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Diagnosing the bioactive compounds in Iraqi garlic (*Allium sativum*) by GC-MS and HPLC

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Abstract This study including diagnosing the bioactive compounds releasing from an action of alliinase with alliin in the Iraqi garlic by GC-MS and HPLC technique. The bioactive compounds resulting from the garlic by GC-MS were heptane,2,4-dimethyl,cyclopropyl bromide, octane,5-ethyl-2-methyl, diallyl disulphide, trisulfide,di-2-propenyl,tetrasulfide,di-2-propenyl,3-vinyl-1,2-dithiacyclohex-4-ene,3-vinyl-1,2 dithiacyclo hex-5-ene and eicosane at different percentages through periods of time ranged between 5-30 minutes when following its activity by GC-MS. The best time for an activity was found at 30 minutes. Diallyl disulphide was the highest percent of 51.92% and tetrasulfide, di-2-propenyl at percent of 19.49%, as well as standard allicin and prepared allicin that is the main product of the enzymatic decomposition was diagnosed by HPLC technique of Iraqi garlic at the same periods with high concentration 39.244%.

KEYWORDS: Alliinase; Iraqi garlic; allicin; GC-MS; HPLC

INTRODUCTION

Garlic comes back to *Allium* genes, which has been disputed for a long time in terms of the taxonomic position, so the following hierarchy of *Allium* genes is adopted: *Liliopsida*, *Liliidae*, *Liliianae Amaryllidales*, *Alliaceae*, *Allioideae*, *Allieae* and *Allium*, the garlic comes second in importance in *Allium* plants, and grows in large parts of the world as a medical plant and important spice (1). Garlic contains high levels of phosphorus, potassium, sulphur and zinc, moderate levels of selenium, vitamin A and vitamin C and low levels of calcium, magnesium, iron, manganese, sodium and B-complex vitamins, as well as about 33 sulphur compounds and 17 amino acids (2). Sulphur compounds include alliin, allicin, ajoene, allyl, propyl disulfide, diallyl, trisulfide, s-allyl-cysteine, vinylidithiines, S-allyl-mercaptocysteine, etc., as well as a number of enzymes such as alliinase, peroxidase, myrosinase and others (3). Allicin (diallyl thiosulfate) is responsible of the sharp ideal aroma and therapeutic properties of the garlic (4-6). The unique flavor and health-promoting properties in the garlic is generally attributed to the rich content of sulfur-containing compounds such as alliin and γ -glutamylcysteine and their derivatives, when smash or crush or cut up cloves of fresh garlic lead to the free enzyme alliinase from gaps and quickly convert alliin to allicin, which is a very unstable compound and turns immediately to a huge number of products containing sulfur and soluble in the oil including compounds diallyl disulfide (DADS), diallyl sulfide (DAS), diallyl trisulfide (DATS), diallyl tetrasulfide (7). Alliinase is a glycoprotein belongs to the wrapped type I from enzymes that dependent on coenzyme pyridoxal-5-phosphate (PLP) from family aminotransferase which plays a role of a



catalyst in stimulating the transformation of the non-protein amino acid alliin (+) S-allyl-L-cysteine sulphoxide to allicin (diallyl thiosulfinate) (8). Explain (9) there are two types of organic sulphur compounds that have been derived from garlic, the first class dissolved in the fat, including diallyl sulfide, diallyl disulfide, diallyl trisulfide, allyl methyl sulphide, (1E)-1-(allyl disulphanyl)-3-(allyl sulphanyl)-1-propene, thiocremonone and 2-vinyl-4H-1,2-dithiin, the sulphur compounds which dissolved in the fat are derived from the sulphur-based compound alliin, which accounts for 80% of cysteine sulphoxides compounds, and the second is dissolved in the water including S-allylcysteine and s-allyl mercaptocysteine that obtained during the long incubation period of the crashed garlic in the aqueous solution by the aging process.

