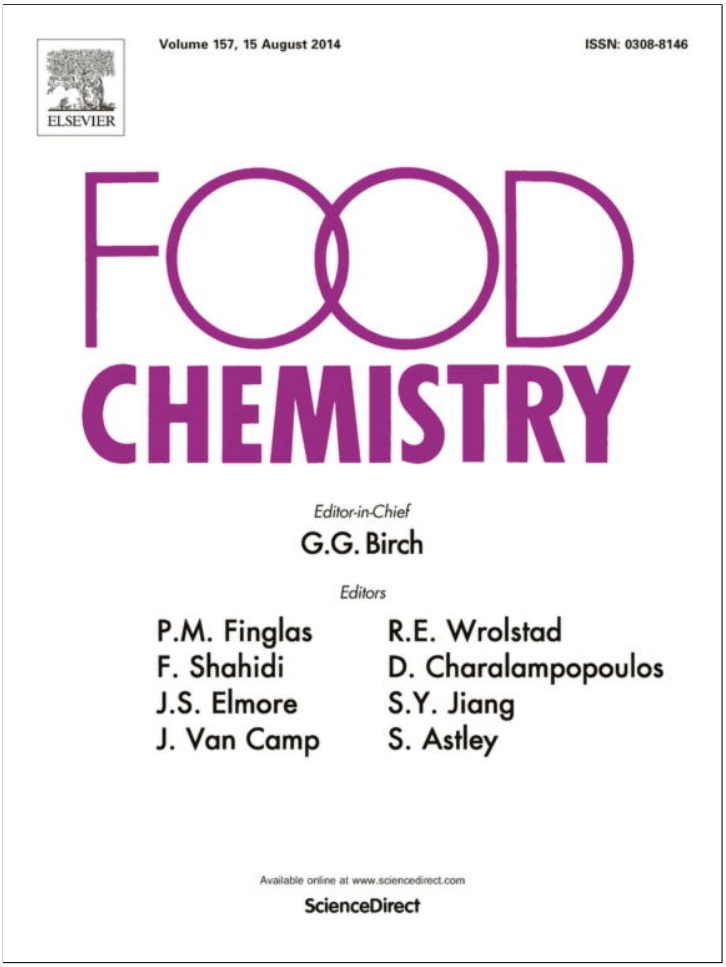

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Analytical Methods

# Solid phase microextraction coupled to liquid chromatography. Analysis of organosulphur compounds avoiding artifacts formation

Daniela A. Locatelli [a,b], Jorgelina C. Altamirano [c,d], Juan M. Luco [e], Rikard Norlin [f], Alejandra B. Camargo [a,b,d,*]

[a] Instituto de Biología Agrícola de Mendoza (IBAM) – CONICET, Mendoza, Argentina
[b] Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Alte. Brown 500, 5505 Chacras de Coria, Mendoza, Argentina
[c] Instituto Argentino de Nivología, Glaciología y Ciencias Ambientales (IANIGLA) – CONICET, Mendoza, Argentina
[d] Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Mendoza, Argentina
[e] Facultad de Química Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco y Pedernera, San Luis, Argentina
[f] Swedish Defence Research Agency, CBRN Defence and Security, Umeå, Sweden

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ABSTRACT

This work proposes the novel application of a microextraction technique, solid phase microextraction (SPME), coupled to liquid chromatography with UV detection (HPLC–UV) for the analysis of organosulfur compounds (OSCs) in garlic samples. Additionally, a comparative study of OSCs profiles obtained by SPME coupled to HPLC–UV and gas chromatography with flame photometric detector (GC–FPD), respectively; was carried out. This study provided complementary evidence about OSCs's lability and "artifacts" formation during the analytical process. Raw, cooked and distilled garlic samples were considered. The target analytes were diallyl disulphide (DADS), diallyl sulphide (DAS), diallyl trisulphide (DATS), allicin, 3-vinyl-4*H*-1,3-dithiin (3-VD), 2-vinyl-4*H*-1,2-dithiin (2-VD) and (E)- and (Z)-ajoene, which are the most important OSCs with biological activities present in raw and processed garlic. The coupling of SPME and HPLC showed to be reliable, fast, sensible and selective methodology for OSCs analysis.



