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Analytical Methods

Development of garlic bioactive compounds analytical methodology based on liquid phase microextraction using response surface design. Implications for dual analysis: Cooked and biological fluids samples

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ABSTRACT

Organosulphur compounds (OSCs) present in garlic (*Allium sativum* L.) are responsible of several biological properties. Functional foods researches indicate the importance of quantifying these compounds in food matrices and biological fluids. For this purpose, this paper introduces a novel methodology based on dispersive liquid-liquid microextraction (DLLME) coupled to high performance liquid chromatography with ultraviolet detector (HPLC-UV) for the extraction and determination of organosulphur compounds in different matrices. The target analytes were allicin, (E)- and (Z)-ajoene, 2-vinyl-4H-1,2-dithiin (2-VD), diallyl sulphide (DAS) and diallyl disulphide (DADS). The microextraction technique was optimized using an experimental design, and the analytical performance was evaluated under optimum conditions. The desirability function presented an optimal value for 600 μL of chloroform as extraction solvent using acetonitrile as dispersant. The method proved to be reliable, precise and accurate. It was successfully applied to determine OSCs in cooked garlic samples as well as blood plasma and digestive fluids.

1. Introduction

Therapeutic properties of garlic (*Allium sativum* L.) have been widely known and employed since ancient times. The consumption of these vegetables has been associated with the prevention of chronic diseases, mainly due to their immunological and cardiovascular functions. It has also been associated with the prevention of carcinogenic diseases. Moreover, garlic has been reported to possesses antioxidant, antithrombotic, antibacterial, antifungal and antiviral activities (Kamel & Saleh, 2000; Kwiecien, Iciek, & Wlodek, 2009). There is evidence from several investigations that suggests that the biological and medical functions of garlic are primarily attributed to its high organosulphur compounds (OSCs) content (Corzo-Martinez, Corzo, & Villamiel, 2007).

During disruption of garlic tissue, such as cutting, crushing or chewing the vacuolar enzyme alliinase, rapidly lyses the cytosolic cysteine sulphoxides (mainly alliin) to form thiosulfinates (TS) (Amagase, Petesch, Matsuura, Kasuga, & Itakura, 2001). Allicin is the predominant, and represents 70–80% of the total TS present in garlic. All of these compounds are reactive molecules and decompose to form OSCs like diallyl sulphide (DAS), diallyl disulphide (DADS) and diallyl trisulphide (DAT), vinyldithiins (E)- and (Z)- ajoene, among others (Lawson, 1992; Yu, Wu, & Liou, 1989).

Since OSCs are responsible of different biological activities, the precise determination of their quali-quantitative levels is of paramount interest. Nevertheless, nowadays, the mere presence of these compounds in food matrices is not enough to guarantee that biological properties would be verified. For these reasons, researches related to functional foods suggest the importance to develop robust analytical methodologies, not only for the analysis of food preparations but also for biological fluids.

In a previous work of our research group (Locatelli, Altamirano, Luco, Norlin, & Camargo, 2014), it was demonstrated the inconvenience to employ gas chromatography (GC) separation technique for OSCs analysis, due to its thermal instability, and it was recommended the use of High Performance Liquid Chromatography

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(HPLC). In addition, this work proposed a Solid Phase Microextraction (SPME) technique, to avoid artifacts formation (analytes not present in the original sample product of undesired reactions) during analytes extraction. Although this methodology showed satisfactory results, we decided to develop an alternative methodology to be in line with new trends.

It is also important to note that although there are literature referred to the determination of these compounds, they were mainly focused on OSCs quantification in fresh garlic and galenic preparations (Bocchini, Andalò, Pozzi, Galletti, & Antonelli, 2001; Ichikawa, Nagatoshi, & Yoshida, 2006; Kamel & Saleh, 2000; Nikolic & Stankovic, 2001; Yoo, Lee, Lee, Seog, & Shin, 2010). Moreover, to the authors' best knowledge, regarding biological fluids very few publications can be found, and they have only determined some of the analytes studied in this work (Lawson, 1993; Rosen et al., 2000). However, validated methodologies that quantify OSCs in biological fluids are still lacking.