Found (10) there was no allicin or its metabolic material in human organs or body fluids when consuming garlic orally by detecting those compounds in saliva after consuming 20 tablets of garlic coated pills for more than 24 hours and 13 different time periods using GC-MS technology but was clarified the vital availability of allicin through respiratory studies. Vinylidithiin is a sulphur compound dissolved in the oil, first was diagnosed as a result of the thermal decomposition of allicin during the analysis of the gas chromatography by (11). Two different types of vinylidithiin were discovered, one of which is 3-vinyl-4H-1,2-dithiin or 1, 2-venyldithiin and the other 2-vinyl-4H-1,3-dithiin or 1,3-venyldithiin, both have an antioxidant activity and reduced cholesterol (12). Ajoene is one of the derived compounds found as a main sulphur compound in oily garlic extract, is described as a strong inhibitor to the assembly of blood platelets inside and outside the body, consisting of the self-condensation of the allicin molecules and not found in the dry garlic powder, and there are two isomers of ajoene, one of which is an cis form called Z-ajoene and the other in trans form, called E-ajoene, and both have different degrees of vital specialization (13). This search was carry out to know the bioactive compounds in Iraqi garlic by GC-MS and the best time for extraction them.

MATERIALS AND METHODS

Materials. Fresh Iraqi garlic is purchased from local markets in Basra, Iraq. Cleaning garlic, remove the outer parts, peels, washed with distilled water and kept at the refrigerator temperature at 4 °C in polyethylene bags until use.

Chemicals. Alliin and allicin were bought from Santa Cruz Biotech.Co., USA, methanol from BHD Co., England.

Apparatuses. Gas Chromatography – Mass Spectrophotometry (GC-MS), Shimadzu Co., Kyoto, Japan; High Performance Liquid Chromatography (HPLC), Shimadzu Co., Kyoto, Japan.

Enzyme extraction. Peeled and chilled of garlic cloves (100 g) were crushed in a plastic mortar and leave for 30 minutes, then added a buffer sodium phosphate 20 mM, pH 6.5 at a ratio of 1:2 (w/v) contains EDTA 5 mM, NaCl 5%, PLP 20 µM and glycerol 10% (14) and also left for 30 minutes, then the mixture was filtered by layers of cheesecloth, followed by centrifugation at 10000 rpm for 30 min. on temperature 4°C. Enzyme concentration by adding ammonium sulfate to the crude extract gradually with saturation ranged 30-70%, stirring continuously for 4 hours, then the precipitate was collected and dissolved in buffer consists

of sucrose 15% (w/v) and NaCl 1% and dialyzed for 24 h at 4°C, the product was lyophilized and kept for studying enzyme characterization later.

Enzyme assay. Enzyme activity was estimated spectrally by measuring pyruvate concentration (Standard curves was prepared by using sodium pyruvate) according to the method of (15), the reaction mixture consists of 0.5 ml enzymatic extract, 0.5 ml standard alliin, 0.5 ml 2,4 dinitrophenylhydrazine (0.0125% of DNPH in 2N of HCl), then put in a water bath at 37°C for 15 min. after the incubation period adding 2.5 ml of 0.6N NaOH, Pyruvate was measured using a UV-Vis spectrophotometer (Apel 303 UV, England) at 420 nm. The final concentration of pyruvate was measured as micromole/min.

Protein assay. A concentration of protein was measured using bovine serum albumin (BSA) as standard (16).

The Best Time for Alliinase Activity in Iraqi Garlic. Estimated enzymatic activity(unit/ml) and specific activity (unit/mg) in Iraqi garlic record for different time periods (5,10,15,20,25,30) min., extracted with ratio 1:2 (w/v) using buffer solution sodium/potassium phosphate 0.2M, pH 6.5 preparation from sodium phosphate dihydrogen (NaH_2PO_4) and potassium phosphate monohydrogen (K_2HPO_4), PLP 0.1M, glycerol 10% (17).

