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PHYTOCHEMICALS IN *ALLIUM* SPECIES AND ITS ANALYTICAL METHODS – A REVIEW

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ABSTRACT

Phytochemicals are purely energetic compounds occurred in edible foods that when ingested, have the prospective to avert or hold-up the inception of ailment. The biologically energetic bioactive compounds are usually seen in roots, stems, barks, leaves, flowers, seeds, tubers etc. The lofty quantity of organosulphur compounds is one of the major characteristics of the genus *Allium*. Numerous health reimbursement of *Allium* species are credited to these compounds, which make up between 1 to 5% of the dry weight of the bulbs. Apart from organosulphur compounds, *Allium* species also include other vital compounds. Except water (ranging between 65-95%), they contain mainly carbohydrates, flavonoids and saponins. In this review, the researches performed to analyse the phytochemicals in *Allium* species were discussed.

INTRODUCTION

Garlic (*Allium sativum*) and onion (*Allium cepa*) are two food ingredients widely used in our gastronomy. Garlic, besides to be used like food, has been used as medicinal plant for over 4000 years for a variety of ailments including headache, bits, intestinal worms and tumors[1]. During the first Olympic games in Greece, athlets ingested garlic as stimulant; in India, garlic has been used for centuries as an antiseptic lotion for washing wounds and ulcers; in China, onion and garlic tea have long been recommended for fever, headache, cholera and dysentery; and in the two world wars, garlic was used as an antiseptic in the prevention of gangrene. Garlic is still being employed in folk medicine all over the world for the treatment of a variety of diseases[2]. Evidence from several investigations suggests that the biological and medical functions of garlic and onions are mainly due to their high organo-sulphur compounds content[3].

The occurrence of fructans in *Allium* species, including *A.cepa, A.sativum* and *A.porrum* has been reported by various authors[4,5,6,7,8,9,10,11]. Ernst *et al*.[12] characterized the fructan series of the bulbs of 13 species of *Allium* and of several *Allium* vegetable crop cultivars. Analyses of ACSOs (S-alk(en)yl-L-cysteine sulphoxide) were performed by thin layer chromatography[13], amino acid analyzer[14], or electrophoresis and gel chromatography[15]. However, the TLC method was not accurate for quantification, and electrophoresis and gel chromatography required complicated procedures for the analysis.

Using gas chromatographic mass spectrometric (GC-MS) analysis of garlic extracts, Brondinitz *et al*.[16] revealed the presence of two isomeric cyclic compounds claimed to be 3-vinyl-1,2-dithi-5-ene and 3-vinyl-1,2-dithi-4-ene. Several methods have been reported for the quantitation of thiosulphinates[17,18,19]. It might appear that the best method for measuring the flavour and aroma is GC under carefully controlled conditions[20]. The individual components can be separated by GC and identified by MS. However, this method is sometimes unsatisfactory, especially when the compound is unstable to heat. For this reason, HPLC may be a better method for separating the heat liable compounds, although it also has the disadvantage of poor resolution. In Yu and Wu[21] study, both GC and HPLC were used to determine the effects of pH adjustment on flavour formation in garlic during the blending of garlic cloves.

The biological effects of additional constituents of intact garlic and onion, such as lectins (the most abundant proteins in garlic and onion), prostaglandins, fructan, pectin, adenosine, vitamins B1, B2, B6, C and E, biotin, nicotinic acid, fatty acids, glycolipids, phospholipids and essential amino acids, have been studies for over several decades[22]. The primary sulphur containing constituents in both whole vegetables are the S-alk(en)yl-L-cysteine sulphoxides (ACSOs), such

as alliin, and g-glutamylcysteines, which besides to serve as important storage peptides, are biosynthetic intermediates for corresponding ACSOs from which, and by different metabolic pathways in each vegetable, volatile, such as allicin, and lipid soluble sulphur compounds, such as diallyl sulphide (DAS), diallyl disulphide (DADS) and others, are originated[23].

