasturtium officinalis* | Watercress young leaves | Mixed |
| *Reseda luteola* | Dyers rocket young leaves | Mixed |
| *Reseda alba* | White mignonette young leaves | Alkyl |
| *Sibara virginica* | Young leaves | Mixed |
| *Tropaeolum majus* | Nasturtium young leaves | Aromatic |

Extracted and modified from Refs. [17,18,89].

tion using acetonitrile–water mixtures such as the mobile phase and tetraoctylammonium bromide as the source of counter ion. With the aid of this method 4-hydroxy-3-indolylmethyl glucosinolate and 4-methoxy-3-indolylmethyl glucosinolate were separated and identified [63,83].

One of the most widely used methods for quantitative analysis of desulphoglucosinolates by reversed-phase HPLC was developed by Fenwick et al. [14]. This method uses an on-column enzymatic desulphation treatment of plant extracts followed by HPLC detection of the resultant desulphoglucosinolates. Adaptation of the sulphohydrolase (sulphatase) desulphation method as an HPLC method, although the most widely used method for glucosinolate separation, is still subject to difficulties in interpretation because of the effects of pH, time and enzyme activity of the desulphation products [17]. Typically, this method uses response factors determined with purified desulphosinigrin and uses desulphobenzyl glucosinolate as an internal standard. Correspondence of glucosinolate retention times, and comparison to standardized rapeseed extracts, are typically used to validate chromatographic profiles. Unfortunately, the biological activity of these molecules is compromised by the removal of the sulphate. After desulphation, they can no longer serve, as substrates for myrosinase and thus their cognate isothiocyanates are not available for bioassay or for direct measurement by cyclocondensation—key tools in the study of the pharmacokinetics, pharmacodynamics and bioactivity of these compounds.

To date, many plant glucosinolates have not been rigorously identified by modern analytical and spectroscopic methods such as HPLC, NMR, mass spectroscopy, near-infrared spectroscopy or supercritical fluid chromatography with light scattering detection [84–87]. There was, and still is, an extreme paucity of high purity chromatographic standard glucosinolates available to researchers. Only the generosity of a handful of leaders in this field has permitted investigators who do not isolate and purify their own standards to perform meaningful research on these compounds [17].

In the last 15 years, characterization and quantification of glucosinolates by HPLC/MS methods in plant extracts have improved [85]. These improvements exploit: (1) pair ion chromatography of alkylammonium salts (e.g. tetraoctyl- or tetradecylammonium bromide) used in conjunction with myrosinase hydrolysis and isothiocyanate assay by cyclocondensation with vicinal dithiols [88]; (2) a novel method for replacement of the counter ion by $NH_4^+$ which is critical for bioassay and mass spectroscopy; (3) improvements in mass spectroscopic analysis by combined fast atom bombardment and chemical ionisation techniques; (4) high resolution nuclear magnetic resonance (NMR) spectroscopy, which provides final confirmation or identity [85]. This combination of steps provides a powerful method for rapidly characterizing and quantifying glucosinolates [17].

HPLC systems using a photodiode array (PAD) detector are very sensitive; levels of desulphoglucosinolates in the nanomolar range can be detected. Whilst spectral data of individual desulphoglucosinolates will allow initial confirmation of structural class, the addition of MS detection increases the discriminatory power of the technique even further. Desulphoglucosinolates are commonly separated using end-capped $C_{18}$ reverse phase columns eluted with water:acetonitrile gradients, whilst isocratic elution with water:methanol phases has also been reported for the separation of both desulphoglucosinolates and intact glucosinolates. Kiddle et al. [89] reported details of species and tissues that are good sources of glucosinolate standards, details of macro- and micro-glucosinolate extraction methods, and optimised desulphation conditions for subsequent analysis by reverse-phase HPLC (Tables 2 and 3). Details of the chemical validations ($^1H$ and $^{13}C$ NMR and chemical ionisation MS) of a range of glucosinolates were also given.

Reverse-phase $C_{18}$ HPLC methods for either intact GLSs or desulphoglucosinolates are preferable, and are generally more accurate for determining glucosinolate content. These methods are especially robust, powerful, and selective when they form a component of an optimised negative ion mass spectrometry LC–MS method [90].

Due to their ionic nature, GLSs are not directly amenable to GC and require a precolumn derivatisation or their conversion to the volatile desulphoglucosinolate derivatives. High-performance liquid-phase separation methods such as HPLC and capillary electrophoresis (CE) are therefore more readily applicable to the analysis of glucosinolates. Recently [91], a CE method for the detection of total glucosinolates in plant extracts was reported. The method was based on the enzymatically released glucose from glucosinolates by the action of the enzyme myrosinase, and the subsequent conversion of the glucose to glucuronic acid in the presence of the enzyme glucose oxidase. The resulting gluconic acid (GA) was then labelled with the fluorescent tag 7-amino-naphthalene-1,3-disulphonic acid (ANDSA). The GA-ANDSA derivative has a short migration time and when combined with laser-induced fluorescence (LIF) detection, the elecropherograms were clean and void of interfering compounds. Thus far, only a few publications have described CE-based methods for the qualitative and quantitative analysis of intact glucosinolates or their degradation products in real plant samples (Table 2). The determination of individual intact glucosinolates in a certain part of a given plant by CE is hindered by the lack of authentic standard glucosinolates, and also, in order to exploit the full potentials of CE in the analysis of glucosinolates, other suitable electrophoretic systems and detection schemes must be developed [91].