Currently, there is a tendency to apply greener and miniaturized extraction techniques. The literature on liquid phase microextraction, shows a variety of approaches, of which we can mention: single-drop microextraction (SDME), continuous-flow microextraction (CFME), dispersive liquid-liquid micro extraction (DLLME), directly-suspended droplet microextraction (DSDME) and hollow-fiber liquid phase microextraction (HF-LPME) (Sarafraz-Yazdi & Amiri, 2010). Accordingly, in terms of organosulphur compounds it is possible to further improve the efficiency of the analysis by employing a liquid microextraction. With this goal we chose a dispersive liquid-liquid microextraction in order to upgrade the sample preparation stage. This technique uses microliters volumes of an organic solvent as the extractant along with few milliliters of a dispersive solvent (Rezaee et al., 2006). Thus, hydrophobic solutes are enriched in the extraction solvent, which is dispersed into the bulk aqueous solution. In turn, abundant studies can be mentioned in which this technique was applied to extract organic compounds from food samples, showing their suitability of application (Bidari, Ganjali, Norouzi, Hosseini, & Assadi, 2011; Viñas, Campillo, López-García, & Hernández-Córdoba, 2014; Wu et al., 2013). The main advantages of DLLME are simplicity of operation, low cost, high recovery and specially, that involve environmental friendly analytical procedures (Sarafraz-Yazdi & Amiri, 2010).

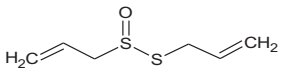
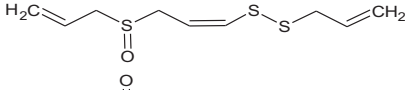
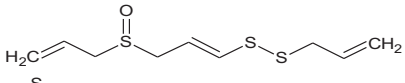
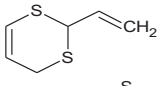
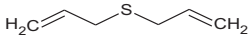
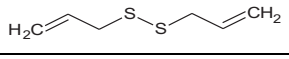
Consequently with the above mentioned, since the methodologies reported for OSCs determination have not shown a modernization, especially at sample preparation stage, and also no precedents can be found related to OSCs validated methodologies in complex matrices, the present work proposes a novel methodology using DLLME for the extraction and preconcentration of OSCs in garlic samples prior to their determination by HPLC-UV, and then evaluate its application in different matrices such as cooked samples and biological fluids (digestive fluids and blood plasma). The names and structures of the target analytes are summarized in Table 1. The optimum extraction conditions were obtained employing a response surface experimental design, and then analytical figures of merit were calculated under optimum conditions. The proposed method was successfully applied to determine the OSCs content from different matrices, therefore achieving the verification of the method suitability. Finally, it was concluded that the present methodology provides an efficient way to extract and purify these compounds from different complex matrices, and also the formation of "artifacts" is avoided. Therefore, the development of this methodology represents a reliable tool for use in bioaccessibility and bioavailability studies, thus enabling respond queries related to the release of the OSCs from the food matrix and their subsequent availability in the body to exert its beneficial effect.

2. Experimental

2.1. Reagents and analytical standards

DAS (97%) and DADS (80%) were purchased from Sigma Aldrich (Buenos Aires, Argentina). Acetonitrile (ACN), methanol (MeOH), acetone, hexane, isopropanol, chloroform and dichloromethane (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 diallyl 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-castrozd, 1986). Vinyldithiin compounds were synthesized by heating allicin in acetone/methanol (60:40 v/v) following the procedure described by Iberl et al. (Iberl, Winkler, & Knobloch, 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 S5W HPLC column and hexane/isopropanol (92:8 v/v) as mobile phase. 2-vinyldithiin (2-VD) was purified by reverse phase-HPLC using the chromatographic conditions described in "Operating conditions" section of this work. The 3-VD purification was not successful, therefore was not included within the validation study. Then, the

Table 1
Chemical structures of the target OSCs.

Name	Abbreviation	Chemical structure
Diallyl thiosulfinate	Allicin	
trans-Ajoene	Z-ajoene	
cis-Ajoene	E-ajoene	
2-Vinyl-4H-1,3-dithiin	2-VD	
Diallyl sulphide	DAS	
Diallyl disulphide	DADS	

synthetized OSCs were concentrated under reduced pressure and characterized 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, 1992). 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 was confirmed by UV and mass spectra; the last one through the confirmation of formed vinyldithiins. Obtained mass and UV spectra agree with bibliography (Ilić et al., 2012; Lawson, 1992).

2.2. Samples

2.2.1. Garlic samples

2.2.1.1. Vegetal material. Red garlic clone "Rubi" from the germplasm collection of Instituto Nacional de Tecnología Agropecuaria (INTA) La Consulta, Mendoza, was used for all the assays. Garlic was grown at INTA's experimental field located in La Consulta, Mendoza, Argentina (33°44′ S, 69° 07′ W) in 2014.

2.2.1.2. Blank samples. Uncrushed cloves lacking the OSCs of interest were used in the development and validation of the method. The blank samples were prepared inactivating alliinase by applying a thermic process (Yoo & Pike, 2001), which consisted in gently boiling whole garlic cloves for 30 min, according to Locatelli et al. (Locatelli, Altamirano, González, & Camargo, 2015). Then, samples were analyzed and it was possible to confirm that organosulphur compounds could not be detected.

