Determination of Active Ingredients (Alliin & Allicin) in Different species of Garlic Extracts By Using High Performance Liquid Chromatography

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Abstract

Alliin and allicin products were measured and determined by RP-LC with UV detection at 210 nm. Compounds were extracted from above various kinds of garlic with methanol / ethyl acetate and chromatographed on ODS with gradient elution .The aqueous Iraqi garlic extract has the highest concentration of alliin and allicin (17.9, 23.94) ppm respectively. The lowest concentration of allicin was found in French garlic extract 0.56 ppm and 4.3 ppm of alliin in Chinese garlic extract.

Allicin and alliin concentrations decreased with time 0.9-0.04%alliin, 1.2-0.00025% allicin after 9 days. Allicin concentration decreases to non- detectable amounts after 12 days.

Keywords: alliin, allicin, garlic, extract, ingredients, HPLC

Introduction

Allium is a genus of some 500 species belonging to the family Liliaceae. However only a few of these are important as food plants, notably onion, garlic, chive, leek, and rakkyo [(1)]. Garlic (Allium sativum) contains a large number of compounds, but only the thiosulfonate (allicin) has been found to have significant activity at levels representing normal garlic consumption (3-5 g/day). Allicin has been shown to be essential to most of the antimicrobial and

hypocholesterolemic effects of garlic and probably to most of the antithrombotic and antioxidant effects [2]. Garlic contains about 1% Alliin(S-allylcysteine sulfoxide), which converts to allicin (diallyl disulfide-oxide) in the presence of the enzyme alliinase [3,4]. Pharmacological research on garlic has shown the thiosulfinates free radical scavenging, inhibition of lipid peroxidation [5], inhibition of platelet aggregation [6], stimulation of fibrinolysis [7], and reduction of serum cholesterol and lipid levels.

Miron T. et al. describe the quantitative analysis of alliin and allicin, as well as of alliinase activity with 4-mercaptopyridine (4-MP) in garlic preparations[8]. Indirect quantitation of allicin by conversion of either diallyl disulfide or allyl mercaptan followed by gas chromatographic (GC) analysis has been reported [9]. The (GC) and (HPLC) methods all require allicin as an external standard [9,10].

The aims behind this study are:

1. To determine the active ingredients in garlic extracts.
2. To study the stability and conversion between unstable forms of Allicin.
3. To establish optimized method in liquid chromatography for quantitative evaluation.

Experimental

- **Pumping System:** A chromatographic system consists of one pump as solvent delivery model LC – 4A from Shimadzu Corporation, it contains a single small plunger reciprocating pump of constant discharging and quick suctioning tube which delivers the mobile phase to the chromatographic column at a flow rate of (0.1-10) ml/min. at a pressure of up to 500 $kgf.cm^{-2}$.

- Solvent Reservoirs: Round-bottomed flasks of (750 ml) capacity were used as reservoirs, (0.2-0.5 μm) stainless steel filter was fitted at the end of the PTFE (Poly Trifluoro ethylene) tubes transferring the mobile phase from the reservoir to the pump to avoid any possibility of damage to the pump caused by the ingress of any foreign particles.

- Connecting Tube: The tube connecting an injector to the pump system was stainless steel tubing (1.6 X 0.8 mm internal diameter (i.d)), connecting between injector and a column which is constructed of stainless steel (2.6 X 0.3 mm i.d) should be kept as short as possible to minimize sample diffusion and band spreading.

- Sample Injection System: The sample was introduced into the column by using a Rheodyne model SIL-1A syringe loading sample injector fitted with a (50**µl**) injection loop.

- Columns: The µ-Banda pack C-18 (250 X 4.6 mm i.d) stainless steel, (5 µm) particle sized as a reversed phase column was used in this study from Supelco. Inc.

- Column Oven: The column temperature was maintained by using column's oven model CTO –2AS temperature setting range $(0\text{-}90)^0$C (in steps of 1^0C) with temperature control range (Ambient temperature $+10^0$C-90^0C).

- **Detectors:** A Shimadzu SPD-2AS Ultraviolet- Visible detector with (8 µL). (1mm i.d X 10mm) flow cell was used. The Ultraviolet source is a deuterium lamp.