One of the major problems in the analysis of glucosinolates has been the lack of suitable standards. The only commercial available glucosinolates are benzylglucosinolate (glucotropaeolin) and 2-propenylglucosinolate (sinigrin). Sinigrin is not a suitable internal standard because of the presence of this compound in most Brassicaceae plants, but glucotropaeolin is not normally present in *Brassica* and has been used as internal standard. Several mass spectrometric techniques have been investigated for structure elucidation of the various (desulpho-) glucosinolates, e.g. direct probing electron impact, chemical ionisation, and fast atom bombardment [63].

Recently, a novel ELISA procedure was reported for the determination of sinigrin and progoitrin in Brussels sprouts extracted with phosphoric acid, using antisera raised against hemisuccinate-linked glucosinolate conjugates [92,93]. The method tended to overestimate glucosinolate content in com-

parison to HPLC methods but seems to offer great potential advantages in terms of the cost and time needed for routine analysis in breeding programmes [63].

Many methods have been proposed to analyse GLSs, a selective and sensitive quantitative method for direct determination of intact glucosinolates was developed using negative ion tandem mass spectrometry (MS/MS) coupled with HPLC separation [94], successfully applied to quantify 10 individual glucosinolates in broccoli, broccoli sprouts, Brussels sprouts, and cauliflower, and a 10-fold improvement in detection sensitivity over conventionally used HPLC techniques.

An example of Crucifer tissues as a good source of high concentrations of glucosinolates for the isolation of standards are broccoli florets [89] where we find the following major glucosinolates (decreasing amounts):

3-Indolylmethyl- > *N*-methoxy-3-indolylmethyl-

> 4-methyl-sulphinylbutyl-

> 4-hydroxy-, 4-methoxy-3-indolyl-methyl-

There is extensive variability in GLS concentrations (i.e. sinigrin and glucoraphanin) among *Brassica* species and genotypes that is available for selection and breeding (Table 3). New elite lines can be produced for use as commercial crops in the health food or nutraceutical industries [66].

Separation and identification of isothiocyanates (ITC) from plant extracts is typically accomplished by HPLC. Based on the reaction of isothiocyanates with 1,2-benzenedithiol to form a cyclic thiocarbinol reaction product, it is possible to measure as few as 10 pmol of isothiocyanates in complex biological fluids such as plant extracts. This method also offers the possibility of quantifying either total or individual glucosinolates or isothiocyanates from plant extracts, from separate HPLC peaks or from clinical samples such as urine or blood [17,88,95].

The application of HPLC to the investigation of glucosinolate breakdown products has been limited due to the volatility of many compounds. Furthermore, thiocyanates and nitriles are not detectable spectrometrically. Isothiocyanates (ITC) and nitriles can be analysed by GLC. HPLC with UV detection may be used for analysis of oxazolidinethiones and indoles [63,96]. Two closely related 4-methyl-sulphinylbutyl-GLS [glucoraphanin] hydrolysis products from *B. oleracea* L. var. *Botrytis*, 1-isothiocyanate-4-(methyl-sulphinyl)butane (sulphoraphane) and 5-(methyl-sulphinyl)pentanenitrile (sulphoraphane nitrile), may have beneficial or deleterious effects on human health, and a GC/MS method suitable for routine screening of plant materials was developed to provide a simple, rapid technique for the analysis of both sulphoraphane and sulphoraphane nitrile [97].

The isolation and purification of GLS hydrolysis products is of particular interest both for their potential use in organic synthesis and for their biological activities. Methods have been developed for the isolation and purification of gram-scale quantities of several ω-(methylthio)alkyl-, ω-(methylsulphinyl)alkyl-, ω-(methylsulphonyl)alkyl-, and substituted benzyl glucosinolate hydrolysis products; the isothiocyanates erucin [1-isothiocyanate-4-(methylthio)butane], iberin [1-isothiocyanate-3-(methyl-sulphinyl)propane], cheirolin [1-isothiocyanate-3-(methylsulphonyl)propane], lesquerellin [1-isothiocyanate-6-(methylthio)hexane], hesperin [1-isothiocyanate-6-(methylsulphinyl)hexane], sulphoraphane [1-isothiocyanate-4-(methylsulphinyl)but-3-ene], 3-methoxybenzyl isothiocyanate, and 4-hydroxybenzyl isothiocyanate; the nitriles iberverin nitrile [1-cyano-3-(methylthio)propane], erucin nitrile [1-cyano-4-(methylthio)butane], and 3-methoxybenzyl nitrile using defatted seedmeal from several different genera within the crucifer family and Limnanthaceae as the source of parent GLSs. The procedures use solvent extraction of autolysed defatted seedmeals from various plant sources, together with variable reaction pHs and solvent partitioning to obtain relatively pure (generally >97%) compounds without the need for chromatographic separation [98].