## 1. Introduction

*Allium* plants are generally consumed from ancient times as a condiment, and due to its therapeutical benefits. Garlic was proposed as a healthy food due to different biological activities as antibacterial, antitumorigenic, antithrombotic, antioxidant, etc. (Omar & Al-Wabel Saudi, 2010). It is one of the most researched medicinal plants since it contribute in reducing the risk of cardiovascular and cancer diseases, as well as cellular oxidation. These properties are mainly attributed to OSCs, in addition to fructans, flavonoids and minerals (Se, Ge, etc.) (Rahman, 2003).

OSCs suffer biochemical pathways along the physiological stages of plant development until the garlic bulb is ready for consumption. The intact garlic cloves contain S-amino acids including cysteine, methionine (traces), as well as γ-glutamyl peptides and the alk(en)yl cysteine sulfoxide (ACSOs). The last are the precursors of the thiosulphinates (TS) compounds, which are formed by enzymatic reaction of allinase when garlic tissue is damaged. Allicin is the dominant and represents 70–80% of the TS in garlic. These compounds are reactive molecules and can undergo a number of transformations depending on temperature, pH and solvent of the medium (Iberl, Winkler, & Knobloch, 1990; Lawson & Hughes, 1991; Yu, Wu, & Chen, 1989). Their reaction could lead to different compounds, including OSCs such as diallyl, methyl allyl, and diethyl mono-, di-, tri-, tetra-, penta-, and hexasulphides, the vinyldithiins and (E)- and (Z)-ajoene. Thus, different type of garlic processing (cooking, distilling, storing and ageing extracts) could lead to different OSCs profiles (Kamel & Saleh, 2000; Lawson, 1993). Since these compounds are responsible of different biological activities and contribute to sensory attributes of the garlic products, the precise determination of the OSCs profiles is of interest. It is important to take into account that TS, including allicin, can also suffer transformations during the different stages of the analytical process (isolation, gas-chromatographic analysis and mass identification – when hot ionisation source are used-or combination of them) due to the reactive characteristics (Auger, Rousset, Thibout, & Jaillais, 1989; Block, 1993). This fact leads to artifacts formation, reporting thus, OSCs profiles no representative of the original sample (Block, 1993, 2013), however there are more recently publications about OSCs analysis employing GC–MS.

* Corresponding author at: Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Alte. Brown 500, 5505 Chacras de Coria, Mendoza, Argentina. Tel.: +54 2614135010.
*E-mail address:* acamargo@fca.uncu.edu.ar (A.B. Camargo).

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Reported analytical methodologies for OSCs analysis in garlic samples, involve different sample preparation techniques along with selective and sensitive instrumentation. The most commonly used sample preparation techniques are distillation, solvent extraction, sorbent extraction and the combination of some of them (Kim, Wu, Kobayashi, Kubota & Okumura, 1995; Kim, Wu, Kubota, & Kobayashi, 1995; Lee, Kim, & Lee, 2003; Yu et al., 1989; Yu, Lin, & Ho, 1994; Yu, Wu, & Ho, 1993; Yu, Wu, & Liou, 1989).

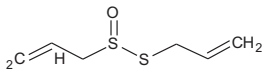
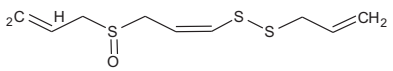
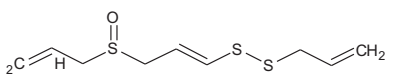
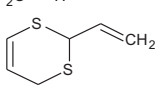
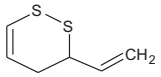
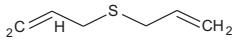
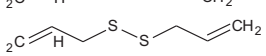
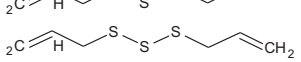
Solid phase microextraction is a solvent free sample-preparation technique, which is based on analyte partition between a stationary phase (sorption polymer supported on a capillary) and the headspace of the sealed vial containing the sample or the sample matrix bulk. SPME coupled to HPLC is receiving increased attention concerning the analysis of labile compounds (Melo, Aguiar, Mansilha, Pinho, & Ferreira, 2012).

