Analytical method for appreciation of garlic therapeutic potential and for validation of a new formulation

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Abstract

The consumption of garlic reduces the risk of cardiovascular disease and cancer, *S*-allylcysteine sulfoxide (alliin), allicin (DATi), diallyl disulfide (DADS), *S*-allylcysteine (SAC) and several storage dipeptides are the organo-sulphur compounds (OSC) involved in the protective mechanism of garlic against cardiovascular disorders and carcinogenesis. Thus it is very interesting to quantify simultaneously all these compounds in different garlic powders obtained in several cultural conditions. The quantification of OSC by a new ion-pair HPLC method allowed showing the general sulphur-dependence positive effect of garlic on cardiovascular disorder and carcinogenesis and the variable specific activity of each implicated OSC. The screening of 11 garlic tablets proposed on the market showed the variability and particularly the differential instability of each OSC. From these results, a new garlic tablet was realised and each step was controlled by this method. This analytical method proved to be a very powerful tool for the understanding of the garlic protective mechanism against cancer and cardiovascular diseases and the development and quality control of garlic tablets.
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Keywords: Garlic; Health; Organo-sulphur compounds; HPLC; Tablets formulation

1. Introduction

Since, ancient times garlic is recognized as an important medicinal plant. The consumption of garlic reduces the risk of cardiovascular diseases and cancer [1]. Recent epidemiologic studies reveal that the high garlic consumption reduces the risk of cancer, including stomach and colon [2].

The compounds implicated or suspected to be responsible in the health effect are well known. Among these substances, organo-sulphur compounds (OSC) are very active. *S*-Allylcysteine sulphoxide (alliin), diallyl thiosulphinate (allicin), diallyl disulfide (DADS), *S*-allylcysteine (SAC) and γ-glutamyl-*S*-alk(en)yl-L-cysteine named storage dipeptides

are the OSC (Table 1) involved in the protective mechanism against cardiovascular disorders and carcinogenesis.

These OSC can be present simultaneously in garlic and garlic products and their transformation fully depends on enzymatic reactions. The chemistry of garlic is quite complex and not yet completely explained. Intact garlic bulbs and certain garlic powders (GP) contain high amount of γ-glutamyl-*S*-alk(en)yl-L-cysteine which are hydrolysed and oxidized to form alliin. Alliin accumulates naturally during storage of garlic bulbs at cool temperature. When bulbs are crushed or cut, the vacuolar enzyme, alliinase, transforms alliin into the very unstable thiosulphinate allicin [3]. In the GP, the conversion of alliin into allicin starts when water is added to the powder. Then allicin is more or less rapidly degraded into DADS, vinyldithiins I, II (Table 1) and ajoenes [4], depending of the conditions: concentration, temperature, pH.

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Table 1
Name and chemical structures of OSC detected in GP by HPLC

Compound	Name	Abbreviation	Chemical structure
1	*S*-Allyl-ʟ-cysteine sulfoxide	Alliin	
2	*S*-Allyl-ʟ-cysteine, deoxyalliin,	SAC	
3	γ-Glutamyl-*S*-allyl-ʟ-cysteine	GLUAlCS	
4	γ-Glutamyl-*S*-(*trans*-1-propenyl)-ʟ-cysteine	IsoGLUAlCS	
5	Diallyl thiosulfinate	Allicin	
6	3-Vinyl-[4*H*]-1,2-dithiin	Vinyldithiin I	
7	2-Vinyl-[4*H*]-1,3-dithiin	Vinyldithiin II	
	Sulfides		
	n = 1, diallyl sulphide (DAS)	DAS	
	n = 2, diallyl disulfide	DADS	
	n = 3, diallyl trisulfide	DATS	

Allicin is a key molecule in garlic and presents a lot of biological properties, medicinal or pesticidal [5], for example, this substance is well known for its antibacterial effect [6], particularly against *Helicobacter pylori* [7].