Seeds of several commonly consumed crucifers, including broccoli, were analysed not only for GLS but also for those GLS-breakdown components that might have negative health implications, such as certain indole-containing GLSs and erucic acid-containing lipids, quantified using HPLC-UV and HPLC–MS methodology [86,99].

Glucobrassicin represents the most widespread indole glucosinolate and is present in cruciferous vegetables of the *Brassica* genus (Table 3; Fig. 2). This indolylmethyl glucosinolate is involved via its breakdown products, such as indole-3-carbinol, 3-3′-diindolylmethane, and subsequent oligomerization products, and these products, also known to induce detoxification enzymes such as cytochrome P-450 or glutathione *S*-transferases (GST), have been analysed by different LC methods using either synthetic [100] or plant glucobrassicin [101], and also plasma samples [102].

Although separation and identification of glucosinolates by HPLC analysis has proven a straightforward and indispensable analytical tool [85,90], sample preparation (e.g. enzymatic desulphation of total glucosinolates on ion-exchange columns, or tissue extraction with organic solvents followed by evapo-

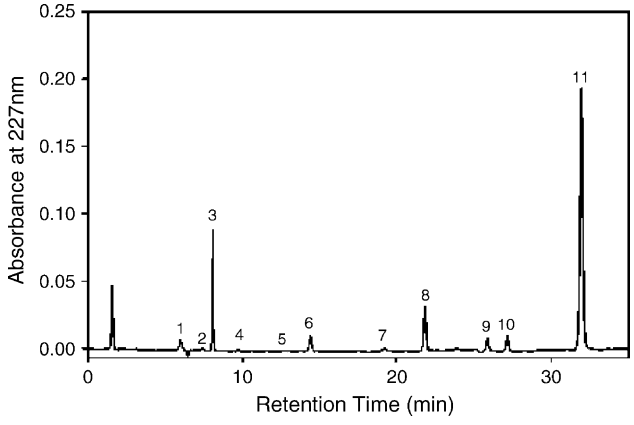



Fig. 2. Chromatographic separation of GLSs from broccoli inflorescences. Typical GLS chromatogram from broccoli florets. (1) 3-Methylsulphinylpropyl-GLS; (2) 2-hydroxy-3-butenyl-GLS; (3) 4-methylsulphinylbutyl; (4) 5-methylsulphinylpentyl; (5) 3-butenyl; (6) 4-hydroxy-3-indolylmethyl; (7) 4-pentenyl; (8) 3-indolylmethyl; (9) 2-phenylethyl; (10) 4-methoxy-3-indolylmethyl; (11) 1-methoxy-3-indolylmethyl [123].

ration and solubilisation of the residue) and cost of analysis can be limiting factors in large-scale screening procedures. The microtitre plate-based colorimetric assay of single leaf disks provides a simple, robust, and inexpensive visual qualitative method for rapidly screening large numbers of plants in mapping populations or mutant collections. Subsequently, a considerably smaller set of candidate plants with altered leaf quinone reductase inducer potency is subjected to rigorous analysis of glucosinolate content and composition by HPLC [103]. This kind of assay may become a valuable tool to estimate rapidly the content of chemopreventive glucosinolates in breeding programs, bioprospecting, or nutraceutical and functional food research and development projects.

For analysis of intact GLSs or desulphoglucosinolates HPLC is most widely used and for identification and confirmation of structures, GLC and HPLC techniques can be coupled to mass spectrometry (MS). Mass spectrometry has proved to be an invaluable tool in the identification and structural elucidation of glucosinolates and their breakdown products. Positive ion fast atom bombardment mass spectrometry (FAB) [84] has yielded mass spectra characterised by abundant protonated and cationised molecular ion (of the glucosinolate anion). This proved especially advantageous in the analysis of crude plant extracts and mixtures of purified GLSs [63]. Zhang et al. [88,104] developed a spectroscopic quantification of organic isothiocyanates. Under mild conditions nearly all organic isothiocyanates (*R*-NCS) react quantitatively with an excess of vicinal dithiols to give rise to five-membered cyclic condensation products with release of the corresponding free amines (*R*-$NH_2$). The method can be used to measure 1 nmol or less of pure ITCs or ITCs in crude mixtures.

Selenium (Se) is a nutritionally essential element and Se deficiency results in disease conditions in humans and domestic livestock [4,105]. Most of the recent interest in Se nutrition, however, is not directed towards over-supplementation in amounts of 3-6-fold beyond the Recommended Dietary Allowance (RDA 55 μg/day) [106], because there is evidence that such intakes are protective against cancer [107]. Predominate forms of Se found in nature include salts such as sodium selenate and selenite and the amino acid selenocysteine; these forms are readily used by Se-deficient animals for production of selenoproteins. The amino acid selenomethionine (SeMet) may randomly substitute for methionine and thus may accumulate in general methionine-requiring proteins. Methylated amino acids such as Se-methyl selenocysteine (SeMSC) are metabolised primarily in the excretory pathway, and limited data suggests that methyl selenol generated in this pathway is the metabolite which is most responsible for preventing cancer [108,109].

