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COMPARATIVE ANALYSIS OF FREON EXTRACTS FROM LIME FLOWERS CROPPED IN 2016 AND 2017 YEARS

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Introduction

Development of analytical procedures for qualitative and quantitative analysis of herbal drug preparations (HDP) is one of the most important stages in creation of medicines. However, as it has been shown in numerous papers, in particular, by our scientists [1–6], variety of difficulties caused by the below-mentioned reasons arises in this process.

It's not always known which biologically active substances (BAS) of a certain plant are exactly responsible for their pharmacological activity, which extractives are inert or ballast and which ones can reduce therapeutic action or even cause side-effects. Therefore herbal raw materials are often standardized by total extractives, and this is not objectively enough to estimate quality of HDP [1–5]. For some plant species there are no techniques of quantitative analysis at all in pharmacopoeias of Ukraine, EP, BP etc. [7, 8, 9], lime flowers can be an example.

Another reason consists in that quantitative content of a sum of extractives or active substances (and even their structure) considerably vary within one kind of herbal raw material depending on harvesting place (and, hence, soils), harvest time, and, especially, weather conditions (which are impossible to change), etc. [1, 3, 10, 11]. Therefore in Ukraine and abroad different agriculture technologies are used for cultivation of herbs in predicted, adjustable "hothouse" conditions. Hence, most of producers of phytochemical preparations prefer cultivated herbal raw materials against wild ones [1, 4]. But here also certain limitations exist. It's impossible to grow up large trees, for example, oak, linden under artificial hothouse conditions because of their sizes and duration of growth. Such species of plants are cropped only wild-growing.

During our previous research of *T. cordata* lime flowers, [12, 13, 14] high anti-inflammatory, in particular antirheumatic, and also febrifugal activities of freon extracts obtained from the said raw material have been found out. Besides, they showed wide spectrum of antimicrobial effect comparable with other herbal antiseptics [15, 16]. In the paper [10] the phenolic complex from the said herbal drug has been withdrawn and studied as well and it has shown appreciable antiulcer activity comparable to that of the reference medicine [17]. As a result of experiments the technology for complex processing of linden blossom [18] has been developed and patented.

Some authors [16, 19, 20, 21] pointed out that the lipophilic extracts taken from various lots of lime flowers consisted of hydrocarbons, aromatic oxygen-containing substances and monoterpenes as the main components of essential oil fractions, however the latter considerably differed in their relative percentage of components and biological activity. This can be explained by differences in geographical location of studied materials and, hence, in environmental conditions. Previously we have found that the herbal raw material cropped in 2007 when there was abnormal dry spring and extremely hot May [22], resulted in 1,7-1,8 % yield of freon extracts. From the raw material of 2008 crop when spring was rather cool and rainy [22] 2,7-2,8 % yield of freon extracts has been obtained. The lime flowers gathered in 2009 after very droughty and warm spring gave about 1,9 % of the specified extract recalculated to dry weight.

The purpose of the present work was to carry out the comparative analysis of the freon extracts taken from lime flowers cropped in 2016 and 2017 years, considering abnormal weather conditions in springs of the specified periods.

Materials and methods

The herbal raw materials were lime flowers cropped from linden Tilia cordata within Kharkiv region in 2016 and 2017, comminuted to particle size 0,5-2,0 mm which were placed into two parallel connected percolators of the experimental equipment.

The entire equipment circuit was hermetically closed, degassed and then the percolators were filled with condensed difluorochloromethane (freon R22). After that two-stage extraction was provided at raw material: extracting solvent ratio 1:8 on each stage under constant circulation of a liquid phase due to temperature and, accordingly, pressure difference between two vessels. The minimum pressure and temperature were 12 atm and +30°C accordingly, maximum ones – 17 atm and +45°C. Total exposure of the process took about 2 hours on each stage. As a result the extracts of dark green colour with intensive fragrant smell have been received.

Precise assay 0,250 g of the extract was dissolved in 25,00 ml of methylene chloride and resulted 1,00 % solution was placed into a vial which was then tightly sealed.

1,00 мкл the aforementioned solution was entered into the front injector of gas-chromato-mass-spectrograph «Agilent Technology 6890» (USA) which included two consequently connected columns: Agilent 19091S-433HP-5MS, filled with adsorbed 5 % Phenyl-Methyl-Silox, and Agilent 165-6626 USB369911A, and also mass selective detector 5973N. Sizes of the first column were 30 m long with internal diameter 250 μm and adsorption layer thickness 0,25 μm. Sizes of the second column were 0,7 m x 150 μm x 0,15 μm respectively.

