Comparison of the essential oil composition of *Acorus calamus* obtained by supercritical carbon dioxide extraction and hydrodistillation methods.

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Sweet flag, *Acorus calamus* L., is a member of the family of Araceae. It grows wild in the temperate zones of Europe, North America and East Asia, along swamps, brooks and lakes. The plant materials under research were collected from three different places in Estonia.
The simultaneous distillation and extraction (SDE) micro-method and supercritical CO_2 extraction have been chosen for the essential oil isolation from the calamus samples. Supercritical extraction was performed on a self-completed equipment under pressure of 100 bar and temperature 50°C. Essential oil compositions were studied using capillary gas chromatographic methods. GC analysis was carried out using a chromatograph with FID on two fused silica capillary columns with bonded stationary phases SPB-5 (poly (5%-diphenyl-95%-dimethyl) siloxane) and SW-10 (polyethylene glycol). The identification of the oil components was based on the comparison of their retention indices RI on two columns with the corresponding data of our RI data bank and with literature data. The obtained results were confirmed by GC/MS.
The yield of calamus oil was in the range of 1.1-3.3% for SFE and 1.0-2.4% in the case of SDE. The extracts obtained by SFE contained mainly β-asarone, isoshyobunone, shyobunone, preisocalamendiol, spirodecanone, acorenone, acarone. A comparison with the SDE oil did not reveal any big differences, except for acarone (13.3% versus 2.8%), acorenone (12.5% versus 22.4%), preisocalamendiol (15.7% versus 8.1%) and spirodecanone (5.1% versus 0.9%). Furthermore, some quantitative differences were observed not only between the extraction methods but also among the calamus samples, collected from different places.

INTRODUCTION

Nowadays the interest for phytoproducts has grown significantly in Estonia and worldwide. Each third product on the international pharmaceutical market has a phyto origin. Nevertheless, most of essential oil plants are not yet well studied. In this work two methods for the isolation of essential oil (SDE and SFE) from plant *Acorus calamus*, collected from different places in Estonia were investigated by using GC.
Acorus calamus grows wild in Europe, East Asia, North America, but it is a native plant of India. The plant could be found on river banks and other reservoirs also in swamps and meadows. The use of calamus could be different because the composition of the essential oil is different. The variability in the composition of calamus has been explained to the existence of four chemotypes with different ploidity. The diploid grows in North America and contains no β-asarone. European triploid cytotypes contain 5-20% β-asarone. In the tetraploid, present in East Asia, India and Japan, the β-asarone content is up to 70%. Hexaploid cytotypes grow in Kashmir Region with β-asarone content of 5% [1-4].

The compound β-asarone [(Z)-1,2-trimethoxy-5-prop-1-enyl-benzene] is of moderate acute toxicity. Because of this property its use in digestive medicine was discontinued in many countries [5]. In the US, calamus oil and its extracts are prohibited from use in food. However, it is allowed in Europe, where the concentration of β-asarone in foods and beverages is limited to 1 mg/kg and in alcoholic beverages limited to 0.1 mg/kg [6]. β-Asarone has been demonstrated to induce duodenal and liver cancer in rats [7], central nervous system inhibitory [8], sedative and hypothermic [9], unscheduled DNA synthesis in hepatocytes [10] and immunosuppressive [11] effects.
G. Mazza [2] found, that Indian calamus oil contained high amount of β-asarone (77.7%) and 6.8% α-asarone, but in European calamus oil acorenone (8.1%), isoshyobunone (6.3%), β-gurjunene (6.7%), calamendiol (5.2%) and β-asarone (5.2%) were found to be major components. In the essential oil of the calamus leaves from Lithuania β-asarone (15.7 - 45.5%) was the most abundant compound, whereas acorenone (20.9%) and isocalamendiol (12.8%) were dominant in the rhizomes [12-13].
The ploidity of Estonian plants has not been investigated to our knowledge.