- **System Control Unit:** The controller solvent delivery, column oven temperature and wavelength unit model Shimadzu SCL- 4A system was used in liquid chromatography.

- Other equipments or instruments used in this study were to filter the solution before use for injection by:

Millipore filter /® 67 Moisheim –France / sterile, 0.45 µm, Cutter Lap, tube machine, model Tc-10, Germany, Blender: Matsushita Electric Industrial (Japan), Distil the water with Distiller blase, Gesellshaft, (Germany), Deionize water with Elgastat Deioniser, Erath essential optimum, flow rate: 2ml/min., England, pH-meter, PWS 420, Philips, Micro syringes Whittier, U.S.A, Ultraviolet – Visible Spectrophotometric Detector, Shimadzu (160 A), Japan.

- Sample Preparation for Separation of Allicin (Extract) [(11)]: Frozen fresh garlic cloves (20g) of each sample were peeled, chopped, blended with absolute ethanol: ethyl ether (1:1)

into blender, and extracted twice with (10ml) of cool mixture about (10min) for each extraction. The combined extracts were dried over anhydrous sodium sulfate and filtered. The extract was immediately subjected to HPLC.

- **Simultaneous Qualitative and Quantitative Determination of Alliin and Allicin by HPLC**: The isolated components were analyzed by ion-pair reversed –phase liquid chromatography with UV detection, using an octadecyl silane column with gradient elution. For the operation conditions see Table (1) [12].

Results & Discussion

- Extraction: Some components in aqueous garlic extracts are unstable at high temperature and especially at the boiling temperature of water that boiling garlic in water results in a loss of its activity and heating depresses alliinase activity, which retards the formation of sulfur compounds [9]. Alliin and enzyme alliinase are quite heat stable, but allicin is not, which is converted to several other sulfur compounds, such as diallyl sulfide, diallyl disulfide, etc. Alliin and alliinase are also stable when dry [13]. Water – soluble vitamins are sensitive to heat, alkali, and light [14]. Therefore concentrated water garlic extract was used under high vacuum, to avoid decomposition events, acid-base reaction, and oxidation.

-**Sample Preparation:** Samples rarely come in a form that can be injected directly into the instrument; some form of sample preparation usually is required [15]. In this study, sample preparation includes any manipulation of the sample prior to analysis, including techniques such as weighing, dilution, concentration, filtration, centrifugation, derivatization, and chromatography.

-Preliminary Tests: To know how to separate amino acids and water-soluble vitamins by RP-HPLC Technique, we have done test to get the best separation as retention times as in table (2). The separation conditions and results are tabulated in table (1).

- **Determination of Allicin in Aqueous Garlic Extract:** A simple and rapid HPLC method suitable for routine analysis of alliin and allicin was developed by Arnault I. et al.[16] using eluent containing an ion–pairing reagent (Heptane sulphonate) and a (150x3) mm column. Allicin was eluted after Alliin and the synthetic reference compounds were characterized by the same chromatographic method using diode-array UV detector.

The adding of an hydrochloric acid solution to garlic extract will inhibit the formation of allicin and in addition to this adding sulfite can be determinated, without interferes of allicin by reversed phase ion –pairing liquid chromatography with post –column detection [17].

Mochizuki E.N. et al. [18]. reported, before Arnault I. et al., that Allicin and alliin in garlic were determined simultaneously by ion-pair reversed liquid chromatography with diode array UV detection. In these articles, alliin is extracted from garlic and applied as external standard after purification by ion-exchange chromatography.

The method that consists of using an octadecyl silane (ODS) column with gradient elution from 0.01M phosphate buffer (pH2.5) with 5mM heptane sulfonic acid (A) to 0.01M phosphate buffer (pH 2.5) – acetonitrile [(1:1); B] can be used to analyze fresh garlic, spices, garlic preparations, and health foods.

The limits of detection were between 1.76 and 9.40 ng for allyl methyl sulphide and dimethyl disulphide, respectively, and percentage recovery rates of aqueous garlic extracts ranged from 74.4% for the first to 90.3% for dipropyl disulphide, by using GC and MS [19].

By applying a developed liquid chromatography technique based on florescent detection of 9-fluorenyl methyl chloroform ate derivatives, Methyl-L-Cysteine sulfoxide and 2-propenyl – L-Cysteine sulfoxide were determinated in garlic with detection limits less up to 2.5mg /100g fresh weight [20].

