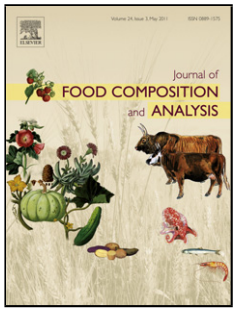

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STUDY REVIEW

Analytical methods for bioactive sulfur compounds in *Allium:* an integrated review and future directions

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HIGHLIGHTS:

- This review covered more than 70 studies about organosulfur compounds determination
- Systematic and comparative bibliographic revisions by OSCs groups were presented
- Alkenyl cystein sulfoxides, thiosulfinates and sulfides determinations were revised
- Advantages and disadvantages of different methodologies were critically discussed

ABSTRACT

Plant organosulfur compounds represent one of the main groups of phytochemicals that evidence an ample spectrum of biological activities. There are two major sources of sulfur-containing compounds in plant foods; *Allium* vegetables, such as garlic, onion, and leek; and cruciferous vegetables, such as broccoli, cabbage, and cauliflower. Among them, garlic is the most studied species, mainly due to the multiple health-enhancing effects attributed to its consumption. Most of these properties have been attributed to organosulfur compounds. Thus, knowledge on the analytical determinations available for the main bioactive sulfur compounds in *Allium* is of interest. In the present review, an extensive bibliographic survey was performed to compile information regarding the different methodologies that can be used for the determination of alk(en)yl cysteine sulfoxides (ACSOs), S-allyl cysteine (SAC), thiosulfinates (mainly allicin), diallyl, mono- di-, and tri-sulfides, vinyldithiins and (E)- and (Z)-ajoene, as influenced by plant matrices and other factors. The gathered information was analyzed and presented in a systemic and comparative way, describing advantages and disadvantages of the methodologies, considering both extractive and separative techniques, the type of matrices, columns and analytical performance data. In addition, new trends and future prospects for the analysis of sulfur compounds in plants were critically discussed.

KEYWORDS

Food composition, food analysis, *Allium*, organosulfur compounds, analytical methodologies, bioactive compounds, alk(en)yl cysteine sulfoxides, allicin, -sulfides, vinyldithiins, ajoene.

CHEMICAL COMPOUNDS

Allyl sulfide (PubChem CID: 11617); Diallyl disulfide (PubChem CID: 16590); Diallyl trisulfide (PubChem CID: 16315); Allicin (PubChem CID: 65036); E-Ajoene (PubChem CID: 5386591); Z-Ajoene (PubChem CID: 9881148); Vinyldithiin (Pub Chem CID: 54113692); Methiin (PubChem CID: 90659041); Propiin (PubChem CID: 91819955); Alliin (PubChem CID: 25200619); Isoalliin (PubChem CID: 90657185); Ethiin (PubChem CID: 91820395); Pyruvate (PubChem CID: 107735)

1. Introduction

The concept of functional food was first introduced in Japan three decades ago. It refers to foods that contain components (whether or not a nutrient) that benefit one or more functions in the body in a targeted way that are relevant to either the state of well-being and health, or the reduction of the risk of a disease (Roberfroid, 2000). Plant-derived compounds that when consumed are responsible for various health-enhancing effects are defined as phytochemicals. Currently, a major goal among researchers in food science is finding objective evidence that demonstrates food functionality. Consequently, studies regarding both the biological properties of putative phytochemicals and the chemical composition of plant foods are of interest. *Allium* vegetables, such as garlic (*A. sativum*), onion (*A. cepa*), and leek (*A. ampeloprasum* var. *porrum*) are widely consumed for their characteristic flavour (as spices) and their health-promoting effects (Block, 2010). Garlic is one of the most extensively studied functional species, not only among *Alliums*, but among all vegetables, and it has been considered a medicinal food for centuries, being used as a traditional remedy for common disorders (Rivlin, 2001). Garlic consumption is associated with decreased risk of some types of cancer, particularly those of the gastrointestinal tract (Guercio et al., 2014; Nicastro et al., 2015; Omar and Al-Wabel, 2010). Preclinical and clinical evidence demonstrated that garlic consumption can reduce risks associated with cardiovascular diseases (CVD), by lowering cholesterol level, inhibiting platelet aggregation, and lowering blood pressure (Bradley et al., 2016; Camargo and Manucha, 2016; Cavagnaro et al., 2007; Sikand et al., 2015). In addition to these cancer- and cardio-protective effects, various other biological activities have been reported for garlic, including antimicrobial, antioxidant, and anti-inflammatory properties (Block, 2010; Borlinghaus et al., 2014; Charu et al., 2014;

Suvarna and Rajagopalan, 2015). Because of all these health-promoting effects associated with garlic consumption, these species have been considered as one of the most important herbs for promoting good health and longevity (Jainarinesingh, 2014). This broad spectrum of health-benefits is mainly attributed to the presence of organosulfur compounds, characteristic of garlic and other *Allium* species. It must be noted that different *Allium* species contain different profile of organosulfur compounds (Block, 2010).

A scheme of OSCs production and subsequent transformations, and their biochemical and physicochemical pathways, is presented in **Figure 1**. Intact garlic bulbs contain S-amino acids, including cysteine and methionine (traces), as well as γ-glutamyl peptides and the alk(en)yl cysteine sulfoxides (ACSOs). Thiosulfinates (TSs), are formed upon enzymatic hydrolysis of the ACSOs, located in the cytoplasm, by the vacuolar enzyme alliinase, when garlic tissues are disrupted (Lancaster and Collin, 1981). Allicin is the predominant garlic TS, and represents 70–80% of the total TSs. TSs are reactive molecules that can undergo a number of transformations depending on temperature, pH and solvent of the medium. These reactions can lead to different OSCs, including diallyl, methyl, allyl, and diethyl mono-, di-, tri-, tetra-, penta-, and hexasulfides, vinyldithiins and (E)- and (Z)-ajoene (Box et al., 1989; Bernhard Iberl et al., 1990; Lawson et al., 1991).

In addition to TSs biosynthesis *via* the ACSOs-alliinase interaction, γ-glutamyl peptides are converted to S-allylcysteine (SAC) through a different pathway (Amagase et al., 2001).

The functional properties attributed to this vegetable have encouraged the manufacturing and marketing of various garlic products for therapeutic purposes. There are various garlic preparations that give rise to different compositions of organosulfur compounds, formulated

for the prevention or treatment of various pathologies. **Table 1**, describes the main organosulfur compounds (OSCs) present in whole raw, crushed raw, cooked, distilled, aged and macerated garlic. As indicated in **Table 1**, garlic preparations vary substantially in their OSCs composition (Kamel & Saleh, 2000; Lawson, 1993).

As previously indicated, OSCs are unstable and reactive compounds. Thus, it is important to consider this condition during the different stages of the analytical process.

The current body of knowledge regarding analytical methodologies available for the analysis of OSCs is scattered, dispersed, and such reports are rarely comparative -among methodologies- and they are non-integrative. In addition, in some cases, results from OSCs analyses using different methodologies have generated controversial discussion, often as a result of an inadequate selection of the method or technique used. Herein, we performed an exhaustive literature review and critically compared and discussed the current methodologies available for determination of OSCs in garlic and other *Allium*. To our knowledge, this is the first review on analytical methodologies for *Allium* organosulfur compounds using a methodical, comparative and integrative approach. .

Finally, it is important to note that the selection of an accurate analytical methodology, considering the different OSCs groups, is important for several reasons. **Figure 2** presentspercentages of publications in which different analytical methodologies were developed for each OSCs group. For example, for breeding *Allium* vegetables, the selection of materials with high content of total OSCs, as evaluated in their raw form, is important for developing cultivars with high nutraceutical value (e.g., high antiplatelet activity). Also, the accuracy and reliability of such analytical procedures are crucial for standardizing the

amounts of active ingredients in galenic formulations, developing and commercializing functional foods (providing reliable data for the consumer), and supporting arguments and claims of functionality.

2. Flavor precursors (ACSOs) and S-allyl cysteine (SAC) determination

S-alk(en)ylcysteine S-oxides (ACSOs) are the precursors of an extraordinary variety of sensory-active and health-beneficial compounds of *Allium* vegetables. At least, seven ACSOs are typically present in commonly consumed *Allium* plants, namely S-methyl-, S-allyl-, S-1-propenyl-, S-propyl-, S-ethylcysteine, S-butyl, and S-1-butenyl-S-oxides (Kubec and Dadáková, 2009). In *Allium*, ACSOs occur naturally as the (+)-L-enantiomers (Block, 2010). Besides these compounds, raw and uncrushed garlic also contains γ-glutamyl peptides (γ-GPs) and S-allyl cysteine (SAC). Important biological properties have been reported for SAC, including antioxidant, anti-cancer, anti-hepatopathic, and neurotrophic activities (Kodera et al., 2002).The focus on γ-GPs is related to their role in the biosynthetic pathway of ACSOs, as the γ-GPs are necessary for ACSOs formation (Jabbes et al., 2012). Alliin, the predominant ACSO in garlic, was first identified by Stoll and Seebeck (1947). After this work, three additional ACSOs present in onion tissues were identified by Virtanen and Matikkala (1959b), namely isoalliin, propiin and methiin. Isoalliin is the major ACSO present in intact onion tissues and is the precursor of the lachrymatory factor (Virtanen and Matikkala, 1959a, 1959b). The lachrymatory factor is produced when alliinase reacts with isoalliin upon disruption of raw onion tissues.