2.2.1.3. Cooked samples. The proposed analytical methodology was applied for the determination of five OSCs in two different cooked garlic samples, Sliced Stir-Frying garlic (SL-SF) and Sliced Rolling-Boiling garlic prepared according to Locatelli et al. (Locatelli et al., 2015). Briefly, sliced Stir-Frying garlic was prepared by cutting the cloves into pieces of ca. 2 mm thickness and fried in 50 mL of sunflower oil (180 °C) for 2 min. Sliced Rolling-Boiling garlic was also prepared by cutting cloves into pieces of ca. 2 mm thickness and next, the samples were immersed in boiling water with large scale movements and cooked for 6 min.

After all treatments, garlic samples were freeze-dried at −58 °C for 72 h (Freeze Dry Systems LabConco Model Freezone 2.5, Kansas, MO, USA). The resulting lyophilized material was ground by using a mortar and stored at −80 °C.

2.2.2. Biological fluids samples

2.2.2.1. Blood plasma. Adults male Wistar Kyoto rats, weighing between 230 and 310 g, from Bioterio de la Facultad de Ciencias Médicas, UNCuyo, were sedated and anesthetized. Subsequently blood was collected by puncture of the abdominal vena cava in heparinized tubes. Afterwards, for OSCs determination in blood plasma, erythrocytes were separated from plasma by centrifugation at 14,000 rpm for 3 min at 4 °C.

To analyze deproteinized plasma, a volume of 200 μL of methanol was added to 800 μL of blood plasma, and then samples were centrifuged at 14,000 rpm for 3 min.

2.2.2.2. Digestive fluids. Simulated gastric and intestinal fluids were prepared according to Hedrén et al. (Hedrén, Diaz, & Svanberg, 2002). A 2.0 pH buffer solution was prepared to mimic gastric fluid, containing 1600 U% mL of porcine pepsin (Sigma-Aldrich P7000-25G), in 0.1 M HCl, with physiological amounts of calcium (3.6 mmol added as $CaCl_2{\cdot}2H_2O$, Biopack), magnesium (1.5 mmol added as $MgCl_2{\cdot}6H_2O$, Merck), sodium (49 mmol added as NaCl, Tetrahedron), potassium (12 mmol added as KCl, Anedra) and phosphate (6.4 mmol added as KH_2PO_4, Biopack). If necessary the pH was adjusted with 2 M HCl solution. A 6.8 buffer solution was prepared to simulate intestinal fluid, containing 2 g L^{-1} of porcine pancreatin (Sigma P7545-25G) and porcine bile salts 25 g L^{-1} (Fluka B8756-10G) dissolved in 0.1 M $NaHCO_3$. If necessary the pH was adjusted by adding 2 M NaOH.

2.3. Sample preparation

A sample preparation step is required in these complex matrices prior DLLME (Viñas et al., 2014).

2.3.1. Garlic sample preparation

From each treatment and blank samples, 5 g of sub-sample were reserved and homogeneized. Then, in a 15 mL tube with conical bottom, an aliquot of 0.13 g of lyophilized garlic was taken and four (4) milliliters of distilled water was added. Continuously, they were spiked at the level of 100 mg L^{-1} of allicin, E-Z ajoene, 2-VD, DAS and DADS. Following, 2.5 mL of MeOH were added to assist the precipitation of solids (fibers and sugars among others) and samples were centrifuged at 10,000 rpm during 30 min. Finally, an aliquot of 3.5 mL of supernatant was subjected to the DLLME procedure.

2.3.2. Preparation of biological fluids for OSCs determination

2.3.2.1. OSCs determination in whole blood, plasma and deproteinized plasma. Standards of allicin, E-Z ajoene, 2-VD, DAS and DADS were spiked to whole blood, blood plasma and deproteinized plasma samples by triplicate. OSCs were added at the levels of 75, 100 and 130 μg mL^{-1}. Immediately done, the proposed DLLME procedure was performed by adding 800 μL of the chloroform-acetonitrile mixture to one mL of each sample, and finally the extracted OSCs were analyzed.

2.3.2.2. OSCs determination in simulated digestive fluids. First, standards OSCs (allicin, ajoene, 2-VD, DAD and DADS) at a concentration of 75, 100 and 150 mg L^{-1}, were added to three samples of 5 ml of gastric solution, and then pH was adjusted to 2. To a second set of three gastric samples, after adding the corresponding OSCs at the concentrations specified above, the pH was adjusted to 5 by adding 2 M NaOH, then 3 mL of intestinal solution were added. After the described procedure, we proceeded to extract the OSCs of all samples using the proposed extraction technique. Finally, the extracted analytes were analyzed using HPLC.

