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Analysis of the diastereoisomers of alliin by HPLC

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ABSTRACT

Garlic has been known for its therapeutic effects for centuries and is used worldwide as a functional food. The concentration of the active molecules could be enhanced by a better knowledge of their biosynthesis. The precursor of these compounds, alliin (a sulfur amino-acid) has been obtained by chemical synthesis. However, this synthesis route also leads to a diastereoisomer as co-product. This work describes the development of an analytical method which allows the separation and quantification of the two diastereoisomers in order to determine in which proportion the natural form can be produced. The HPLC method which was optimized and validated by accuracy profile exploits an original stationary phase consisting of porous graphitic carbon (PGC). Furthermore, the developped method was used to separate the diastereoisomers of methiin, another cysteine sulfoxide, and to analyze an aqueous extract of garlic. The ability to quantify the amount of natural alliin is valuable for further work on garlic molecules and their application for health protection.

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1. Introduction

Garlic has been recognized for centuries for its health benefits, mostly linked to its sulfur-containing components. Antimicrobial and anticancer properties, as well as effects on cardiovascular diseases and diabetes have been proved (reviewed in Ref. [1]). Among the products that can be extracted from the cloves, alliin retained our attention. Alliin can undergo an enzymatic reaction to produce allicin. Allicin is itself the precursor of interesting molecules that show therapeutic properties (Fig. 1).

Alliin was chemically synthesized in two steps (Fig. 2) in order to study its subsequent enzymatic modification in depth. Firstly deoxyalliin is produced by the combination of allyl bromide and L-cysteine. Then, its sulfoxidation leads to alliin but the product differs from the natural compound. The sulfur is indeed a stereocenter. The chemical synthesis leads to two stereoisomers: the L-(+) and L-(−)-alliin although natural alliin is only found as the (+) form. The analytical separation and quantification of these stereoisomers was the aim of this study.

HPLC methods have already been developed for the analysis of L-(+)-alliin and other cysteine sulfoxides, but none of them sought to separate and quantify each diastereoisomers. Most of the methods found in the literature involve the formation of a derivative to improve the retention or allow UV, visible or fluorimetric detection [2–7]. The generation of a derivative presents disadvantages: it requires time, reagents and could cause a bias in the quantification if the reaction is not complete. Methods avoiding derivatives have been developed for the garlic molecules [4,8–11]. In particular, Ichikawa et al. [9] proposed a methodology using an amino stationary phase without derivation (UV detection at 210 nm), and Kubec and Musah [11] developed a method for particular aromatic cysteine sulfoxides, but none of them was designed for the separation of L-(±)-alliin.

Chaimbault et al. [12] developed a method which allows the separation of the twenty protein amino acids thanks to an original porous graphitic carbon (PGC) stationary phase. This particular phase, composed of a crystalline array of hexagonally disposed carbons, forms planar sheets without residual functional groups. PGC shows interesting retention properties because it is more hydrophobic than classical reversed-phases [13,14]. The delocalized electrons of the graphite also allow the separation of polar compounds and structurally close molecules show varying retention as they interact differently with the PGC planar structure [15]. Furthermore, PGC is resistant to extreme temperature and pH ranges. For all of these reasons, PGC was selected as a good candidate for the separation of L-(±)-alliin.

After optimization of the HPLC separation of the two alliin diastereoisomers, the performances were compared to the amino phase. Finally, the method developed was validated according to the accuracy profile approach.

Abbreviations: HT-HPLC, High temperature liquid chromatography; HPLC, High-performance liquid chromatography; PGC, porous graphitic carbon; TFA, trifluoroacetic acid