The toxicity of a carcinogen substance depends on the balance between detoxication and metabolic activation. The protection of garlic against cancer arised from several mechanisms including the blockage of nitrosamines formation and bioactivation [8]. These substances are much suspected to be carcinogens and influence the cancer risk in humans. Dion et al. [9] reported that their formation are retarding by SAC. Garlic decreases also the bioactivation of carcinogens. Cytochrome P4502E1 (CYP2E1) is an hepatic phase I enzyme implicated in the metabolism of nitrosamine, and others carcinogens, and its activity is modulated by OSC [10] such as DADS [11,12]. OSC from garlic block the bioctivation and carcinogenicity of non-nitrosamines such as aflatoxin B1 (AFB1), a cancer agent for the liver. Phase I enzymes CYP1A1, 1A2, 2B1 and 3A4 involved in carcinogen bioactivation show a modified activities after diets supplementation in rats with garlic or several sulphur compound such as DADS [13]. The induction of phase II enzyme detoxication such as glutathione-*S*-transferase (GST), quinone reductase (QR) and uridinediphosphoglucuronate glucuronosyltransferase (UGT) by OSC, such as DADS [14,15] is demonstrated and similar effects are observed with garlic consumption [16,17].

The antiproliferative effect of garlic is also clear. Allyl sulphur compounds are some important antitumorigenic agents [18] and DADS reduces the size and the number of preneoplastic foci in rats liver induced by AFB1 [19].

Cardiovascular diseases include a great number of factors such as for example high cholesterol, hypertension and increased platelet aggregation. The role of garlic in the reduction of cardiovascular diseases was historically proved but contradictory clinical studies emerged from different methodologies. The health benefit observed varies with the garlic products tested, i.e. GP, aged garlic extract, garlic oil and garlic oil macerate [20], with the state of cooked garlic, raw, fried or boiled [21,22] and with the percentage of active components [23]. The contradictory results may be due to several factors and the lack of knowledge about the substances present is certainly one of them.

Inflammatory diseases such as atherosclerosis lead to coronary thrombosis. The platelet aggregation contributes to minimize the atherosclerosis and it is reduced by allicin and

two others thiosulphinates from onion [24]. The antiplatelet activity in onion was significantly positively correlated with high sulphur level [25]. GP also inhibits the platelet aggregation [26] and Lawson et al. [27] reports the variation of the antiplatelet activity of different garlic preparation, cloves or commercial products. These variations are due to the different nature of OSC present.

Garlic decreases certain diseases caused by immune dysfunction. Thus, aged garlic extract presents an immunomodulation effect [28]. The immunomodulatory effect of garlic or garlic constituents shows a modulation of cytokine production as mediator of inflammation. The nuclear factor-κB (NF-κB) is a central transcription factor and has a central role in the expression of genes that control immune response. NF-κB is strongly involved in the activation and regulation of key molecules associated with inflammatory diseases and cancer [29]. It increases the expression of the genes of some cytokines. The inhibition of NF-κB by GP is indirectly controlled by a modulation of pro- and anti-inflammatory cytokines [30].

The certitude that garlic provides beneficial effect on health leads the industry to propose some garlic market product for human consumption in the last decades. There are too major types of market garlic products on the European pharmaceutical and food supplement market and a third one on the American market: (a) the oil macerates of fresh garlic, formulated commonly in soft gelatine capsules, (b) the dry powder products of fresh garlic formulated either as sugar or film coated tablets, and (c) dry powder products of aged garlic formulated either as sugar or film coated tablets [31]. It is the task of the pharmaceutical science to formulate each active principle individually in such a way that the active principle is fully available under the physiological condition of the application. The appearances and characteristics of medicinal products are significantly influenced and controlled by the formulation technology (galenic) and the analytical technology. Both determine the ability to manufacture quality/stability, efficacy, and consumer acceptance of the final medicinal product.

The major aim of a garlic pharmaceutical formulation of high quality is to conserve the activity of the alliinase and the content of its substrate and mask the mostly unwanted smell and taste of garlic. Most market products are based on sugar or polymer film coated tablets. These tablets are packed either in polymer/aluminium blister packs or in glass or polyethylene bottles.

In this work a successful galenical development work is described leading to a "natural" GP product with superior pharmaceutical characteristics.

The development of a new garlic tablet requires few steps. One of them is the dissolution assay for garlic products. This kind of model assay simulates the release of active compounds in pharmaceutical formulations and it mimics the release in the gastro-intestinal compartments of the human body and gives information about which and how much of a compound is released for absorption in these compartments.

In the case of garlic preparation, the release of OSC is of interest, especially in the case of GP formulations where a complex mixture of active compounds is released which additionally are partly unstable in the release media.