GC-MS. Diagnosing the bioactive compounds resulting enzymatically from the Iraqi garlic extracts prepared for different periods (5,10,15,20,25,30) min. by GC-MS. Crashed 5 g of the Iraqi garlic in a plastic mortar and left for the periods above, added 10 ml of petroleum ether solvent to the extracts and left at room temperature for 30 minutes, then filtered by Millipore filter 0.22 μm and injected 1 μl of the Iraqi garlic extract in GC-MS (Split at ratio 1:30 ; Rtx-5MS capillary column (cross band 5% diphenyl-95% dimethylpolysiloxane) , 30 m(L) \times 0.32(i.d.) with a 0.25 μm film thickness; injection temperature 250 °C ; Electron Impact Ionization (EI) ; recorded in intervals from 30-170 m/z), separated peaks was matching spectra database in the program library NIST08.LIB.

HPLC. Allicin was extracted from the Iraqi garlic for different periods (5,10,15,20,25,30) min., crushed 10 g of garlic in 12 ml of combination from distilled water and methanol at ratio 1:1 (v/v) and then centrifuged at 5000 rpm for 10 min. (18). The supernatant was filtered by Millipore filter 0.22 μm and injected 1 μl of the extract in HPLC (column C18with a size of 4.6 mm \times 250 mm; column temperature 40 °C; UV detecting at 240 nm; column was equilibrated with distilled water and methanol (for HPLC) at ratio 50:50; flow rate 0.5 ml/min.). Allicin concentration in the garlic extracts was measured from standard allicin curve.

RESULTS AND DISCUSSION

Best Time for an Alliinase Activity in Iraqi Garlic. Note from the **Figure 1** increasing enzyme specific activity gradually starting from the fifth minute of appreciation which reached 122.66 (unit/mg protein) even thirty minute with specific activity 166.76 (unit/mg protein), so the time of 30 min. was chosen as the best

time for enzyme activity, while enzymatic activity ranged between 182.4-220 (unit/ml). Gradually increasing activity over time may be due to the effect of the pH of the buffer solution through its effect on the ionization groups for the effective site or the effect on an active groups of substrate or effecting on the composition of the enzyme or substrate, so the increasing in activity may be the result from the use of high concentrations of substrate or possibly of temperature effects on the reaction speed, as well as increasing the temperature 10 °C sped the reaction, which effect on the activity of the enzyme (19).

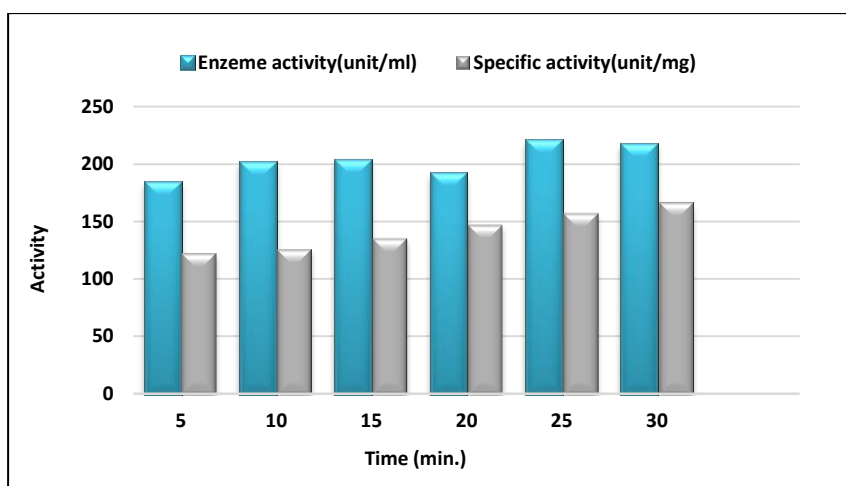


Figure 1. Alliinase activity in an extract of the Iraqi garlic during different periods

Diagnosing the Bioactive Compounds in the Iraqi Garlic by GC-MS. Results of the analysis by GC-MS described in **Figure 2**. The bioactive compounds resulting from the garlic by GC-MS were heptane,2,4-dimethyl; cyclopropyl bromide, octane,5-ethyl-2-methyl, diallyl disulphide, trisulfide,di-2-propenyl ,tetrasulfide,di-2-propenyl,3-vinyl-1,2-dithiacyclohex-4-ene,3-vinyl-1,2 dithiacyclohex-5-ene and eicosane at different percentages through periods of time ranged between (5-30) minutes. Several sulfurous compounds derived from garlic, but there are two different compounds found in all periods. Diallyl disulfide ratio rising gradually in the extraction began from the fifth minute 24.31% until the thirty minute 51.92%, while the other compound was 3-vinyl-1,2-dithiacyclohex-5-ene which was declined gradually over the time from 34.31% in the fifth minute to 5.52% in the thirty minute of extraction.

