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RESEARCH ARTICLE

Development of Method for Identification and Determination of limit Content of Asarone in *Acorus calamus* Rhizomes

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ABSTRACT:
A method for identification of asarone in *Acorus calamus* rhizomes by thin-layer chromatography (TLC) with further determination of its limit content was developed. Determination of asarone in test solutions was performed by comparing presence of zones equivalent as for their location and staining to the asarone reference standard. Determination of asarone limit content in acorus calamus rhizomes was performed by comparing intensity of staining of spots zones between test solutions and dilution series of the asarone reference standard with different concentrations (0.25, 0.5, and 0.75%). The method was tested on 7 batches of the raw material. According to results of the set of experiments, it has been proven that the method is reproducible and at the same time allows for valid identification of the raw material by presence of one of the main components, asarone, and for control of raw material quality beyond its content. The method is proposed for introduction to the draft monograph "*Acorus calamus* rhizomes".

KEYWORDS: *Acorus calamus*, rhizomes, identification, asarone, TLC techniques.

INTRODUCTION:
Acorus calamus (*Acorus calamus* L.) is a perennial herbaceous plant found almost all over the territory of Ukraine. *Acorus calamus* originates in the Eastern Asia, from where it was brought to the Middle East and Eastern Europe, and in the middle of the 18th century, it came to North America. Since the second half of the 20th century, active pharmacological research of acorus calamus has begun, which continues till nowadays.

Pharmacological activity of acorus calamus rhizomes is primarily associated with the fact that essential oil contains one of the main components, namely asarone, which exhibits antimicrobial, fungicidal, analgesic, anthelmintic, sedative, tranquilizing, antiepileptic and spasmolytic properties[1,2,3].

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Asarone is ether, which has alpha (IUPAC name: 1,2,4-trimethoxy-5-[(E)-prop-1-enyl]benzene) and beta (IUPAC name: 1,2,4-trimethoxy-5-[(Z)-prop-1-enyl]benzene) isomers, the latter one being more toxic. The acorus calamus raw material recommended for phytotherapy should have asarone content (the sum of 2 isomers), not exceeding 0.5%. This is due to the fact that at high concentrations it possesses mutagenic and teratogenic properties[4,5,6,7,8].

Depending on acorus calamus species, the content of essential oil and asarone in it varies. Literature data suggests that a triploid species of acorus calamus is widespread over the territory of Ukraine. It contains about 3.1% of essential oil and up to 10% of asarone in oil. Composition of diploid plants found in the territory of North America includes 2.2% of essential oil, which contains no asarone. Essential oil (about 6.8%) from a tetraploid Indian chemotype contains up to 90% of asarone[5,6,7,8,9].

Analysis of applicable regulatory documents, describing methods for determination of asarone in acorus calamus rhizomes, has demonstrated the following. According to DAC "Kalmuswurzelstock" monograph, raw material identification is performed by TLC using anethole, linalol and thymol as reference substances. Chromatographic conditions: a TLC plate Kiiselgel, the mobile phase: toluene/ ethyl acetate (93:7), the developing agent: anisaldehyde solution. According to this monograph, content of cis-asarone (not more than 0.5%) is determined by HPLC with a C8 column and a spectrophotometric detector at a wavelength of 303 nm, the mobile phase is acetonitrile/water (60:40)[10].

According to the Austrian Pharmacopoeia monograph "Kalmuswurzel", identification of the raw material is also performed by TLC using anethole and thymol as reference substances. Rf describes the specified zones in the chromatogram of the test solution, under the following conditions: a TLC plate Kiiselgel H, the mobile phase is ethyl acetate/hexane (10:90), the developing agent is anisaldehyde solution. This monograph also specifies cis-asarone limit content (not more than 0.5%). It is determined by spectrophotometry with measurement of optical density of the test solution at a wavelength of 303 nm and calculation of the specific absorption rate[11].

Apart from that, TLC techniques for identification of acorus calamus rhizomes are provided in the State Pharmacopoeia of the Republic of Belarus[12] and Chinese Pharmacopoeia 2005 (CPh)[13]. In the first case, the technique is completely similar to the technique described in the Austrian Pharmacopoeia; and in the second case, the reference standard of acorus calamus rhizomes is used as a tracking substance, and the mobile phase is represented by chloroform, and the developing agent is represented by 10% sulfuric acid solution in ethanol; and the main zone specified in the chromatogram of the test solution is similar to the same zone in the chromatogram of the standard solution. Both documents lack description of techniques for determination of asarone content.