The major sulphoxide component that is found in garlic was (+)-S-allyl-L-Cysteine sulphoxide (>95%) can be determinated by HPLC on two spherisorb columns (ODS) in series with elution of extracted garlic, alliinase was inhibited by addition of 10mM of hydroxyl

amine during extraction and eluted with 2M NH_4OH through an Amberlite IR-120 anion exchange column (H^+ form) [21].

Kasuga S. et al. [22] found, in Japanese garlic, that RGJ contained 0.162% allicin but no alliin, HGJ contained 0.266% alliin and 0.001% allicin, DGP contained 0.462% alliin but no allicin, and AGE contained 0.003% alliin but no allicin.

In our work we applied Mochizuki E.N. et al [23] method to determine simultaneous alliin and allicin by ion- pair reversed liquid chromatography using UV detection [See Table (1)].

As shown in Table (2), it is seen that fresh Iraqi garlic extract is high in alliin (17.9 ppm, 0.9%) and allicin (23.94 ppm, 1.2%) concentrations. [See Fig. (3) to Fig. (7)].

The concentration of alliin and allicin in Iraqi garlic extract was observed to decrease gradually through a period of 3 days internal for 9 days, from [0.9%alliin, 1.2% allicin, as a fresh to 0.04% alliin, 00.25% allicin after nine days], then another new peak arose in the analysis chromatogram, this might belong to conversion of alliin and allicin to more other stable sulfur compounds, probably like dithiines, alkyl sulfides, ajones, and others. [See Table (3)], [See Figures (Fig. (3), and Fig. (8) to Fig.(11)].

The optimum condition for separation of standard alliin and allicin were applied as shown in typical chromatograms [See Fig. ((1)-(2))]. The same conditions were used for separation of Iraqi, Iranian, Lebanese, French, and Chinese garlic extracts as shown in chromatograms [See Fig. ((3)-(7))]. The same conditions were applied for separation of Iraqi garlic extract as a fresh and after 3,6,9, and 12 days [See Fig. ((3), (8-11))].

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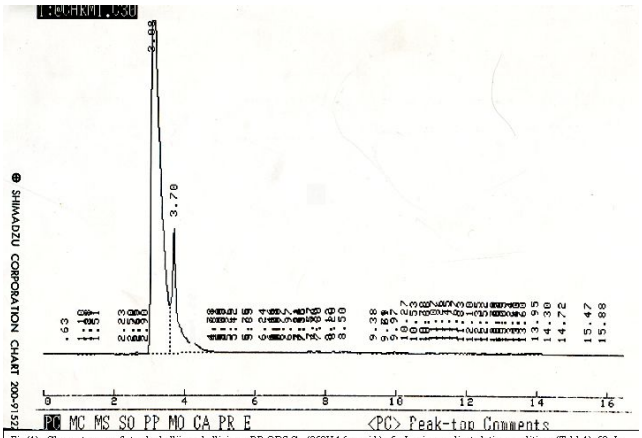

Fig.(1) : Chromatogram of standard alliin and allicin on RP-ODS C_{18}(250X4.6 mm id) , 5 µL using gradient elution conditions (Table1) ,50µL was injected.

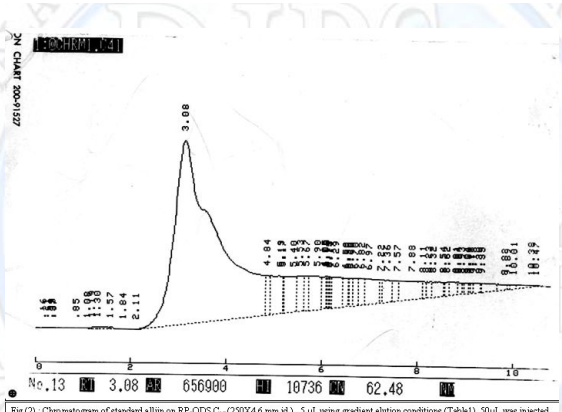

Fig.(2) : Chromatogram of standard alliin on RP-ODS C_{18}(250X4.6 mm id) , 5 µL using gradient elution conditions (Table1) ,50µL was injected.