As a first approach, the amount of all the OSC was evaluated by a recent ion-pair HPLC method [32].

The aim of the present study was to demonstrate the usefulness of the ion-pair HPLC method in the investigation of the anticancer and anti-inflammatory effect of some GP with different OSC composition. This analytical method is also a necessary tool for the control of the quality and stability of different pharmaceutical garlic tablets formulation for human consumption but also for the development of a new galenical formulation, which has to be dry enough so that to provide sufficient alliinase inhibiting condition.

2. Experimental

2.1. GP

2.1.1. Field trial

Garlic bulbs were produced at Crest in France. Certified seeds material variety Printanor were supplied by National Institute of Agronomy Research of Avignon in France. Sulphur fertilisation was provided by dehydrated $CaSO_4$ (50% SO_4) applied before bulb formation. Four levels of SO_4 were experimented: 0, 100, 200 and 400 kg/ha (S0 to S400). The treatments were designed in a randomized block experiment with four replicates of 100 plants each. They were naturally air-dried and cured when completely dry (3–4 weeks later).

2.1.2. Powdering

The bulbs were mechanically peeled in a four-step process, which consisted in heating (3–5 h at 50 °C), cracking, blowing and fine cutting with high pressure air blow. Slicing produced 0.5-mm thick pieces of garlic that were then dehydrated in the following hours. The dehydrating process started at 70 °C the first two hours, followed by 65 °C overnight and 60 °C with 10% air renewal for the last two hours. Samples were dehydrated separately to control the evolution of the dry matter content. When stabilized, the dry matter content was registered and the dry slices were cooled before to be powered in a mill (<25 μm particles).

2.2. Chemicals

Alliin, allicin, *S*-allyl-L-cysteine, γ-glutamyl-*S*-allyl-L-cysteine and γ-glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine were synthesized as described before [32]. Acetonitrile and DADS were purchased from Sigma–Aldrich (St. Louis, MI, USA).

2.3. Analytical procedure

Analysis of GP were performed with a Waters 616 pump and DAD 996 diode-array detector (Waters Corporation, Milford, MA, USA). Compounds were separated on a 150 mm × 3 mm i.d. × 3 μm particle C18 Hypurity Elite Thermo Quest column, at 38 °C (Thermo Hypersil Division, Keystone, Bellefonte, PA, USA) and quantified with UV at 208 nm. The column flow was 0.4 ml/min. The mobile phase consisted of: (a) 20 mM sodium dihydrogen phosphate +10 mM heptane sulfonic acid-pH 2.1 (adjusted with orthophosphoric acid 85%); and (b), acetonitrile −20 mM sodium dihydrogen phosphate +10 mM heptane sulfonic acid pH 2.1 (50/50) [32].

2.4. Sampling for GP analysis

For sample preparation 100 mg of GP was carefully ground and (a) incubated in 2 ml of 20 mM phosphate at pH 6.8 as a 1% (m/m) suspension for 20 min in a shaker (activating enzyme condition) or (b) incubated in 2 ml of methanol/water 80/20 (v/v) + 0.05% formic acid (inhibiting enzyme condition) or (c) incubated in 2 ml of methanol/water 50/50 (v/v) + 0.05% formic acid (partially inhibiting enzyme condition). The suspensions were centrifuged and 1–20 μl from the supernatant were injected to the HPLC. For dissolution assays 20 μl of the dissolution medium is injected directly to HPLC.

2.5. Commercial garlic tablets

Eleven commercial garlic products (Table 2), containing GP as active ingredient, obtained in various European countries (2 × Austria, 1 × Germany, 2 × Italy, 3 × Sweden, 1 × UK) were analysed for different quality parameters: the content of alliin, allicin and the total dipeptides to describe their quality. Furthermore, the stability of the products at ambient conditions (room temperature) over a period of 18 months was estimated.
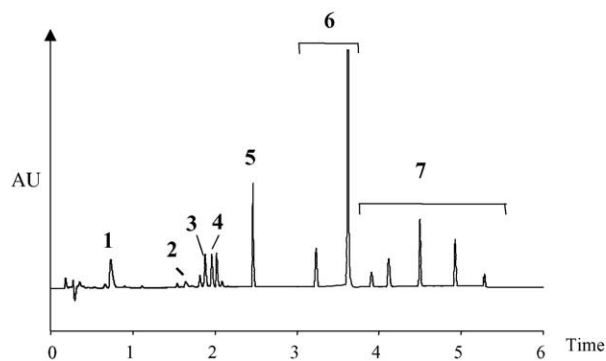

Fig. 1. HPLC profile of a garlic powder in partially inhibiting enzyme condition. See Table 1 for the identity of the compounds.