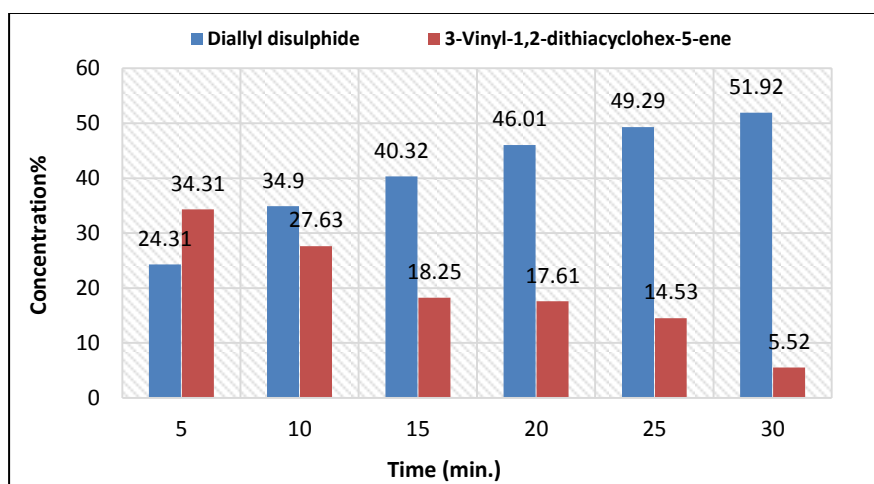


Figure 2. Concentration of diallyl disulphide and 3-vinyl-1,2-dithiacyclohex-5-ene in the Iraqi Garlic diagnosed by GC-MS during different periods

Allicin is considered a thermally unstable compound and degraded rapidly by heat to many sulphides compounds, although allicin volatilizes rapidly at room temperature but exposure the garlic extract to the high injection temperature of GC-MS (280°C) considered a catalyst for the disintegration of thiosulfinate compounds where evaporated garlic compounds after crushing by heat and produces mostly disulfur compounds (18,20), other studies proved the disintegration of compound allicin in garlic extract using high injection heat of GC-MS yield vinylidithins compounds (18). A few studies dealt with the search of 3-vinyl-1, 2-dithiacyclohex-5-ene and did not delve in garlic accurately or its therapeutic effects in the long term, however, the compound is lowering cholesterol and antioxidation (21). The cause of declining of 3-vinyl-1, 2-dithiacyclohex-5-ene with the high proportion of diallyl disulfide over time may be attributed to the quick speed analysis by the injection temperature of GC-MS and graduating the time period used in the extraction or may be its a solubility degree in petroleum ether and its volatile less than diallyl disulfide. (**Observing Figure 3 and Table 1**).

Table 1. Retention time and concentration% of bioactive compounds in the Iraqi garlic during different times diagnosed by GC-MS

Compounds	R.time (min.)	Concentration%					
		5 min.	10 min.	15 min.	20 min.	25 min.	30 min.
Heptane, 2,4-dimethyl-	3.512	1.21	1.56	13.64	1.35	0.85	14.46
Cyclopropyl bromide	6.138	6.24	7.92	1.42	8.79	13.69	1.78
Octane, 5-ethyl-2-methyl-	10.158	2.04	2.57	1.99	1.8	1.28	1.69
Diallyl disulphide	10.673	24.31	34.9	40.32	46.01	49.29	51.92
Tetrasulfide, di-2-propenyl	11.033	2.05	1.62	1.4	1.37	1.14	19.49
Diallyl disulphide	11.201	13.61	9.85	7.9	12.69	11.23	2.36
3-Vinyl-1,2-dithiacyclohex-4-ene	13.300	14.00	11.81	12.79	7.65	6.12	1.41
3-Vinyl-1,2-dithiacyclohex-5-ene	13.917	34.31	27.63	18.25	17.61	14.53	5.52
Trisulfide, di-2-propenyl	15.803	1.36	1.04	1.3	1.14	1.32	0.58
Eicosane	18.499	0.88	1.1	0.99	1.58	0.55	0.77