Broccoli hyperaccumulate Se in methylated forms and many other Brassicaceae species also accumulate Se [110]. Data has demonstrated that Se in soil can be reliably transferred to plants and ultimately to humans, and the Se uptake from soil and accumulation in plants is affected by soil type, soil humidity and sulphur status, soil temperature [111], and soil and air temperature, light conditions and humidity, as found in Chinese cabbage (*B. rapa* L. [Pekinensis group]) [112]. Broccoli (*B. oleracea* var. *italica*) is known for its ability to accumulate high levels of Se with the majority of the selenoamino acids in the form of Se-methylselenocysteine [113].

The complexity of Se chemistry in the environment and in living organisms presents broad analytical challenges. The selective qualitative and quantitative determination of particular species of this element is vital in order to understand selenium's metabolism and significance in biology, toxicology, clinical chemistry and nutrition [114]. These authors reported a wide review on analytical techniques for selenium species in environmental and biological systems (inorganic Se, simple organic Se, Se amino acids and low molecular mass species) and other Se compounds (selenopeptides, proteins, enzymes, sugars, and Se-metal metallothionines) using reversed-phase, paired ion reversed-phase, ion exchange and exclusion HPLC modes for Se speciation, as well as coupled electrophoretic or chromatographic separation with an atomic spectrometric or other selenium specific measurement (HPLC–ICP-MS, GC with ICP-MS detection, capillary zone electrophoresis (CZE) with ICP-MS detection).

In some nutraceutical segments the discovery of a new ingredient can lead to rapid growth. But, the discovery of a new mineral is highly unlikely, and that is why companies are working on new mineral forms which are more efficient for bioactivity, according to different international monitoring entities of this sector. Broccoli is a good vegetable source of minerals for human nutrition (Ca and Mg) and studies have shown that broccoli is an important alternative source of Ca in segments of the population that consume limited amounts of dairy products, and as demonstrated with USDA inbreds and hybrids there was a significant environmental influence on phenotypic expression of Ca and Mg concentrations that may complicate genetic improvement of head mineral concentration [115].

When analysing plant material for nutritional status, fully developed young leaves are usually the material of choice, especially during the full production stage of any given plant crop. In the laboratory, plant tissue samples are washed three times in distilled water after decontamination with 1% (v/v) non-ionic detergent (e.g. Decon 90, Decon Laboratories, Hove, East Sussex, UK), then blotted on filter paper and oven-dried for at least 48 h at 60–80 °C. After grounding in a Wiley Mill, the dried powdered samples (0.2–0.3 g), kept in plastic bags, are usually subjected to wet digestion with concentrated acids or mix of acids (e.g. sulphuric acid, nitric acid, perchloric acid) and hydrogen peroxide to determine their micronutrient (Fe, Mn, Zn and Cu) and cationic content (Ca, Mg, K and N) using atomic absorption spectrophotometry as specified elsewhere [112,116]. Dry matter (0.150–0.200 g) extracted with 1 M HCl for 30 min, when filtered, is usually an extraction procedure to determine soluble or easily extractable element form, more representative of the physiological availability of element for the plant status [116,117].

The main elements (Na, K, Ca, Mg, Cl and P) are essential for human beings in amounts >50 mg/day, while trace elements (Fe, Zn, Cu, Mn, I, F, Se, Cr, Mo, Co, Ni) are essential in concentrations of <50 mg/day. These main and trace elements have very varied functions, e.g. as electrolytes, as enzymes constituents and as building materials, e.g. in bones and teeth. Nevertheless,

human dietary nitrate and nitrite exposure should be controlled as they may be considered a health risk factor. Although nitrates are relatively harmless to humans, their conversion to nitrites or other *N*-nitrose compounds might produce toxic products [118]. Broccoli, as other Brassicaceae, should be rich in the previously mentioned organic anions and cations, even if the mineral composition of this vegetable has not been studied in depth. Oven-dried cooked broccoli samples (65 °C for 5 days after cooking) has been used for the determination of cations (López-Berenguer, Carvajal, and García-Viguera, unpublished results), and chemical analyses were carried out after a digestion $HNO_3$–$HClO_4$ (2:1). Cation concentrations were determined by atomic absorption spectrometry (Perkin-Elmer ICP 5500, Norwalk, CT, USA) in a dilution with an extract aliquot and $LaCl_3$ + CsCl. For ion concentration a Dionex D-100 ion chromatograph with an ionpac AS 124-4mm (10–32) column and AG 14 (4 mm × 50 mm) guard column was used. The flow rate was adjusted to 1 mL $min^{-1}$ with an eluent composition of 0.5 mM $NaCO_3$ and $NaHCO_3$. Ion concentration was measured with Peaknet 6.1 chromatography software, by comparing peak areas with those of known standards. Broccoli is a good source of the main mineral elements, such as Na, K, Ca, Mg, Cl, P and S, and trace elements, such as Fe, Zn, Mn and Cu, essential for the human being [119,120].

## 3. Influence of processing on the phytochemical composition of broccoli

Industrial processing would affect the levels of glucosinolates and breakdown products in *Brassica* vegetables in a very similar way to domestic processing. The main food products produced commercially from *Brassica* vegetables are washed, pre-cut or fresh-cut broccoli, cauliflower, and cabbage, fermented cabbages and frozen sprouts and veggies, or minimally processed foods. All the factors and operations through the postharvest chain will trigger complex reactions mechanisms, and physical and physiological processes change the levels of GLSs and, subsequently, their breakdown products [63,74,121,122]. However, as indicated in previous parts of this review, GLS levels do not necessarily decline rapidly after chopping and even induction can take place, but washed chopped vegetables show optimal conditions for myrosinase activity.