ACSOs composition in *Allium* is influenced by different factors. Among them, the species determine the type of ACSOs, whereas intra-specific genetic variation (e.g., different cultivars) accounts for variation in ACSOs concentration, as well as their relative proportions (González et al., 2009; Khar et al., 2011; Hirata et al., 2014). Besides the genetic component, environmental influence, such as growing conditions, sulfur fertilization, and postharvest conditions can also affect ACSOs profiles (Randle and Lancaster, 2002; Ichikawa et al., 2006; Csiszár et al., 2007; Diriba-Sshiferaw et al., 2014). Thus, the resulting ACSOs profiles are species- and genotype-specific, and such profiles determine the plants aroma and flavor profiles. On this basis, numerous methods for quantitation of flavor precursors have been developed, as shown in **Table 2**. These methods involve techniques that range from a simple quantitative screening to the use of modern chromatographic ones, and they can be classified as indirect or direct methods. Indirect methods are based on determination of various products arising after enzymatic conversion of flavor precursors (e.g. thiosulfinates, pyruvate or ammonia). However, these methods do not determine the profile or relative amounts of ACSOs present in the samples. On the other hand, direct methods allow the determination of ACSOs before their enzymatic decomposition, as described below.

Thin layer chromatography (TLC) is one of the most commonly used techniques. In early reports, ninhydrin detection reagent was used to optimize the differentiation between ACSOs (Hörhammer et al., 1968). Later Kanaki and Rajani (2005) and Keusgen (1997) improved this methodology and introduced High Performance Thin Layer Chromatography (HPTLC). Over time, TLC was replaced by HPLC, mainly by reverse-phase (HPLC-RP) coupled to a UV or fluorescence detector (**Table 2**). Several HPLC techniques for the determination of ACSOs, especially of alliin, have been described (Iberl et al., 1990; Keusgen, 1997; Ziegler and Sticher, 1989). The drawback of these techniques was the extensive sample clean-up

steps necessary to separate ACSOs from concomitants. To solve that disadvantage, pre-column derivatization was proposed, using o-phthaldialdehyde (OPA)/tert-butylthiol (Doran et al., 2007; Iberl et al., 1990; Krest et al., 2000; Mütsch-Eckner et al., 1992), fluorenylmethyl chloroformate (FMOC) (Thomas and Parkin, 1994), much less frequently, dansyl chloride (Dns-Cl) (Yoo and Pike, 1998) or phenyl isothiocyanate (PITC) (Auger et al., 1993; Randle et al., 1995). These reagents have been successfully applied to the analysis of ACSOs, being OPA/tert-butylthiol the most frequently used. The derivatives formed exhibit very high extinction coefficients, allowing sensitive and specific detection. In addition, the derivatization protocols do not involve time-consuming clean-up steps, which enable high sample throughput in routine applications (Kubec and Dadáková, 2009). It must be noted that most of the above methods can quantitatively determine S-substituted cysteine derivatives, including γ-GPs.

As an alternative to the pre-column derivatization, Arnault et al. (2003) reported a method based on sodium heptanesulfonate, used as an ion-pairing reagent. This made possible the quantification of alliin and a few other sulfur secondary metabolites such as allicin and SAC. However, determination of other cysteine derivatives, like methiin and isoalliin could not be performed by this method. Yoo et al. (2003) optimized the method proposed by Arnault et al. (2003) and validated it.

Later, Ichikawa et al. (2006) developed a procedure using a single-step sample preparation, followed by normal phase (NP) and reverse phase (RP) HPLC to determine ACSOs, SAC and γ-GPs in garlic cloves and its powder. In this case, the use of 90% (v/v) methanol with 0.01 N HCl, allowed a better sulfoxides extraction.

Although HPLC generally allows relatively simple, reproducible and accurate determination of ACSOs, its resolving power is the most limiting factor for identification of traces quantities

of minor alliin analogues. This can be due to the fact that most of alliin analogues are (+)-diasteroisomers and under some HPLC conditions they co-elute. In addition, slight variations of the retention time can lead to possible misidentification of peaks. On the other hand, compounds having a minor UV absorption may be overlooked.

As an alternative to minimize these problems, a biosensor method based on immobilized alliinase was developed (Keusgen et al., 2003). This flow-through method presents certain advantages, such as reduced time of analysis compared to the corresponding HPLC method; and the fact that no complex sample pre-treatment is required (only small volumes of organic solvents are used), and no organic solvents are necessary for the operation of the flow-through apparatus. Therefore, the flow-through method has ecological advantages.

Regarding LC-MS-MS, Lundegardh et al. (2008) reported a method which involved isocratic elution and O-(carboxymethyl) hydroxylamine hemihydrochloride (OCMHA) as an alliinase inhibitor (to avoid enzymatic lysis of the ACSOs when *Allium* extracts are prepared), resulting in successful quantifications of the individual ACSOs.

Gas chromatography is another separative technique commonly used for ACSOs determination (**Table 2**). This technique has an excellent sensibility and resolution capability. However, ACSOs are thermolabile and, therefore, the application of this type of analysis is very controversial, as it can lead to ACSOs erroneous estimations. For that reason, it is only possible to find in the literature a few studies where GC analysis was used, most of them employing derivatizing agents coupled to Flame Ionization Detector (FID) or Flame Photometric Detector (FPD) (Saito et al., 1988;Hayashi et al., 1993). More recently, Kubec et al. (2009) reported a method based on the isolation of the amino acid fraction by ion-exchange chromatography followed by derivatization with ethyl chloroformate at ambient temperature and reduction of derivatized ACSOs by sodium iodide. This novel method

allowed, for the first time, the identification of ethiiin sulfoxide, an ACSO not previously reported in *Allium* (Kubec et al., 2009).

Other procedures have been developed for the quantitative determination of ACSOs, such as spectrophotometric methods or capillary electrophoresis (CE). Regarding spectrophotometric methods, one publication reports a simple determination procedure for the quantitative analysis of alliin, allicin and alliinase, based on their reaction with 2-nitro-5-thiobenzoate (NTB) (Miron et al., 2002). Regarding CE technique, it has been used for the separation of alliin and methiin using a non-derivatized method (Horie and Yamashita, 2006) and it was also employed for the separation of FMOC derivates of alliin, isoalliin, methiin and propiin (Kubec and Dadáková, 2008, 2009).

ACSOs extraction procedures mainly rely on a simple solid-liquid extraction. They generally involve the use of organic solvents, like methanol, either at room temperature or heated under reflux steeped over 24 hours. In some cases, methanol is acidified with chloride acid, trifluoroacetic or formic acid in order to ensure the complete inactivation of alliinase. Alternatively, a mixture of methanol:water (50:50 v/v), ethanol:water (80:20) or methanol:chloroform:water (12:5:3 v/v/v) at -20 °C can be used. After simple extraction, the obtained extract can be cleaned upusing solid phase extraction with Sep-Pak C18 cartridge (Tsuge et al., 2002); or it can be eluted through a cation-exchange resin column (Kubec et al., 1999), or Amberlite IR 120 anion exchange column (Edwards et al., 1994).

ACSOs have been detected and quantified in various tissues of *Allium* vegetables, including fresh cloves and bulbs of garlic and elephant garlic (*Allium ampeloprasum* var. *ampeloprasum*), white and yellow onion bulbs; leaves, stems and bulbs of leek, shallot, and garlic; and dehydrated powder of garlic and onion.

Although the methods detailed in **Table 2** allow a reliable and sensitive quantitative determination of ACSOs, not all of them include a study of the analytical figures of merit, such as selectivity, linearity, accuracy, etc.

From the literature revision, it was revealed that High Performance Liquid Chromatography by reverse-phase (RP-HPLC) coupled to a UV detector plays a leading role among the direct methods, despite the fact that this method cannot completely resolve the separation of some ACSOs and that extensive sample clean-up steps are required. HPLC determination after pre-column derivatization is -less frequently- used as an alternative to the previous method. Chromatographic techniques that use high temperature, such as Gas Chromatography, are not recommended for ACSOs analysis, due to the thermal instability of the latter.

The development of analytical methods which allow detection and quantification of ACSOs is very important to estimate the nutraceutical value of garlic and other "*Alliums*". Changes observed in ACSOs composition, have been associated with elicitation of different biological activities. This has also been related to some processing methods that can lead to decomposition of ACSOs and therefore to the formation of new organosulfur compounds.