2.6. Dissolution testing of GP

The dissolution equipment is an apparatus according to the United States Pharmacopoeia (USP); apparatus 2 "paddle method". The temperature was 37 °C, the rotation speed is 100 rpm, the total volume is 500 ml, the sample amount is 10 g, the dissolution media is 66 mM phosphate buffer (for pH 5–9 experiments), 66 mM citrate buffer (for pH 1–5 experiments) and the dissolution additives are starch, microcristalline cellulose, cysteine, argenine, tyrosine, glutamic acid and ferric sulphate.

3. Results and discussion

3.1. GP for health experiment

Fig. 1 shows the typic chromatogram profile of a GP obtained under partially inhibiting enzyme condition. The alliinase is partially inhibited which allows to observe all OSC in a single run.

Some chromatogram profiles obtained under inhibiting enzyme condition and activating alliinase condition are presented in our previous work [32].
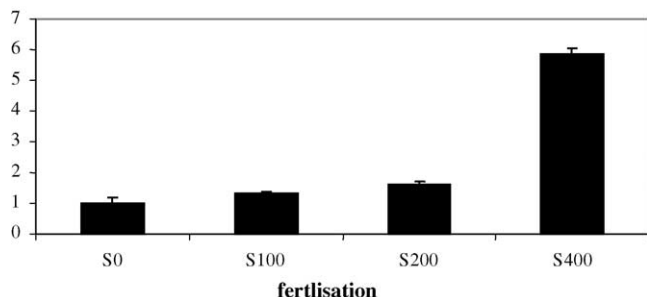
Table 2
List of commercial garlic products

Ref.	Country	Trademark	Company	Typ	Amount active ingredient	+ additional supplements	Legal status
AUT1	Austria	KnoVit™	MCM Klosterfrau GmbH	Sugar coated tablet	317 mg dry powder	Yes	Food suppl.
AUT2	Austria	Dr. Böhm™	Apomedica Graz	Sugar coated tablet	400 mg dry powder	No	Food suppl.
UK1	UK	Boots™	Boots Company PLC	Sugar coated tablet	400 mg dry powder	No	Food suppl.
NL1	Netherlands	Knoflox™	NL Pharma B.V.	Sugar coated tablet	80 mg dry powder	No	Food suppl.
NL2	Netherlands	Knoflook™	Optimax	Hard capsules	100 mg dry extract	Yes	Food suppl.
SWE1	Sweden	Kyolic Extra™	Anjo	Tablets	100 mg dry extract	Yes	Food suppl.
SWE2	Sweden	Kyolic Extra Plus™	Anjo	Tablets	100 mg dry extract	Yes	Food suppl.
SWE3	Sweden	Friggs™	Friggs	Sugar coated tablet	300 mg dry powder	Yes	Food suppl.
ITA1	Italy	Aglio™	Arkopharma	Hard capsules	310 mg dry powder	No	Food suppl.
SWI1	Switzerland	AVC Plus™	Whitehall-Robins AG	Sugar coated tablet	112 mg dry powder	Yes	Food suppl.
GER1	Germany	Kwai™	Lichtwer Pharma AG	Sugar coated tablet	300 mg dry powder	No	Drug

Fig. 2. Alliin contents in GP used for cancer experimentation.

Fig. 4. OSC content in GP used for the anti-inflammatory experiments. Bars represent the mean ± S.E.M., $n = 2$. *Different from unfertilized garlic, $p < 0.05$.

Fig. 2 shows the concentration of OSC (in g/100g GP) in the GP used to study the effect of OSC content on the hepatic drug metabolizing enzymes in rats [33]. The alliin content in GP was positively correlated with the increase of UGT activity. Actually, the pattern of enzyme modifications by consumption of GP was similar of that DADS, namely decrease of CYP2E1 and increase of UGT and GST activities.

The analytical method allowed showing the importance of the characterization GP on the anticancer effect. Actually, the number and the size of pre-neoplastic foci induced by AFB1 in rat liver decreases when supplementary diets was S400 (Fig. 3). These results are described elsewhere [34].