Although Brussels sprouts and broccoli are not storable for more than a few weeks, many types of cabbage are stored for long periods at low temperatures. Storage of heads of white and red cabbage for up to five months at 4 °C does not seem to affect the levels of GLSs [63], but in general there is still little information about the influence of storage on total or individual glucosinolate content of *Brassica* vegetables.

Broccoli is a commodity that benefits from storage under increased $CO_2$ and reduced $O_2$ concentrations and short term storage of broccoli under CA (controlled atmospheres) or in film wraps was found to extend shelf life and maintain quality by delaying yellowing and reducing loss of chlorophyll and ascorbic acid [123,124]. In order to determine the total and individual GLSs in broccoli stored under low $O_2$ and high $CO_2$ to understand better GLS metabolism in *Brassica* vegetables after harvest, Hansen et al. [124], maintained freshly harvest 'Marathon' broccoli florets during 7 days storage under air and 0.5% $O_2$ + 20% $CO_2$, and they found increased total GLS content by 42% and 21%, respectively, as compared to freshly harvested broccoli (47 μmol glucosinolate/g d.w.). Treatment with 20% $CO_2$ in the absence of $O_2$, resulted in visible injury and water soaking of the tissue. In contrast, 4-methoxyglucobrassicin content analysed by HPLC increased during storage under low $O_2$ atmosphere and increased further after transfer to air. Oxygen and $CO_2$ concentrations were verified daily by analysing gas samples using electrochemical and infrared analysers [124].

Low $O_2$, controlled atmosphere (CA), modified atmosphere (MA), and MAP (modified atmosphere packaging) made with argon (Ar) instead of $N_2$ have been reported to improve the storage life of fresh fruits and vegetables [125]. Total aliphatic and indole GLSs, phenolic compounds (flavonoids and hydroxycinnamoyl derivatives), and Vitamin C contents were evaluated in freshly harvested broccoli (*B. oleracea* L. var. *italica* cv. Marathon) inflorescences using HPLC-UV methods. The broccoli florets were stored for 7 days at 1 °C to simulate a maximum period of commercial transport and distribution. After cold storage, inflorescences were kept for 3 days at 15 °C to simulate a retail sale period. The respective losses, at the end of the cold storage and retail periods, were 71–80% of total GLSs, 62–59% of total flavonoids, 51–44% of sinapic acid derivatives, and 73–74% caffeoyl-quinic acid derivatives. The conditions scarcely affected Vitamin C content of the florets at the end of both periods [123].

The aroma compounds in broccoli stored in different modified atmospheres were studied using different packaging materials (oriented polypropylene, OPP; poly(vinyl chloride), PVC; low-density polyethylene, LDPE) containing ethylene-absorbing sachet and broccoli heads packed individually were analysed raw and after cooking. All samples were stored for either 1 week at a constant temperature of 10 °C or for 3 days at 4 °C, followed by 4 days at 10 °C and the analysis with regard to volatile compounds (dimethyl sulphide, dimethyl disulphide, dimethyl disulphide, hexanal, 3-*cis*-hexen-1-ol, nonanal, ethanol, thiocyanates) was carried out using gas-phase (headspace) extraction followed by gas chromatography–mass spectrometry (GC–MS) and GC-olfactometry [126]. The storage in OPP (14% $O_2$, 10.5% $CO_2$) resulted in most of the off-odours and heat treatment of the broccoli increased the content of aroma compounds as well as the number of compounds containing sulphur.

Domestic cooking has the potential to alter the nutrient quality (ascorbic acid, proteins, fat, and carbohydrates) of plant foods [127,128]. Before consumption, *Brassica* vegetables are usually chopped up. Cutting the fresh plant tissues creates optimal conditions for myrosinase, so a high degree of glucosinolate hydrolysis can be expected, and in the extreme case, pulping of plant tissues results in the complete breakdown of glucosinolates by autolysis [63]. However, Verkerk et al. [129] observed elevated levels of all indolyl GLSs and some aliphatic after chopping and prolonged exposure to air of different *Brassica* vegetables, something which could have large influences on quality factors such as flavour and anticarcinogenicity [130].

The total GLS content of processed cabbage is possibly a reflection of two opposing mechanisms, namely breakdown of GLSs by myrosinase, and formation of especially indolyl GLSs caused by an unknown mechanism [63].

The effect of cooking on glucosinolates has received a relatively large amount of attention. Cooking reduces GLS levels by approximately 30–60%, depending on the method (e.g. conventional, microwave, high pressure), cooking intensity (e.g. temperature, time), and on the type of compound. Also thermal degradation and washout occur, leading to large losses of intact GLSs. Glucosinolate breakdown products are apparently hardly detectable after prolonged cooking, with the exception of the thiocyanate ion and ascorbigen [131].