In conclusion, ACSOs play an important role in *Allium* biochemistry and contribute significantly to the health-benefits attributed to their consumption. Therefore, efficient analytical methods are required to determine the content of individual ACSOs to characterize both *Allium* vegetables (for fresh consumption) and their herbal and pharmaceutical products.

3. Pyruvate determination

Pyruvate analysis as an estimator of Allium organosulfur content, antiplatelet strength and flavor intensity

As described above, the flavor and aroma (Lancaster and Boland, 1990) as well as the antithrombotic properties of garlic and onion (Augusti 1990; Lawson et al., 1992) are attributed to a suite of organosulfur compounds formed after the lysis of ACSOs by the enzyme alliinase and subsequent reactions (Figure 1) (for a comprehensive review on *Allium* biochemistry see Block, 2010). The pyruvate produced by alliinase upon crushing raw garlic (Cavagnaro et al., 2005; Gonzalez et al., 2009) is significantly correlated with *in vitro* antiplatelet activity (IVAA) and, therefore, is considered a good predictor of antiplatelet strength (Goldman et al., 1996). Pyruvate levels are also significantly and positively correlated with individual and total thiosulfinates content in garlic (Cavagnaro et al., 2005; Cavagnaro et al., 2007a; Gonzalez et al., 2009), onion (Sance et al., 2008; Beretta, 2016), and other *Allium* vegetables (Beretta, 2016). In addition to predicting TS content, pyruvate levels can predict onion flavor, as indicated by the strong and positive correlations found between pyruvate levels and the intensity of the pungency sensory perception in raw onions (Cavagnaro et al., 2007b; Schwimmer and Guadagni, 1962; Wall and Corgan, 1992).

Taken together, these previous studies demonstrate the importance and widespread use of pyruvate analysis for estimating functional value, flavor intensity and the concentration of bioactive organosulfur compounds (e.g., TSs) in raw *Allium* vegetables. Due to its relatively simple, fast and inexpensive analytical procedure, as compared to the direct measurement of these traits [e.g., determinations of antiplatelet activity by electrical impedance of whole blood aggregometry (Cardinal and Flower, 1980) or quantitation of TSs by HPLC analysis (Block, 1992)], pyruvate analysis is widely used among food technologists, biochemists and plant breeders dealing with *Allium* vegetables.

The most widely used method for pyruvate determination is that of Schwimmer and Weston (1961). To date, more than 70 scientific publications have used this procedure for estimating pyruvate levels in *Allium* species, most of them in onion and garlic. This spectrophotometry-based procedure determines total 2,4-dinitrophenyl-hydrazine-reacting carbonyls, resulting from the addition of excess 2,4-dinitrophenyl-hydrazine (DNPH) to pyruvate-containing aqueous extracts of *Allium* (pyruvate is water soluble). Color development in the solution, due to the formation of chromogenic DNPH-pyruvate adducts, is measured at 420 nm with a spectrophotometer. Thus, color intensity –estimated by light absorbance at 420 nm- is directly related to the pyruvate concentration in the aqueous extract. A calibration curve using commercial standards of sodium pyruvate is generally used to estimate pyruvate content in the samples.

It must be noted that this procedure quantifies total pyruvate, without discriminating pyruvate produced as a result of enzymatic (alliinase) lysis of the ACSOs from pyruvate derived from non-enzymatic sources, such as the pyruvate generated during the respiratory Krebs cycle. This non-enzymatic pyruvate, or "basal pyruvate", generally represents a small proportion of the total pyruvate content, and varies among *Allium* species. While in onion bulbs non-enzymatically produced pyruvate may account up to 10-30% of the total pyruvate (Schwimmer and Weston, 1961), in garlic it rarely exceeds 2% of total pyruvate (Cavagnaro, 2007; Natale, 2003). Thus, while in onion extracts basal pyruvate needs to be determined and subtracted from total pyruvate content, as it represents a substantial amount of the total pyruvate, in garlic its low relative content is considered negligible by some authors and, therefore, is often not estimated.

In order to estimate non-enzymatically produced pyruvate a common methodology is to inactivate the enzyme alliinase by heat treatment prior to preparation of the aqueous extracts. Alliinase is heat-sensitive (Jansen et al., 1989). Thus, when tissues are crushed the ACSOs cannot be cleaved by alliinase and, therefore, enzymatically produced pyruvate is not formed, remaining in these extracts only basal pyruvate to be determined. The content of enzymatically-produced pyruvate results from subtraction of basal pyruvate from the total pyruvate content, both estimated generally using the Schwimmer and Weston (1961) method.

A number of minor modifications have been proposed to the original Schwimmer and Weston (1961) procedure, aiming at improving linearity, sensitivity and specificity, as well as throughputness of the analysis. These modifications include reading light absorbance at a different wavelength, modification of the samples size and reagents concentrations, and the use of a microplate reader (Anthon and Barrett, 2003; Boyhan et al., 1999; Yoo et al., 1995). Despite these proposed modification, the original method of Schwimmer and Weston (1961) is, to date, the most widely used procedure for *Allium* pyruvate analysis.

4. Thiosulfinates determination (allicin)

Thiosulfinates (TSs) are the most studied and well known compounds derived from *Allium* vegetables. They are found in all the species from this genus, and the differences among species are due to the type and relative content of their precursors (i.e., the ACSOs) (Lanzotti, 2006).

Quantification of allicin, the predominant garlic TS, is important because it is considered a quality indicator of commercial garlic varieties. According to the British Pharmacopeia (1998) the minimum allicin content necessary to ensure bioactive activities in garlic powder

products should be 4.5 mg g^{-1} (Wang et al., 2009). However, allicin determination entails certain difficulties given its instability and reactive nature. Its half-life time varies with temperature, concentration and type of solvent in which is stored (Rivlin, 2001). Due to the large number of TSs and other organosulfur compounds present in both fresh garlic and different garlic formulations, and given their biological importance, several analytical methodologies have been developed to determine and quantify them.

Table 3 presents different studies related to allicin determination in various garlic samples, indicating the extraction, separative and detection techniques, and including some analytical figures of merit of the methodologies. The main findings of these investigations are summarized below.

Cavallito and Bailey (1944) were the first to describe the isolation and characterization of allicin from garlic, obtained by ethanol extraction at room temperature (Block, 1992). Stoll and Seebeck (1950, 1948) determined allicin structure and demonstrated that this compound was formed enzymatically from alliin. The first report that evaluated total TSs content used an indirect determination of pyruvic acid as previously described (Freeman and McBreen, 1973) . Another methodology to determine total thiosulfinates is based on spectrophotometric detection of colored compounds formed from reaction with 5,5-dithio-bis-(2-nitrobenzoic acid) (DNTB) (Miron et al., 2002).

The first reference to Gas Chromatography (GC) was in the 60's when Carson and Wong (1960) proposed a technique to separate alkyl di- and tri- sulfides. However, Bernhard et al. (1964) noticed that many of the compounds detected in *Allium* species by GC analysis corresponded to "artifacts of the analysis". Brodnitz et al. (1971) proposed a method based

on Gas Chromatography coupled to a Mass Spectrometer (GC-MS) to determine allicin, and also provided evidence for its thermal decomposition. Despite GC and GC-MS excellent identification and resolution capabilities for stable volatile compounds, it has been proved that these separative techniques are not adequate for TSs determination, as they are thermolabile.

During 1980s and early 1990s, due to inconsistencies found in the results obtained by GC-MS, HPLC analyses were developed to identify and quantify the volatile compounds of garlic extracts avoiding thermal degradation. Miethig (1984) reported an HPLC analysis to determine allicin in garlic ether extracts, using normal phase HPLC, and later, Jansen et al. (1987) reported a RP-HPLC technique to determine allicin in aqueous extracts (Block, 1992; Jansen et al., 1987). During the same period, Iberl et al. (1990) and Lawson et al. (1990) performed quantitative determinations of allicin and other organosulfur compounds from garlic homogenates, employing RP-HPLC analysis. In subsequent years, HPLC-based methods employing both reverse phase and normal phase were improved, and the analysis was also extended to commercial garlic products (de Diego et al., 2007; Olech and Zaborska, 2012; Valle et al., 2008) and different biological matrices, such as blood, plasma and simulated body fluids (Freeman and Kodera, 1995; Ramirez et al., 2017; Rosen et al., 2000).