Currently, Ukrainian regulatory documentation applicable for this type of medicinal plant raw materials is represented by the Monograph of the USSR Pharmacopoeia, XI edition, which specifies content of essential oil in the raw material and gives no description for determination of asarone presence[14]. Considering broad application of acorus calamus rhizomes in medicine and the fact that national regulatory documentation is lacking the method to control asarone content in the raw material, development of methods for identification of this substance in the raw material and determination of its limit content appears to be of great current interest.

MATERIALS AND METHODS:

For this experiment, we used batches of the raw material, *Acorus calamus* rhizomes, gathered in Kharkiv (RS 464, 469), Sumy (RS 465), Poltava (RS 466), Kyiv (RS 467), Chernihiv (RS 468), and Zhytomyr (RS 470) regions.

The test solution was prepared as follows: 1.00 g of the raw material milled and sieved through the sizing screen (355) was weighed into a 25-mL round-bottom flask, 5 mL of methanol were added, and the flask was heated in a water bath under reflux at 60° C for 1 min. After cooling, the resulting extract was filtered through a paper filter and was used for application. For identification of asarone in different batches of the raw material, an optimal aliquot of test solutions of 15 µL was experimentally chosen.

Previous studies of the raw material/extractant ratio have shown that 1/5 ratio chosen is satisfactory, since at additional extraction of the remaining raw material with methanol, only trace amount of the asarone zone (less than 0.02%) develops in the chromatogram of the test solution, which does not affect conclusion about asarone limit content (not more than 0.5%) in raw material samples subject to analysis.

Identification was performed by thin-layer chromatography (TLC) using chromatographic plates TLC silica gel 60 F254 (Merck).

The reference standard of α-Asarone manufactured by Sigma-Aldrich Chemie Gmb H, Germany (serial number BCBJ9108V), was used as a tracking substance. For

identification of asarone in acorus calamus rhizomes, a reference solution in methanol at a concentration of 3 mg/mL was prepared.

Chromatographic plates were activated by incubation at 100° C for 30 min, then after cooling the plates to room temperature, samples of the reference solution and test solutions were applied in 1-cm strips. After passage of the mobile phase, the chromatographic plate is removed from the chamber and dried in the air. A solution of anisaldehyde was used as the solution for chromatogram development.

For better clearness and uniformity of staining of zones in chromatograms, we have chosen the development method, involving immersion of the plate into the developing solution using Immersion Device (Camag) at immersion speed of 50 mm/sec and immersion time of 3 sec. To compare staining intensity between the standard solution zone and zones of test samples, we used the solution of the asarone reference standard at a concentration of 1.5 mg/mL, which was subsequently applied on the plate in three aliquots: 5 µL (corresponding to 0.25 % of asarone in the raw material), 10 µL (0.5 %) and 15 µL (0.75 %).
Further, chromatograms were heated at 100°-105° C for 5-10 min, and were immediately examined in daylight.

RESULTS AND DISCUSSION:

Results of chromatographic runs of test samples of *Acorus calamus* rhizomes and the asarone reference standard are presented in Fig.1. After treatment of chromatograms with the anisaldehyde solution, as compared to zone of the asarone standard, zones (Rf close 0.43) with pink-purple staining, changing over time to gray-green, were detected in all samples.

Determination of limit content of asarone in samples was performed by comparing staining intensity between zones of asarone standard solutions and test samples. When treating plates by spraying the developing agent, we obtained staining intensity that was non-uniform and unclear. This was associated with non-uniform application of the developing solution on the chromatographic plate. Therefore, further treatment of plates was performed by immersing them into the anisaldehyde solution. (Fig. 2, 3).

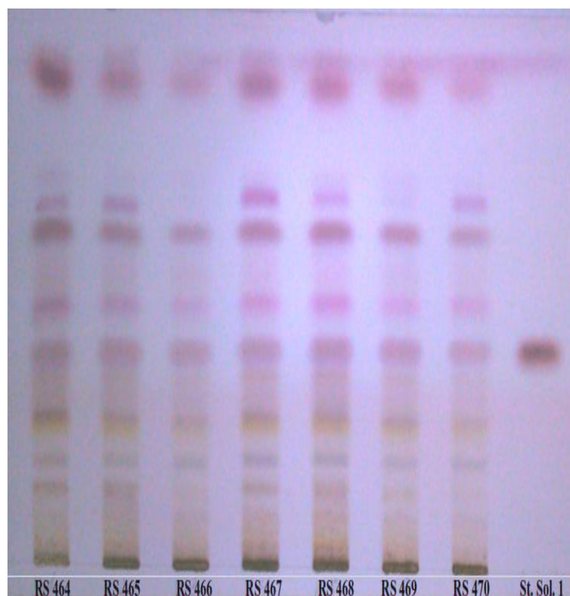

Fig.1. A TLC chromatogram of test samples of *Acorus calamus* rhizomes and the asarone reference standard, where RS 464 - 470 = 15 µL of test solutions of acorus calamus rhizomes; St. Sol. 1 = 10 µL of the asarone standard solution.