Broccoli (*B. oleracea* L. var. *Plenck*) was used as a testing material and sampled in four groups, including fresh, precooked (50 °C, 10 min), cooked (boiling, 8 min), and precooked followed by cooking (precooked + cooked), to investigate the effect of the cooking treatment on the textural change in the vegetable as well as on the antioxidant properties of this plant food. The results showed that precooked tissue could show a higher resistance to softening during cooking. The extracts from the precooked, cooked, and precooked + cooked broccoli were analysed using different spectrophotometric methods and exhibited high reducing powers and high DPPH radical-scavenging activity (2,2-diphenyl-2-picryl-hydrazyl method of Zhang and Hamauzu [132], with slight reductions with respect to fresh material and lower inhibitory effects against the peroxidation of a linoleic acid emulsion system than α-tocopherol and butylated hydroxyanisole (BHA) [133].

Green leafy and flower vegetables including broccoli (raw or cooked) contained >100 μg phylloquinone/100 g f.w. vegetable [54], determined by reversed-phase high-performance liquid chromatography (HPLC) as described elsewhere. Potential factors affecting phylloquinone (Vitamin $K_1$) concentrations include processing and varietal type of leafy vegetables.

Effects of microwave (1000 W oven, 1.5 min) and conventional cooking methods significantly affected the total phenolic content – determined by Folin–Ciocalteu reagent [134] but not the total antioxidant activity – determined by the DPPH method of fresh broccoli [135].

Total flavonoid and individual hydroxycinnamoyl derivative (sinapic and caffeoyl-quinic acid derivative) contents were evaluated by HPLC-DAD and HPLC/MS analyses by Vallejo et al. [136] in the edible portions of freshly harvested broccoli (cv. Marathon) inflorescences before and after cooking and in the cooking water. High-pressure boiling, low-pressure boiling (conventional), steaming and microwaving were the four domestic cooking processes used in this work. Clear disadvantages were detected when broccoli was microwaved, namely high losses of flavonoids (97%), sinapic acid derivatives (74%) and caffeoyl-quinic acid derivatives (87%); authors concluded that a greater quantity of phenolic compounds will be provided by consumption of steamed broccoli as compared with broccoli prepared by other cooking processes.

The influence of common frying methods (frying in an oven, in a pan deep frying) on cooking time and nutritive value of vegetables compared to other cooking methods (boiling, steaming, stewing) revealed that after frying vegetable food, the content of protein, carbohydrates, vitamins, and minerals was almost fully retained, while boiling and steaming reduced the mineral content by 25–50% [137]. Fresh broccoli and cauliflower were cooked by boiling, steaming, microwave boiling and microwave steaming to equivalent tenderness as measured by a shear press and analysed for sensory attributes using panellists and Vitamin C, Vitamin $B_6$, Mg, Ca and moisture content of the vegetables determined initially in the raw or frozen state as well as after cooking by AOAC [138] methods. The retention of Vitamin C, Vitamin $B_6$, Mg, and Ca was highest in broccoli and cauliflower cooked by microwave steaming, followed by microwave boiling, followed by steaming, and then by boiling, as examples of how important the method of cooking is in affecting the nutrient content of the vegetables as consumed [139].

Nitrite and nitrate levels in broccolis coming from field experiments were analysed in order to study the effect of cooking on both types of product industrially frozen and cooked broccoli [118]. Nitrate levels determined by sulphanilamide and *N*-(1-napthyl) ethylenediamine hydrochloride (recording absorbance at 540 nm; references elsewhere) in broccolis from industrial freezing gave rise to an increase in the nitrate levels, probably as a consequence of high levels in the processing water. Cooking (using distilled water) reduced nitrate levels (22–79%), but there was no difference in the levels of reduction between fresh and frozen vegetables and nitrite levels were scarcely affected either by freezing or by cooking.

Anions (chloride, nitrate, sulphate, and phosphate), cation plant and human macronutrients (sodium, potassium, calcium, and magnesium) and cation micronutrients (iron, zinc, manganese, and copper) contents were evaluated in the edible parts of broccoli cv. Marathon (florets) using different domestic cooking methods (high-pressure boiling, low pressure boiling, steaming and microwaving) and results indicated that a regular daily serving (200 g of raw broccoli) could provide, in general, over 20% of the total requirements (mg/day) of these nutrients, even if during the different cooking processes, a percentage of loss might occurs (López-Berenguer, Carvajal, and García-Viguera, unpublished results). For the cations, chemical analyses were carried out after acid digestion using AAS (atomic absorption spectrometry) of diluted samples in $LaCl_3$ + CsCl (references elsewhere). For ion concentration we used an ion chromatograph equipped with guard column and results were obtained by comparison with external standards.

## 4. Bioavailability of broccoli phytochemical compounds

Human consumption of bioactive natural products is not limited to pharmaceutical products. A much greater number is ingested as foods or dietary supplements (functional foods and nutraceuticals). While these are just as likely to exert biological effects that go far beyond providing calories and essential nutrients, the pharmacological properties of foods and dietary supplements are much more difficult to define and study. This is because the reductionism approach of modern pharmacology is not designed to study pleiotropic effects produced by complex mixtures of compounds and a better understanding of

health-related phytochemical bioactivity is both necessary and is a current challenge in Food Science and Technology. It should lead to a more sophisticated, holistic approach to disease prevention and treatment [40,140].