OSCs and allicin extraction from pulverized or finely cut vegetable samples, is based on any of the traditional methods, such as distillationor steam distillation, although mainly liquid phase extraction is employed (Iranshahi, 2013). In the latter, since TSs are water soluble, aqueous extractions are the most common, although some authors have reported solvent extraction techniques employing methanol (Al-Dulimyi et al., 2013; Arnault et al., 2003; Jansen et al., 1987), ethanol (Cavallito and Bailey, 1944a; Zhou et al., 2015),

dichloromethane (Block et al., 1992; Bocchini et al., 2001; Yoo et al., 2010) and ethyl acetate (Al-Dulimyi et al., 2013). Currently, there is a tendency to develop and use "environmentally-friendly" extraction procedures, including Supercritical Fluid extraction (SC-CO_2) (Calvey et al., 1997; Rybak et al., 2004; Valle et al., 2008), Solid Phase Microextraction (SPME) (Locatelli et al., 2014) and Dispersive Liquid-Liquid Microextraction (DLLME) (Ramirez et al., 2017).

Supercritical Fluids are more efficient solvents with better transport properties (diffusivity, mass transfer coefficient, penetration capacity) than usual extraction solvents in liquid state. Particularly, when carbon dioxide (CO_2) is used, high selectivity can be achieved for microconstituents. An additional advantage for substances susceptible to oxidation is that they are not exposed to oxygen or high temperatures during treatment with supercritical CO_2. However, this technique is not frequently used in routine analysis as it is relatively costly and involves the use of especial materials (Valle et al., 2008).

Solid phase micro-extraction is a sample preparation technique that employs a fused silica fiber, coated with a suitable stationary phase. The analytes in the sample are extracted on the fiber coating material, which can then be analyzed by GC (Calvo-Gómez et al., 2004), or allicin and OSCs can be desorbed from the coating material in a proper solvent, such as methanol, and finally analyzed by HPLC (Locatelli et al., 2014).

Regarding chromatographic methods for characterizing and quantifying unstable compounds, such as the TSs formed during garlic tissue disruption, some considerations should be taken into account. Milder chromatographic methods like HPLC provide a reliable qualitative and quantitative measurement of both head space volatiles and room temperature

extracted compounds from garlic (Block, 1992). Identification techniques usually include spectroscopy and spectrophotometry, including in most cases UV detectors (with or without diode array) and mass spectrometers (Iranshahi, 2013).

It is important to note that although there is abundant literature related to the determination of these compounds, most of published studies lack analytical figures of merit, which are of utmost importance, since they allow answering inquiries about the analytical performance of the proposed methods. Of the available literature, only the studies by Bocchini et al. (2001); de Diego et al. (2007); Locatelli et al. (2014); Wang et al. (2009); Ramirez et al. (2017) and Yoo et al. (2010) have validated the methodologies they introduced. The rest of the studies have only determined allicin and other thiosulfinates with the goal of chemically characterizing different garlic samples and garlic preparations, or to investigate associations between the compounds and a particular biological effect.

As mentioned above, TSs are unstable and reactive, they are easily oxidized, and can undergo changes under thermal processes. These properties should be considered when choosing an adequate method for TSs determination. Thus, the ideal analytical methodology should be sensitive, of moderate cost and avoid chemical and thermal transformations of the analytes. Because of these drawbacks, it is common that TSs degradation products (e.g., ajoenes, vinyldithiins and polisulfurs) are determined, instead of analyzing the formers.

5. Ajoenes, vinyldithiins and volatile sulfurs

Garlic products and medicinal extracts have been used since ancient times. Nowadays, the consumption of such products is growing due to an increased awareness of the health benefits associated with OSCs consumption, such as decreased incidence of chronic diseases.

Because of the biological importance of these compounds, health organizations have targeted *Allium* vegetables as candidates for the development of nutraceutical foods. Thus, it is highly relevant to characterize the OSCs composition in several garlic preparations.

Selection of OSCs extraction and isolation procedures depends on the type of matrix, and the relative volatility and polarity of the compounds to be analyzed. There is no universal method to determine these type of compounds but, as depicted in **Table 4**, there is abundant literature describing the use of different analytical methodologies for the isolation and quantification of volatile sulfur compounds present in garlic samples (McGorrin, 2011). **Figure 3** shows a 3D chromatogram obtained from HPLC-DAD analysis of a solution of OSCs commercial standards, with the corresponding molecular structures of allicin, E-Z-ajoene, vinyldithiins and polisulfides.

The earliest investigations regarding the determination of garlic OSCs date back to the late nineteenth century, when Semmler (1892) identified diallyl disulfide in garlic distilled oils. Several decades later, Block (1992) demonstrated that these compounds and other flavor compounds from garlic were formed enzymatically from precursors present in whole garlic cloves.

As already mentioned in the previous section, Cavallito and Bailey (1944) isolated and characterized allicin for the first time, and demonstrated that the decomposition of allicin leads to the formation of diallyl disulfide. Later, Brodnitz et al. (1971), by means of GC-MS analysis, provided evidence for another mode of allicin decomposition, which gave rise to the degradation products 3-vinyl-3,4-dihydro-1,2-dithiin and 3-vinyl-3,6-dihydro-1,2-

dithiin. The studies by Block et al. (1984, 1986) allowed the first identification of ajoenes from the decomposition of allicin in solvent mixtures.

To date, there is a great variety of techniques available for the extraction of ajoenes, vinyldithiins and sulfides from their matrices, without an evident preference for a particular one.. Most of the reported studies employed distillation, with some variations in the process (steam distillation, hydrodistillation, microwaved-assisted distillation or simultaneous distillation and extraction) prior to GC-MS or GC-FID (Gas Chromatography with Flame Ionization Detector) analysis. The most common distillation procedure employs Lickens-Nickerson apparatus. However, although it has been reported that ajoenes, vinyldithiins and sulfides are more stable than TSs, the formers are also reactive against temperature changes, and therefore, thermal decomposition should always be considered when this technique is used, especially when analyzing raw garlic samples. In these cases, the high temperatures required for distillation may cause the formation of artifacts, leading to chromatographic profiles unrepresentative of the composition in the original samples.

Unlike TSs, in the case of liquid phase extractions, since ajoenes, vinyldithiins and sulfides have low polarity, aqueous extractions are not employed for their isolation. Instead, solvent extraction techniques are commonly used, employing different non polar organic solvents such as hexane (Yoo et al., 2013), diethyl ether (Artacho Martin-Lagos et al., 1995; Block et al., 2010; Mochizuki and Yamamoto, 1998; Yu et al., 1994), acetonitrile (Lawson et al., 1991; Yan et al., 1992), isooctane (Yan et al., 1993), pentane (Weinberg et al., 1993) and dichloromethane (Locatelli et al., 2015; Mondy et al., 2001). Nevertheless, these procedures present the following disadvantages: they are time consuming, require large amounts of

solvents, generate abundant waste and may lead to distortion of the results due to both, impurities present in the reactants and/or by prolonged manipulation of the compounds.

To prevent such issues, some authors have employed modern extraction techniques, including SPME, DLLME, Supercritical Fluid extraction and cryotrapping. Of these, SPME is the most frequently used, particularly for the study of headspace volatile compounds, while cryotrapping is used for the isolation of garlic volatile compounds at low temperatures and low pressure (Calvo-Gómez et al., 2004; Ferary et al., 1996; Kim et al., 2011, 1995a; Lee et al., 2003; Locatelli et al., 2014; Mondy et al., 2001, Ramirez et al., 2017).

During the last 30 years, GC has been the main separative technique used for the analysis of garlic samples, although the negative effects of high temperatures on *Allium* compounds were known since the 1960's. GC analysis is still used to date, mainly because of the advantages it has in terms of high resolution chromatographic profiles and ease of compound identification by interfacing with Mass Spectrometer. Flame Ionization Detector and Flame Photometric Detector have also been employed to identify and quantify these compounds (Box et al., 1989; Locatelli et al., 2014; Mochizuki and Yamamoto, 1998). In order to minimize errors in the quantitation of *Allium* OSCs by GC analysis, it is advisable to first test GC properties of known compound standards, and compare the analytical results with those obtained by milder separative techniques, such as HPLC. If a certain compound is unstable under GC conditions, it is possible to achieve better results by cooling the injection port, or even using cryogenic injection conditions (Arnault et al., 2000; Block, 2010). In the early 90's, the low reliability of OSCs profiles analyzed by GC, led to the development of HPLC-based methodologies. Since then, RP-HPLC has been the most commonly used methodology, mainly employing methanol-water or acetonitrile-methanol-water mobile phases. In addition

to Mass Spectrometers, UV detectors are of frequent use since the functional groups of these compounds are UV active.In addition to the previous methodologies, a new family of techniques, called Ambient Mass Spectrometry, has been developed to examine *Allium* chemistry. These techniques allow direct sampling of molecules in their native environment without sample preparation or separation, by creating ions outside the Mass Spectrometer. Direct Analysis in Real Time (DART) is one of them, and there is reference of its use for OSCs determination in garlic samples (Block et al., 2010).