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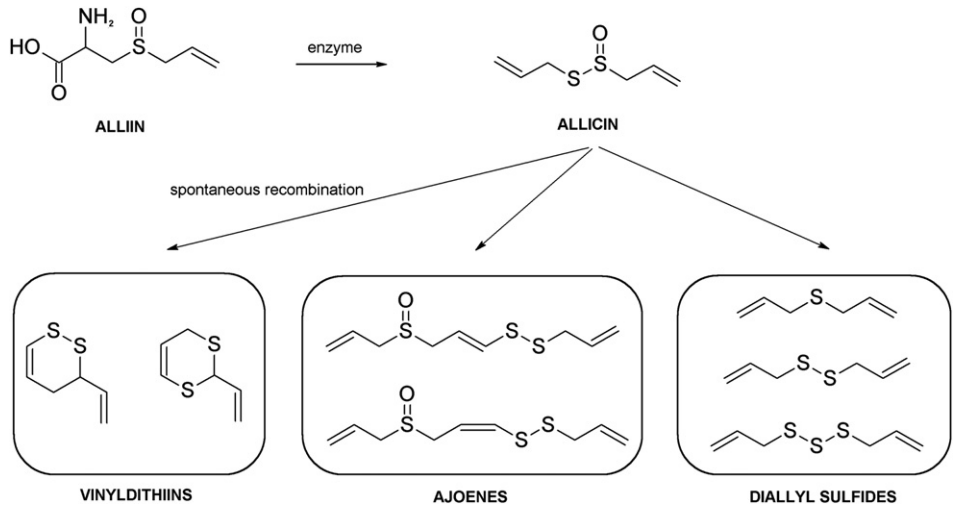

Fig. 1. Alliin enzymatic transformation into allicin and the latter's spontaneous recombination into molecules with therapeutic effects.

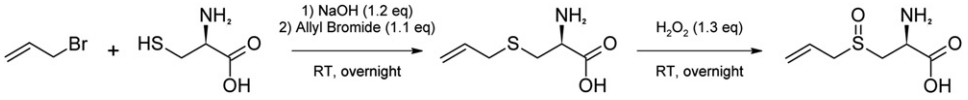

Fig. 2. The chemical synthesis of alliin is completed in two steps.

2. Material and methods

2.1. Instrumentation

The HPLC system used for the study consisted of a Hewlett-Packard 1100 series (Agilent Technologies, USA), with the following specifications: a quaternary pump, an online degasser, an autosampler, a column heater and a diode-array detector. The data treatment was performed on the ChemStation software (Agilent Technologies).

Two different columns were tested: an amino-bonded silica gel column Nucleosil® 100-NH2 RP (125 × 3 mm, particle size 5 μm) provided by Macherey-Nagel (Düren, Germany) and a porous graphitic carbon column (PGC, Hypercarb® 150 × 3 mm, particles 3 μm, Thermo scientific, Waltham, USA).

2.2. Reagents and samples

All reagents were of analytical grade. Acetonitrile provided by Scharlab (Barcelona, Spain) was HPLC grade (> 99.85%). Pure water was obtained from an Elix system (Millipore, Milford, USA). Trifluoroacetic acid (TFA) (SDS, Peypin, France), phosphoric acid (Merck, Darmstadt, Germany) and formic acid (VWR, Fontenay sous Bois, France) were all of analytical grade. Nylon syringe filters of 0.20 μm were provided by Macherey-Nagel. Garlic extract was prepared from fresh cloves bought on the local market: 20 g of cloves were carefully peeled and boiled in 200 mL of deionized water during 15 min, then crushed and boiled for 15 more minutes. The preparation was then filtered. L-(±)-alliin standard was purchased from LKT Laboratories (Saint Paul, USA) and the solutions were prepared in water (0.1, 0.5, 1.0, 1.5, 3.0 and 4.0 mg mL^{-1}). Finally, (±)-methiin was synthesized as described by Stoll and Seebeck [16].

2.3. Optimization of the chromatographic conditions

On the basis of the conditions developed by Ichikawa et al. [9], an isocratic elution with acetonitrile–water (84:16 v/v) at 1 mL min^{-1} was used with the amino column. The percentage of phosphoric acid in the mobile phase was changed (0.05%, 0.1% and 0.2%) to optimize the separation of the alliin diastereoisomers. The UV detection was performed at 210 nm.

Table 1
Factors examined for the optimization of the separation. When studying the effect of a factor, the others were fixed at the underscored level.