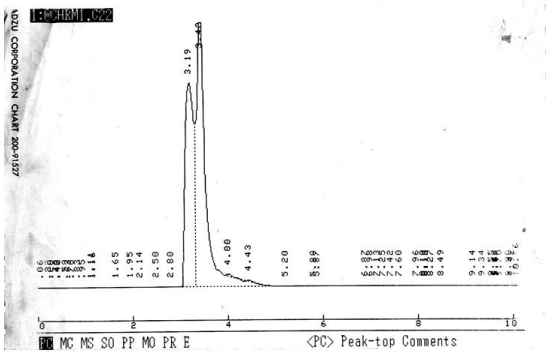

Fig. (3) : Chromatogram of alliin and allicin in fresh Iraqi garlic extract on RP-ODS C_{18}(250X4.6 mm id), 5 µL using gradient elution conditions (Table 1) ,50µL was injected.

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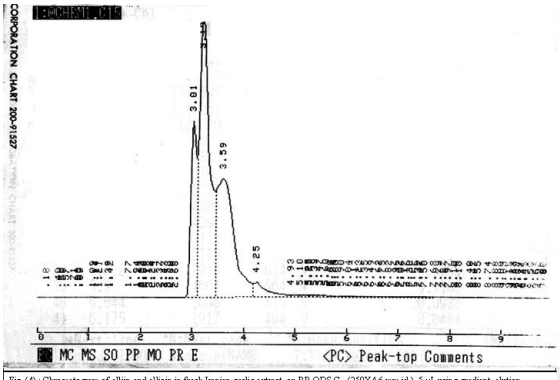

Fig. (4) : Chromatogram of alliin and allicin in fresh Iranian garlic extract on RP-ODS C_{18} (250X4.6 mm id), 5 µL using gradient elution conditions (Table 1) ,50µL was injected.

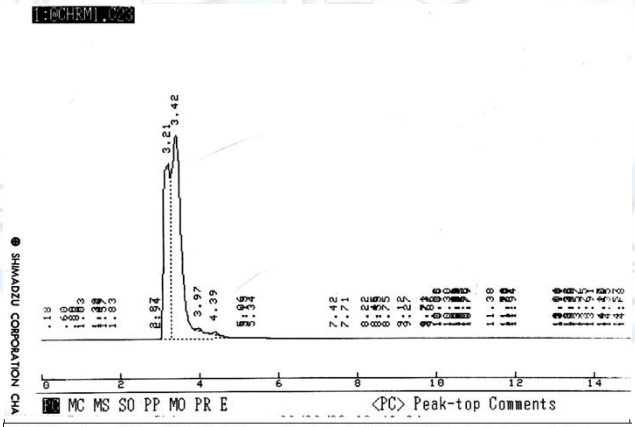

Fig. (5) : Chromatogram of alliin and allicin in fresh Lebanese garlic extract on RP-ODS C_{18} (250X4.6 mm id), 5 µL using gradient elution conditions (Table 1) ,50µL was injected.

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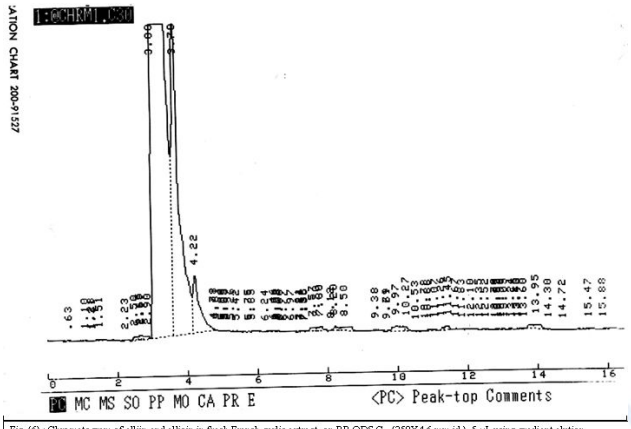

Fig. (6) : Chromatogram of alliin and allicin in fresh French garlic extract on RP-ODS C_{18} (250X4.6 mm id), 5 µL using gradient elution conditions (Table 1) ,50µL was injected.