Regarding matrix types, these OSCs have been analyzed in fresh garlic, culinary garlic preparations, and garlic oil (Kim et al., 2011, 1995b; Kimbaris et al., 2006; Lee et al., 2003; Locatelli et al., 2015, 2014; Yoo et al., 2013; Yu et al., 1994, 1993). Vinyldithiins and ajoenes were found in garlic samples macerated in vegetable oil. The disulfide compounds (diallyl disulfide, allyl methyl disulfide or dimethyl disulfide) were found in steam-distilled garlic oil. Comparisons of the results from oil-macerated garlic and garlic powder revealed substantial variation in the OSCs composition and concentration of both types of samples (Lanzotti, 2006).

Noteworthy, most published studies related to ajoenes, vinyldithiins and sulfides determinations, did not perform a method validation, as indicated in Table 4.

In conclusion, the reviewed literature on the analytical determination of *Allium* OSCs points to the importance of their accurate and reproducible determination when it is desired to address the study of these bioactive compounds and their relation with biological activities.

6. New trends

Recent advances indicate that there is a tendency for the application of "environmentally-friendly", fast, and inexpensive methodologies. This is evidenced, for example, during sample preparation steps, where miniaturized microextraction techniques are the current method of choice. The recently developed separative techniques, such as Ultra Performance Liquid Chromatography (UPLC) and Ultra-Fast Liquid Chromatography that came from the evolution of packing materials used to improve resolution, also contributed to such advances. The reduction of the packing particle size improved, consequently, the efficiency and resolution of the analysis. Moreover, Mass spectrometry (MS) is, arguably, the most important analytical spectroscopic tool of modern times, especially when coupled to GC and LC. While it has been widely used for OSCs determinations, when coupled to GC this methodology generates technical artifacts, as discussed above. Nevertheless, this detection technique allows obtaining abundant information related to the identification of new compounds, varietal and species characterization and metabolomic studies, as discussed below.

Quite recently, considerable attention has been brought to the "omic" sciences. Various disciplines are grouped under this term and they are aimed at the universal detection, identification and study of DNA and genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a specific biological sample (Horgan and Kenny, 2011).

Metabolites are low molecular weight molecules that are chemically and enzymatically transformed during metabolism and, as such, they provide a functional readout of cellular state. Unlike genes and proteins, metabolites work as direct signatures of biochemical activity

and, consequently, they are easier to correlate with phenotype (Patti et al., 2012). In this context, metabolite profiling, or *metabolomics*, has become a powerful tool in multiple research areas such as medicine, pharmacology, plant analysis and human nutrition, among others. Most metabolomic studies are carried out by Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) and help provide an overview of the metabolome and compound elucidation.

In the area of food analysis, metabolomic approaches have been used to study the quality, safety and processing of raw and processed products, as well as to perform characterizations and distinctions among vegetables species and cultivars (reviewed by Cevallos-Cevallos et al., 2009). Among these studies, only a few are related to *Allium* vegetables, and they mainly focused on untargeted analysis to discriminate different species, including leek (Soininen et al., 2014b), onion (Soininen et al., 2014a, 2012) and wild *Allium* vegetables (Kusano et al., 2016). Garlic metabolome has also been investigated (Corsaro et al., 2015; Ritota et al., 2012), but only amino acids, sugars, organic acids and flavonols have been reported. However, a detailed study of the metabolic profile of all organosulfur compounds in *Allium* vegetables is still pending. Thus, the development of new methodologies based on liquid or gas chromatography coupled to mass spectroscopy and / or NMR analysis to study OSCs metabolome is necessary. Such studies may provide valuable information about new metabolites or patterns related to their formation and/or degradation. In addition, predictive studies would allow identifying relationships between OSCs and biological activities.

7. Conclusions

Since the first OSCs determinations in the 40's to the present days, the number of research studies in this area have been constantly growing. This review presents a broad spectrum of analytical methods that have been developed for determining *Allium* OSCs in several

matrices. Each method has its own advantages and disadvantages. They were developed and used to standardize commercial preparations, to characterize species and cultivars, and as an important tool to assess and investigate functionality in different systems.

However, although there are abundant studies related to the development of analytical methods for OSCs determination, further validated investigations, in lines with new trends, are needed to improve the efficiency and the performance of the entire analysis.

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Figure 1: Scheme of OSCs biochemical pathways, including synthesis and subsequent transformations upon different processing stages. Major OSCs groups sharing common structures and physicochemical characteristics are differentiated from each other by closed dashed lines. ACSOs: Alk(en)ylcysteine sulfoxides; SAC: S-allylcysteine; DAS: diallyl sulfide; DADS: diallyl disulfide; DATS: diallyl trisulfide).

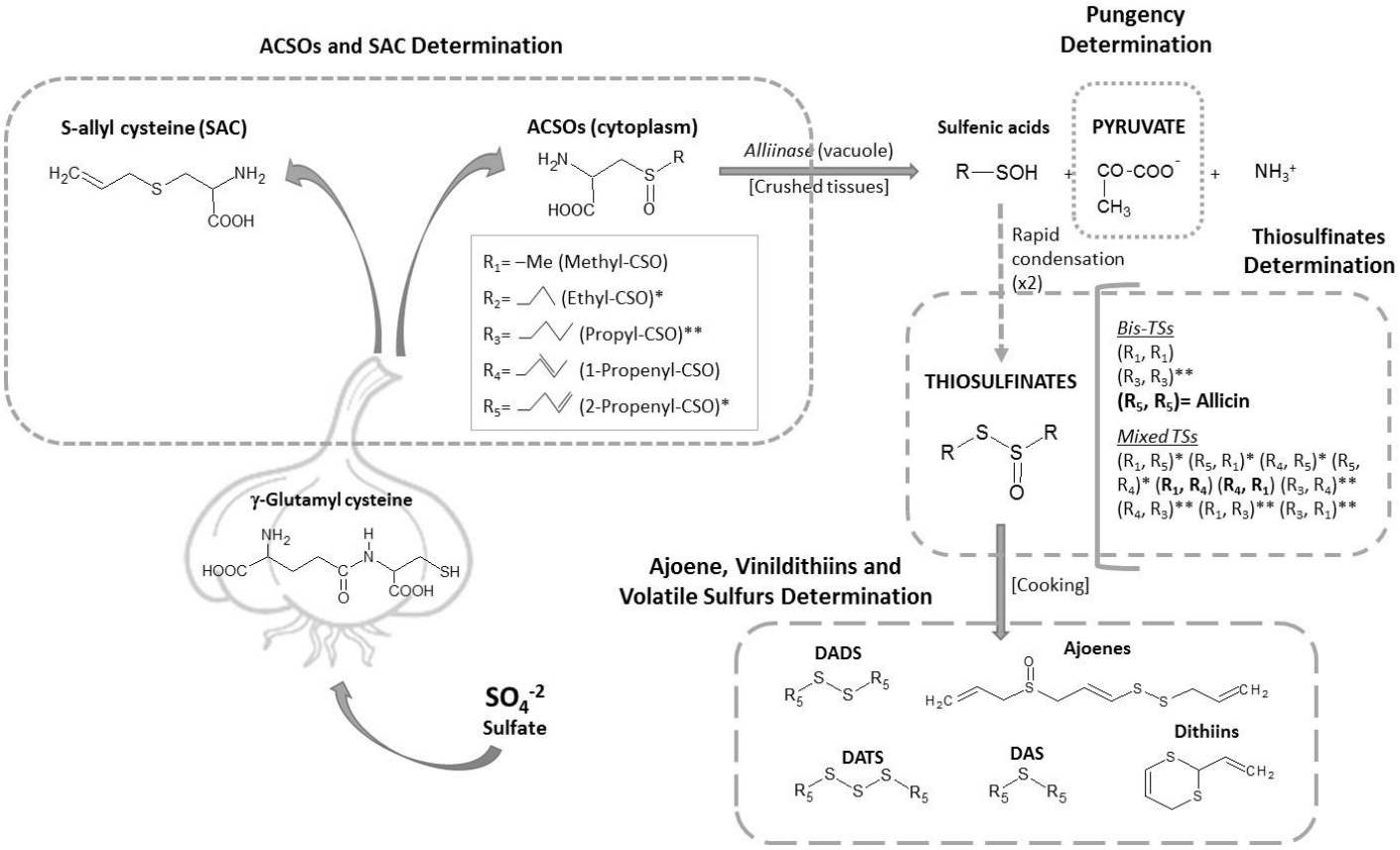
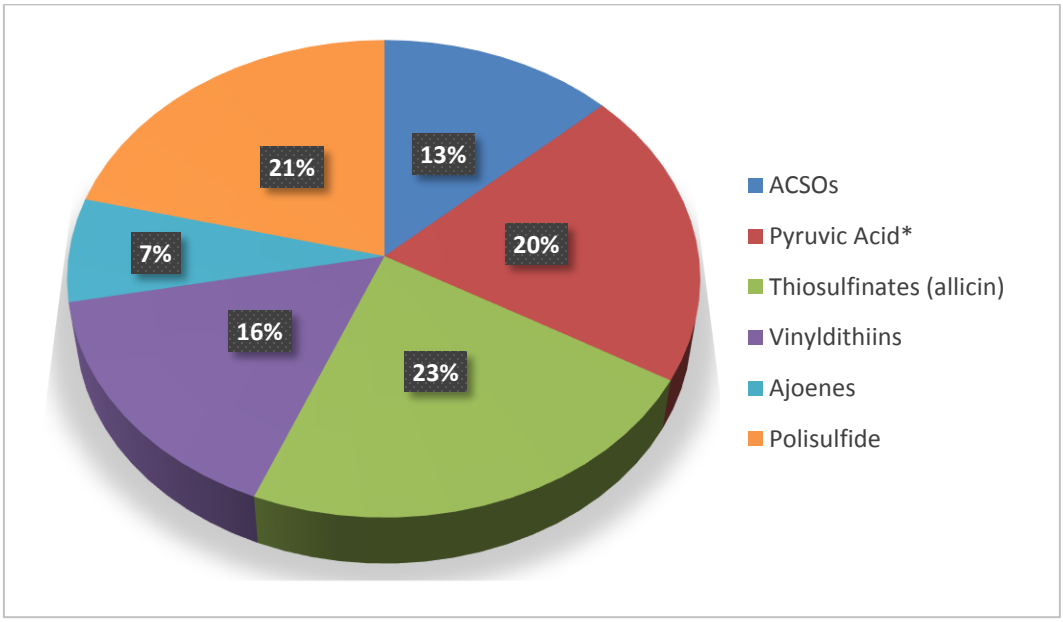