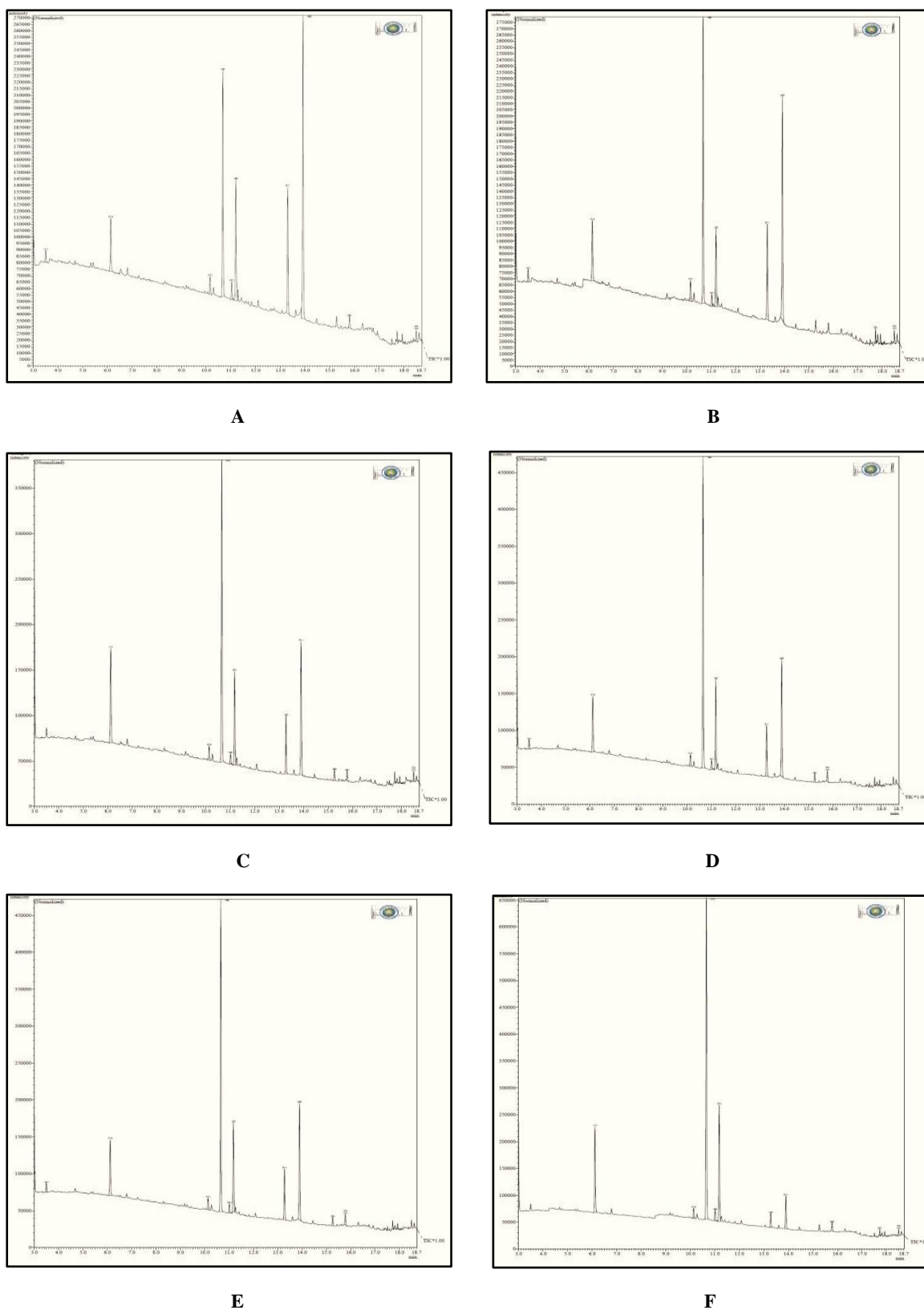


Figure 3. Chromatogram of the Iraqi garlic extract diagnosed by GC-MS for the periods (A: 5 min.; B: 10 min.; C: 15 min.; D: 20 min.; E: 25 min.; F: 30 min.)