The oven was programmed as follows: equilibration time – 0.5 min, temperature – from 50°C to 250 °C with rate 4°C/min, then 30°C/min to 300 °C, maximum temperature was 325°C, runtime – 51,667 min.

Front inlet carrier gas – helium: in the first column the flow rate - 1 ml/min under 14,313 psi, and in the second one – 3,8 ml/min at initial pressure 3,8 psi, and in

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the outlet vacuum was provided.

Transferring from gas-liquid chromatograph (GLC) to mass spectrometer occurred at 230°C. The detector and evaporator temperature was supported at 150°C.

Electron ionization was spent at 70 eV in range of masses from m/z 29 to 450. Identification of compounds was carried out by comparison of the acquired mass spectra with the data of existing libraries (about 500000 substances). Pentadecane was used as the internal standard. Quantitative content of each substance expressed in percentage of total assay weight of the sample was determined by areas of corresponding peaks considering that 10^9 standard units of an area corresponded to 0,002 mg of a substance in the assay (1 μL of 1,00 % freon extract solution in ethyl alcohol with ρ =0,812 g/cm^3). Besides, relative percentage content of each substance in the assays was calculated. All solvents and chemicals were of analytical grade.

Chromatographic experiments were reproduced in triplicate.

Results and discussion

Chromatograms of the difluorochloromethane extracts (obtained by freon R22) from lime flowers cropped in 2016 and 2017 on the territory of Kharkiv region are presented on Fig. 1 and Fig. 2 accordingly.

As it can be seen from the data received, the analyzed samples contained 80 and 68 volatile substances most of which have retention times more than 35 minutes.

Samples of 2017 contained rather noticeable quantities of more volatile substances with shorter retention times that can be explained by appreciable differences of meteorological conditions in spring and beginning of summer in the said period: dry and cold weather in 2017 and very wet one – in 2016 (Table 1). Besides, total yield of the freon extract in 2016 was 2,75 %, and in 2017– 1,42 % recalculated on dry weight of raw materials studied.

Table 1. The data from archives of weather service concerning Kharkiv city in 2016 and 2017 [22].

Period observed	Month average temperature, °C		Month total rainfall (snowfall), mm	
	2016	2017	2016	2017
April	+12,2°C	+8,9°C	46,0	37,1
May	+15,6°C	+14,6°C	148,1	33,0
June	+20,3°C	+19,6°C	53,8	17,5