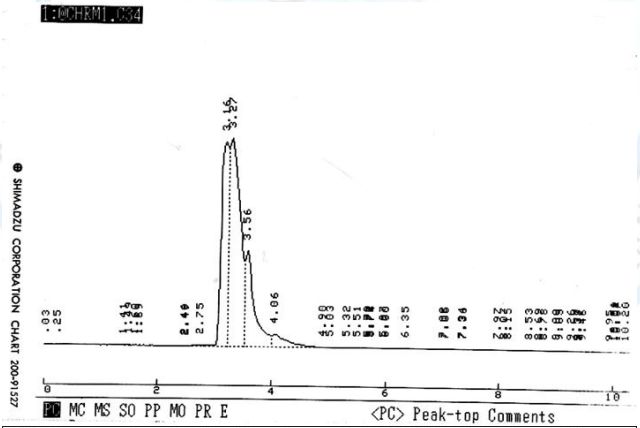

Fig. (7) : Chromatogram of alliin and allicin in fresh Chinese garlic extract on RP-ODS C_{18} (250X4.6 mm id), 5 µL using gradient elution conditions (Table 1) ,50µL was injected.

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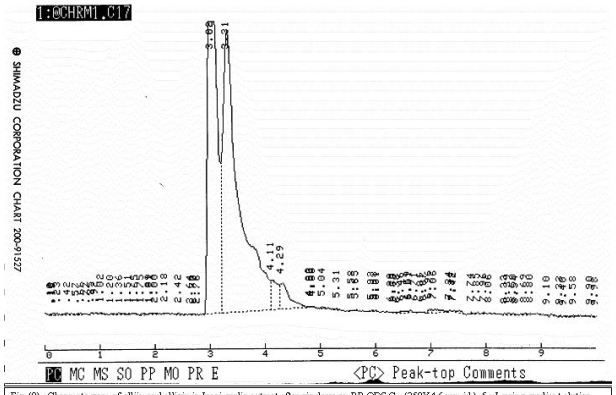

Fig. (9) : Chromatogram of alliin and allicin in Iraqi garlic extract after six days on RP-ODS C_{18}(250X4.6 mm id), 5 µL using gradient elution conditions (Table 1) ,50µL was injected.

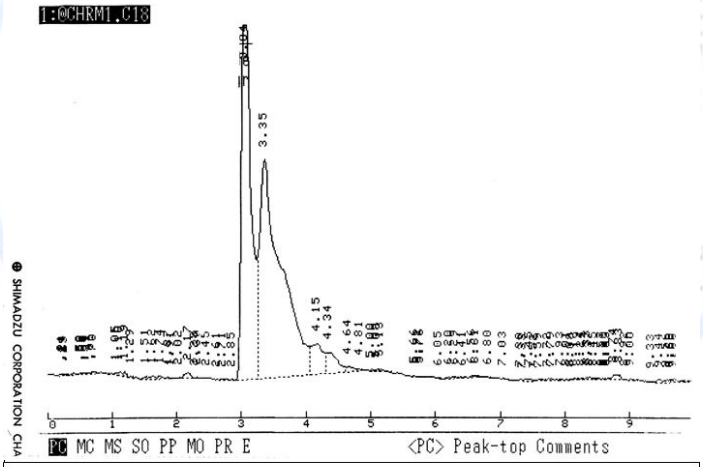

Fig. (10) : Chromatogram of alliin and allicin in Iraqi garlic extract after nine days on RP-ODS C_{18}(250X4.6 mm id), 5 µL using gradient elution conditions (Table 1) ,50µL was injected.

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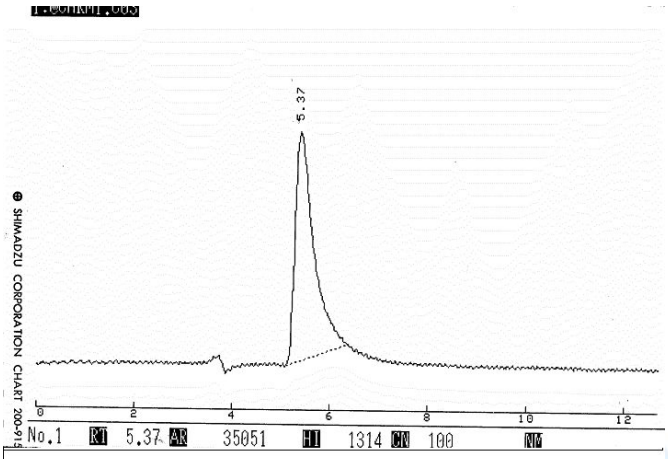

Fig. (11) : Chromatogram of Iraqi garlic extract after twelve days on RP-ODS C_{18} (250X4.6 mm id), 5 µL using gradient elution conditions (Table 1) ,50µL was injected.