Less work has been done on the more stable, genuine constituents of garlic, particularly the sulphur containing γ-glutamyl peptides. The first studies relating to these compounds were performed in the early 1960s[24,25]. In these studies the structures of the isolated compounds were proposed on the basis of infrared spectroscopy, chemical degradation, chromarographic detection of the degradation products and comparison to reference substances (e.g.glutamic acid and S-allyl-cysteine). These sulphur containing v-glutamyl peptides are considered to be storage products for nitrogen and sulphur[26]. Four γ- glutamyl peptides were isolated from a hydrophilic extract of garlic bulbs. γ-L- Glutamyl – S- (tra-1propenyI) – L- cysteine has been isolated as a genuine constituent of *A.sativum*. For the first time detailed NMR data for γ –r- glutamyl – S-allyl-L-cysteine, γ-L-glutamyl-S- (trans – 1-propenyl) – L- cysteine and γ- L-glutamyl –S-allylthio-cysteine are reported by Margrot *et al*.[27]. Martin -Lagos *et al*.[28] used gas chromatography and mass spectrometry to study organic sulphur compounds present in extracts of *A.sativum* plants.

Block and O'Connor[29], in 1974, summarised the complex chemistry of alkyl thiosulfinates and Block *et al*.[30] proposed a suitable HPLC method for the quantification for alkyl thiosulfinate in garlic as well as in other *Liliaceae*. Kaku *et al*.[31] described two new mannose binding lectins isolated from garlic (*Allium sativum,*ASA) and ramsons (*Allium ursinum* AUA) bulbs, of the family Alliaceae by affinity chromatography on immobilized mannose. The carbohydrate binding specificity of these two lectins was studied by quantitative precipitation and hapten inhibition assay. More recently allicin and related thiosulfinates in garlic were analysed by HPLC with MS detector[32,33,34]. Gas chromatography is not used due to the easy formation of thermal decomposition products unless precautions to minimise the thermal degradation of sulphur compounds are taken[30,32,35]. Garlic, *A.sativum* L.[36,37,38] has been known since antiquity for its medicinal properties and its characteristic flavour. The chemical composition of garlic cloves has been described in numerous studies and its pharmacological properties have been documented and established. These properties are associated with the presence of sulphur containing molecules which are the main components of garlic.

Numerous sulphur-containing molecules are present in garlic cloves, alliine being the most important. The characteristic aroma of garlic is obtained after the reaction of the enzyme

alliinase which converts alliine into allicine. The two stage analytical strategy used for this kind of analysis is described: (i) location of the sulphur-containing compounds from the specific sulphur chromatograms obtained by monitoring the sulphur atomic emission line with an atomic emission spectrometer coupled to a gas chromatograph and (ii) structural analysis of the located and selected compounds by mass spectrometry after transposing the chromatographic method to GC-MS[39]. Thomas and Parkin[40] analyzed the flavour precursors from onion, garlic, and leek using HPLC method after derivatization. However, peaks of Me, Pe, and AICSO peaks were fused and separation of those peaks was not clear. Edwards *et al*.[41] was able to quantify the intack flavour precursor compounds using an HPLC method after extracting through an ion exchange column. Even though this method has an advantage to measure the content without derivatization, it requires several steps of extraction before analysis by an HPLC.

