(BIO)SYNTHESIS, EXTRACTION AND PURIFICATION OF GARLIC DERIVATIVES SHOWING THERAPEUTIC PROPERTIES

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CONTEXT AND OBJECTIVES

Plants of the Allium genus, for example garlic and onion, are relatively odourless as long as they are intact. But if they are crushed or cut, a bunch of strong aromas are released. This is the result of a reaction between the enzyme alliinase and its substrates, alk(en)yl cysteine derivatives, probably as a defence against pests (Ellmore et al., 1994; Ankri et al., 1999). This occurs after cell damage as before, both compounds are in separate cell compartments

The thiosulfinates formed during this reaction are volatile and unstable: they quickly turn into a range of molecules depending on the conditions. The main cysteine derivative in garlic is alliin, or S-allyl cysteine sulfoxide (Stoll and Seebeck, 1951). Its reaction with alliinase and the transformation of the resulting thiosulfinate, allicin, are described in Figure 1.

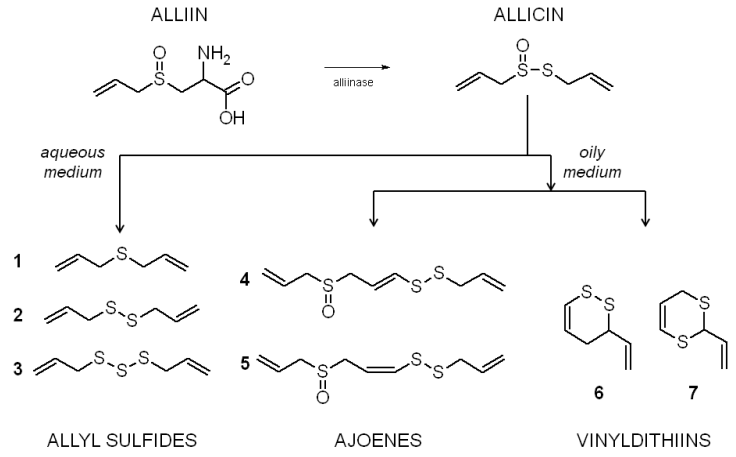


Figure 1: Alliin is the substrate of the enzyme alliinase. Allicin, the product, quickly turns into various groups of molecules according to the conditions and medium.

Garlic is not only used for its flavour: the organosulfur compounds obtained when garlic is crushed have shown health protective effects (reviewed by Block, 2010). For example, vinyldithiins showed interesting properties against adipocytes differentiation (Keophiphath et al., 2010), alliin showed antioxidant properties (Bopanna et al., 1999), and diallyl disulfides antioxidant and antitumor properties (Dwivedi et al., 1992), as well as cholesterol level lowering activity (Liu et al., 2000). The stereochemistry of the compounds might greatly influence these properties and special attention must be given to this aspect of the production of these bioactive molecules.

The development of processes producing these high-value added compounds could lead to new applications as nutraceutical or to the discovery of new

therapeutic molecules. The aims of this work were to optimize the extraction of vinyldithiins from garlic (Figure 1, compounds 6 and 7) and to synthesise alliin (Figure 1, starting substrate) and diallyl mono-, di- or trisulfides (Figure 1, compounds 1-3). The enzymatic resolution of the stereoisomers of alliin is also presented.

EXTRACTION OF VINYLDITHIINS FROM GARLIC

Vinyldithiins are formed in oily media. The influence of the conditions and of the medium was investigated for the extraction of 1,2- and 1,3-vinyldithiins in edible oil.

Crushed garlic was allowed to macerate in edible oil under determined conditions. The parameters considered for the extraction were the following (in parenthesis, the number of levels tested): the origin of the cloves (5), the nature of the edible oil (8), the garlic/oil proportion (5), the extraction temperature (5), the stirring (on/off), and assisted extractions by microwaves or ultrasounds. Two repetitions of each level have been performed, as well as a kinetic assay to optimize the extraction time. Vinyldithiins were then extracted with an equal volume of acetonitrile, and the samples analyzed by RP-HPLC (C18 column, 45/55 ACN/water in isocratic mode, UV detection at 210nm) and quantified with a calibration curve (standards provided by BioXtract SA).

