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RAPID AND SIMPLE ANALYSIS OF ALLICIN IN *ALLIUM* SPECIES BY LC-CIS-MS/MS

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ABSTRACT. A simple liquid chromatography-coordination ion spray-mass spectrometry method (LC-CIS-MS/MS) for analysis of allicin in *Allium* species extracts has been developed. Allicin was chromatographic separated under isocratic conditions using a mobile phase of 1 mM ammonium acetate. A silver nitrate solution was post-column added to enhance the allicin detection by formation an ionized coordinated complex. The overall time of one analysis was 1 min. The detection of allicin was performed in multiple reaction monitoring mode using an ion trap mass spectrometer with electrospray positive ionization. The linearity domain was established between 18 and 864 µg/mL. Inter-day accuracy and precision were less than 11% and 2.2%, respectively.

Keywords: *allicin, Allium extracts, liquid chromatography, coordination mass spectrometry*

INTRODUCTION

Allium species have been used for food and medicine for thousands of years, especially *Allium sativum* (garlic) and *Allium cepa* (onion), and recently interest in other species has been increasing [1,2]. Garlic is considered as a medicinal plant and especially one of the best disease-preventive foods against some forms of cancer and cardiovascular disorders. Its beneficial widespread effect on health is attributed to sulphur-containing compounds, and particularly to thiosulfinates [3]. When garlic is cut or crushed, the enzyme alliinase is released from its compartment and transforms S-allyl-L-cysteine sulfoxide (alliin) into diallyl thiosulfinate (allicin, **ALC**, Fig.1), the characteristic compound of garlic flavor. The hypocholesterolaemic activity of garlic has been attributed to diallyl disulphide, a decomposition product of ALC [4]. Ajoene (a secondary degradation product of alliin) inhibits platelet aggregation by altering the platelet membrane via an interaction with sulphydryl groups [4]. Antimicrobial activity is well documented for garlic, and antifungal activity is more effective

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than nystatin (ALC is the main active component by inhibition of lipid synthesis). In vitro antiviral activity was attributed to ALC and its derivatives, and alliin has anti-hepatotoxic activity in vitro and in vivo [4,5].

Several methods for qualitative or quantitative determination of ALC have been reported. Mainly, high performance liquid chromatography methods with UV or MS detection were described [6-10]. However, the main drawback of the previously reported methods for analysis of ALC are the long analysis time – about 10-30 minutes per sample and the poor specificity in case of UV detection (Table 1).

The analysis by liquid chromatography-coordination ion spray-mass spectrometry method (LC-CIS-MS/MS) has been used as an alternative technique instead of classical LC/MS, especially when a compound does not readily ionize in the ion source of mass spectrometer due low proton affinity. In the LC-CIS-MS/MS, the molecule is ionized by attachment of a metallic ion with coordination capabilities (usually silver) instead of a proton. The site of coordination is usually a carbon-carbon double bond [11] or sulfur atom [12].

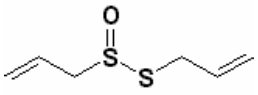

Figure 1. Molecular structure of ALC

The aim of present study is the development of a rapid and specific LC-CIS-MS/MS method for ALC quantification from *Allium* extracts. In comparison with previously published HPLC methods (Table 1), the proposed method is rapid and specific.

RESULTS AND DISCUSSION

Although ALC absorb UV light at 220 nm and in the literature there are reported analytical methods using this type of detection [6-8, 10], frequent interferences may appear at this wavelength because of lack of selectivity, leading to measurement errors.

Regarding mass spectrometry analysis, because ALC does not have ionizable chemical groups (either acidic or basic) it cannot be readily ionized under the influence of the pH of the mobile phase, so it cannot be detected „as it is" by LC/MS-electrospray with good sensitivity.

The sulphur-containing compounds have the ability to form adduct complexes with some transitional metals. Because of the metallic ion, such complex has an electric charge and it can be analyzed by mass spectrometry with electrospray ionization. For ALC and some of its derivatives, their complexes with silver were already reported, but only for qualitative analysis [12]. In order to obtain the selectivity in quantitative measurement of ALC by LC/MS we used for quantification the adduct complex formed by ALC and the silver ion. An aqueous silver nitrate 1mM solution at a flow rate of 10 µL/min was post-column added to effluent.