Factors	Levels
Gradient length (min)	5, 7, *10*, 13
Acids	TFA, phosphoric acid, formic acid
Acid concentration (% in the water phase)	0, 0.01, 0.05, *0.1*, 0.2, 0.5
Temperature (°C)	10, 20, *25*, 30, 40, 50, 60
Flow rate (mL min^{-1})	0.05, 0.1, 0.2, *0.3*, 0.4, 0.5, 0.6

An acetonitrile–water gradient was used on the PGC column. The initial program was set as follows: time (min)/%ACN (v/v): 0/0, 10/16, 12/100, 17/100, 20/0, 30/0. The molecules were also detected at 210 nm. Different gradient slopes were tested: the time for the ACN % to change from 0% to 16% was varied (5, 7, 10 and 13 min). The separation was then optimized according to the following parameters: the nature of the acid and its concentration in the mobile phase, and the column temperature. The conditions tested are summarized in Table 1. A Van Deemter plot was performed for seven flow rates: 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mL min^{-1}.

2.4. Method validation

The optimized HPLC method was validated according to the accuracy profile procedure [17,18]. The optimized conditions were those of the initial program with the following improvements: the water in the mobile phase was acidified with 0.1% of TFA, the column temperature was set at 30 °C and the flow rate was 0.3 mL min^{-1}.

Calibration standards were exploited to establish the response function, while validation standards were used as samples of unknown concentration to validate the analytical method. Three replicates of four calibration standards (concentrations: 0.1, 0.5, 1.0 and 4.0 mg mL^{-1} of L-(±)-alliin in water) were prepared and

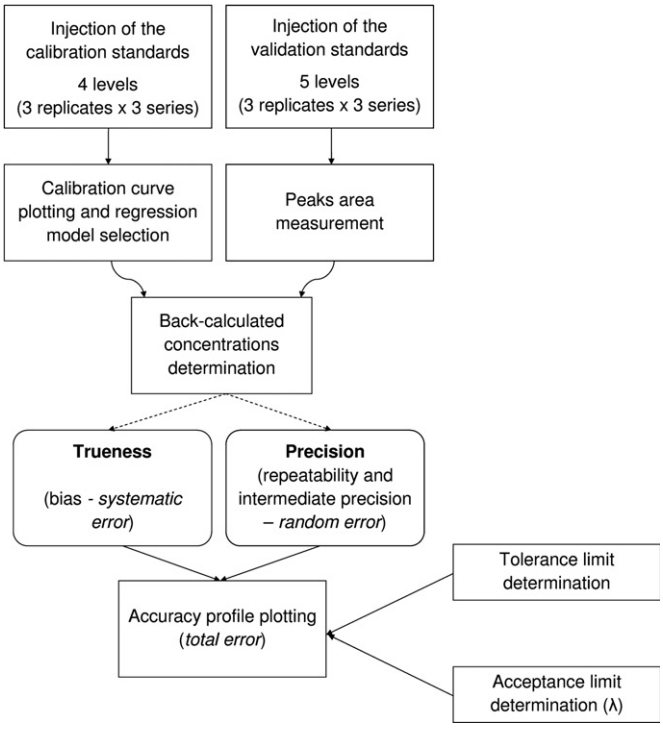

Fig. 3. Validation process for the HPLC method.

analyzed. This manipulation was repeated on three different days by three different operators (three series). The same principle goes for the validation standards: five standards (0.1, 0.5, 1.0, 1.5, 3.0 mg mL^{-1}) underwent the same process.

In a first step of the method validation, the accuracy criteria (trueness and precision) were checked for concentrations between 0.1 and 4.0 mg mL^{-1}. The second step allowed representing the accuracy of the method through its accuracy profile. After the injections, the data were processed according to the following procedure (Fig. 3):

1. The three series of calibration standards were injected, the peaks integrated and the calibration curves plotted. The regression model that fits the best was selected. Then, the response function (relationship between the peak area and the concentration) was established through the chosen regression model.
2. The validation standards were then injected, and the peaks areas were measured.
3. The concentrations of the validation standards were determined thanks to the response function chosen in 1.
4. The trueness (the mean bias) was estimated for each concentration level of the validation standards as well as the precision.
5. The accuracy profile was drawn according to the regression model chosen in 1.