Among the reported instrumentation gas chromatography (GC) and liquid chromatography (LC) with different detectors were mentioned. For GC, flame photometric detector (FPD) (Monchizuki & Yamamoto, 1998), flame ionisation detector (FID) (Yan, Wange, & Barlow, 1992, 1993; Yu et al., 1994; Yu, Wu, & Ho, 1993) and mass spectrometry (MS) (Artacho Martin-Lagos, Olea Serrano, & Ruiz Lopez, 1995) were reported. On the other hand, ultraviolet (UV) and mass spectrometric (MS) were coupled to LC (Arnault et al., 2003; Block, Dane, Thomas & Cody, 2010; Block, Naganathan, Putman & Zhao, 1992; Iberl et al., 1990). Regarding to MS, different ionisation sources, like electron impact ionisation (EI) (Lee et al., 2003), electrospray ionisation (ESI) (Ogra, Ishiwata, & Suzuki, 2005) and atmospheric pressure chemical ionisation (APCI) (Calvey et al., 1997) were mentioned.

There are some antecedents on SPME coupled to GC with different detectors for analysis of OSCs in garlic samples (Lee, 2003; Calvo-Gómez, Morales-López, & López, 2004; Kim, Park, Jang & Lee, 2011; Warren, Parkinson, & Pawliszyn, 2013); however there is none about SPME coupled to HPLC for this purpose.

In view of the analytical potential of the SPME–HPLC–UV, the purpose of this study was the development and validation analytical methodology base on SPME–HPLC–UV for OSCs screening in cooked garlic samples. Target compounds, and their structure are summarized in Table 1. Additionally, it was considered interesting to evaluate the GC potential for determining OSCs profiles in samples which had undergo heating treatment (distillation, cooking) along its processing. This type of samples has the distinction of having been processed including one or more thermal steps. Therefore, it is expected that labile compounds that have undergone a transformation, do not further suffer transformations along the GC analysis. In this sense, a comparative study of OSCs profiles (HPLC vs. GC) was carried out, considering the use of SPME for avoiding artifacts formation along the analytical process.

**Table 1**
Target organosulfur compounds.

| Compound | Chemical structure |
|---|---|
| Diallyl thiosulfinate (Allicin) |  |
| Trans-ajoene (Z-ajoene) |  |
| Cis-ajoene (E-ajoene) |  |
| 2-vinyl-4H-1,3-dithiin (2-VD) |  |
| 3-vinyl-4H-1,2-dithiin (3-VD) |  |
| Diallyl sulphide (DAS) |  |
| Diallyl disulphide (DADS) |  |
| Diallyl trisulphide (DATS) |  |