Table 1. Analytical characteristics of several reported HPLC or LC/MS methods for the determination of ALC in vegetal extracts

Extraction	Mobile phase	Detection	Run time (min)	Observations	Ref.
Extraction at room temperature	Sodium dihydrogenphosphate+ heptanesulfonic acid / acetonitrile, gradient	HPLC-UV, 208 nm	30	Other ALC derivatives also analysed	**6**
Ultrasonic extraction/ centrifugation	Water-methanol, isocratic	HPLC-UV, 254 nm	20	-	**7**
Turboextraction, liquid-liquid extraction	Water-methanol, isocratic	HPLC-UV, 254 nm after post-column photochemical derivatisation	10	-	**8**
Supercritical fluid extraction	Water-acetonitrile gradient	LC-APCI-MS	25	Other ALC derivatives also analysed	**9**
Vortex-sonication	Water-methanol, isocratic	HPLC-UV, 220 nm	15	-	**10**

The pseudo-molecular mass spectra (MS^1, no fragmentation applied) obtained for ALC-silver complex (Fig. 2) shows two main ions with m/z 449 and 451, corresponding to a molecular formula $[2*ALC+H_2O+Ag]^+$, in which the silver ion is surrounded by two ALC molecules and one molecule of water. The two major ions from the spectra (m/z 449 and 451) are the adducts formed by the two silver isotopes. The mass spectrum of ALC-silver complex (MS^2, fragmentation applied) is shown in Fig. 3 and the proposed main fragmentation pathways in Fig. 4.

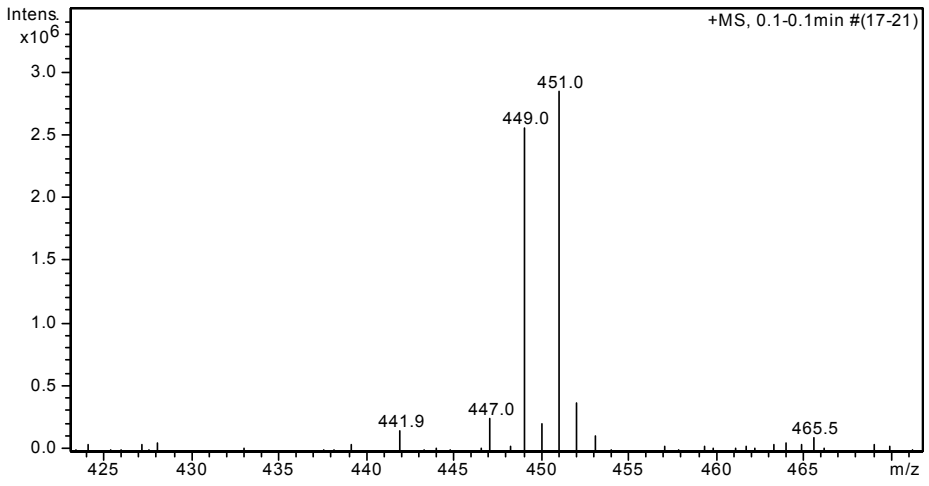

Figure 2. Pseudo-molecular (MS^1) non-reactive ion on mass spectra of ALC-silver complex