3. Results and discussion

3.1. Amino column

The method provided a separation of the two forms of alliin in 12 min. The maximal resolution (R) of 1.08 between (+) and (−) alliin was obtained with 0.05% of phosphoric acid in the mobile phase. This result is satisfactory, but as we will see later, PGC provides better results (resolution > 3). Furthermore, the flow rate of 1 mL min^{-1} used on the amino column led to a smaller response factor than the one on PGC (flow rate 0.3 mL min^{-1}). The second method is thus more sensitive.

3.2. Method development on porous graphitic carbon (PGC)

The interpretation of the quality of the separation is based on the following parameters: the resolution between the peaks, their symmetry and their number of theoretical plates. The retention factor was also considered during the evaluation of the temperature applied on the column.

3.2.1. Gradient

A gradient elution was necessary to separate the diastereoisomers. A ten minute gradient seemed to be well-adapted for the separation. Indeed, a shorter gradient decreased peak resolution, symmetry and number of theoretical plates, and lengthening it was unnecessary and solvent consuming. The cleaning step was too long as no more impurities were detected at the end of column reconditioning. The reconditioning was also too long as no modification of the retention time was observed during successive injections. The final program was set as presented in Table 2.

3.2.2. Nature of the acid in the mobile phase

The acidification of the mobile phase is often suggested in HPLC as the protonation state of the compounds affects their retention. The most common acids used for that purpose are formic, phosphoric and trifluoroacetic acid (TFA). All three were tested.

At pH lower than the pKa of alliin, the compound is mostly protonated (acid function –COOH and amine function $-NH_3^+$). The pKa was therefore measured in our lab by pH-metry and its value was 3.67. The acidification with formic, phosphoric and trifluoroacetic acid at 0.1% in water resulted in pH of 2.88, 2.33 and 1.98 respectively, which were all under the pKa value.

Firstly, no separation of the diastereoisomers was obtained without acidifying the mobile phase. The use of phosphoric acid does not result in any separation, only one broad peak was observed. Formic acid leads to encouraging results, but the second peak underwent an important tailing. Only TFA provided a good resolution and satisfactory peak symmetry. The improvement in the separation obtained with TFA did not seem to be due only to acidification of the medium. TFA seemed to play the role of an ion pairing agent for cationic compounds as is often the case with the perfluorocarboxylic acids used in chromatography [19].

3.2.3. Acid concentration

A percentage of 0.1% TFA is the most interesting. At that value, the resolution was maximal, while the number of theoretical plates and the symmetry of the peaks were satisfactory. Furthermore, the baseline underwent a deviation when the TFA concentration exceeds 0.1%: at that percentage, TFA absorbance becomes significant.

Table 2
Optimized timing for the separation of L-(±)-alliin.

Time (min)	ACN (%)	Aim of this step
0	0	Separation
10	16	
12	100	Washing
13	100	
15	0	Reconditioning
22	0	

3.2.4. Temperature

The effects of temperature on the separation on a PGC column were studied and compared to the results obtained at room temperature. Contradictory results can be found in the literature. Investigations have been conducted on PGC between −13 and 250 °C (see Ref. [15] for a review), high temperature liquid chromatography (HT-HPLC) being achievable on PGC as the phase is resistant to extreme conditions (up to 200 °C) [20]. Optimal temperatures of 160 and 150 °C have been reported respectively for the separation of polymers and lipid molecular species on PGC [21,22], as well as temperature of 80 °C for the separation of small anions [23]. Besides that, PGC appears to provide good resolution for aminoalcohol enantiomers below 0 °C [24], and the best separation of the 20 amino acids was achieved at 10 °C [12]. The prediction of the effect of temperature is thus complex and an optimization of the temperature during the separation of alliin appeared mandatory.

A range between 10 and 60 °C was investigated. Obviously higher temperatures had to be avoided because they required stainless steel tubing instead of peek, a strong preheating of the mobile phase and a post column cooling [25]. The use of water in the mobile phase restricted the lower temperatures. The resolution, the number of theoretical plates and the symmetry have been processed. The resolution was satisfactory over the whole range of temperatures even if a maximum was observed at 30 °C. On the other hand, the number of theoretical plates increased rapidly when the temperature decreased, especially for (+)-alliin. Symmetry of the (+)-alliin peak is maximal at 10 and 30 °C.