Table (1):- The HPLC gradient conditions of Separation of Allicin and Alliin.

Mobile Phase (A) at pH 2.5 by Phosphoric Acid	((10mM) Potassium dihydrogen Phosphate + (5mM) 1- Heptane Sulfonic Acid)		
Mobile Phase (B) at pH 2.5 by Phosphoric Acid	[10mM Potassium Dihydrogen Phosphate + Acetonitrile (ACN)] (1 :1)(v :v)		
Flow Rate	1.0 mL. $Min.^{-1}$		Injector Volume: **50 µl**
*Detection: **210 nm***	**Temperature:** 40 ºC		
Time (min)	0.01	20	25
Mobil phase (B) %	0	100	Stop

Table (2): Retention Times, Area, and Concentration of Alliin and Allicin in Standard, Iraqi, Iranian, Lebanese, French, and Chinese Garlic Extracts.

Garlic Extract	Alliin				Allicin			
	t_R, min.	I. Area	Conc., ppm	Conc., %	t_R, min.	Area	Conc., ppm	I. Conc., %
Standard	3.080	337105	2.50	0.00025	3.700	569480	2.50	0.00025
Iraqi	3.197	2413007	17.90	0.900	3.425	5452475	23.94	1.200
Iranian	3.190	1776597	13.18	0.660	3.590	1316251	5.78	0.290
Lebanese	3.210	788764	5.85	0.293	3.420	1523505	6.69	0.335
French	3.080	681753	5.10	0.260	3.700	126612	0.56	0.030
Chinese	3.16	579170	4.30	0.22	3.27	1333754	5.90	0.290

Table (3): Retention Times , Area, and Concentration of Alliin and Allicin in Standard and Iraqi Garlic Extracts After Period of Times.

Iraqi Garlic Extract	Alliin				Allicin			
	t_R, min	Area	Conc., ppm	Conc., %	t_R, min.	Area	Conc., ppm	Conc., %
Standard	3.080	337105	2.50	0.00025	3.700	569480	2.50	0.00025
Fresh	3.197	2413007	17.90	0.900	3.425	5452475	23.94	1.200
3 days	3.023	137300	1.02	0.05	3.258	270562	1.20	0.060
6 days	3.024	130323	0.97	0.05	3.318	159137	0.70	0.040
9 days	3.043	93948	0.70	0.04	3.351	116831	0.50	0.025

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تقدير المكونات الفعالة (الالييين و الاليسين) في أنواع مختلفة لمستخلصات الثوم باستخدام تقنية كروموتوغرافيا السائل العالي الأداء

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الخلاصة

تم قياس وتعيين المركبين (alliin , allicin) بتقنية كروموتوغرافيا السائل ذات الاداء العالي بكاشف الأشعة فوق البنفسجية عند طول موجي (210 nm). استخلصت المركبات لأنواع الثوم الخمسة أعلاه بمزيج absolute ethanol / ethyl ether (1:1) وقيست كروموتوغرافيا باستخدام عمود [ODS C_{18} (250X4.6 mm id)]. تبين إن تركيز الاليسين والالييين يكون الاعلى في المستخلص المائي للثوم العراقيppm (23.94, 17.9) على التوالي . بينما اعطى مستخلص الثوم الفرنسي اقل نسبة من الاليسين (0.56 ppm) في حين المستخلص الصيني الاقل نسبة من الالييين (4.3 ppm) .

ان تركيزي الاليسين والالييين يتناقص مع الزمن من (0.9-0.04%) الالييين ،(1.2-0.00025%) الاليسين بعد تسعة ايام) حتى تكون كمية الاليسين المتناقصة غير محسوسة بعد اثنى عشر يوما، بسبب تفكك الاليسين المتكون مع الزمن.

الكلمات المفتاحية: الالينيين, الاليسين, الثوم, مستخلص, مكونات فعالة, كروماتوغرافيا السائل العالي الاداء.