MATERIALS AND METHODS

All four samples were bought or collected in Estonia. Three samples were bought in retail pharmacies. Two there of them were collected in Estonia namely: Sample 1 in Raplamaa and Sample 3 in Järvamaa harvest time May and November, 2006. The third one was grown in Russia (Sample 4, harvest 2006). Sample 2 was gathered in October 2007 near Põlva (Lake Koiola) and was dried at room temperature. Voucher specimens have been deposited at the Institute of Pharmacy, University of Tartu, Estonia.
Supercritical fluid experimentation (SFE) was performed on a self-completed equipment which allows the operating pressure up to 30MPa and temperature up to 70ºC. Apparatus consisting of high-pressure pump HPP 4001, Czechia, thermostat: Intersmat IGC 121 C FL, France, and high-pressure 10-mL cell was constructed in the laboratory. CO_2 (99.8%) was obtained from Eesti AGA. Experiments were carried out at constant temperature of 50ºC and pressure of 10MPa. Extraction time was 120 minutes. Collecting solvent was n-hexane from Sigma-Aldrich (≥95%). About 1.5g of calamus was used for each run. Two replicate extractions were performed for every plant sample.
Simultaneous distillation and extraction (SDE) with n-hexane (Fluka, >99%) as a solvent (0.5 mL) was performed using Marcusson type microapparatus. The SDE process was carried out during 120 minutes. The oil amount (%) was determined using n-tetradecane (Reachim, >99%) as internal standard (2 μL). About 10 g of dried ground calamus rhizomes was used for each run. The reproducibility of three parallel SDE procedures with a single sample showed the variation coefficient below 20%.
Volatile compounds in the extracts were analysed by gas chromatography. GC analysis was carried out using a Chrom 5 chromatograph with FID on two fused silica capillary columns with bonded stationary phases SPB-5 (30m × 0.25mm, Supelco) and SW-10 (30m × 0.25mm, Supelco). Film thickness of both stationary phases was 0.25μm. Carrier gas helium with split ratio 1:150 and the flow rate 30 – 35 cm/sec was applied. The temperature program was from 50° - 250°C at 2°C/min, the injector temperature was 250°C. A Hewlett-Packard Model 3390A integrator was used for data processing.
The identification of the oil components was accomplished by comparing their retention indices (RI) on two columns with the RI values of reference standards, our RI data bank and with literature data [14-15]. The results obtained were confirmed by GC/MS.

The percentage composition of the oils was calculated in peak areas using normalization method without correction factors. The relative standard deviation of percentages of oil components of three repeated GC analysis of single oil sample didn't exceed 5%.

RESULTS

The yields of oils, relative percentage and RI values of main compounds of SFE and SDE extracts from *A. calamus* rhizomes on two columns are reported in Table 1.

The total yields of SFE fractions for the four samples from grinded calamus rhizomes at pressure 10 MPa and at temperature 50ºC were 1.1 – 3.3%. The hydrodistillation (SDE) of grinded rhizomes gave the similar oil yields (1.0 – 2.4%).

In the studied oils, 85 compounds were identified, representing more than 95% of the total oils. The quantitatively most important compounds in calamus SFE extracts were oxygenated sesquiterpenes β-asarone (7.6 – 82.8%), preisocalamendiol (2.1 – 15.7%), acorenone (tr – 13.5%), acorone (0.8 – 14.0%) isoshyobunone (0.4 – 4.0%) and shyobunone (1.8 – 6.3%).

The amount of monoterpenes in calamus SFE oils was small (1.0 – 2.7%). Camphene content was the highest (0 – 1.7%). Predominant oxygenated monoterpenes (total 1.0 – 7.1%) identified in calamus samples were camphor (0 – 1.5%) and methyl eugenol (0 – 3.5%). The sesquiterpene hydrocarbons (0.7 – 16.6%) were represented by β-sedrene, aristolene, α-humulene, α-selinene and δ-cadinene.

The SFE extracts of one Estonian calamus Sample 3 and Russian Sample 4 were characterized by a high amount of β-asarone (to 83%). The predominant compounds (over 12%) in the other Estonian samples 1 and 2 were preisocalamendiol, acorenone and acarone. The amount of β-asarone in these samples was 5.4 – 7.6%.

A comparison between the SFE and SDE extracts of Sample 3 did not reveal any big differences. To our knowledge, there is no data about SFE of calamus rhizomes of β-asarone rich samples. Chromatogram of SFE extracts from *A. calamus* rhizomes of Sample 3 are reported in Figure 1.

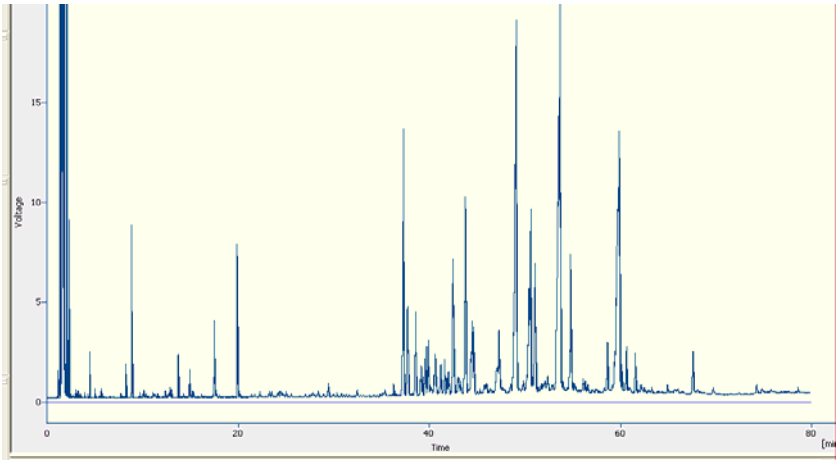
It can be seen, that hydrodistilled oil of Sample 2 contained more monoterpenenoic compounds compared with SFE oils, but the amounts of acarone (13.3% versus 2.8%), preisocalamendiol (15.7% versus 8.1%), and spirodecanone (5.1% versus 0.9%) were found to be higher in SFE extracts.