2.4. DLLME procedure

One milliliter of acetonitrile (dispersive solvent) containing 600 μL of chloroform (extraction solvent) was injected rapidly into each sample solution using a syringe. A cloudy solution (aqueous garlic sample, acetonitrile and chloroform) was formed in the test tube. Subsequently, the mixture was centrifuged for 3 min at 2000 rpm. Accordingly, the dispersed fine particles of extraction solvent settled in the bottom of the conical test tubes. 600 μL of the extractant phase were removed using a syringe. The extracted phase was dried under nitrogen stream, and then it was dissolved in 500 μL of MeOH, filtered and injected into HPLC-UV for analysis.

2.5. Instrumentation and operating conditions

Chromatographic analyses were performed using a liquid chromatograph (Konik KNK-500-series) with a UV/Vis detector (Konik, Barcelona, Spain). The HPLC column used was a 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.

Operating conditions were adapted from those previously reported by Iberl et al. (Iberl et al., 1990) as follows: isocratic

elution using as mobile phase ACN/water/MeOH (50:41:9 (v/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.

2.6. Statistical software

The computer software Design-Expert 7.0.0 was used to design the experiments and to model and analyze the results.

2.7. Method development

In order to determine the optimum conditions to work, the effect of different factors such as type of dispersive solvent, type of extraction solvent, and extraction solvent volume were tested using a D-Optimal response surface model. Peak areas of each analyte were investigated as responses in order to optimize the independent variables (factors).

Miscibility in both, organic phase (extraction solvent) and aqueous phase is the key point for selection of dispersive solvent (Farajzadeh, Bahram, Vardast, & Bamorowat, 2010). Therefore, acetonitrile and methanol were studied for this purpose.

The extracting solvents were selected on the basis of higher density than water and extraction capability of the studied compounds (Farajzadeh et al., 2010; Rezaee et al., 2006; Viñas et al., 2014). Thus, chloroform and dichloromethane were chosen to test as extraction solvents.

To examine the effect of the extracting solvent volume on the performance of the presented DLLME procedure, different volumes of the extraction solvents (300–600 μL) were used according to the experimental design.

In addition, others parameters of interest were investigated such as the precise volume of dispersive solvent and the salting out effect on the DLLME performance.

3. Results and discussion

3.1. Method development

3.1.1. Experimental design and response surface modeling

Three parameters (factors) were used for DLLME optimization using experimental design. D-Optimal model was selected to minimize the generalized variance of the parameter estimates for a pre-specified model. Peak areas of each analyte were investigated as responses in order to optimize the independent variables (factors).

Fig. 1, illustrates the graphic analysis of the global desirability function. These plots were generated and studied to obtain an optimum set of conditions (maximum area) for each response, based on the scale of desirability function from d = 0, for complete undesirable response, to d = 1, for a full desirable response. All these plots show the interaction between two factors when the remaining factor have been kept in the fixed amount using the proposed model.

Fig. 1.a, showed a decrease in the response (expressed as desirability function) when approaching the coordinates −1 from variable C and −1 from variable B. This means, working under such conditions that MeOH is the dispersive solvent and DCM is the extractant. On the contrary, desirability reached its optimum value (0.894) when approaching coordinates 1 from C and 1 from B, this is equivalent to using ACN as dispersant and Chloroform as extractant solvent. In this case, the constant variable is volume of extracting solvent, for which 600 μL was found as optimum value.

In both Fig. 1.b and c, it was observed that the maximum response, was achieved for the largest volume of extracting solvent