## 2. Experimental

### 2.1. Equipment, analytical standards and methods

#### 2.1.1. Analytical standards

DAS (97%), DADS (80%), and garlic oil blend, artificial (GOB) were purchased from Sigma Aldrich (Buenos Aires, Argentina). DATS (98%) was purchased from LKT Laboratories, Inc. (St. Paul, USA). Acetonitrile (ACN), methanol (MeOH), acetone, hexane, isopropanol and dicloromethane (DCM) were chromatography grade purchased from Merck (USA). Ultrapure water (18 MΩcm) was obtained from a Milli-Q water purification system (Millipore, France). Allicin was synthesized by oxidation of diallil disulphide DADS with hydrogen peroxide following the previously reported by the group (González, Camargo, & Burba, 2007). To obtain E-Z Ajoene isomers, synthetized allicin was heated while stirring in acetone: water (40:60 v/v) (Block, Ahmad, Catalfamo, Jain, & Apitz-Castro, 1986; Soto et al., 2007). Vinyldithiin compounds were synthesized by heating allicin in acetone: methanol (60:40 v/v) following the procedure described by Iberl et al. (1990); with slight modifications. The synthetized OSCs were further isolated by fractions collection after HPLC separation. Allicin and E- and Z-ajoene were purified using a normal phase Waters Spherisorb S5 W HPLC column and hexane: isopropanol (92:8 v/v) as mobile phase. 2-VD was purified by reverse phase-HPLC using the chromatographic conditions described in "Operating condition" section of this work. The 3-VD purification was not successful, therefore was not included within the validation study. Then, the synthetized OSCs were concentrated under reduced pressure and characterised by UV-spectroscopy and GC–MS analyses. UV spectra were obtained by using a Varian's Cary 50 UV–Vis Spectrophotometer and quantification was carried out by using the extinction coefficient (Lawson, Wang, & Hughes, 1991). For GC–MS analyses a Perkin Elmer Clarus 500 was used. Vinyldithiins as well as E- and Z-ajoene were confirmed by mass spectra. Allicin by UV and mass spectras; the last, through the confirmation of formed vinyldithiins. Obtained mass and UV spectra agree with bibliography (Ilic et al., 2012; Lawson & Hughes, 1991).

#### 2.1.2. Equipment

Gas chromatography: Hewlett Packard 5890 Serie II GC with FPD detector (Agilent Technologies, USA), HP5 column (30 m × 0.30 mm, 0.25 μm film thicknesses, Hewlett Packard, (Agilent Technologies, USA). The GC data was processed by HP-ChemStation Series II software. Liquid chromatography: Konik KNK- 500-series, UV/Vis detector (Konik, Barcelona, Spain). The HPLC column used was Waters $C_{18}$ column (254 × 4.6 mm I:D. 5 μm particle size) (USA), HPLC data was processed by EZChrom Chromatography Data System Version 6.8 software. SPME holder and fibre (30 μm polydimethylsiloxane (PDMS) coat were purchased from Supelco (Bellefonte, USA). Microwave oven (ATMA MR806, Noblex, Argentina).

#### 2.1.3. Operating conditions

GC–FPD: $N_2$ (99.999%) flow 2 mL $min^{-1}$, injection port: 250 °C, oven program was: 35 °C (1 min), increased to 75 °C at 10 °C $min^{-1}$ (1 min), then; increased to 100 °C (0 min) at 2 °C $min^{-1}$, and finally to 280 °C (5 min) at 10 °C $min^{-1}$. HPLC–UV/Vis conditions were adapted from those previously reported by Iberl et al. (1990) as

follows: isocratic elution using as mobile phase ACN:water:MeOH (50:41:9 (v/v)) at 1.0 mL $min^{-1}$; and a wavelength of 254 nm for detection. Peak identification in samples was carried out by comparing retention times with reference standards.

*2.1.4. Sample preparation and cooking procedures*

Garlic bulbs were purchased in local market. A ca. 1 kg aliquot was peeled and mixed. Sub-samples of ten cloves were smashed, homogenised and let to stand for 15 min for natural allicin synthesis. A 0.5 g cooked garlic aliquot, or 1 μL (46 mg) GOB standard, was placed into a 15 mL vial and tightly sealed with a septum-cap. The assayed cooking procedures were the following ones: (1) microwave cooking (800 W, 2450 MHz, 40 s); (2) steaming cooking (food steamer, 3 min); and (3) raw homogenate (control sample). The assays were carried out by four replicates.

*2.1.5. SPME procedure*

The fibre was conditioned in GC injection port at 250 °C for 5 min, or in 5 mL MeOH with stirring, 15 min for GC or HPLC analysis, respectively. After cooking procedure, the sample vials were equilibrated at 40 °C for 15 min. SPME was carried out by exposing the fibre to the head space, while kept at 40 °C for 15 min. After extraction stage, analytes were desorbed from the fibre by following different procedures depending on the oncoming type of analysis. For GC, the fibre was exposed at 250 °C into the GC port for 5 min. When analysed by HPLC, the fibre was exposed into 40 μL ACN aliquot contained into an 80 μL capillary tube for 5 min; injecting then a 20 μL aliquot for its analysis.