The content of OSC in the GP powder used to investigate their immunomodulatory effect is presented in Fig. 4. Actually, the activity of the NF-κB is reduced more than 1.5 times when blood samples were treated with fertilized garlic (S200) than with the unfertilized ones [30]. These results suggest that the positive effects of GP on cancerogenesis and the inflammatory diseases are strongly linked to the content of OSC and particularly to the allicin, release potential and DADS production. These results are described elsewhere [33].

3.2. Analysis of European garlic products

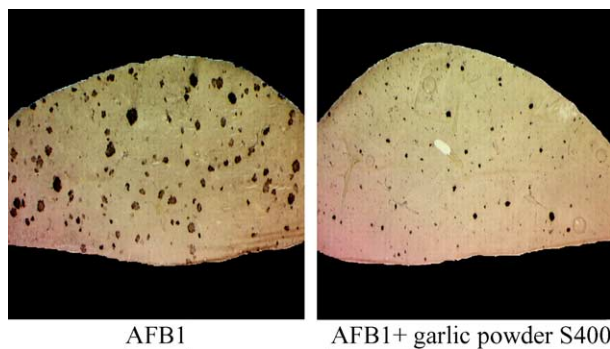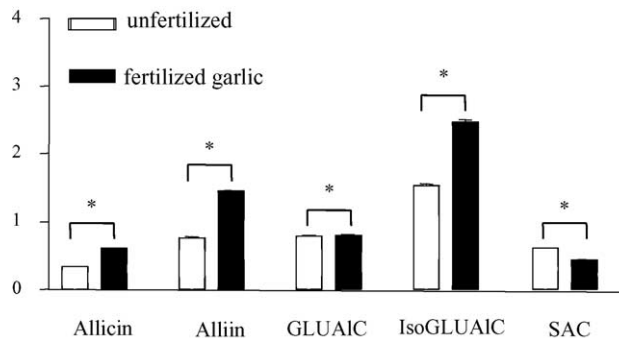The analytical test results show a very high variability of the quality of the manufactured GP. We analysed the content of alliin, allicin and storage dipeptides in the 11 European products (Fig. 5) and the majority of the products present a very different composition from what is claimed by the companies.

There may be two explanations for it: the first one is the legal status of the majority of the products that are sold as food supplements. For supplements the regulatory demands are obviously lower than for products possessing a drug (medicinal product) status. This seems to have a significant negative influence on the quality of the products. Only one product possesses the legal status of a drug (Germany). For this product values are found as claimed on the package.

Another important quality parameter for GP products is their stability. It is known that their content of alliin and allicin decreases in correlation to their storage time. This can be explained by the residual activity of the alliinase. The results clearly show the instability of GP products when stored under ambient conditions for 18 months (Fig. 6).

None of the products meets the 90% limit criterion for pharmaceutical products. The best products have a remaining activity of 70–90%. Most of the products fall below the 50% value.
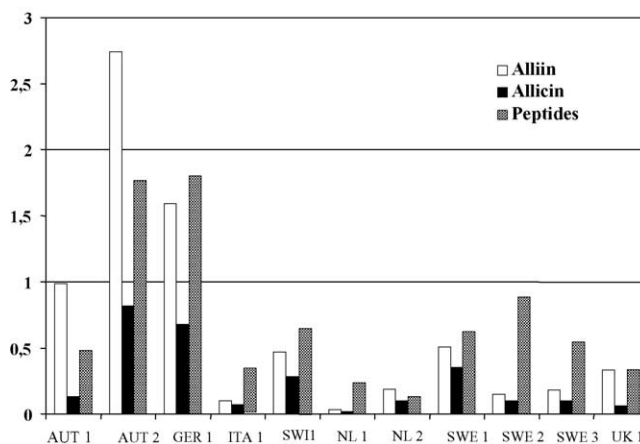

Fig. 3. Effect of garlic in the appearance of pre-neoplastic foci induced by carcinogen administration aflatoxin B1 (AFB1) in the rat liver.