Understanding the bioavailability, transport and metabolism of GLSs after consumption of *Brassica* vegetables as food is a prerequisite for understanding the mechanisms of their protective effects in humans. "Bioavailable" nutrient or Bio-nutrient (e.g. Bio-iron) corresponds to the fraction of ingested nutrient used to meet functional demand in target tissues. Sweetness, crispness and intensity (intense bitter, pungent and green/grassy notes) of broccoli flavour are important attributes for broccoli acceptability, and glucosinolate content affects these sensory profiles, ultimately affecting consumer acceptance [141]. Evidence suggests that when plant myrosinase is present in the diet, glucosinolates are rapidly hydrolysed in the proximal gut. If myrosinase is deactivated, for example by cooking the vegetables prior to consumption, the ionised nature of glucosinolates may be expected to enable them to reach the distal gut where they could be metabolised by bacterial enzymes [63]. Myrosinase releases glucose and breakdown products, including isothiocyanates (ITCs). Glucosinolates are broken down by plant myrosinase in the small intestine or by bacterial myrosinase in the colon, and metabolites are detectable in human urine 2–3 h after consumption of *Brassica* vegetables. Interpretation of epidemiological data and exploitation of *Brassica* vegetables for human health requires an understanding of GLS chemistry and metabolism, across the whole food chain, from production and processing to the consumer [11,12,142].

The cancer-chemopreventive effects of broccoli may be attributed, in part, to ITCs, hydrolysis products of GLSs. Glucosinolates are hydrolysed to their respective ITCs by the enzyme myrosinase, which is inactivated by heat. The metabolic fate of GLSs after ingestion of 200 g of fresh or steamed broccoli showed an average 24-h urinary excretion of HPLC detected ITC equivalents amounted to $32.3 \pm 12.7\%$ and $10.2 \pm 5.9\%$ of the amounts ingested for fresh and steamed broccoli, respectively, occurred as the *N*-acetyl-L-cysteine conjugate of sulphoraphane (SFN-NAC); thus, bioavailability of ITCs from fresh broccoli is approximately three times higher than that from cooked broccoli, in which myrosinase is inactivated, then cooking broccoli may markedly reduce its beneficial effects on health [143]. Cabbage contains the GLS sinigrin, which is hydrolysed by myrosinase to allyl ITC. The effect of cooking cabbage on ITC production from GLSs during and after ingestion was examined in human subjects and the results indicated that ITC production is more extensive after consumption of raw vegetables but that ITCs still arise, albeit to a lesser degree, when cooked vegetables are consumed. The lag in excretion on the cooked cabbage treatment suggested that the colon microflora catalyse GLS in that case [144].

Little is known about the direct effect of broccoli sprouts on human health, even though in vitro and in vivo data provided evidence that supports the belief that young cruciferous sprouts with their high concentrations of phytochemicals may be a potent source of protective chemicals against cancer [36]. Recently a phase I study of multiple biomarkers for metabolism and oxidative stress after 1-week intake of broccoli sprouts was carried out, and it revealed that only one week of broccoli sprouts intake improved cholesterol metabolism and decreased oxidative stress markers [145]. Broccoli sprouts are a rich source of GLSs and ITCs that induce phase II detoxication enzymes, boost antioxidant status, and protect animals against chemically induced cancer. The disposition of broccoli sprout GLSs and ITCs in healthy volunteers was studied through growing and processing sprouts, and analysing them for quinone reductase inducer activity (microtitre plate murine cell assay); quantification of ITCs using a modification of an HPLC-based assay of urinary ITCs [146], using the cyclocondensation reaction of 1,2-benzenedithiol with ITCs present in human plasma, followed by extraction and analysis by HPLC-UV to detect ITCs and their dithiocarbamate metabolites; GLSs after quantitative conversion to ITCs by treating with purified myrosinase, then submitted to cyclocondensation), and paired-ion chromatography of GLSs; myrosinase activity [146–148]. Dosing preparations included uncooked fresh sprouts (with active myrosinase) as well as homogenates of boiled sprouts that were devoid of myrosinase activity and contained either GLSs only or ITCs only. In subjects fed four repeated 50-μmol doses of ITCs, the intra- and inter-subject variation in dithiocarbamate excretion was very small, and after escalating doses, excretion was linear over a 25–200-μmol dose range. The ITCs are about six times more bioavailable than GLSs, which must first be hydrolysed. Thorough chewing of fresh sprouts exposes the GLSs to plant myrosinase and significantly increases dithiocarbamate excretion. These findings will assist in the design of dosing regimens for clinical studies of broccoli sprout efficacy [148–150].

More recently a solid phase extraction-high-performance liquid chromatography-electrospray ionisation mass spectrometry/mass spectrometry method (SPE-HPLC–MS/MS) for the specific analysis of individual ITC mercapturic acids in urine has been developed [151]. This method reflects the dose of ITCs absorbed after intake of ITCs or after breakdown in the human gut of GLSs and can thus be used to determine the health-promoting potential of cruciferous species in animal studies and clinical trials. Taking together the evidences in this respect [29,152], variation in *Brassica* bioactive compounds (i.e. GLSs) and their respective contents in the food matrix and the methods used to prepare these foods, underscores the multiple layers of complexity that affect the study of gene–diet interactions and cancer risk in humans.