*2.2. SPME–GC/SPME–HPLC comparative study*

Microwaved, steamed, raw and GOB samples were analysed by SPME–GC–FPD and SPME–HPLC–UV following the described analytical methodologies. The reported data is the relative chromatographic area (A%) of each target analyte considering the sum of all studied OSCs as 100%. Data was analysed by ANOVA statistical tool using STATGRAPHIS Centurion software. Means of each group were compared by Tukey HSD test.

## 3. Results and discussion

*3.1. Analytical performance of SPME–HPLC–UV*

The calibration curves for each target OSCs were made under operation conditions (described in Section 2) using garlic homogenate aliquots with inactivated allinase microwaving 2 min/100 g 1000 W, (Natale, Camargo, & Gamarini, 2005). In Fig. 1 it is possible to observe that the target analytes can be chromatographically resolved by reverse phase- liquid chromatography, except for E- and Z-ajoene, which coeluted, therefore were considered together (Iberl et al., 1990) along quantification analysis. The calibration curves were built up considering expected concentrations in real samples. The range of concentration tested and analytical Figures of merits are summarized in Table 2. The detection limit (LOD) of the analytes for 0.5 g garlic homogenate, calculated as three times signal/noise ratio (S/N = 3), ranging between 9 and 31 mg $kg^{-1}$. The precision was evaluated over five replicates resulting values of RSDs ⩽ 4.1%. The resulting regression coefficient of the calibration

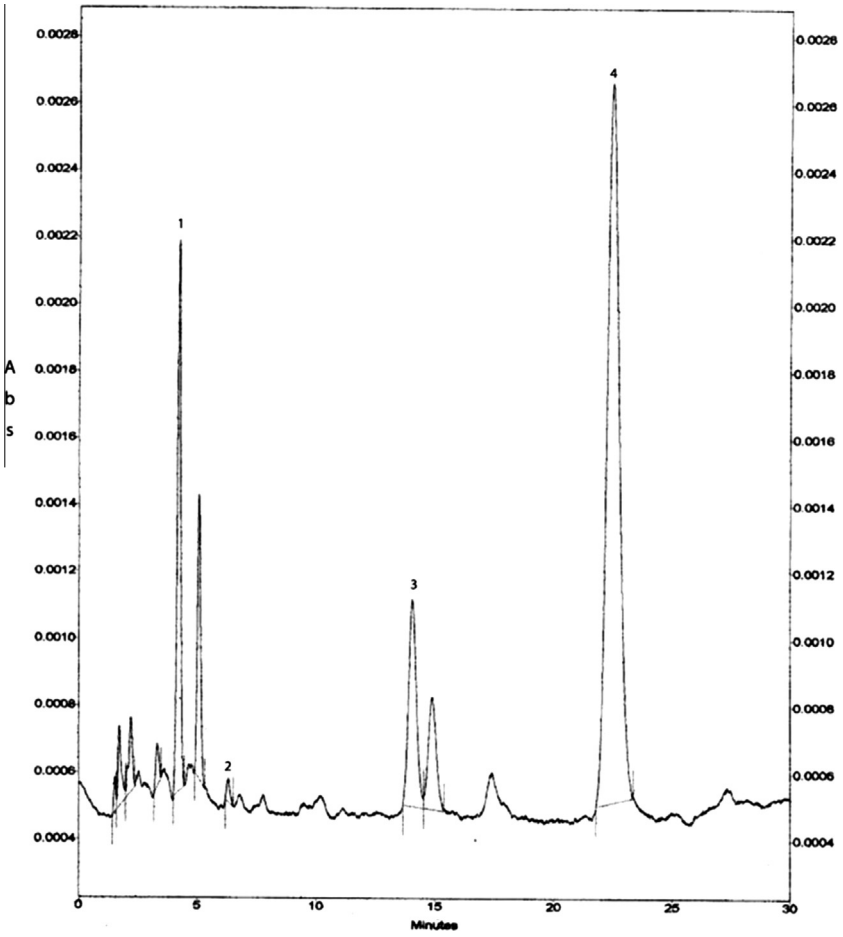



**Fig. 1.** SPME–HPLC chromatogram of OSCs in a steamed garlic sample. SPME and HPLC conditions were described in Section 2.1. 1-allicin, 2-E-Z-Ajoene, 3-DADS and 4-DATS.