The aforementioned results led to the selection of a temperature of 30 °C for the analysis. Although 10 °C give the best efficacy (i.e. the theoretical plate number) with a correct resolution, this temperature is harder to reach and maintain by the system; therefore, the best compromise was fixed at 30 °C.

The retention time decreases when the temperature is increased. Furthermore, the retention factors of each diastereoisomer tend to equalize at the extreme temperatures, which is coherent with the evolution of the resolution. The retention factors at 30 °C show a greater separation.

3.2.5. Flow rate

The Van Deemter plot allows selection of the best flow rate for the separation. It has been drawn for (+) and (−)-alliin (Fig. 4). The efficiency was higher for the (+)-stereoisomer and the plot gave an optimal flow rate of 0.1 mL min^{-1}. However the flow rate was fixed at 0.3 mL min^{-1} because this value provided a good efficiency while allowing shortening of the analysis time.

3.2.6. Final program

The chromatogram obtained for the separation of L-(+) and L(−) alliin with the optimized method is shown in Fig. 5. Thanks to a garlic extract the major peak (6.7 min) was identified as (−)-alliin and the second (8.1 min) as (+)-alliin, the natural form of alliin. Two impurities were identified as by-products of the synthesis: some remaining L-cysteine was detected at 4.3 min and deoxyalliin after 11.5 min. The last impurity at 13.1 min could be a sulfone, corresponding to alliin with the sulfur double-bonded to two oxygen atoms. The LC–MS analysis confirmed this hypothesis.

Tests on methiin showed that the method also succeeds in the separation of other diastereoisomers of cysteine sulfoxides: the resolution between (+) and (−)-methiin is worth 1.08.

Finally, the method was able to provide a good separation of other organosulfur compounds in an aqueous garlic extract, as shown in Fig. 6. Peaks were identified as methiin, cycloalliin, and isoalliin by comparing the retention times and UV spectra with those of reference compounds (synthesized standards and/or molecules identified by the HPLC method developed by Ichikawa et al. [9]). The last peaks could probably be identified as the γ-glutamyl cysteine derivatives.

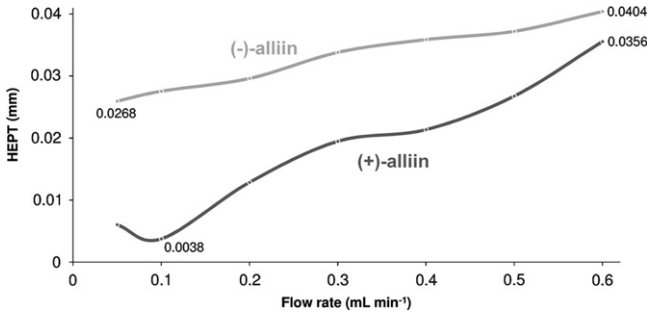

Fig. 4. Van Deemter plot. The optimal flow rate is 0.1 mL min^{-1}.

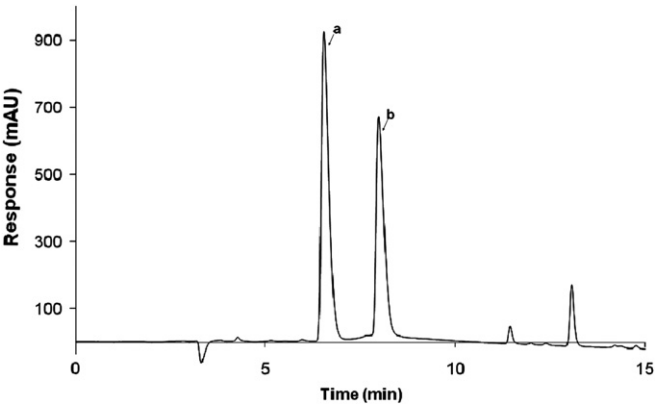

Fig. 5. Optimized separation of (−) and (+)-alliin with the PGC column (flow: 0.3 mL min^{-1}, gradient: ACN 0%–16% in 10 min, T°: 30 °C, acidification of water: 0.1% TFA). Peaks: a, (−)-alliin; b, (+)-alliin.