Onion (*A. cepa*) is a versatile vegetable that is consumed fresh as well as in the form of processed products. More recently, there has been renewed attention given to the antioxidant content of onions because many epidemiological studies suggested that regular consumption of onions in food is associated with a reduced risk of neurodegenerative disorders, many forms of cancer, cataract formation, ulcer development, reduction in symptoms associated with osteoporosis (NOA), prevention of vascular and heart diseases by inhibition of lipid peroxidation (LPO) and lowering of low density lipoprotein (LDL) cholesterol levels[42,43,44,45]. Onion is one of the major sources of various biologically active phytomolecules, eg. phenolic acids, flavonoids, cepaenes, thiosulfinates and anthocyanins[46]. White, yellow and red onions are known to contain a large amount of flavonoids[47,48,49,50,51] especially elavonols with wide range of quercetin, isorhamnetin and kaempferol conjugates[48]. From the approximately 20 detectable flavonols in onion species, the two quercetin conjugates quercetin-3,4'-O-diglucoside (QDC) and quercetin-4'-O-monoglucoside (QMG) are the main flavonols, which make up to 80-85% of the flavonoid content[52,53]. Tsushida and Suzuki[54] reported that QMG can be found in higher concentrations than QDG. Reason for these varying results may be the differences in onion cultivars, soil type, growth location and storage, which all can influence the flavonoid content[51,55]. On the other hand, much less attention has been paid to the analysis of the γ-glutamyl dipeptide forms. This situation has been mostly due to commercial unavailability of these compounds and the necessity of their laborious synthesis or isolation. Only a few reports on their quantitative determination have thus far been published with HPLC being the most commonly employed method[56,57,58,59]. However, these methods do not usually allow simultaneous determination of the whole range of S-substituted cysteine derivatives in a single run.

The main pigment, anthocyanin was observed in the red onion. *A.cepa* L., cultivars 'Ruby', 'Southport Red Globe' and the Japanese variety 'Kurenai' was first identified as cyaniding 3-glucoside[60,61,62,63,64,65,66]. Cyanidin 3-(3''-glucosylglucoside-3-laminariobioside) and some uncharacterised cyaniding derivatives were also reported. Flavonoids contain phenolic groups, which can interact with many proteins in human organism. The main representative of the flavonoids is quercetin[67]. Steroid saponins are common Liliaceae family and closely related families. In *Allium*, spirostanol and furostanol types of steroid saponins were found[68]. Later Moore *et al.*[69,70] indicated that the main anthocyanins of red onion were acylated, however, without determination of the acyl group nor the substitution pattern. Then the main anthocyanins of the red onion cultivars 'Kurenai', Red Bone' and 'Red Granex' were identified as the 3-(6''-malonylglucoside), 3-(3''-glucosyl -6''-malonylglucoside), 3-(3''-glucosylglucoside) and 3-glucoside of cyaniding[71,72,73]. The flavonoids quercetin 4'-glucoside, quercetin 7,4'-diglucoside and quercetin 3,4'-diglucoside have been reported from white onions[47] and the anthocyanins peonidin 3-arabinoside, cyaniding 3- glucoside and cyaniding 3-laminariobioside have been reported to occur in the red varieties Sutton's Blood, Ruby and South Post[62,64,65,74]. The occurrence of acylated anthocyanins in onion was detected by Moore *et al.*[69] in 1982 and one of these pigments was identified as a malonated cyaniding 3-glycoside[75]. Due to the high flavonoid content of onion, it has been suggested as a healthy component of the diet in order to prevent coronary heart disease mortality[76]. From the edible parts of the Spanish red onion, the anthocyanins cyanidin 3 glycoside, cyaniding 3-arabinoside, cyaniding 3-malonylglucoside and cyanidin 3-malonylarabinoside and the flavonoids quercetin 3,4'-diglucoside, quercetin 7,4'-diglucoside, quercetin 3-glucoside, dihydroquercetin 3 glucoside and isorhamnetin 4'-glucoside were identified by Fossen *et al.*[72] (1996) and Terahara *et al.*[71]. Fossen *et al.*[72] also identified some of the minor anthocyanins to be the 3-(3'', 6''-dimalonylglucoside) and 3-(3''-Malonylglucoside) of cyaniding as well as the 3, 5-diglucosides of cyaniding and peonidin. Additionally, Donner *et al.*[73] identified cyaniding 3-(3''-malonylglucoside), peonidin 3- glucoside and peonidin 3-malonylglucoside, however without determination of the linkage between the acyl group and the sugar of the latter pigment. They also indicated minor occurrence of a cyaniding 3-laminariobioside derivative with both glucose units substituted with malonic acid. In contrast to previous report of Du *et al.*[65], who identified cyaniding 3- glucoside and cyaniding 3-laminariobioside as main anthocyanins in Spanish red onion, Ferreres *et al.*[77] have without support from NMR or MS data identified the 3-arabinoside and 3-malonylarabinoside of cyaniding among the main anthocyanins of Spanish red onion (cultivar '*Morada de Amposta'*).