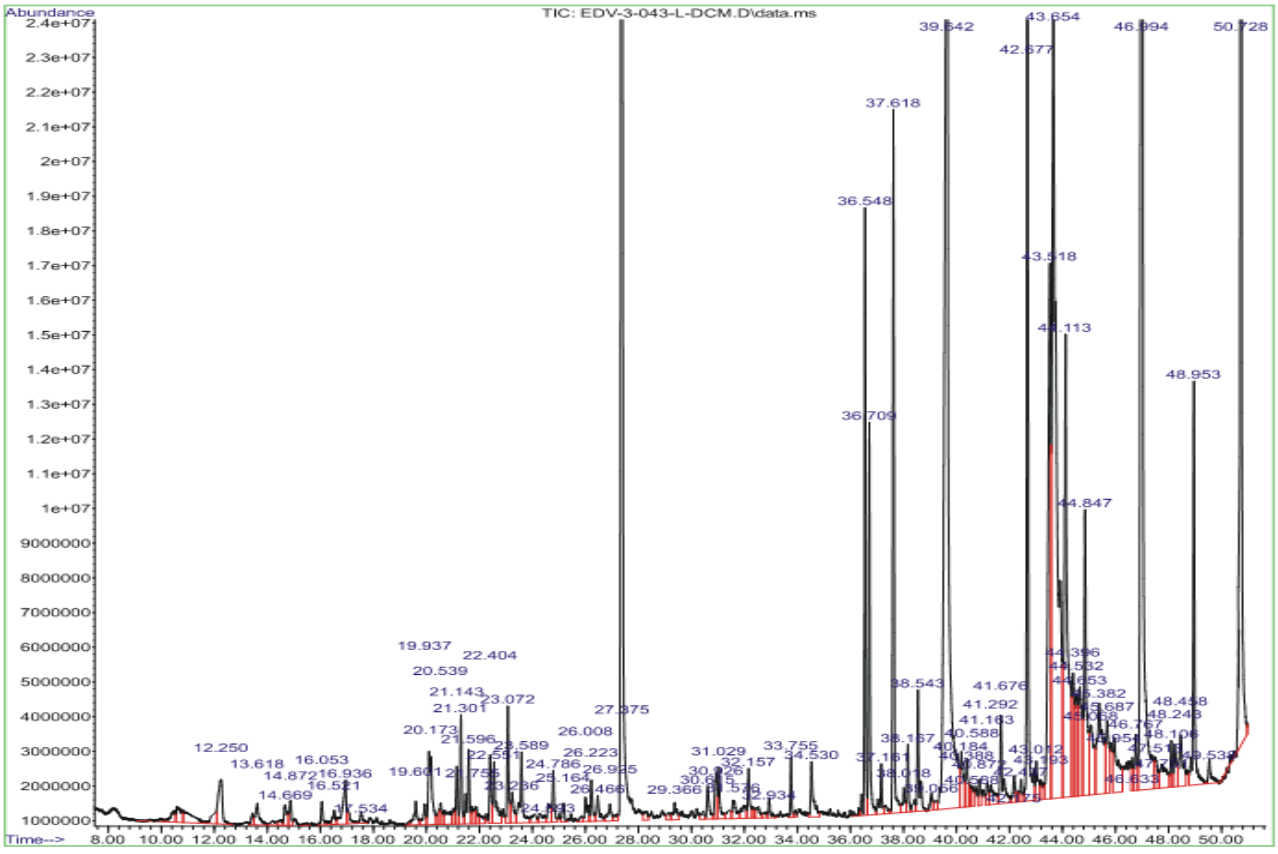

Fig. 1. Gas-liquid chromatogram of the freon extract from lime flowers cropped in 2016 in Kharkov region.

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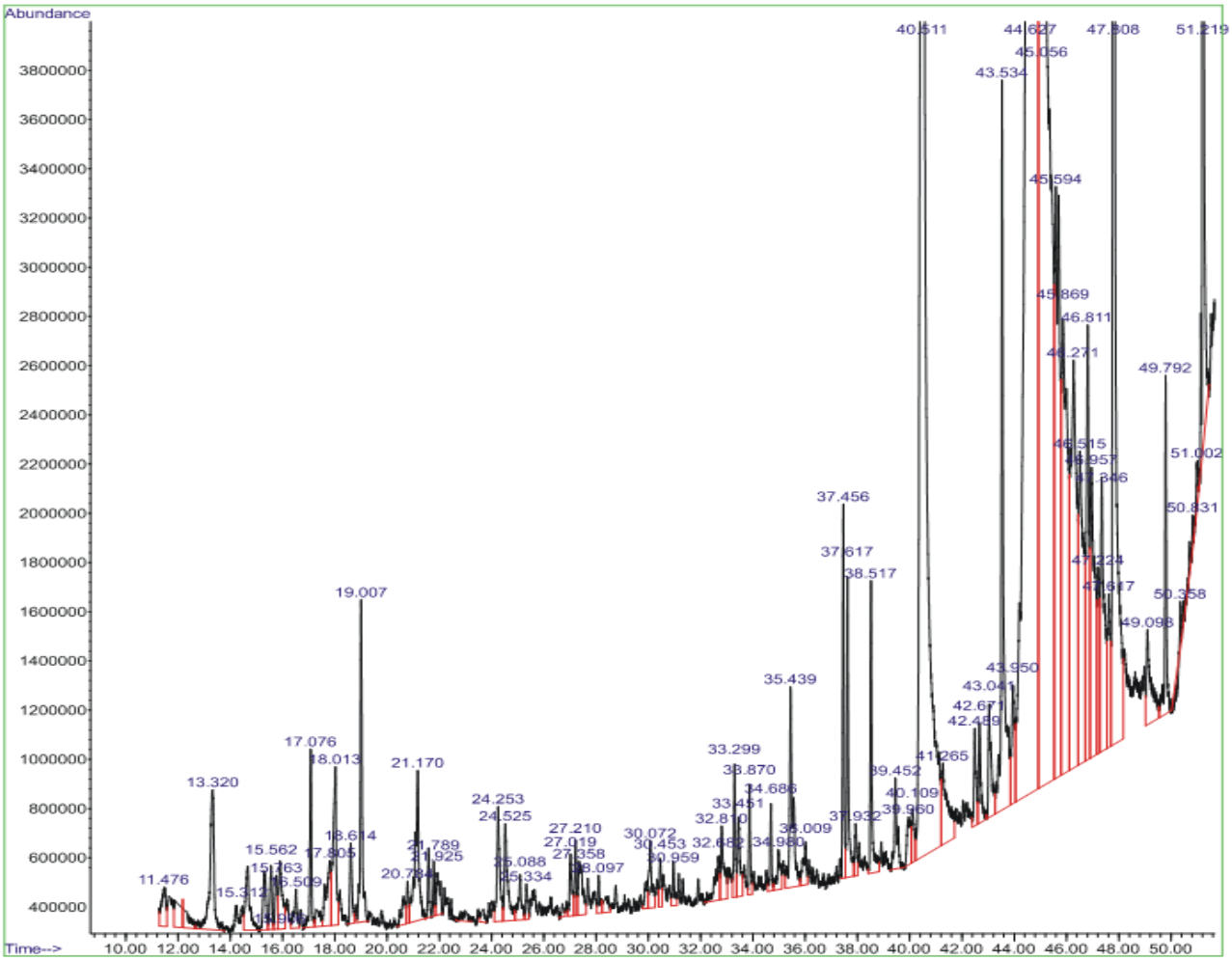