**Table 2**
SPME–HPLC–UV analytical performance for OSCs determination in garlic samples.

| Target analyte | Range of assayed concentration (mg kg$^{-1}$) | $r$ | LOD (mg kg$^{-1}$)[a] | RSD (%)[b] | Recovery (%)[c,d] |
|---|---|---|---|---|---|
| Allicin | 21–84 | 0.9857 | 9 | 3.0 | 86 |
| E-Z-ajoene | 33–134 | 0.9941 | 9 | 1.2 | 105 |
| 2-vynildithiin | 53–213 | 0.9378 | 16 | 3.3 | 99 |
| DAS | 89–357 | 0.9798 | 31 | 4.1 | 92 |
| DADS | 68–272 | 0.9818 | 14 | 0.3 | 108 |
| DATS | 100–400 | 0.9857 | 25 | 0.6 | 105 |

Analysis procedure as described in Section 3.2.
[a] 95% Confidence interval; $n = 5$, expressed as wet weight.
[b] OSCs concentration 20% and 80% FSD.
[c] [Found/Added] × 100.
[d] Average.

curves ($r$) exceeded 0.9378. Recovery values of the spiked samples at two different concentrations (20% and 80%) of the full scale deflection (FSD), were estimated. The R% was ⩾68% indicating satisfactory robustness of the methodology.

### 3.2. Comparison of SPME–HPLC vs. SPME–GC for processed garlic samples analysis

Fig. 2A and B summarize the results of SPME–HPLC (A) and SPME–GC (B) analysis considering different cooking processes assayed. The data (Area% for each analyte obtained after the assayed processing) was statistically analysed by Tukey HSD test at $p \leqslant 0.05$ ($n = 4$). In SPME–HPLC analysis (Fig. 2A), allicin values showed significant differences among the considered processing. DADS values obtained showed differences between cooked and raw processing; however, the differences between steamed and microwaved were not significant according to Tukey test. DATS levels showed significant differences for the cooked processes, and could not be detected in raw samples. In SPME–GC analysis (Fig. 2B), 2-VD, 3-VD and DADS were observed in all analysed processing. DADS and 2-VD showed significant difference between cooked and row samples; however no differences were observed between steamed and microwaved. 3-VD did not show significant differences along the analysed samples.

Regarding the OSC's profile showed in Fig. 2A allicin, DADS and DATS were the majority in SPME-HPLC chromatograms, while (E)- and (Z)-ajoene, 2-VD and 3-VD were not observed. Comparing profiles of raw against cooked samples, it is possible to observe that DATS is present only in the heated samples. Additionally, allicin shows predominance in raw samples profile, and displays a lower concentration in cooked samples. These results agreed whit previous reports (Ali, Bordia, & Mustafa, 1999; Cavagnaro, Camargo,