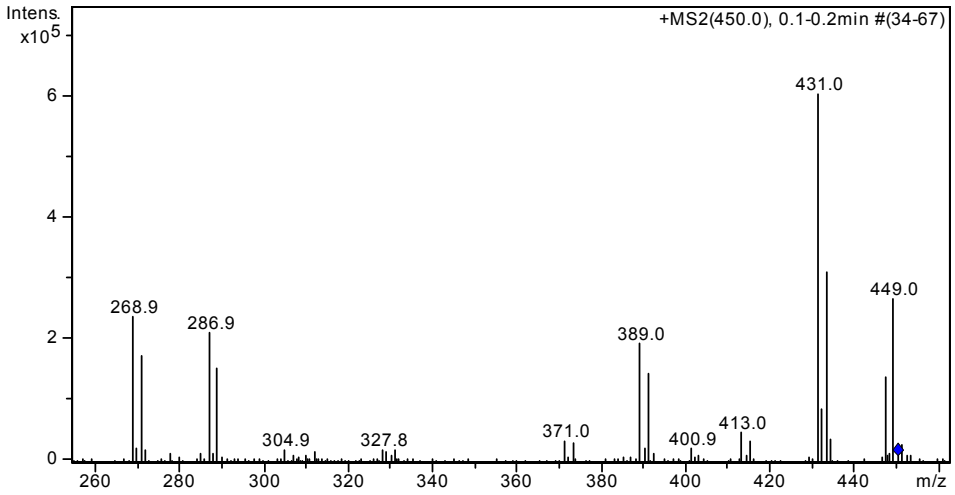

Figure 3. MS/MS mass spectra of ALC-silver complex

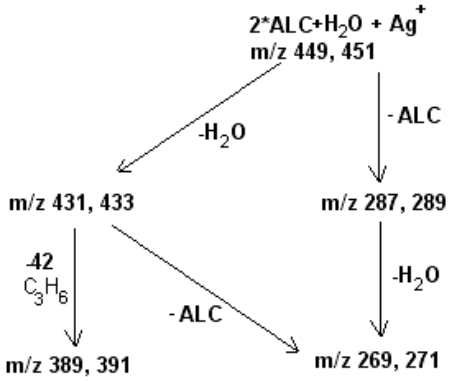

Figure 4. The proposed fragmentation pathways of ALC-silver complex

The peak of ALC was observed at RT = 0.9 min (Fig. 5).

The calibration curves showed linear response over the entire range of concentrations used in the assay procedure. The calibration curve for ALC was in the concentration range 18.0-864.0 µg/mL, using 7 calibration levels, n = 3 days, with a coefficient of correlation greater than 0.999. The residuals had no tendency of variation with concentration and were between ±13.1% values. The bias and precision of calibration curves are presented in Table. 2.

The developed analytical method was applied for analysis of ALC in five *Allium* species extracts. The found concentrations are presented in Table 3. The extracts prepared by heating at 60 ºC ("C" extracts) are richer in ALC content than "R" extracts, proving that extraction at higher temperatures favors the transformation of alliin to ALC.

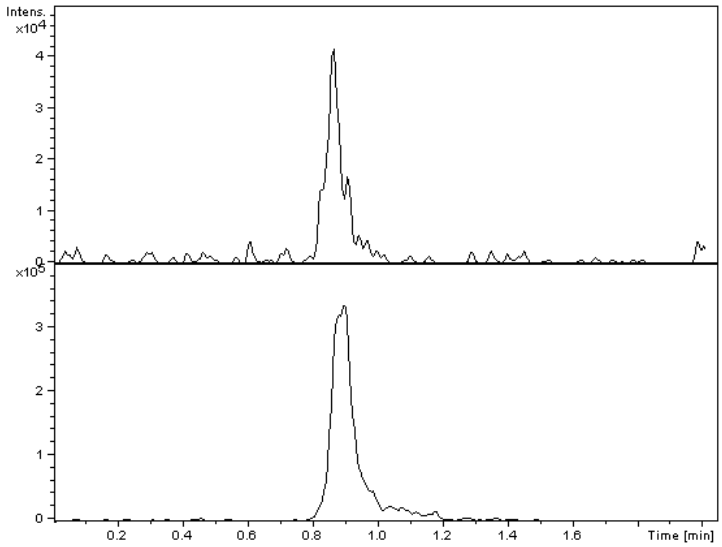

Figure 5. Typical chromatogram of the ALC standard (upper image) and *A. obliquum* extract (lower image)

Table 2. The bias and precision for ALC determination method

	Calibration 1		**Calibration 2**		**Calibration 3**		**Inter-day precision**	**Inter-day bias**
Cnominal µg/mL	Cfound µg/mL	Bias %	Cfound µg/mL	Bias %	Cfound µg/mL	Bias %	%	%
18.0	18.7	4.0	18.6	3.3	18.8	4.4	0.56	3.91
36.0	33.0	-8.2	32.8	-8.9	32.3	-10.2	1.09	-9.11
72.0	75.0	4.1	77.5	7.7	78.4	9.0	2.34	6.92
144.0	160.8	11.6	162.9	13.1	155.9	8.3	2.24	11.00
288.0	266.4	-7.5	267.0	-7.3	267.3	-7.2	0.17	-7.33
576.0	576.5	0.1	563.9	-2.1	570.2	-1.0	1.11	-1.00
864.0	874.0	1.2	871.9	0.9	869.7	0.7	0.25	0.91
Slope	5630.80		5576.19		5690.72			
Interrcept	44976.69		34772.45		48727.85			
r	0.99931		0.99915		0.99935			