During a development of a fast method to measure the precursor content in many numbers of onion breeding lines, Sun and Pike[78] modified an amino acid analysis method and obtained a satisfactory result. The new method is simple and can be used in screening of a large number of samples with automation. Sun and Pike[78] reported the method for separation and quantification of ACSOs and discusses the distribution of ACSOs among *Allium* species with regard to flavours of each species.

Cysteine sulphoxides possibly play a critical role in determining the characteristic smell and taste of these plants. Starting with odourless, non volatile cysteine sulphoxide derivatives, such as (+)-S-(2-propenyl)-L-cysteine sulphoxide (alliin) or (+)-S-(1-propenyl)L-cysteine sulphoxide (isoalliin), which undergo reactions to yield alk(en)yl thiosulphinates in the presence of the enzyme alliinase. Finally, the corresponding alk(en)yl (poly)sulphides are formed from the thiosulphinates especially if the *Allium* species are heated/cooked. The thiosulphinates play a very important role in flavour and aroma of fresh garlic. Additionally, the cepeanes which are also formed from thiosulphinates in onion provide a 'sweet flavour note'[79]. These substances are physiologically active and are used as antibiotic and antitumor agents, especially in the context of stomach cancer treatment[80].

Furthermore agronomic parameters also cause variations in phytochemical levels[81]. According to British pharmacopoeia[82], the minimum allicin content to ensure pharmaceutical and economical viability of garlic powder products should be 4.5 mg/g[83]. Hence it is important to standardize garlic, ie. breeding a garlic clone with suitable content of allicin and agronomical traits which are needed for the large-scale culture and drug production. Garlic is a sterile species and reproduces only by vegetative propagation. A series of different ecotypes have been established over time in various areas of cultivation. Considerable morphological and biochemical variations between and within ecotypes are displayed[84,85]. These differences were described with the objective of selection the best quality of active substance[85]. Baghalian *et al.*[83] studied 24 ecotypes collected from the main production regions. Since the study of genetic variability in different traits of available ecotypes is a prelude to crop improvement, genetic variation and diversity for different traits including botanical characteristics, allicin content and molecular studies were conducted.

Antifungal proteins have been isolated from various *Allium* species including onion seeds[86], bulbs of the round cloved (alternatively called single cloved) garlic[87], chive shoots[88], shallot bulbs[89] and leek[90]. Xia and Ng[91] isolated a protein alliumin with a molecular mass of 13KDa and an N-terminal sequence similar to partial sequence of glucanase, and demonstrating antifungal activity against *Mycosphaerella arachidicola,* but not against *Fusarium oxysporum*, was isolated from

multiple cloved garlic (*A.sativum*) bulbs. The protein, designated as alliumin was purified using ion exchange chromatography on DEAE-cellulose, CM-cellulose and mono S, affinity chromatography on Affi-gel blue gel and gel filtration on Superdex 75. An analytical method for the determination of allicin (diallylthiosulfinate) in garlic samples using reversed phase HPLC with both UV and electrochemical detection (ED) and on-line post column photochemical reaction is reported by Bocchini *et al.*[92].

Flavonoids, abundant in onion but practically absent in garlic, and a small amount of non-volatile water soluble sulphur compounds found in garlic, as S-allyl cysteine (SAC)[93], are also responsible for a great part of the health benefits of both vegetables. Several analytical methods have been reported for the determination of S-allyl cysteine (SAC) using HPLC[58,94,95], HPLCeMS[96,97,98] and thin layer chromatography (TLC)[99]. In general, Arnault's method[58] using HPLC-UVD (ultraviolet detection) was used for determination of SAC in garlic.