The number of literature data concerning SFE of calamus rhizomes is not sufficient [4]. Marongiu et al. supposed that by SFE it is possible to avoid the partial hydrolysis of iso-acorone, acorone, and cryptoacorone which occurs when water is used as in SDE. Our results were in a good agreement with this opinion. Content of acorone in SFE extracts was 0.8 – 13.3%, but in SDE extracts 0 – 2.8%. (Marongiu: 2.0% versus 0%). The composition of SFE and SDE extracts of Samples 1 and 2 was in a good agreement with data of calamus oils of Marongiu et al. They found acorenone (SFE 13.4% and HD 21.6%), dehydroxy isocalamendiol (SFE 7.7% and HD 3.5%), (Z)-sesquilavandulol (SFE 11.0% and HD 13.0%), shyobunone (SFE 2.6% and HD 7.0%), shyobunone isomer (SFE 3.3% and HD 2.5%)to be the main components in their samples.. The β-asarone content in SFE extract was 5.5% and SDE extract was 5.1% [4].

Table 1: **Concentration of main compounds of SFE and SDE extracts of *Acorus calamus* rhizomes**

Compound	RI		Concentration, %					
			SFE				SDE	
	SPB-5	SW-10	Samples 1	Samples 2	Samples 3	Samples 4	Samples 1	Samples 3
Camphene	941	1067	0.2	1.7	-	0.4	0.7	-
Camphor	1136	1510	1.1	1.5	-	0.2	2.6	0.1
Methyl eugenol	1396	2048		3.5	0.2	tr	1.4	
β-Sedrene	1408	1580	0.9	2.1	-	0.2	0.8	tr
Aristolene	1420	1572	0.8	1.6	tr	0.2	1.2	0.1
β-Gurjunene	1438	1621	0.4	1.2	-	0.2	0.6	-
α-Humulene	1441	1656	0.2	1.4	-	0.2	0.1	-
(E)-Geranyl acetone (Z)-Methylisoeugenol	1454 1454	1869 2103	0.2	1.3	0.4	0.6	0.6	0.8
Isoshyobunone	1483	1846	2.8	4.0	0.4	0.9	3.9	0.6
cis-β-guaiene α-Muurolene	1492 1494	1700 1720	0.7	0.5	0.3	0.3	0.1	tr
Shyobunone α-Selinene	1506 1504	1891 1710	4.5	6.3	1.8	2.4	13.7	2.7
δ-Cadinene	1515	1749	1.5	0.8	0.2	0.4	1.0	0.2
Ledene	1518	1695	0.4	1.6	-	-	-	-
Ledene oxide I β-Sesquiphellandrene	1521 1520	2073 1821	3.9	0.8	0.7	0.8	2.3	0.5
Spathulenol	1566	2117	1.2	0.9	-	0.2	1.3	0.1
Caryophyllene oxide	1570	1973	1.7	1.6	0.8	0.6	1.8	1.3
Preisocalamendiol	1600	2010	15.7	13.5	2.1	7.1	8.1	1.0
β-Asarone	1630	2359	7.6	5.4	82.8	64.9	10.2	85.3
Dehydroisocalamendiol	1640	2151	2.6	3.4	1.0	1.5	3.5	1.0
(E)-α-Cadinol	1652	2227	1.3	tr	-	0.3	0.1	0.2
α-Farnesol	1665	2252	0.4	-	0.4	1.0	1.4	0.5
α-Asarone	1670	2490	5.1	5.6	1.1	3.5	0.9	tr
Spiro[4,5]decan-7-one, 1,8-dimethyl-4-(1-methyl-ethyl)	1672	2169					0.9	
Acorenone	1682	2175	12.5	13.5	tr	0.1	22.4	0.8
n-Heptadecane	1700	1700	0.4	0.3	0.4	0.5	-	-
Hexahydrofarnesol	1704	2287	2.8	3.2	tr		0.2	-
Longipinocarvone	1775	2310	1.3	0.9	0.1	0.4	1.0	-
Acarone	1794	2590	13.3	14.0	0.8	4.8	2.8	-
Total, %			83.5	90.6	93.5	91.7	83.6	95.1
Oil yield, %			1.1	3.3	1.2	0.9	1.0	2.4

Figure 1: **Chromatogram SFE extracts of *Acorus calamus* rhizomes**



CONCLUSIONS

It is proposed that there could be the correlation between the amount of β-asarone and ploidity [1-4]. From our results as the content of β-asarone in Samples 3,4 was 82.8% and 64.9% and in Samples 1,2 7.6% and 5.4%, it could be concluded that *Acorus calamus* of tetraploid and triploid cytotypes respectively were investigated.
As it is seen from our results, SFE method in mild extraction conditions gives similar SFE and SDE oil composition of calamus rhizomes with high β-asarone content (tetraploid chemotype). This work is the first study using SFE from calamus rhizomes of β-asarone rich samples. Some differences were seen in the composition of SFE and SDE extracts from triploid calamus rhizomes. SFE method affords to avoid the hydrolysis of spirodecanones as it was seen in earlier work [4].

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