Table 3. The ALC content found in various *Allium* extracts

Allium species	**ALC (mg/ml in "R" extracts)**	**ALC (mg/100 g vegetal product, "R" extracts)**	**ALC (mg/ml in "C" extracts)**	**ALC (mg/100 g vegetal product, "C" extracts)**
A. obliquum	2.819	272.806	5.579	426.629
A. senescens subsp. *montanum*	0.919	82.474	5.880	435.931
A. schoenoprasum	3.968	320.001	3.410	947.222
A. fistulosum	0.122	5.275	4.481	896.225
A. ursinum (leaves)	0.028	1.965	-	-
A. ursinum (flowers)	1.946	175.614	-	-

CONCLUSIONS

This is the first reported quantitative analytical method for determination of ALC by using LC-CIS-MS/MS. The developed method is fast (1 minute run-time), selective and provides high-throughput in analysis of ALC from *Allium* species extracts.

EXPERIMENTAL SECTION

Reagents

Reference standard of Allicin (ALC) was purchased from "Allicin International", Great Britain. Methanol, ammonium acetate and silver nitrate were Merck products (Merck KgaA, Darmstadt, Germany). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system.

Standard solutions

A stock solution of ALC with concentration of 4 mg/mL was prepared by dissolving appropriate quantity of reference substance in 10 mL metanol. The working solution was obtained by diluting a specific volume of stock solution with water. This solution was used to prepare 7 calibration solutions with concentration range between 18-864 µg/mL.

Chromatographic and mass spectrometry systems and conditions

The HPLC system was an 1100 series model (Agilent Technologies) consisted of a binary pump, an in-line degasser, an autosampler, a column thermostat, and an Ion Trap SL mass spectrometer detector (Brucker Daltonics GmbH, Germany). Chromatograms were processed using QuantAnalysis software. The detection of ALC was in MS/MS mode using electrospray positive ionisation (ESI+). The monitored ion transition was m/z (449 + 451) > m/z (269 + 271 + 287 + 289) Chromatographic separation was performed at 40˚C on a Synergy Polar 100 mm x 2 mm i.d., 4 µm column (Phenomenex, Torrance, SUA), protected by an in-line filter.

Mobile phase

The mobile phase consisted in 100% ammonium acetate, 1mM in water, isocratic elution, flow 0.6 mL/min. A silver nitrate solution 1mM in water was post column added, with a flow of 10 µL/min.

Sample preparation

Fresh stems and leaves of *Allium obliquum*, *A. senescens* subsp. *montanum*, *A. schoenoprasum*, *A. fistulosum*, leaves of *A. ursinum* and flowers of *A. ursinum* were crushed and then extracted with ethanol 70% by repercolation method at room temperature ("R" extracts). In order to observe the influence of the temperature on the allicin extraction it were also performed extractions by heating the mixtures at 60˚C on water bath for 30 min ("C" extracts). All extracts were filtered and adjusted up to the final volume. All plants were identified and voucher specimen (CL 659564, CL 659563, CL 659561, CL 659761

and CL 659750) was deposited at the Herbarium of "A. Borza" Botanical Garden, "Babes-Bolyai" University of Cluj-Napoca. Before analysis, the vegetal extracts were diluted 10 folds with distilled water, then 1 µL was injected in the chromatographic system.

Method validation

In three different days, a calibration curve was run. The linearity of the peak area against standard concentration was verified between 18-864 µg/mL ALC by applying least-squares linear regression. The applied calibration model was y = a*x+b, 1/y weight, where y is peak area and x, concentration. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated. The calibration model was accepted if the residuals were within ±20% at the lower limit of quantification and within ±15% at all other calibration levels and at least 2/3 of the standards meet this criterion [13-15]. The limit of quantification was established as the lowest calibration standard with an accuracy and precision less than 20%.

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