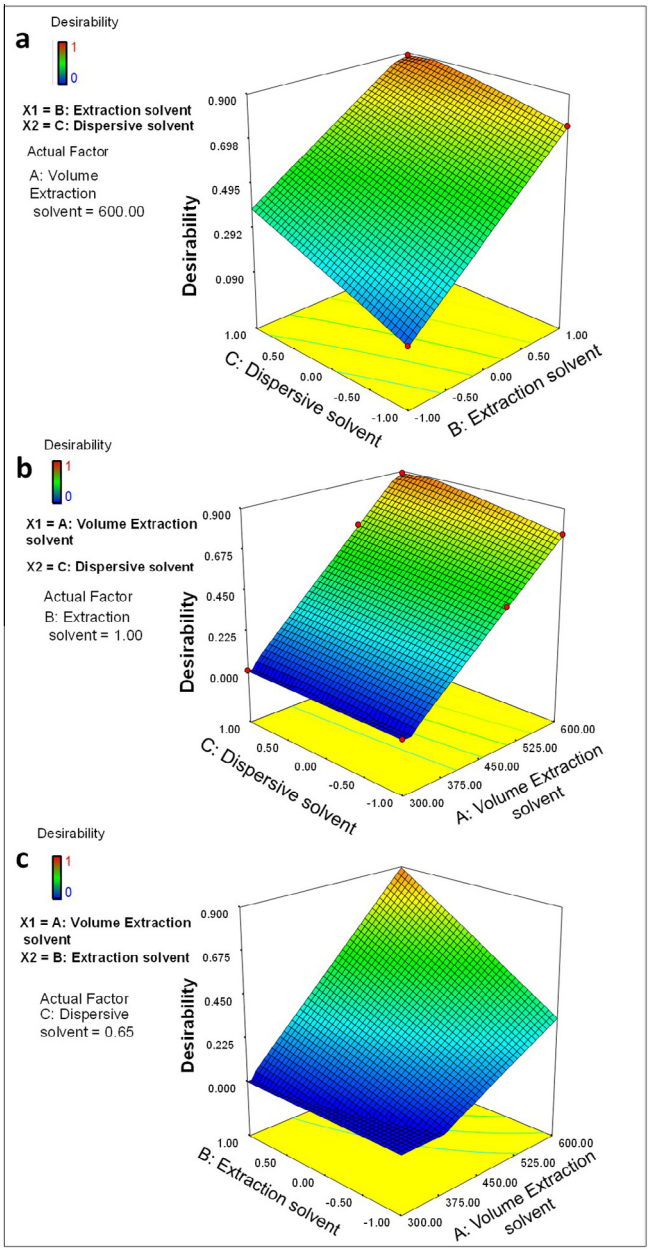


Fig. 1. 3-D graphics of the global desirability function of the variables C (Type of dispersive solvent) vs. B (Type of extraction solvent) – **a**, C vs. A (Volume of extraction solvent) – **b** and B vs. A – **c**. In each case, not represented variables remain constant with the value corresponding to the optimum of the global function.

(600 μL). Particularly in Fig. 1.b, desirability function value increased when approaching coordinate 1 from variable C that is, using ACN as dispersive solvent. The type of extraction solvent is the constant variable in this graph (Chloroform optimum solvent).

Regarding Fig. 1.c, the optimum value of the desirability function was obtained when approaching coordinate 1 from B (Chloroform), keeping constant the variable C (dispersive solvent) for which the optimum was ACN.

The total desirability function showed an optimum of coordinates 600; 1 and 0.65 in codified variables, this means, 600 μL (volume of extraction solvent), Chloroform as extractant and ACN as dispersive solvent. At this point desirability function reached its maximum value.

3.1.2. Study of dispersive solvent volume and methanol addition during sample preparation

When dealing with biological or complex samples, it is necessary to eliminate or minimize matrix effects. In this sense, precipitation it is a simple technique that generates insoluble compounds which can be separated from the media. With this goal, a volume of a solvent with low solubility for selected components (proteins, carbohydrates) is added to samples. The insoluble components formed within the solvent precipitate, can be subsequently separated from the soluble phase, by centrifugation.

Therefore, prior to microextraction, MeOH was added to each sample maintaining a proportion equivalent to 60% (v/v), 25% (v/v) and 10% (v/v) of the initial volume of sample. Then it was observed, which of these volumes favored solids precipitation without altering the extraction process, endeavoring that extractant phase settled effectively. Consequently, 2.5 mL of MeOH (as solid precipitant during sample preparation) showed best results.

The volume of dispersant solvent is restricted to the volume of methanol used above for sample preparation since both determine the polarity of the aqueous phase and thus condition the dispersion process of the extraction solvent, its interaction with the analytes and subsequent decanting.

So in order to study this interaction, to six garlic samples, a mixture of different volumes of dispersants (methanol or acetonitrile) and 500 μL of chloroform was added. The studied dispersant volumes were 1 mL, 1.5 mL and 2 mL.

Results showed that 1 mL was the optimum volume of dispersant solvent, because during the injection of the dispersant-extractant mixture to the aqueous solution, the formation of the dispersant cloud, could be visually observed immediately using both solvents. It could also be appreciated how fine droplets began to settle to the bottom of the tubes after a few seconds. In turn, the volume of sedimented phase was large enough to be separated from the aqueous phase, and it could be analyzed correctly by HPLC-UV, verifying the presence of OSCs. However, this study was not conclusive about which of the dispersants was optimal for the technique; this could only be concluded with the experimental design made.

3.1.3. Salting out effect on the DLLME

Generally the presence of electrolytes in a two-phase liquid system can influence analytes partition mainly by decreasing the solubility of hydrophobic compounds in the aqueous phase. This phenomenon is often used to increase the sensitivity of an analytical method and is called "salting out effect".

In the present work the salting out effect on the microextraction performance was studied by adding NaCl in the range of 0.05–0.2 g to each sample. The DLLME procedure included 1 mL of ACN as dispersive solvent and 600 μL of chloroform as extractant, according the results of method development, and two replicates were analyzed for each sample. Results exhibited that there was an increase in settled volume of the extractant phase and recovery values by adding 0.05 g of NaCl. Recovery values increased a 20% for allicin, 6% for ajoenes, 12% for vinyldithiin and DAS, and 2% for DADS. No changes were observed in terms of recovery adding larger amounts of salt (over 0.1 g).