Investigations of various wild species of the genus *Allium* have shown that some contain higher amounts of the cysteine sulphoxides than the cultivated species, and thus they may have considerable potential as spice, vegetable, and medicinal plants[100,101]. Gennaro *et al*.[102] have recently reported the presence of minor amounts of delphinidin and petunidin derivatives in pigmented scales of the red onion cultivar 'Tropea'. Recently Fossen *et al*.[103] have reported the presence of several C-4-substituted anthocyanins isolated in minor amounts from pigmented scales of red onion. Their structures were established by extensive use of NMR spectroscopy and electrospray MS.

Abu Lafi *et al*.[104] used different GC-MS injection port conditions, and also on –column injection together with 'cryogenic' injector/oven conditions, and serially coupled capillary columns with different stationary phases. Additionally they also used HPLC analysis for the isolation and GC-MS identification of major organic sulphur compounds. High-performance liquid chromatography (HPLC) with UV detection is the method of choice for the separation and quantification of flavonols in plant extracts[52,105]. More recently, HPLC-mass spectrometry has been used to aid in the separation, quantification and structure elucidation of flavonoids found in many plants, vegetables and fruits[106,107,108,109,110,111]. This technique was used by Bonaccorsi *et al.*[112] to identify seven flavonols in samples of Southern Italian Red Onion (*Allium cepa*L.). Quercetin 4'-monoglucoside and quercetin 3.4'-diglucoside were the most abundant flavonols in the samples. Five minor flavonols were also identified and this provided a characteristic profile for the red onions used by Caridi *et al.*[113]. The individual flavonols were quantified and expressed as quercetin 4'-monoglucoside.

Pearl onion and leek (*A.ampeloprasum*) have higher relative amounts of methiin and propiin respectively. Alliin dominates in the widely used "garlic type", which includes wild leek (*A.obliquum*) and sand leek (*A.scorodoprasum*). Alliin and isoalliin rarely codominate, being only found in the cultivated Chinese leek (*A.tubersoum*). A triple mix of almost equal amounts of methiin, alliin and isoalliin is present in ramson (*A.ursinum*)[114].These compounds provide to garlic and onion their characteristic odour and flavour, as well as most of their biological properties[115]. The importance of biological and pharmacological activities, such as antifungal, antibacterial, antitumor, anti-inflammatory, antithrombotic and hypocholesterolemic properties of certain steroid saponins and sapogenins such as b-chlorogenin, has been recently demonstrated[115]. Other characteristic chemical constituents of garlic include allixin and organo-selenium compounds. These chemical compounds are reported to exhibit several biological effects, including cholesterol reduction, cancer prevention and others, and probably work synergistically with organo-sulphur compounds[116]. The major flavonoids found in dry peel of onion that has been considered usually as waste, contain large amounts of quercetin, quercetin glycoside and their oxidative product which are effect antioxidants against the lethal effect of oxidative stress[117,118]. They are also reported to have liver protective effect, immune enhancement potential and anti-infection, anti-stress, anti-cancer and other pharmacological properties[119,120]. Singh *et al*.[121] optimized the antioxidant extracting procedure, based on their contents of total phenolics (TPC), flavonoids (TFC), antioxidant activity (AOA), free radical scavenging activities (FRSA), and reducing power (RP) using standard invitro antioxidant assays. Specific phenolics composition using HPLC and MS in the various extracts/fractions was also performed. Bonaccorsi *et al*.[122] analyzed the flavonol composition of the edible portion of six different varieties and consumption typologies of onion bulbs and two varieties of shallot bulbs, employing the high-performance liquid chromatography-diode array detector (HPLC-DAD) coupled with electron spray mass spectrometry (ESI-MS-MS), a fast and accurate technique that allows quantitative and qualitative analyses of these compounds[123,124,125,126].