Diagnosing Allicin in the Iraqi Garlic by HPLC. The resulting peaks were diagnosed by HPLC using UV detector at wavelength 240 nm, matching peaks separated with standard allicin which diagnosed through retention time 16.789 min. (**Figure 4**), as well as a concentration of allicin was measured in the extract exactly through an equation of the allicin standard curve ($r^2=0.9982$). Showing in the **Figure 5 and Table 2** increasing concentration of allicin in different times by increasing the area under the curve, and got high concentrations of allicin in the prepared extract higher than found in the standard sample. In the fifth minute allicin concentration was 5 (mg/ml), then increased concentration of allicin in the minutes (10, 15, 20, 25) up to 5.33, 5.43, 5.53, 6.78 (mg/ml) respectively, while the highest concentration was at thirty minute reached to 6.80 (mg/ml). Alliin and allicin were diagnosed and assayed in different types of garlic (Iraqi, Iranian, Lebanese, French and Chinese), compare with standard samples diagnosed by reversed phase HPLC technology, found that the concentration of allicin in Iraqi garlic was higher than the rest types of garlic, its concentration reached to 23.94 ppm followed by the Chinese, Iranian, Lebanese and French garlic with 6.69, 5.90, 5.78 and 0.56 ppm respectively, under the same conditions of extraction and preparation of the samples and the working conditions (22), the obtained results agree with the conclusions who reached (22) about containing Iraqi garlic a high concentration of allicin despite of differing conditions of extraction and type of HPLC apparatus using in this experiment. A presence of high concentrations of allicin in the prepared extracts was attributed to the effect of ultraviolet radiation on allicin and its high responsiveness to changes in absorbance at this wavelength depending upon the amount of allicin in the prepared sample (23). HPLC technique is the ideal techniques using to diagnose allicin not only for being given a high concentration of allicin as well as not effect on allicin structure due to the low injection temperature which reduce a chance of the rapid decomposition for allicin in high temperatures and prevents breakage inside the column and keeps it as much as possible from the decomposition to variety sulphurous products.

Table 2. Retention time and concentration% of prepared allicin during different times diagnosed by HPLC

Time	R.time(min.)	Concentration%
5 min.	16.965	30.245
10 min.	16.975	31.838
15 min.	16.770	32.212
20 min.	16.770	32.259
25 min.	16.948	33.328
30 min.	16.951	39.244