3.2. Analytical performance of DLLME-HPLC-UV

In all essays, one blank garlic was used, adding known amounts of allicin, E-Z ajoene, 2-VD, DAS and DADS. Thereafter, under optimum conditions; (volume of dispersive solvent (ACN), 1 mL; extracting solvent (chloroform) volume, 600 μL; and 0.05 g of salt, analytical figures were validated according to Quattrocchi et al. (Quattrocchi, Abelaira de Andrizzi, & Laba, 1992).

Table 2, summarizes the retention times of the analytes and the analytical figures of merit. E- and Z-ajoene coeluted when reverse phase is used (see Fig. S1 in Supplementary material), therefore they were considered together throughout the analysis (Iberl et al., 1990). The calibration curves were found to be linear in a concentration range of 10–200 μg mL^{-1} (10, 35, 75, 100, 200 μg mL^{-1}), with correlation coefficients (r) > 0.99 for all analytes. The detection limits (LOD) of the target OSCs, calculated as three times signal/noise ratio (S/N = 3), ranged between limits of 0.16 and 1.13 μg mL^{-1} and LOQ, calculated as ten times signal/noise ratio (S/N = 10), ranged between 0.5 and 5.27 μg mL^{-1}. To evaluate the precision of the analytical method, the dispersion of six replicates was determined and RSD (Relative Standard Deviation) was calculated. The critical parameters were found within the acceptance criteria (%RSD ⩽ 3.9). To evaluate the extraction recovery of the technique, samples were contaminated with three levels of OSCs (75, 100 and 150 μg mL^{-1}) and they were extracted in triplicate. Calculations were performed according to Rezaee et al. (Rezaee et al., 2006), the enrichment factor (EF) was defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration of analyte (C_0) within the sample: $EF = \frac{C_{sed}}{C_0}$. The C_{sed} was obtained from calibration graph of direct injection of OSCs standard solution at the range of 10–200 mg L^{-1}. The extraction recovery (R%) was defined as the percentage of the total analyte amount (n_0) which was extracted to the sedimented phase (n_{sed}). $(R\%) = \frac{n_{sed}}{n_0} \times 100 = \frac{C_{sed}}{C_0} \times \frac{v_{sed}}{v_{aq}} \times 100$. Results showed extraction recoveries ranged between 80 and 106%.

3.3. Application of the method to cooked garlic samples

To test the applicability of the presented method in real sample analysis, Sliced Stir-Frying garlic (SL-SF) and Sliced Rolling-Boiling garlic were prepared. To each sample, analytes were added at the level of 75 mg L^{-1} and the DLLME method was applied. In order

Table 2
Retention times and Quantitative results of DLLME-HPLC-UV of target OSCs.

Analyte	Retention times (min)	r[a]	LOD[b]	LOQ[c]	RSD (%)[d]	%R[e]
Allicin	4.45	0.99674	1.13	5.27	1.2	106
E-Z-ajoene	6.07	0.99876	0.39	1.18	1.4	88
2-vinyldithiins	8.55	0.99562	0.38	0.5	2.7	95
DAS	10.79	0.99717	1.05	1.5	3.9	80
DADS	15.65	0.99626	0.16	1.13	0.9	86

[a] r, correlation coefficient.
[b] LOD, limit of detection (μg mL^{-1}).
[c] LOQ, limit of quantification (μg mL^{-1}).
[d] RSD, (C = 75 μg mL^{-1}, n = 6).
[e] Average.

Table 3
Relative recoveries and founded concentrations of OSCs in cooked samples.[a]

OSCs	Sliced Stir-Frying garlic		Sliced Rolling-Boiling garlic	
	Founded Concentration (μg mL^{-1})	Relative Recovery (%)	Founded Concentration (μg mL^{-1})	Relative Recovery (%)
Allicin	nd[b]	–	19 ± 0.15	120
E-Z-ajoene	30 ± 1.43	116	7.5 ± 4.44	105
2-Vinyldithiins	20 ± 3.76	107	4.15 ± 0.93	99
DAS	nd	–	nd	–
DADS	14 ± 0.71	108	nd	–

[a] Relative Recovery calculated as Rezaee et al. (2006). Results expressed as mean ± SD.
[b] Not detectable.

to compare the obtained results, Relative Recoveries (RR) were calculated according to Rezaee et al. (Rezaee et al., 2006).

The data shown in Table 3, indicate that relative recoveries were between 108 and 116% for Sliced Stir-Frying garlic and 99–120% for Sliced Rolling-Boiling garlic, which indicated DLLME technique for OSCs extraction in cooked garlic samples was suitable. Notably, the analytes found in the different cooked samples as well as the undetected ones agree with those reported in other studies (Locatelli et al., 2015).