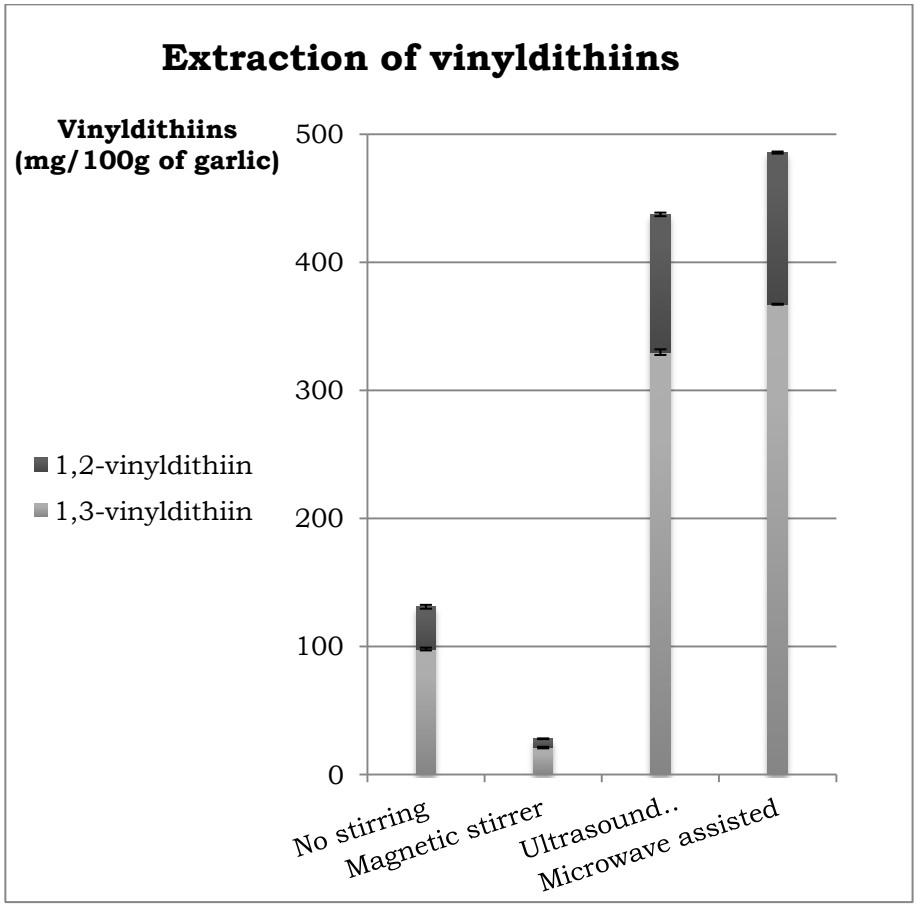


Figure 2: Influence of the conditions on the extraction of vinyldithiins. We recommend the absence of stirring and the use of microwaves to increase the yield of both 1,2- and 1,3-vinyldithiins. RP-HPLC conditions: C18 column, 45/55 ACN/water in isocratic mode, UV detection at 210nm.

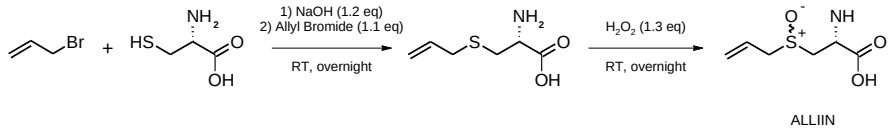
Important differences have been found between samples that were stirred or not, and between classical and assisted-extractions. The optimisation leads up to a 4-fold higher yield (Figure 2). This result might be explained by a higher number of interactions between the enzyme and its substrate. On the one hand, the absence of stirring could allow better binding between alliinase and alliin, and on the other hand, the rupture of the cell walls, favoured by the high pressure developed by the microwaves (Tatke et al, 2011), could increase the quantity of both partners of the reaction. The kinetic experiments led to choose an extraction time of 6h (longer times led to degradations). The other studied parameters had a smaller influence on the yield and no clear tendency could be drawn. For example, no correlation could be established between the extraction yield and the physico-chemical properties of the oil (data not shown).

SYNTHESIS OF BIOACTIVE COMPOUNDS

Synthesis and resolution of alliin stereoisomers

Extractions of the bioactive compounds from garlic generally lead to low yields. Higher amounts of target molecules, necessary for the biological activity studies, could be obtained by synthesis. However, this latter route can lack specificity (cfr alliin synthesis). The development of processes combining these two routes could be interesting.

The synthesis of alliin was performed according to Stoll and Seebeck's method (1951). The two-step process shown in Figure 3 leads to two diastereoisomers (the sulfur becomes a stereocenter when it is oxidized). 58% of (-)-alliin and 42 % of (+)-alliin were respectively measured in the synthesis mixture. This lack of stereospecificity might have consequences on the bioactivity of the products. Furthermore, (+)-alliin is the only natural isomer in garlic (>99%). These observations led us to elaborate a process to produce alliin stereospecifically.



ALLIIN

Figure 3: The chemical synthesis of alliin from L-cysteine leads to two stereoisomers.