3.3. Validation of the method

The validation approach consists of setting the response function, calculating the performance criteria and finally drawing the accuracy profile. The validation was achieved on L-(+)-alliin, the natural molecule of garlic.

3.3.1. Response function and linearity

The response function is the mathematical relationship between the concentration and the response (the signal) within a concentration range. It is determined thanks to a calibration curve and is often linear even if exceptions exist (e.g. with particular detectors or wide concentration ranges) [17]. The UV-detector should provide a linear response according to Beer–Lambert's law.

The calibration curve is sometimes confused with linearity, which actually describes the relationship between the concentrations injected and the back-calculated concentrations (even if the curve is not a straight line) [18]. It is important to check the suitability of the model but also the linearity of the response function.

The curve was drawn and the linear model for the response function fit with a mean correlation factor (R^2) for the three curves of 0.9999. The choice of the model can also be corroborated with the linearity profile (Fig. 7). The regression line of the relation between the introduced concentrations and the back-calculated concentrations fits, because the ß-expectation limits (with ß=95% in the Student's *t*-test) are fully included in the acceptance limits (set at 10%).

3.3.2. Accuracy of the method

The accuracy of an analytical method depicts the closeness between the observed value and the true value (fixed by another tool, here with scales). It includes the systematic error (i.e. trueness) and the random error (i.e. precision). The performance

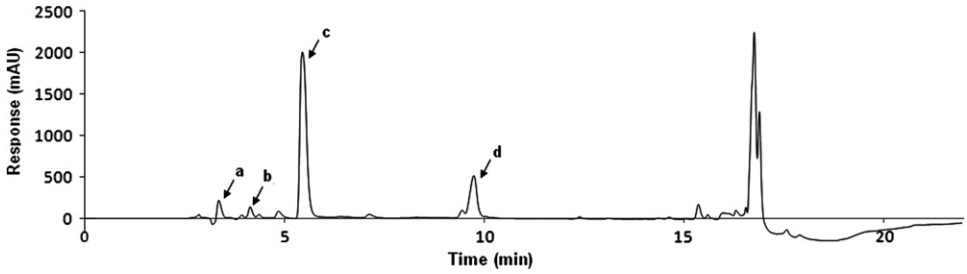

Fig. 6. Typical PGC-phase chromatogram of garlic aqueous extract. Peaks: a, methiin; b, cycloalliin; c, alliin; d, isoalliin.

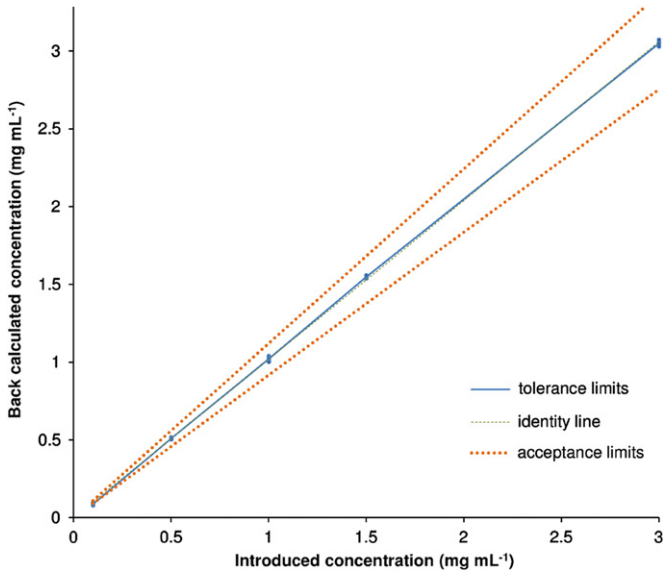

Fig. 7. The linearity of the chosen response function is satisfactory: The ß-expectation limits are included in the acceptance limits (10%).