Figure 2: Percentages of publications for each group of *Allium* organosulfur compounds



* Pyruvic acid, although not an organosulfur compound, is produced together with TSs in the ACSOs-alliinase reaction and is considered an estimator of the total TSs content.

Figure 3: Three dimension chromatogram and molecular structures of the main OSCs present in garlic. Experimental conditions according to Ramirez et al., (2017).

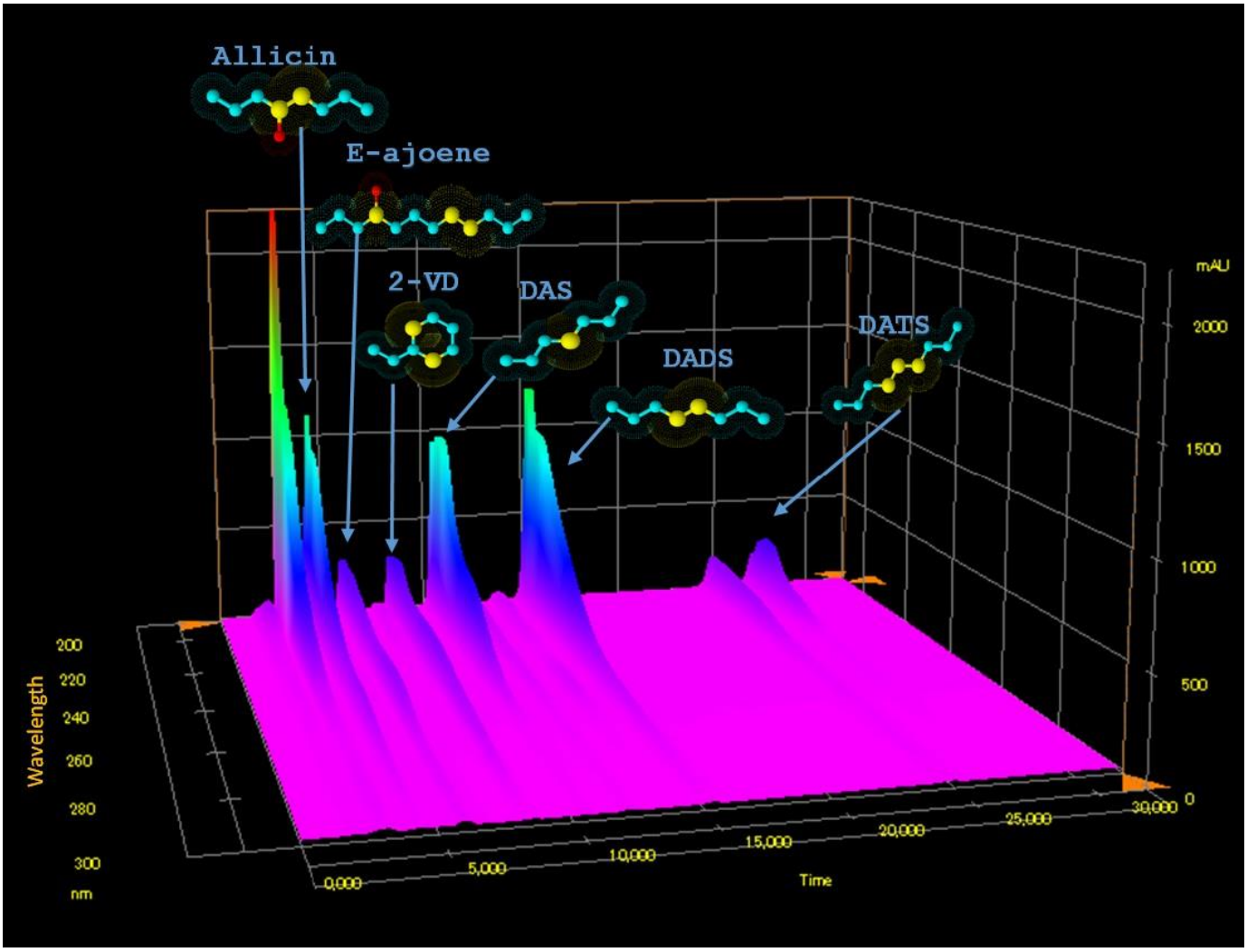


Table 1: OSCs groups analyzed in different garlic and garlic sub products.

	ACSOs	SAC	TS	Polisulfides	E- and Z-ajoene	Vinyldithiins
Whole raw garlic	■	■			■	■
Crushed/sliced raw garlic		■	■		■	■
Crushed/sliced cooked garlic		■	■	■	■	■
Dehydrated/lyophilized garlic	■	■	■		■	■
Capsules/tablets			■			
Oil macerated garlic				■	■	■
Ageing garlic	■	■			■	

Highlighted cells indicate matrices in which the different organosulfur compounds have been found.

Table 2: List of the principal chromatographic methods used to ACSOs analysis

Chromatographic technique/detection	**Column type/wavelengths /elution mode**	**Pre-chromatographic steps**	**Samples**	**Recovery**	**Compounds**	**Precision (%RSD)**	**Limit of detection**	**References**
HPLC-UV	RP-TMS/210 nm/isocratic	simple sample preparation	garlic and garlic products	> 90%	alliin	not reported	not reported	Mochizuki et al., 1988
HPIPC-FL	RP-C18/405-480 nm/isocratic	ion-pairing chromatography with tetra-n-butylammonium bromide		> 90%	alliin	not reported	not reported	
HPLC-UV	RP-C18/337 nm/ gradient and isocratic	sample clean-up using Bond Elut C18 or SCX cartridges. Pre-column derivatization OPA-*ter*. BuSH	fresh garlic, dried extracts, and garlic preparations	not reported	alliin, methiin	not reported	not reported	(Ziegler and Sticher, 1989)
HPLC-UV	RP-C18/337 nm/isocratic	pre-column derivatization OPA-ter.BuSH	Fresh garlic	not reported	alliin	not reported	not reported	(Iberl et al., 1990)
HPLC-DAD	RP/360-337 nm/gradient and isocratic	pre-column derivatization OPA-ter.BuSH	garlic and garlic preparations	not reported	alliin, γ-l-glutamyl-S-alk(en)yl-l-cysteins	not reported	not reported	(Mütsch-Eckner et al., 1992)
HPLC-FL	RP-C18/230-420 nm/ gradient and isocratic							

GC-FPD-FID	SE-30 fused silica capillary column	trimethylsilylation	cultured tissues of *A. sativum*	not reported	alliin and other sulfur amino acids	not reported	not reported	(Hayashi et al., 1993)
HPLC-DAD	RP-C18/254 nm/gradient	pre-column derivatization PITC	*Allium* species	not reported	alliin, methiin, propiin, isoaliin	not reported	not reported	(Auger et al., 1993)
HPLC-FL	RP-C18/264-340 nm/isocratic	pre-column derivatization- 9-fluorenylmethyl chloroformate reagent (FMOC)	garlic cloves, onion bulb white dehydrator, leek bulbs, cabbage leaves	not reported	alliin, methiin, propiin, glutamic acid, glycine, valine	not reported	5 pmol	(Thomas and Parkin, 1994)
HPLC-UV	RP-C8/254 nm/isocratic	pre-column derivatization- Dns-Cl reagent	garlic cloves, giant garlic cloves, leek leaves, chinese chive leaves	not reported	alliin, methiin, isoalliin	not reported	not reported	(Yoo and Pike, 1998)
GC-FID	HP-5 or HP-INNOWax fused-silica capillary column	pre-column derivatization ECF-NaI+acetyl choride	garlic	not reported	alliin, methiin, isoalliin, ethiin, propiin, cicloalliin	not reported	1ppm	(Kubec et al., 1999)
HPLC-UV	RP-C18/335 nm/gradient	pre-column derivatization OPA-2-methylpropanethiol	rhizomes and bulbs belongs to different Allium family	not reported	alliin, methiin, ethiin, propiin, isoalliin, buthiin	not reported	not reported	(Krest et al., 2000)