Resolution of stereoisomers can be achieved enzymatically if the biocatalyst can react more specifically with one isomer. Alliinase has a 4-fold higher affinity for (+)-alliin in comparison with (-)-alliin (Krest and Keusgen, 1999), and this property was used to separate a mixture of the diastereoisomers. The following conditions were used to resolve a mixture of alliin stereoisomers :100 U of alliinase were allowed to stand at 37°C with 0.03 mmol of synthetic (+/-)-alliin (42/58, mol/mol) in presence of 0.25 nmol of pyridoxal phosphate (the enzyme cofactor) in a potassium phosphate buffer (pH 6.2). Samples were collected every 2 min during 10 min, then at increasing intervals (Figure 4). Immediately after sampling, alliinase was deactivated by decreasing the pH under 3 with HCl. The resulting solution was analysed by HPLC according to the method developed and validated by the accuracy profile approach in our laboratory (Dethier et al., 2012). Briefly, this method allows the separation and quantification of both stereoisomers of alliin by using a porous graphitic carbon column (retention times of 7.4 min and 9 min for (-)- and (+)-alliin respectively).

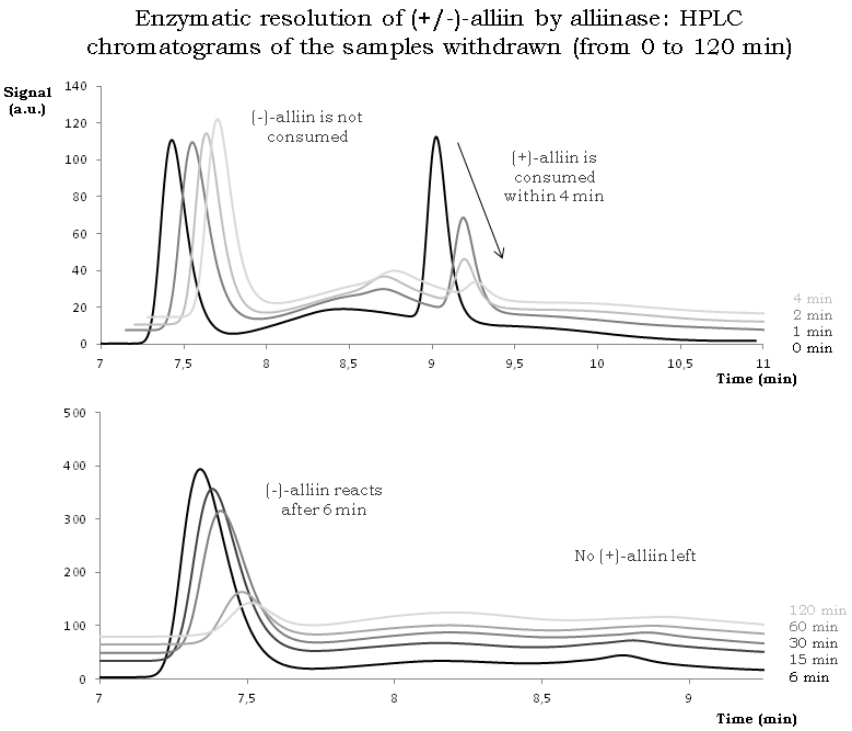


Figure 4: Enzymatic resolution of the diastereoisomers of alliin. The injection volume for the second part of the assay (2nd graph) was increased 4-fold. HPLC conditions: porous graphitic column (150×3 mm, particles 3 µm); solvents ACN/H2O + 0.1% trifluoroacetic acid (gradient: ACN % increases from 0% to 16% in 10 min); 0.3 mL/min; detection at 210 nm.

Figure 4 shows that the enzymatic resolution of the diastereoisomers of alliin is possible. Under the proposed conditions, (+)-alliin reacts totally within 6 min, and (-)-alliin in about 2 h. This result is promising for a resolution at larger scale in optimized conditions.

Other methods could be applied to separate the isomers of alliin: chromatographic separation or crystallisation (Stoll and Seebeck, 1951) but the process has a poor yield. A stereospecific synthesis has been developed (Koch and Keusgen, 1998) and avoids the need for a separation, but numerous steps are required.

Diallyl sulfides chemical synthesis

Diallyl sulfides (DAS) are formed when alliin reacts with alliinase in an aqueous medium. Three molecules are obtained: diallyl monosulfide (DAMS, 1 on Figure 1), diallyl disulfide (DADS, 2 on Figure 1) and diallyl trisulfide (DATS, 3 on Figure 1). DADS is the major allyl sulfide in garlic aqueous extracts and essential oil.

Two similar processes can be found in the literature for the chemical synthesis of diallyl disulfides (Figure 5): a classical synthesis (Maloney et al., 2006) and a microwave-assisted one (Yuan et al, 2006). They use a phase transfer catalyst (PTC): tetrabutylammonium bromide or fluoride. Both routes lead to a mixture of DAMS, DADS and DATS. Our objective was to optimize the yield and ratio in DADS.