Fig. 5. Content in percentage (%) of alliin, allicin and dipeptides of various European market products.
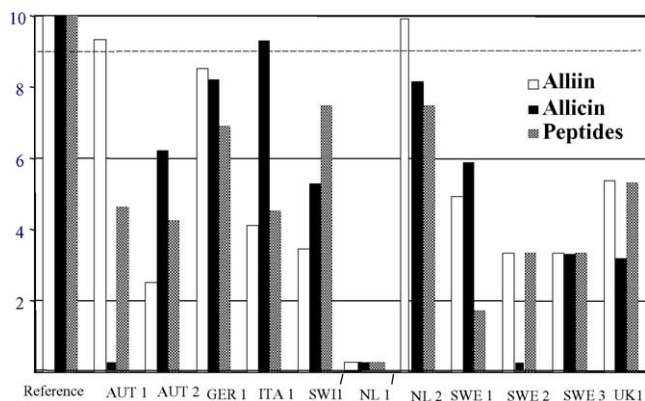
Fig. 6. Stability of garlic dry powder products estimated by analysing the content of alliin, allicin and dipetides after 18 months of storage.

Fig. 8. Alliin release profiles of from ground garlic powder related to pH.

3.3. Dissolution results of GP under different test conditions

The release behaviour of GP concerning OSC were tested under different release conditions (media). Typical release profiles obtained from pure GP are pH dependent as alliinase is pH dependent enzyme.

Under physiological conditions (pH 6.8) it can be clearly shown that GP once incubated releases very fast the highly water soluble compounds, amino acids and dipeptides (Fig. 7).

Within less than 5 min nearly 100% of these compounds are released from GP. The dipeptides are released without further transformation, whereas the alliin is enzyme transformed immediately into allicin after its release into the media. Not the complete theoretical amount of allicin was released, since advancing tablet dissolution the enzyme substrate contact becomes rate limited.

It was found that alliin is very fast released from the powder (dissolved in the media) nearly to a complete extend (100%) and immediately metabolised to allicin from the powder matrix within the pH range of 3.5–8.4 (Fig. 8). At pH 2.0, the alliin is completely released within a minute without further degradation to allicin.
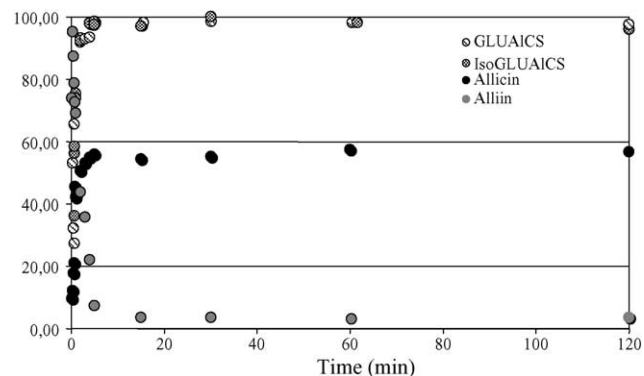
The formation/release of allicin from GP into the surrounding media occurs also very fast within the pH range of 3.5–8.4 (Fig. 9). The extend of release is found within a range from 45% to 60% of the maximum release potential as a function of pH. It can be shown very clearly that allicin is degraded in significant amounts, as a function of pH, too. Higher pH values seem to stabilise the allicin, whereas lower pH values accelerate its degradation. Interestingly, no further degradation products of allicin (e.g. sulfides, dithiines) can be detected in the release media under these conditions.

3.4. Development of a novel dosage form with superior stability

As a consequence of the disappointing stability results of the market products, a development concept for a novel dosage form was made.

The task of development should be focussed therefore on (a) a production process which avoids accumulation of humidity in the final tablet and (b) 100% water vapour tight packaging material which goes conform to consumer expectations.
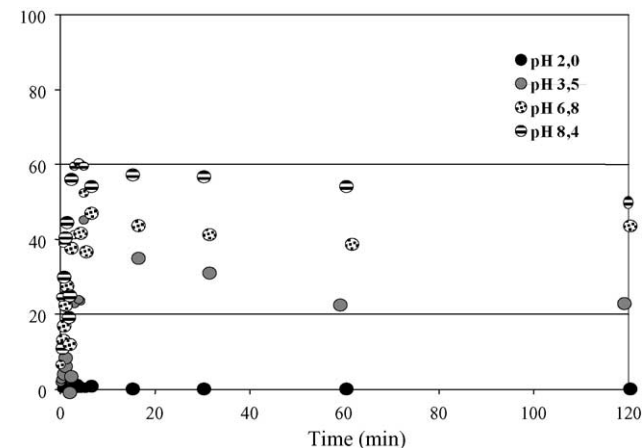

Fig. 7. OSC release profiles (%) of pure, ground garlic powder (pH 6.8).