Polyphenols are abundant micronutrients in our diet and the health effects of polyphenols depend on the amount consumed and on their bioavailability. Recently, the nature and contents of the various polyphenols present in food sources and the influence of agricultural practices and industrial processes have been reviewed. Research is needed on polyphenol bioavailability to allow us to establish correlations between polyphenol intakes with one or several accurate measures of bioavailability (such as concentrations of key bioactive metabolites in plasma and tissues) and with potential health effects in epidemiological studies, despite the difficulties linked to the high diversity of polyphenols, their different bioavailabilities, and the high interindividual variability observed in some metabolic

processes, especially those in which the microflora is involved [153].

Bioavailability differs greatly from one polyphenol to another, so that the most abundant polyphenols in our diet are not necessarily those leading to the highest concentrations of active metabolites in target tissues [154]. The data from bioavailability and bioefficacy of polyphenols in humans on the kinetics and extent of polyphenol absorption among adults, after ingestion of a single dose of polyphenol provided as pure compound, plant extract, or whole food/beverage, showed that the metabolites present in blood, resulting from digestive and hepatic activity, usually differ from the native compounds. The plasma concentrations of total metabolites ranged from 0 to 4 μmol/L, with an intake of 50 mg aglycone equivalents, and the relative urinary excretion ranged from 0.3% to 43% of the ingested dose, depending on the polyphenol. Gallic acid and isoflavones are the most well-absorbed polyphenols, followed by catechins, flavanones, and quercetin glucosides, but with different kinetics. The least well-absorbed polyphenols are the proanthocyanidins, the galloylated tea catechins, and the anthocyanins. Data are still too limited for assessment of hydroxycinnamic acids and other polyphenols [155].

Broccoli flavonols [153] are now studied to indicate the type and magnitude of effects among humans in vivo, on the basis of short-term changes in biomarkers [153]. The flavonoid composition of broccoli inflorescences has been studied by high-performance liquid chromatography coupled with on-line mass spectrometry with electrospray ionisation source (LC/UV-DAD/ESI-$MS^n$) [55]. Quercetin (the main representative of the flavonol class, found at high concentration in broccoli) influences some carcinogenesis markers and has small effects on plasma antioxidant biomarkers in vivo, although some studies failed to find this effect. Generally, quercetin is not found in the plasma as the free form or as the parent glucoside. At the doses used in the intervention studies with humans (21–100 mg/day), it would be found exclusively as methyl, sulphate, or glucuronic acid conjugates; when added together, these compounds would represent the equivalent of approximately 1–5 μmol/L aglycone equivalents at the highest dose. The length of human intervention studies should be increased, to reflect more closely the long-term dietary consumption of polyphenols [152].

Bioavailable Se from Se-enriched plant foods is usually assessed by measuring blood Se and glutathione peroxidase enzyme activity [2,156]. Selenium enriched broccoli reduced preneoplastic lesions in rat colon, spontaneous intestinal tumours in the multiple intestinal neoplasia (Min) mouse line and carcinogen-induced mammary tumours in mice, and increased the activity of proapoptotic genes. However, the bioavailability of Se from broccoli, when determined by improvement of Se status in rats, was much lower than for selenite or selenomethionine; studies in humans gave similar results [2 and references therein]. On the other hand, selenium-enriched broccoli – by selenium fertilization – decreases the production of phenolic acids and also resulted in a modest decrease in indole, aliphatic, and total glucosinolates and glucoraphanin, but greatly depressed sulphoraphane production as determined by HPLC electrospray mass spectrometry [2].

Ascorbic acid (or Vitamin C) physiological functions are attributed to its capacity to provide reducing equivalents for biochemical reactions. Because 35–95% of the antioxidant capacity of Vitamin C-rich fruits and vegetables has been attributed to Vitamin C content, the in vivo antioxidant protection afforded by fruit and vegetable consumption may be adversely affected by the storage and handling of food [157]. Plant foods can be improved as sources of essential micronutrients or microminerals (generally occurring at relatively low concentrations in living tissues) either by increasing the concentrations of nutrients in the food or by increasing the bioavailability of micronutrients in the food, or both, and this subject has been reviewed for specific trace elements (e.g. Co, Cu, Fe, I, Mn, Se, and Zn) [119,120]. Treatments including manipulation of the soil nutrient supply and the effects of processing (freezing, canning, etc.) and cooking on the maintenance of vitamins and minerals in plant foods are crucial to this respect in order to improve the quality and potential bioactive properties of the plant products, but it is very important to consider the appearance of high levels of undesirable elements such as nitrate [158,159] or toxic heavy metals [112]. Use of either stable or radioactive isotopes incorporated intrinsically into edible portions of plant foods during plant growth will likely provide the most reliable estimates of the bioavailability of micronutrients consumed in mixed diets [120].

These results emphasize the complex interactions of bioactive chemicals in a food; attempts to maximize one component may affect accumulation of another, and consumption of high amounts of multiple bioactive compounds may result in unexpected metabolic interactions within the body [110,140].

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