GC-MS	capillary DB-5MS column	tert-butyldimethylsilylation (TBDMS), cleanup sample method using solid phase extraction	onion	methiin 74.4%, propiin 74.0%,	alliin, methiin, propiin	not reported	not reported	(Tsuge et al., 2002)
HPLC-UV	RP-C18/208 nm/gradient	ion-pairing chromatography. It not require particular sample preparation and run time is short (30 min)	garlic bulbs	not reported	alliin, allicin, γ-glutamyl-S-allyl-L-cysteine	not reported--	0.1-0.5 nmol	(Arnault et al., 2003)
HPLC-UV	NP- aminopropyl-bonded column/210 nm/isocratic	simple sample method	garlic bulbs	97.1−102.3 %	alliin, methiin, isoalliin, γ-glutamyl peptides	not reported	not reported	(Ichikawa et al., 2006)
LC-MS-MS	RP-C18/isocratic	o-(carboxymethyl) hydroxylamine hemihydrochloride (OCMHA)-alliinase inhibitor	leek	not reported	methiin, alliin, isoalliin, propiin	4.0% for methiin, 3.5% alliin, 3.5% isoalliin, 3.6%, propiin	10 ppm for methiin, 20 ppm for alliin and isoalliin, and 40 ppm for propiin.	(Lundegardh et al., 2008)
HPLC/Dns-Cl	RP-C-18/250 nm/gradient	pre-column derivatization- Dns-Cl reagent	Garlic, onion, shallot, leek	not reported	methiin, alliin, isoalliin, propiin, ethiin,	3.5–5.2% (mean 4.4%)	1.5 pmol	(Kubec and Dadáková, 2009)

HPLC/OPA/t-BuSH	RP-C-18/337nm/gradient	pre-column derivatization- OPA-ter.BuSH			γ-glutamyl-S-alk(en)ylcysteines, γ-glutamyl-S-alk(en)ylcysteine S-oxides	4.7–9.0% (mean 6.6%)	1.1 pmol	
HPLC/FMOC	RP-C18/265 nm/gradient	pre-column derivatization-FMOC reagent				5.5–6.5% (mean 5.9%)	2.0 pmol	
GC/-FID	HP-5MS fused silica capillary column	pre-column derivatization-Ethyl chloroformate (ECF)				3.4–6.1% (mean 4.8%)	not determined	
CE/FMOC	fused-silica capillary/265 nm/	derivatization with FMOC reagent				1.6–8.1% (mean 5.4%)	0.3 pmol	

OPA: o-phthaldialdehyde; PITC: phenylisothiocyanate; DAD: diode array detection; FL: fluorimetric detection; RP: reversed-phase; NP: normal-phase; ECF: ethyl chloroformate; FPD: flame photometric detector; FID: flame ionisation detection; –: no data.

Table 3. Thiosulfinates determination.

Samples	Extraction Technique	Separative Technique/ Detection[a]	Recovery %	Concentrations found	Limit of detection	Precision[b]	Reference
-Fresh garlic	-Ethanol extraction -Distillation	not reported	not reported	not reportednot reported	not reported	not reported	(Cavallito and Bailey Hays, 1944)
-Fresh garlic	-Trichlorofluoromethane extraction	GC-MS	not reported	not reported	not reported	not reported	(Brodnitz et al., 1971)
-Fresh garlic -Dry garlic powder	-Aqueous and Methanol extraction	RP-HPLC-UV	not reported	0.3537 mg/mL *(fresh garlic)* 0.107 mg/mL *(dry garlic powder)*	not reported	0.004mg[c] *(fresh garlic*) 0.0085mg[c] *(dry garlic powder)*	(Jansen et al., 1987)
-Fresh garlic -Garlic powder -Coated tablets	-Aqueous extraction	RP-HPLC-DAD	not reported	0.006 – 0.26 wt%[d] (*coated tablets)* 0.06 – 2.45 wt%[d] (garlic powder) 0.12 – 0.47wt% [d] *(fresh garlic)*	not reported	not reported	(Iberl et al., 1990)
-Fresh garlic	-Aqueous extraction	RP-HPLC-UV	not reported	0.45 mg/g dry garlic[e]	not reported	>1.1 CV%	(Lawson et al., 1990)
-Fresh garlic	-Room temperature vacuum distillation -Dichloromethane extraction	**RP-HPLC-UV** HPLC-^{1}H NMR NH-HPLC-UV GC-MS	not reported	13 µmol/g garlic *(extract)*[f] 6.7 µmol/g garlic *(distillate*)[f]	not reported	not reported	(Block et al., 1992)
-Commercial garlic preparations under simulated digestive conditions and blood	-Enzymatic extraction -Dichloromethane extraction	RP-HPLC-UV	not reported	90 µg/g garlic *(simulated gastric fluid)* 64 µg/g garlic *(simulated intestinal fluid*) - not detected in blood	not reported	not reported	(Freeman and Kodera, 1995)
-Fresh garlic	-Supercritical Fluid extraction (SC-CO_2)	RP-LC-APCI-MS	not reported	53 % mol	not reported	not reported	(Calvey et al., 1997)

-Dehydrated granular garlic and enteric coated tablets under simulated digestive conditions	-Enzymatic extraction	RP-HPLC-DAD	not reported	not reported	not reported	not reported	(Rosen et al., 2000)
-Fresh garlic	-Aqueous extraction -Dichloromethane extraction	RP-HPLC-UV and ED *(on-line photochemical reaction)*	not reported	1.7 – 4.6 mg/g fresh garlic[g]	0.1 mg/L *(UV)* 0.01 mg/L *(ED)*	2.7%RSD *(UV)* 2.1%RSD *(ED)*	(Bocchini et al., 2001)
-Powdered garlic	-Aqueous extraction -Methanol/water extraction	RP-HPLC-DAD	not reported	not reported	not reported	not reported	(Arnault et al., 2003)
-Fresh garlic -Powdered garlic	-Supercritical Fluid extraction (SC-CO_2)	RP-HPLC-DAD	96%	0.73 – 4.13 mg/g garlic [h]	not reported	3% RSD	(Rybak et al., 2004)
-Garlic powder -Garlic tablets	-Aqueous extraction and sonication	RP-HPLC-UV	>89.11%	1.27 wt% (garlic powder)	0.27 µg/mL	<6.14% RSD	(de Diego et al., 2007)
-Dehydrated garlic -Fresh garlic -Garlic capsules	-Supercritical Fluid extraction (SC-CO_2)	RP-HPLC-UV	not reported	0.21 g/kg sample	not reported	not reported	(Valle et al., 2008)
-Lyophilized dry garlic	-Aqueous extraction	RP-HPLC-UV	not reported	not reported	not reported	<2% CV	(Díaz and Jiménez, 2008)
-Dry garlic	-Aqueous extraction	RP-UPLC-UV	98.9 %	1.14 – 2.42 wt%	0.79 µg/mL	0.76% RSD	(Wang et al., 2009)
-Fresh garlic pulp	-Dichloromethane extraction	NP-HPLC-UV	81.89%	not reported	6.63 µg/mL	1.54% RSD	(Yoo et al., 2010)
-Fresh garlic	-Aqueous extraction	SPE cartridge and elution with different solvents UV detection	not reported	1724 – 1815.8 µg/mL of aqueous extract [g]	not reported	not reported	(Wanyika et al., 2010)
-Fresh garlic -Garlic supplements	-Aqueous extraction	UV detection *(spectrophotometric method*)	not reported	0.35-2.22 mg/Tablet	not reported	not reported	(Olech and Zaborska, 2012)