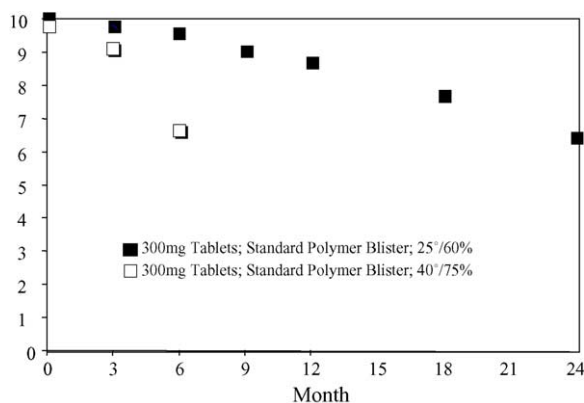Fig. 9. Allicin release profiles of from ground garlic powder related to pH.

Fig. 10. Allicin release profiles from 300 mg commercial available degrees in standard blister material.

The residual water has to be limited to less than 3%. Standard GP possess 5–6% residual water. When taking freeze–dried qualities together with very dry or pre-dried excipients tablets cores of sufficient quality could be obtained. Since freeze drying triples the cost of the GP, we succeeded too, by taking standard GP together with a sufficient amount of dry excipients, each contained less than 2% residual water.

The freeze-dried approach causes more difficulties in the core compression process, as the lack of humidity in the powder obviously weakens the important binding forces between the components in the tablet. Tablet cores with sufficient hardness (binding forces) could be obtained without problems. We gained a stable compression process on high speed machines for 100 mg and a 300 mg tablets.

A coating process reducing the water access to potential hygroscopic tablet cores to a minimum can be designed by using polymer suspensions in organic solvents instead of water.

We tried the "organic" way by producing organic film coated tablets and obtained a stable and reproducible coating process for 100 mg and 300 mg tablets. The production was run fully under the cGMP and ICH guidelines framework.
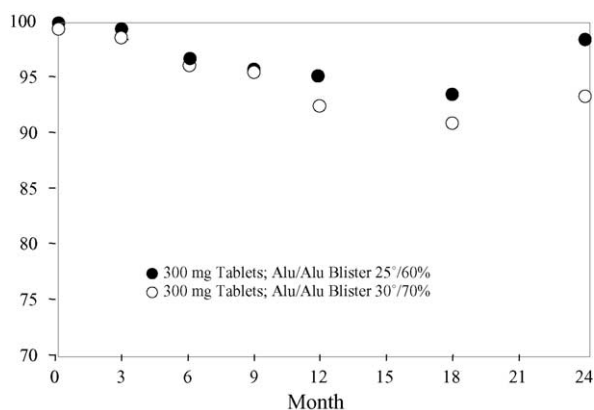

Fig. 11. Allicin release profiles from the novel film coated tablet containing 300 mg GP.

In a cGMP and ICH orientated approach, we re-developed the tablet core formulation for the needs of a water-based process by using more pre-dried excipients and chose coating polymer formulation providing a highly water vapour barrier. The choice of packaging material was orientated at the strict potential water barrier function of the material. Any kind of polymer blister packaging material failed the tests within 6–12 months (Fig. 10). The results of the novel film coated tablet formulation in different packaging material show clearly its stability up to 24 months (Fig. 11).

4. Discussion

The HPLC method allowed pointing the influence of OCS compounds composition on the health benefit effect of garlic. It also demonstrated its usefulness to improve the comprehension of the mechanism involved in health protective effect of garlic as well as for the quality control of different garlic pharmaceutical formulations.

This analytical method can be regarded as very economic to follow process development. The quality and stability of European GP containing market products are poor. The results presented within this paper were obtained by using the successfully developed HPLC method as a scientific tool. It is very useful and flexible in many different aspects of measuring OSC in garlic.

We can envisage extending the applications of this analytical tool to other *Allium* spp. as onion (*Allium cepa*) that was reported to possess beneficial effect on atherosclerosis [24,25]. Actually, its therapeutic potential could be more investigated and from the results, some high quality tablet formulation with a base of onion powder could be developed.

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