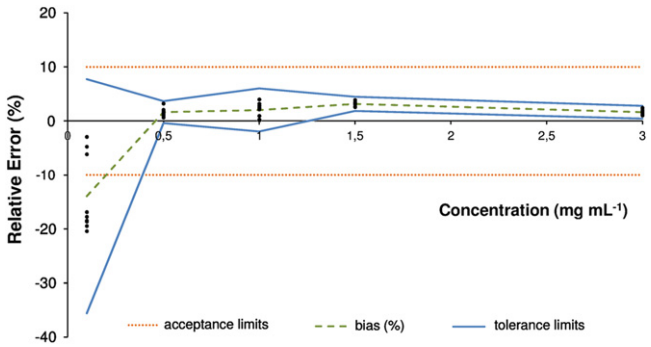

Fig. 8. Accuracy profile. The relative error is within the acceptance limits, except for the lowest concentration. The method can be validated between 0.5 and 3 mg mL^{-1}.

Table 3
Performance criteria at five concentrations. The lowest concentration has to be excluded; its accuracy and intermediate precision are not satisfactory.

Theoretical concentration (mg mL^{-1})	Accuracy		
	Trueness (relative bias, %)	*Precision*	
		Repeatability (%RSD)	Intermediate precision (%RSD)
0.100	13.9	1.66	9.47
0.500	1.6	0.54	0.57
1.000	2.0	0.43	1.48
1.500	3.2	0.62	0.49
3.000	1.6	0.41	0.45

criteria calculated are the trueness and the precision, the last one including the repeatability and the intermediate precision.

The trueness represents the systematic error (or relative bias) and depicts the closeness between the theoretical value (accepted as the true value) and the mean value within series of measurements. It is expressed in absolute or relative value. Values lower than 5% are usually considered as acceptable [25]. It is the case for the four highest concentrations tested (Table 3).

The precision expresses the closeness between the series of measurements obtained from multiple analysis of one sample. Measurements done the same day by the same operator provide the repeatability, while those on different days by various operators provide the intermediate precision. The repeatability expresses the random error and should be less than 2%. Our analyses are excellent for all the tested concentrations. Finally, the intermediate precision, that illustrates the intra-laboratory variation and is supposed to be less than 2.5%, is tolerable except, once again for the lowest concentration (Table 3). This approach to assess the method would exclude the theoretical concentration of 0.100 mg mL^{-1}, but the accuracy profile, which combines all the information, is a better tool to make a decision and it may lead to a different conclusion (see below).

3.3.3. Accuracy profile

Contrary to the one-by-one factor validation, the accuracy profile takes the global error into account. It provides the ability of the method to give a result within acceptation limits. The acceptance limits are arbitrarily set at 10%. The usual value for biological samples is 15%, and for pharmaceuticals 5% [26].

The trueness and the precision are assessed simultaneously: the trueness via the relative bias, and the precision through the ß-expectation tolerance limits. The latter were set through a Student's *t*-test (ß=95%).

The accuracy profile (Fig. 8) gives the final information to decide if the method can be validated in the concentration range. The lowest concentration did not fulfill the fixed criteria: the low tolerance limit exceeded the acceptance limit. The method is fully validated for concentrations between 0.5 and 3 mg mL^{-1}. This concentration range is satisfactory for the present analysis.

The accuracy profile and the validation criteria are consistent and show that the HPLC method is fully validated for concentrations between 0.5 and 3 mg mL^{-1}.

4. Conclusions

The porous graphitic carbon (PGC) column gave excellent results for the separation of structurally very close molecules such as diastereoisomers. Compared to the amino phase, the resolution was up to four times higher. Furthermore, the quality of the developed method is clearly demonstrated. Parameters such as the resolution, the symmetry of the peaks, or the number of theoretical plates have been optimized and are totally satisfactory.

The method has been fully validated for concentrations between 0.5 and 3 mg mL^{-1} and was already applied in the lab. This range is suitable for our next experiments.

The presented method is also versatile: the separation of other diastereoisomers such as (+) and (−)-methiin was achieved, as well as the analysis of the main organosulfur compounds in garlic aqueous extracts.

This original method will allow further investigation on alliin, its synthesis, and its transformation into the high value-added biologically active molecules of garlic. Better knowledge of garlic organosulfur molecules production could help to provide natural ways to fight cardiovascular diseases.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.09.058.

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