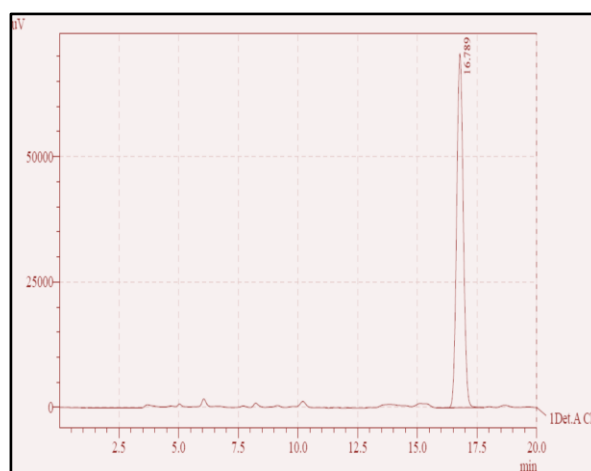


Figure 4. Chromatogram of standard allicin diagnosed by HPLC

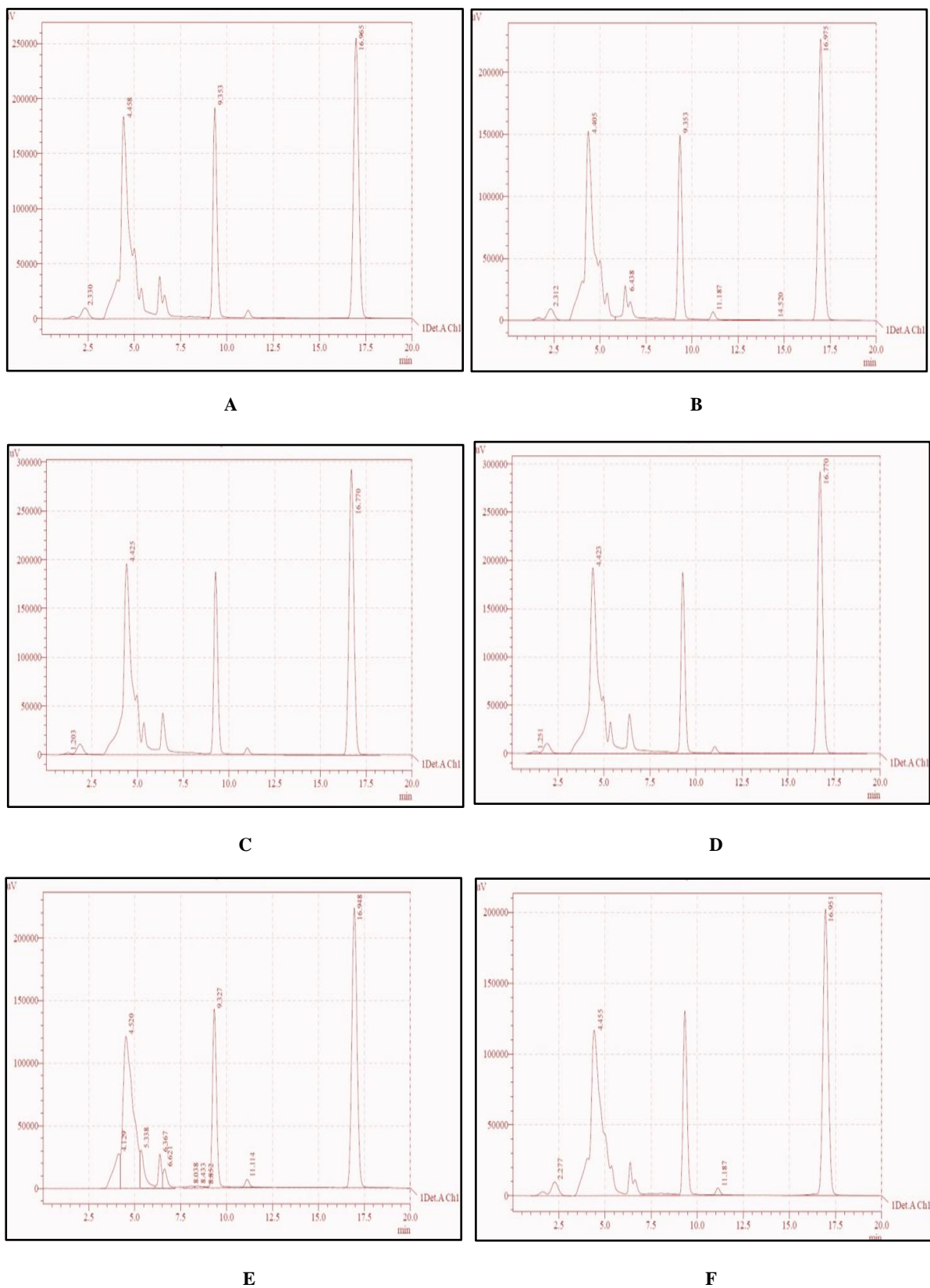


Figure 5. Chromatogram of allicin in the Iraqi garlic extract diagnosed by HPLC for the periods (A: 5 min., B: 10 min., C: 15 min., D: 20 min., E: 25 min., F: 30 min.)

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