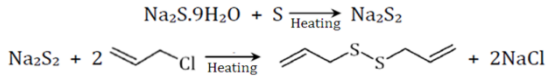


Figure 5: Synthesis of diallyl disulfides (also leading to diallyl mono- and trisulfides).

For the first step of the synthesis, a solution containing 1.3 mol/L of sulfur and an excess of sodium sulfur is heated at 60 °C for 2 hours. Allyl bromide is added (sodium disulfide in excess), then the PTC, and the mixture is stirred for 15 min at the chosen temperature. Four temperatures, controlled by classical (oil bath) and microwave-assisted heating, were compared (2 repetitions). After this first screening, a Box-Behnken experimental design was applied. The influence of the temperature, the stirring rate and the amount of PTC were assessed. Three levels (table 1) and two repetitions were performed. Products were identified by GC-MS on an HP-5ms column and by 1H and 13C NMR. DAS was quantified by GC-FID.

Parameters	Levels		
Stirring	no	moderate	high
Temperature	40°C	60 °C	80 °C
PTC/allyl bromide ratio (mmol/mol)	10	25	40

Table 1: Levels attributed to each parameter for the Box-Behnken experimental design.

Preliminary results led to higher DADS purity at low temperature in both oil bath and microwave oven (Figure 6). At temperatures above 80°C, DADS is probably degraded into other compounds, mostly DAMS. The influence of the temperature on the degradation is greater with microwaves. Homogeneous heating in microwave vessels could enhance this effect, since the core of the sample reaches instantly the requested temperature (Eskilsson et al., 2001).

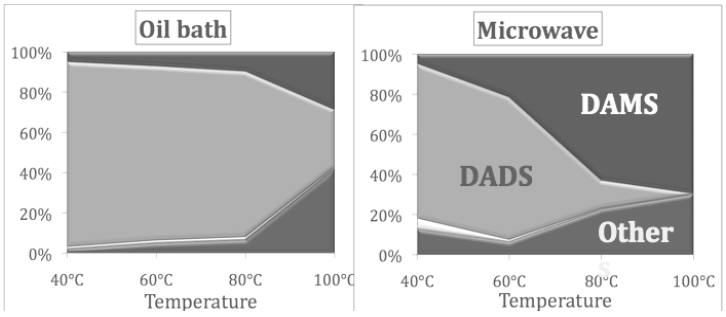


Figure 6: Selectivity towards DADS decreases with the temperature, especially in the microwave-oven. The white surface represents DATS.

The response surface design (Figure 7) allowed to reach several interesting conclusions. The presence of a PTC is recommended to increase both the quantity of DADS synthesized and the purity of the final product. The use of other PTC could eventually improve these results. At the opposite, elevated temperatures lead to a loss of purity and an important decrease in yield. Stirring had antagonist effects: strong stirring leads to higher yields probably

by increasing the contacts between allyl bromide and sodium disulfide, and by enhancing the effect of the PTC. No stirring provided a better DADS purity, but decreased the global yield (data not shown).

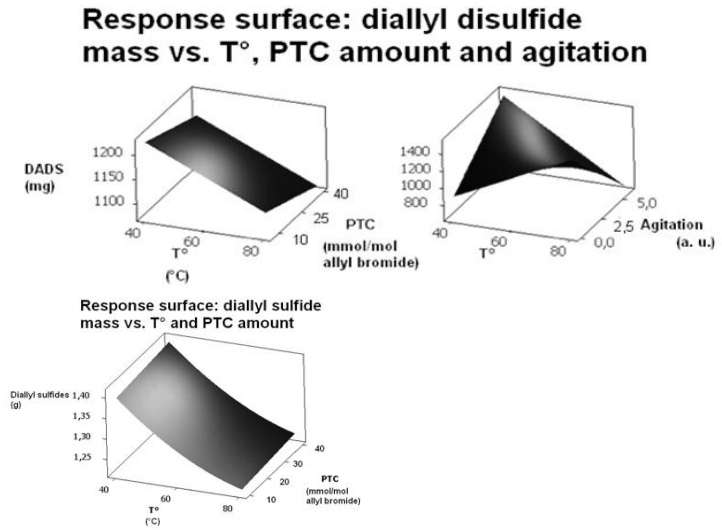


Figure 7: The DADS amount increases at low phase transfer catalyst concentration, low temperature and strong stirring OR at high temperature with no stirring (left). The global yield of diallyl sulfides increases at high phase transfer catalyst (PTC) concentration and low temperature.

PERSPECTIVES

Numerous garlic molecules can still be explored, and their extraction or synthesis optimized. The stereochemical approach leads to new garlic-like compounds that could demonstrate positive effects on human health. Investigations on molecules close to natural products are very promising in the field of new drugs discovery, and alliin stereoisomers are an interesting starting point in this context.

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