3.4. Application of the method for OSCs determination in biological fluids. Implications

Under optimum conditions, the validated proposed methodology was applied in two different biological fluids. The results are shown in Table S1 (Supplementary material) and Fig. 2.

Results concerning blood and plasma analysis, showed low recovery values. In the first case (OSCs added directly to blood) OSCs levels are the lowest, as can be seen in Fig. 2, the non-extracted fraction of each compound is bigger than the recovered one. This might be due to OSCs reactions with both, erythrocytes as proteins present in plasma, so the percentage of free compounds, available to extract, is very low (Lawson, 1993).

Regarding OSCs determination in blood plasma samples (Fig. S2 in Supplementary material shows the corresponding chromatogram), previous studies have shown the present analytes react rapidly with the sulfhydryl groups of aminoacids belonging to plasma proteins, to form first, S-allylmercaptocysteine, following allylmercaptan (an intermediate) and finally allyl methyl sulphide (AMS) (Kwiecien et al., 2009; Lawson, 1993). However, with the present extraction technique it was possible to obtain recoveries that ranged from 10 to 20% (Table S1), despite OSCs degradation and reactions with components of the medium. The fact that these recovery values are higher than those found in blood, reinforce the theory that reactions occur among OSCs and erythrocytes, so in this case that erythrocytes were removed from the medium, values of recovery partially increase, as shown in Fig. 2. Furthermore, the possibility of having extracted the OSCs under study from blood plasma, employing the proposed DLLME technique, is important, since most studies reported in literature, fail to determine the analytes without a prior deproteinization step.

The reaction products of organosulphur compounds became evident in chromatographic runs, where it could be observed the presence of a peak at 3.6 min that did not correspond to any of the OSCs under study. Furthermore, we must highlight that unlike what has been reported in some studies in which it was not possible to extract allicin from plasma samples, the application of our method based on DLLME allow the extraction of this analyte, without requiring a prior deproteinization step.

Regarding to OSCs addition, after eliminating the protein content of the matrix, results showed high recovery values (87–98%). In this case, it is possible that the reaction between the analytes and cysteine, does not occur, and therefore OSCs remain intact and can be extracted.

The results of the analysis of gastric and gastrointestinal fluid samples are also shown in Table S1 and Fig. 2. Recoveries were calculated and values ranged between 28 and 87%; the highest value in gastric fluid correspond to allicin, while in gastrointestinal fluid, the lowest value correspond to this compound. These agrees with Freeman et al. (Freeman & Kodera, 1995) and Yu et al. (Yu, Wu, & Chen, 1989) investigations, which indicated that allicin is more stable at low pH, and therefore suffer lower degradation, while DAS and DADS stability growths with increasing pH, according with our results. Recoveries of E- and Z-ajoene and 2-VD, were highest in both fluids, indicating a greater degree of stability of these compounds at both pH. A chromatogram corresponding to gastrointestinal samples is shown in Fig. S3 in Supplementary material. To our best knowledge, it was not possible finding data to compare recovery results, related with biological fluids, so this is the first time that this type of study has been carry out.

In complex matrices, absolute recovery percentage is not always critical, but the method must be reproducible, so it is important to determine the precision of the analytical method. As seen in all biological matrices, the repeatability of the method was found within accepted parameters, with RSD values below 3.9% (refer to Table S1).

3.5. Comparison of the presented DLLME with other sample preparations techniques

In our case, although there is abundant literature on analytical methodologies to determine organosulphur compounds in garlic