-Fresh garlic	-Methanol/ethyl acetate extraction	RP-LC-UV *(ion pair)*	not reported	0.56-23.94 ppm[h]	not reported	not reported	(Al-Dulimyi et al., 2013)
-Fresh garlic	-Aqueous extraction (with sonication or microwaves) -Dichloromethane extraction	RP-HPLC-UV	not reported	3.33 µg/mL extract *(maceration)* 9.53 µg/mL extract *(probe sonication)*	not reported	0.41-0.75% RSD	(Bose et al., 2014)
-Fresh and cooked garlic	-Solid Phase Microextraction -Methanol desorption	RP-HPLC-UV	86%	0.17 mg/g garlic *(microwaved)* 0.12 mg/g garlic *(steamed)* 0.29 mg/g garlic *(raw)*	9 mg/kg garlic	3% RSD	(Locatelli et al., 2014)
-Garlic powder -Garlic oil -Garlic tablets	-Water/ethanol extraction	GC-FID Spectrophotometric (VIS) HPLC-UV	not reported	61.15 - 76.39 wt% *(garlic oil)* 0.61 - 0.65 wt% *(garlic tablet)* 0.43 – 0.44 wt% *(garlic powder)*	not reported	3.44 CV% *(VIS)* 8.1 CV% *(GC)* 4.68 CV% *(HPLC)*	(Zhou et al., 2015)
-Fresh garlic -Garlic pearls	-Aqueous extraction	RP-HPLC-UV	not reported	not reported	not reported	not reported	(Sekar et al., 2015)
-Raw and cooked lyophilized garlic -Digestive fluids -Blood, plasma	-Dispersive Liquid Liquid microextraction	RP-HPLC-UV	106%	19 µg/mL *(sliced rolling-boiled garlic sample)*	1.13 µg/mL	1.2% RSD	(Ramirez et al., 2017)

[a] RP-HPLC-UV: Reverse Phase High Performance Liquid Chromatography with Ultraviolet detection. SPE: Solid Phase Extraction. UV: Ultraviolet detection. RP-HPLC-ED: Reverse Phase High Performance Liquid Chromatography with Electrochemical detection. NP-HPLC-UV: Normal Phase High Performance Liquid Chromatography with Ultraviolet detection. HPLC-^{1}H NMR: High Performance Liquid Chromatography with Hydrogen Nuclear Magnetic Resonance analysis. LC-MS: Liquid Chromatography coupled to Mass Spectrometer. GC-MS: Gas Chromatography coupled to Mass Spectrometer. RP-HPLC-DAD: Reverse Phase High Performance Liquid Chromatography coupled to a diode-array ultraviolet detector. RP-LC-APCI-MS: Reverse Phase Liquid Chromatography coupled to a Mass Spectrometer with atmospheric pressure chemical ionization.

[b] RSD: Relative Standard Deviation. CV%: Coefficient of variation percentage

[c] Precision, measure as Standard deviation

[d] Weight percentage (g allicin/ 100g sample)

[e] Mean of n=16 fresh garlic samples from different varieties

[f] Results from HPLC analysis

[g] Concentration range found for different cultivars samples

[h] Concentration range found for different garlic samples

Table 4. List of published extraction, separative and detection techniques used for vinyldithiins, ajoenes and polysulfides determination.

Samples	Extraction Technique	Separative Technique/ Detection	Concentrations found	Recovery %	Precision (%RSD)	Limit of detection	Reference
- Fried garlic - Oil-Cooked Garlic - Microwave-Fried Garlic - Baked Garlic - Microwave-Baked Garlic	Steam-distilled/solvent extraction (SDE)	GC-FID GC-MS	not reported	not reported	not reported	not reported	(Yu et al., 1993)
- Blanched garlic - Blanched Fried Garlic - Baked Blanched Garlic	Diethyl ether extraction	GC-FID GC-MS	not reported	not reported	not reported	not reported	(Yu et al., 1994)
- Crushed Garlic	Steam-distilled/solvent extraction (SDE)	GC-FPD GC-FID GC-MS	not reported	not reported	not reported	not reported	(Yu et al., 1989)
- Macerated garlic oil	Solvent extraction	RP-HPLC-DAD	0.03 – 1.35 %	not reported	not reported	not reported	(B Iberl et al., 1990)
- Oil-macerates garlic - Steam-distillated garlic oil	Solvent extraction (Acetonitrile)	RP-HPLC NP-HPLC	34-435 (µg g^{-1}) (*Oil-macerates garlic*) 5-1134 (µg g^{-1}) (*Steam-distillated garlic oil*)	not reported	not reported	not reported	(Lawson et al., 1991)
- Garlic Oil	Acetonitrile extraction SPE	GC-FID	23 – 195 (mg g^{-1})	88-114	not reported	not reported	(Yan et al., 1992)
- Powder Garlic	Isooctane extraction	GC-FID	0.019-2.457 (mg g^{-1})	not reported	not reported	not reported	(Yan et al., 1993b)
- Aged Garlic Extract	Pentane extraction	GC-MS	86-11383 (ng g^{-1})	65-96	15-50	not reported	(Weinberg et al., 1993)
- Raw Garlic	Ether extraction	GC-FID GC-MS	0.122 – 830.069 (mg 100 g^{-1})	74.39 – 90.31	1.52 – 3.03	1.76 – 9.40 (ng 0.2 μL^{-1})	(Artacho Martin-Lagos et al., 1995)
- Stir-Fried Garlic	Supercritical CO2 extraction Simultaneous distillation and extraction (SDE)	PTI-GC-MS	0.04 – 3.78 (mg 100 g^{-1})	not reported	not reported	not reported	(Kim et al., 1995a)
- Garlic in soybean oil	Steam distillation and Extraction	GC-FID GC-MS	0.12-38.34 (mg 100 g^{-1})	not reported	not reported	not reported	(Kim et al., 1995b)
- Garlic, leek and onion	Volatilization and cryotrapping	HPLC-MS	not reported	not reported	not reported	not reported	(Ferary et al., 1996)
- Garlic, onion, rakkyo and caucas	Diethyl ether LLE	GC-FPD	not reported	not reported	not reported	not reported	(Mochizuki and Yamamoto, 1998)

- Garlic and onion	HS-SPME Dichloromethane (LLE)	GC-MS HPLC	not reported	not reported	not reported	not reported	(Mondy et al., 2001)
- Fresh garlic	Simultaneous distillation and solvent extraction (SDE) Steam distillation (SD) Solid-phase trapping solvent extraction (SPTE) Head space Solid-phase microextraction (HS-SPME)	GC-MS	not reported	not reported	not reported	not reported	(Lee et al., 2003)
- Garlic - Garlic tablets	Hydroalcoholic extraction	RP-HPLC-UV	not reported	not reported	not reported	not reported	(Arnault et al., 2003)
- Garlic	Hydrodistillation SPME	GC-MS	not reported	not reported	not reported	not reported	(Calvo-Gómez et al., 2004)
- Garlic	Simultaneous distillation solvent extraction (SDE) Microwave-assisted hydrodistillation extraction (MWHD) Ultrasound-assisted extraction (USE)	GC-FID GC-MS	0.1 – 43.9 (%)	not reported	not reported	not reported	(Kimbaris et al., 2006)
- Garlic	Diethyl ether extraction	Direct Analysis in Real Time Mass Spectrometry (DART-MS)	not reported	not reported	not reported	not reported	(Block et al., 2010)
- Autoclaved Garlic - Aged-black Garlic - Roasted garlic - Crushed garlic	SPME	GC-MS	not reported	not reported	not reported	not reported	(Kim et al., 2011)
- Macerated garlic oil	Hexane and 2-propanol	NP-HPLC	3.78 – 158.72 (µg mL^{-1})	80.23 – 106.18	0.55 – 11.67	0.11 – 3.16 (µg mL^{-1})	(Yoo et al., 2013)
- Microwave cooked garlic - Steam cooked garlic	SPME	GC-FPD HPLC-UV	0.09-0.28 (mg g^{-1}) (*Microwave cooked garlic*) 0.1-0.48 (mg g^{-1}) (*Steam cooked garlic*)	86 – 108	0.3-4.1	9 – 31 (ppm)	(Locatelli et al., 2014)
- Stir-fried garlic - Rolling boil garlic - Simmered garlic	Dichloromethane extraction	HPLC-UV	12.5-2964.5 (µg g^{-1}) (*Stir-fried garlic)* 3.8 -415.2 (µg g^{-1}) (*Rolling boil garlic)* 2.6-222.7 (µg g^{-1}) (*Simmered garlic)*	not reported	not reported	not reported	(Locatelli et al., 2015)
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-Raw and cooked lyophilized garlic -Digestive fluids, blood, plasma	-Dispersive Liquid Liquid microextraction	RP-HPLC-UV	14-20 µg/mL *(sliced stir-fried garlic sample)* 4.15-7.5 µg/mL *(sliced roiling-boil garlic sample)*	80-95%	0.9-3.9% RSD	0.16-1.05 µg/mL	(Ramirez et al., 2017)

SDE: Simultaneous distillation and solvent extraction. SD: Steam distillation. SPTE: Solid-phase trapping solvent extraction. HS-SPME: Head space Solid-phase microextraction. MWHD: Microwave-assisted hydrodistillation extraction. USE: Ultrasound-assisted extraction. LLE: Liquid-liquid extraction. SPE: Solid-phase extraction.

GC: Gas Chromatography, FID: Flame ionic Detector, FPD: Flame Photometric Detector, MS: Mass Spectrometer, HPLC: High Performance Liquid Chromatography, RP: Reversed phase, NP: Normal Phase, DAD: Diode Array Detector