Kubec and Dadakova[127] developed a novel HPLC method for determination of a wide variety of S-substituted cysteine derivatives in *Allium* species has been developed and validated. This method allows simultaneous separation and quantification of S-alk(en)yl cysteine S-oxides, γ-glutamyl-S-Alk(en)ylcysteines and γ-glutamyl-S-Alk(en)yl cysteine S-oxides in a single run. Cepaic acid was isolated as a novel xanthylium yellow pigment from the dried outer scales of the yellow onion *Allium cepa* Linne. Its structure was elucinated by Ito *et al*.[128] on the basis of ESI-MS and 2D NMR spectroscopy as a 9-carboxy-1,3,6,8-tetrahydroxyxanthylium, which suggests

that cepaic acid and other yellow pigments in the dried outer skin of onion were formed by the nucleophilic reaction of phloroglucinol derived from quercetin, a flavonol in onion scales, by autooxidation to glyoxylic acid.

Perez-Gregoria *et al.*[129] developed a methodology based on high performance liquid chromatography method coupled with diode array detection (HPLC/DAD) to determine flavonols and anthocyanins. Beesk *et al.*[130] analysed 16 different onion cultivars, cultivated and consumed mainly in the local area of Germany with regard to their varietal differences in their total flavonoid content and the distribution of the flavonoids in different parts of the bulb and this content is higher in pigmented onions (yellow or red) than in the white cultivars. Fossen and Andersen[131] isolated four anthocyanins with the same novel 4-substiuted aglycone, carboxypyranocyanidin have been isolated from acidified, methanolic extracts of the edible scales as well as from the dry outer scales of red onion, *A.cepa* L.

A sensitive and reproducible analytical method to measure SAC in black garlic was developed by Eun *et al.*[132] using the HPLC-FLD (fluorescence detection) with prior derivatization with AccQ-Fluor Reagent. The results were then compared with those from obtained using HPLC-UVD that has been convectionally used for measuring SAC in raw garlic.

Dethier *et al.*[133] describes the development of an analytical method which allows the separation and quantification of the two diastereoisomers in order to determine in which proportion the natural form can be produced. The HPLC method which was optimized and validated by accuracy profile exploits an original stationary phase consisting of porous graphitic carbon (PGC). Furthermore, the developed method was used to separate the diastereoisomers of methiin, and another cysteine sulphoxide, and to analyze an aqueous extract of garlic.

Soininen *et al.*[134] described a convenient NMR method based on metabolic profiling of cerebrospinal fluid (CSF) and present a CTLS approach developed for NMR quantification of onion metabolites, including sugars, lipids, amino acids and phenolic compounds. The CTLS approach is a novel approach that has been demonstrated to yield reliable quantification of overlapping metabolites signals in $_1$H NMR[135].

Ritota *et al.*[136] characterized Italian garlic (*A.sativum* L.) belonging to two varieties, ie. red and white, and cropped in different Italian regions, by using HRMAS-NMR spectroscopy and multivariate analysis. Ashwini *et al.*[137] evaluated the phytochemical components and antioxidant potential of methanolic and hydrophilic extracts isolated from invivo and invitro cultures of onion varieties of Bellary and CO 3. A qualitative phytochemical analysis was performed by Rekha and Shruti[138] for the detection of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and

reducing sugars. Ashwini and Sengar[139] studied the physical parameters of bulbs of *Allium sativum* for their phytochemical constituents.Udu-Ibiam *et al*.[140]studied the phytochemical and antioxidant analyses of selected garlic from Ebonyi State, Nigeria were carried out using dried extracts of sample. Huzaifa *et al.*[141] investigated the qualitative and quantitative analysis of the major bioactive constituents of medicinally important plant *Allium sativum* (garlic) in its aqueous of bulb. The phytochemical tests were conducted using standard methods of analysis.

CONCLUSION

Climatic, geographic and varietal differences might also play an important role in the composition of phytochemical components of onions and garlic. Even though many researches had been performed to determine the phytochemicals in various plants, still more research is required to determine the specific phytochemicals quantitatively.

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