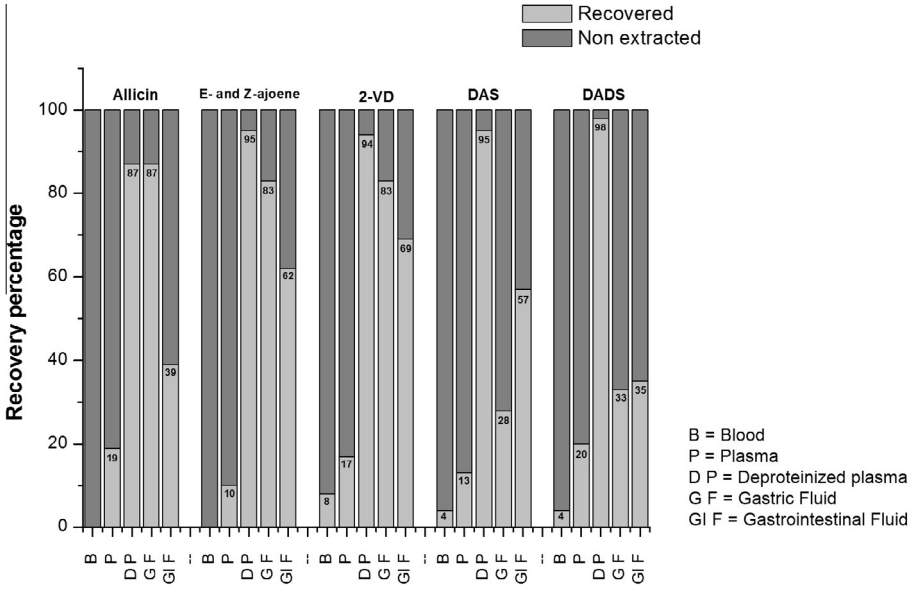


Fig. 2. Graphical representation through a stacked graph of the results from the OSCs determination in the different biological fluids. Light gray bars represent the recovered fraction and dark gray bars represent the unextracted (or in the case of blood undetected) fraction.

Table 4
Comparison of different extraction methods for OSCs in garlic samples.

Methodology	OSCs	Linear Range (μg mL^{-1})	LOD (μg mL^{-1})	RSD(%)	Recovery%	Extraction time (min)	Reference
LE-NP-HPLC-DAD	DADS E- and Z-ajoene Vinyldithiins	0.3–9.6	0.1–3.2	<3.0	>80	>6	Yoo et al. (2013)
SPME-RP-HPLC-UV	Allicin E- and Z-ajoene Vinyldithiins DAS DADS	0.6–11.8	0.3–0.8	<4.1	>80	20	Locatelli et al. (2014)
DLLME-RP-HPLC-UV	Allicin E- and Z-ajoene Vinyldithiins DAS DADS	10.0–200.0	0.2–21.3	<3.9	>80	0.5	Proposed Method

(Lawson, 1992; Yu, Wu & Chen, 1989; Yu, Wu & Liou, 1989), only a small number of them focuses in the study of the analytes present in our study and further only a few investigations have determined the method performance by calculating the analytical figures of merit.

Consequently to the literature search, our methodology based on microextraction liquid-liquid dispersive coupled to high performance liquid chromatography performance with UV detector, was compared with others methods based on traditional liquid extraction and solid-phase microextraction (SPME), in all cases coupled to HPLC-UV.

Table 4, indicates the linear range, limit of detection (LOD), precision (as RSD), recoveries and extraction time for determination of OSCs in garlic samples. It is possible to observe that extraction time in DLLME is the shortest (only considering the extraction stage, regardless of the time of sample preparation), and the method proved to be precise, simple and accurate, without presenting many differences in relation to the other methods. However, the proposed procedure has the advantage of its practicality, implying lower costs, and reduced solvent consumption compared to traditional liquid-liquid extraction employed by Yoo, Kim, Lee, and Shin (2013), and it is also a cheaper alternative to the SPME based methodology proposed by Locatelli et al. (2014). It is important to highlight that the methodology based on DLLME prevents the formation of artifacts that may occur by the interaction of the OSCs with organic solvents for a large period of time, and furthermore it allows to work with samples where the analytes are found at low concentrations, since DLLME helps to pre-concentrate the compounds for further determination by HPLC. On the other hand, sample pre-treatment is quite simple despite the matrices complexity, it only involves centrifugation and filtration, and as shown in the previous sections, the application of the analytical method developed in this work, proved to be reliable on different types of samples.

4. Conclusions

The present work propose a novel methodology based on DLLME coupled to HPLC-UV for the extraction, pre-concentration and determination of organosulphur compounds with biological properties present in garlic samples. An experimental design was developed for the optimization of the presented method.

Under optimized working conditions, target analytes can be detected clearly and precisely, without requiring a prior pre-concentration stage, thus analysis time is greatly reduced. Additionally, the proposed methodology represents a modern and environmentally friendly procedure, since it requires lower volumes of organic solvents in comparison to methodologies previously reported. Furthermore, the developed DLLME provides good linearity, precision and quantitative recoveries. As well as SPME coupled to HPLC-UV, this new methodology avoid artifacts formation along the analytical procedure, therefore the proposed methodology becomes a useful and practical alternative for the effective determination of OSCs.

Also, it has been efficiently applied for the extraction and determination of OSCs in different matrices such as cooked garlic and biological fluids. Therefore, the proposed method based on DLLME proved to be a helpful analytical tool to complement previous studies conducted on bioaccessibility and bioavailability of organosulphur compounds. Furthermore, it is important to highlight this is the first time that is reported a DLLME-HPLC-UV methodology for OSCs determination from complexes matrices.

5. Ethical statement

All animals used in the present work were cared in accordance with the Guiding Principles in the Care and Use of Animals by the Institutional Animal Care and Use Committee of the School of Medical Science, Universidad Nacional de Cuyo (Protocol approval N° 66/2015).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.07.170.

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