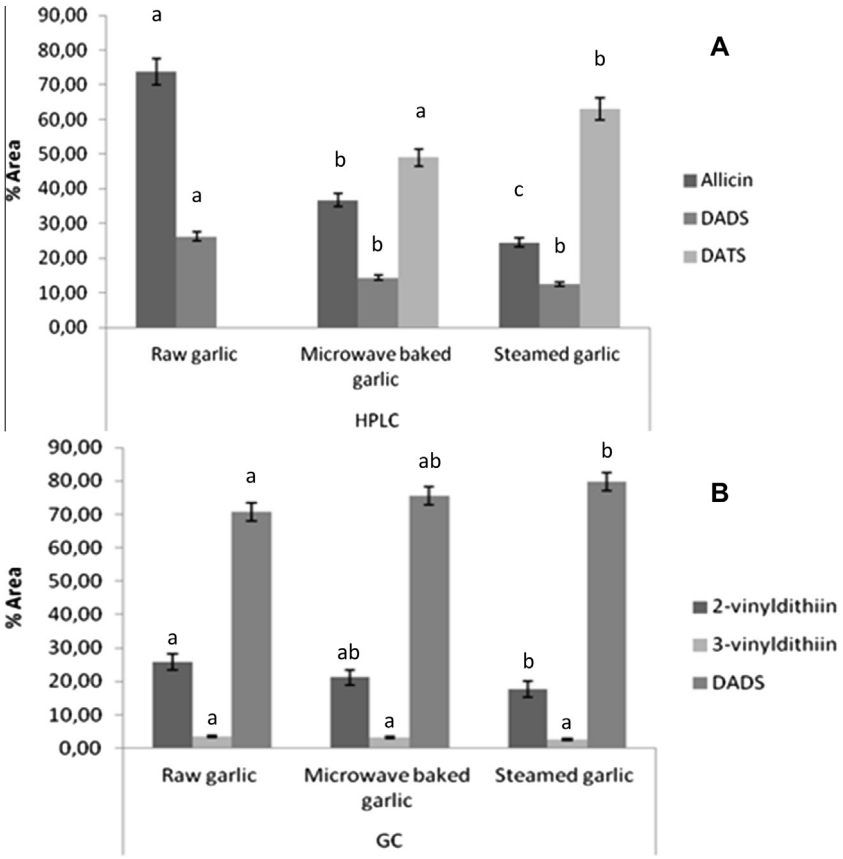



**Fig. 2.** OSCs profiles (including errors bars) for three types of garlic samples processing analysed by (A) SPME–HPLC–UV and (B) SPME–GC–FPD. Each value is the mean ± SD ($n = 4$). Values followed by the same letter were not significantly different according to the Tukey test ($p < 0.05$).

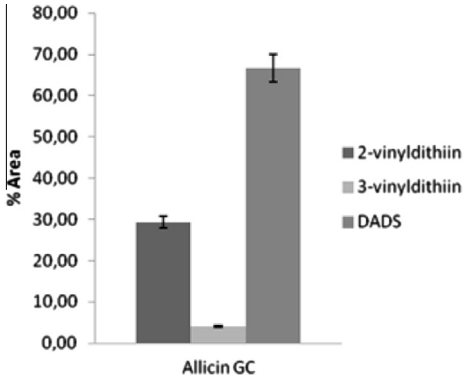



**Fig. 3.** Allicin analysis by SPME–GC.

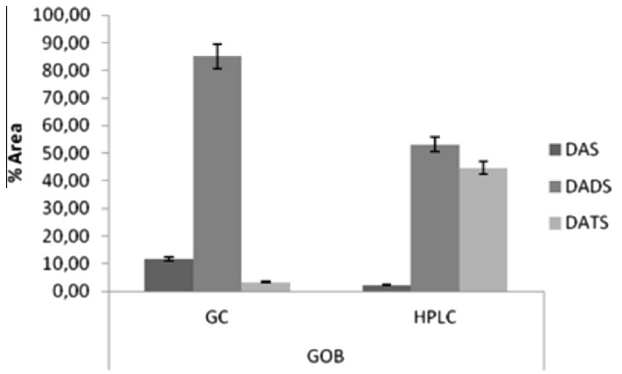



**Fig. 4.** OSCs profiles of GOB by SPME–GC and SPME–HPLC–UV (and their errors bars).

Galmarini, & Simon, 2007). On the other hand, in SPME–GC profiles 2-VD, 3-VD and DADS were observed, being DADS the majority in all analysed samples independently whether or not were cooked (Fig. 2B). It is interesting to highlight that SPME-GC profile for analysed samples is comparable with that obtained for allicin standard under the same analysis conditions (Fig. 3). In a previous work (Cavagnaro et al., 2007) was reported that allicin remains in garlic at detectable concentrations, even after some cooking process. 2-VD and 3-VD were not detected neither when analysed allicin standard nor cooked samples by SPME–HPLC. Therefore, it is evident that the OSCs profiles obtained by SPME–GC were dominated by the "artifacts" resulting from allicin thermodegradation. This evidence strengthens previous reports regarding the artifacts formation (Block, 1993, 2010, 2013), confirming that GC technique is inadequate for determining the real OSCs profiles.