We have proved experimentally that all zones are clear, colors are saturated, and the amount of asarone in all batches tested does not exceed 0.5 % (Fig. 2, 3). According to results of a series of experiments, it was suggested to perform post-chromatographic derivatization by immersing the plate in the developing solution.

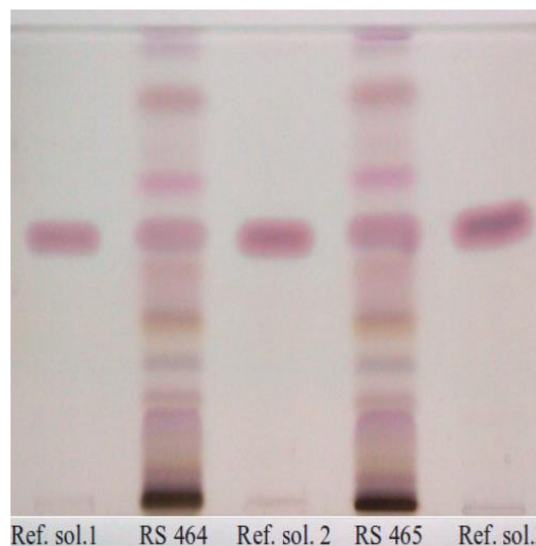

Fig.2. A TLC chromatogram obtained when studying limit content of asarone in acorus calamus rhizomes, where RS 464-465 = 10 µL of test solutions of acorus calamus rhizomes; Ref. Sol. 1 = an aliquot of the standard solution, corresponding to 0.25% of asarone in the raw material; Ref. Sol. 2 = an aliquot of the standard solution, corresponding to 0.5% of asarone in the raw material; Ref. Sol. 3 = an aliquot of the standard solution, corresponding to 0.75% of asarone in the raw material.

With 7 batches of the raw material it has been proved experimentally that the method is reproducible and valid. The results obtained with regards to asarone content in raw material samples were confirmed by densitometry, in particular, by measuring absorption of zones at 550 nm using a TLC Scanner 3 and by processing results using winCATS software, which will be covered in subsequent publications.

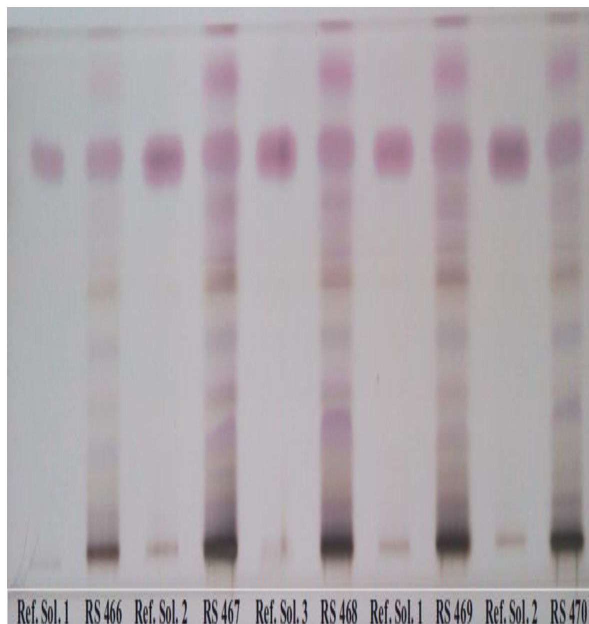

Fig.3. A TLC chromatogram obtained when studying limit content of asarone in *Acorus calamus* rhizomes, where RS 466-470 = 10 µL of test solutions of acorus calamus rhizomes; Ref. Sol. 1 = an aliquot of the standard solution, corresponding to 0.25% of asarone in the raw material; Ref. Sol. 2 = an aliquot of the standard solution, corresponding to 0.5% of asarone in the raw material; Ref. Sol. 3 = an aliquot of the standard solution, corresponding to 0.75% of asarone in the raw material.

CONCLUTION:

The method for identification of asarone in *Acorus calamus* rhizomes with further determination of its limit content in the raw material by TLC has been developed.

The method developed allows for both valid identification of the raw material by presence of one of the main components, asarone, and for control of raw material quality beyond its content.

The method developed will be suggested for inclusion to the national monograph of the State Pharmacopoeia of Ukraine "*Acorus calamus* rhizomes".

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