However, as it was mentioned into the introduction section, it is expected to find out whether GC is suitable for the analysis of samples that have suffered some heating treatment such as garlic oil blend. Fig. 4, shows the results of comparative study between SPME–HPLC and SPME–GC for garlic oil blend samples analysis. It is possible to observe that the OSC's profile in SPME–HPLC; differ quantitatively from that obtained by SPME–GC. The predominant analytes were DAS, DADS and DATS; being DADS the dominant in both profiles, followed by DAS and DATS in SPME–GC, while DATS and DAS in SPME–HPLC. The difference could be attributed to intrinsic parameters of each technique including, gradient temperature, detector sensitivity, chromatographic efficiency, (Skoog, Holle, & Crouch, 2007), as well as thermolability of DADS (Granroth, 1970) or the combination of them. Based on these results, it is possible to say that an analytical methodology based on SPME–GC, is no suitable for determining OSCs profiles; neither for raw samples nor those that pass through a thermal treatment along the preparation process, such as GOB.

**Table 3**
OSCs levels found in garlic samples by SPME–HPLC–UV wet weight.

| Analytes | Raw garlic (mg $g^{-1}$)[a] | Microwave baked garlic (mg $g^{-1}$)[a] | Steamed garlic (mg $g^{-1}$)[a] |
|---|---|---|---|
| Allicin | 0.29 ± 0.02 | 0.17 ± 0.02 | 0.12 ± 0.02 |
| E-Z-ajoene | n.d. | n.d. | n.d. |
| DAS | n.d. | n.d. | n.d. |
| DADS | 0.05 ± 0.01 | 0.09 ± 0.01 | 0.10 ± 0.01 |
| DATS | n.d. | 0.28 ± 0.05 | 0.48 ± 0.08 |

n.d.: Not detectable.
[a] Results expressed as mean ± SD; 95% confidence interval; mg $g^{-1}$ ww.

### 3.3. Application of the method to real samples

SPME–HPLC was applied for the determination of OSCs in different garlic samples. The sample results were carried out in triplicate and expressed as wet weight (Table 3). In raw garlic samples only allicin and DADS were detected, being allicin the dominant (0.29 ± 0.02 mg $g^{-1}$). In microwaved and steamed garlic samples, the analytes found were DATS, allicin and DADS being the first the predominant OSCs (0.28 ± 0.05 and 0.48 ± 0.08 mg $g^{-1}$, respectively). DAD, DATS and DAS were detected in GOB samples. Although cooked garlic samples show the same OSCs profiles the concentrations of the analytes differ depending of the cooking process carried out. It is interesting to highlight that allicin was detected in cooked garlic samples under experimental conditions assayed. This fact together with the presence of DADS and DATS, enhances the potential health benefits effects for consumer of cooked garlic in order to prevent chronic diseases such as cancer and cardiovascular risk.

## 4. Conclusion

This work presents the novelty application and use of SPME coupled to HPLC–UV for the analysis of OSCs in garlic samples. The SPME technique results in an efficient, fast and simple analytical tool for extraction of OSCS with minimum manipulation of the samples. Additionally, it is proposed an alternative approach to the known difficulty for coupling SPME and HPLC techniques. It is important to highlight that the use of the coupled methodology avoid the artifacts formation during the analytical process, thus resulting in a viable and reliable alternative. The analytical Figures of merit obtained in the study of methodology performance were satisfactory. By comparing the use of SPME coupled to GC or HPLC, it is possible to conclude that artifacts were formed for all types of samples tested by GC. This confirms that the use of this technique is inappropriate for determining profile CSOs in garlic samples under different technological processes. The proposed methodology results in a useful and reliable analytical tool for determining the OSCs analytes with potential beneficial health effects on a wide variety of garlic preparations.

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