Fig. 2. Gas-liquid chromatogram of the freon extract from lime flowers cropped in 2017 in Kharkov region

Quantitative distribution of the components which have been revealed by GLC in investigated samples by chemical groups are presented in Table 2.

Apparently from received data (Table 2), prevailing chemical groups of substances were aliphatic hydrocarbons and fatty acids, and also there were alcohols and terpenoids in appreciable quantities. Moreover, percentage of the chemical groups of BAS found in freon extracts obtained from lime flowers cropped in different years considerably differed between each other that points out on strong influence of weather conditions on their accumulation in the studied herbal drug, especially in climatically abnormal 2016 and 2017 years.

Table 2. Quantitative characteristics of the freon extracts from lime flowers cropped in 2016 and 2017

Chemical groups	Extract from lime flowers cropped in 2016		Extract from lime flowers cropped in 2017	
	Amount of substances found	Content of BAS by their chemical groups in the extract, %	Amount of substances found	Content of BAS by their chemical groups in the extract, %
Aliphatic hydrocarbons: - saturated - unsaturated	 7 4	 30,42 % 11,64 %	 7 5	 14,68 % 1,30 %
Terpenoids	16	9,00 %	6	3,02 %
Fatty acids: - saturated - unsaturated	 8 2	 16,69 % 5,32 %	 9 5	 31,52 % 30,04 %
Alcohols	7	14,51 %	6	4,78 %
Aldehydes and	3	0,77 %	9	5,01 %

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ketones				
Esters	–	–	5	7,06 %
Nitrogen-containing substances	14	2,44 %	7	1,64 %
Unidentified substances	19	6,20 %	9	0,95 %
IN TOTAL	80		68	

In the sample of 2016 total quantitative contents of aliphatic hydrocarbons, aromatic alcohols and terpenoids were approximately 3 times more in comparison with 2017. At the same time, fatty acids, especially unsaturated, and also aldehydes and ketones were found to be much less, and esters were absent at all that is probably explained by their hydrolysis under high humidity and warmer weather.

The main substances which relative content exceeding 1 % in investigated extracts are resulted in Table 3 and Table 4.

It's apparently from Table 3, that the freon extract from lime flowers cropped in 2016 consisted of 14 substances with total contain 78,7 % of the whole assay, where the following BAS were dominating: hexatriacontane - 18,73 %, n-hexadecanoic acid - 12,75 %, linoleic alcohol - 10,68 %, 1-pentadecene - 10,57 %.

On the basis of the data in Table 4, it's possible to make a conclusion that the freon extract from the raw material of 2017 crop included 13 substances with total contain 84,14 % of the whole assay weight from which the prevailing ones are: oleic, octadecanoic, hexadecanoic acids with concentrations 27,68 %, 15,02 % and 12,91 % respectively.

Table 3. Substances with relative content more than 1 % in the extract from lime flowers of 2016

List №	Name of the substance	Percentage of the substances in the extract, %
1	Hexatriacontane	18,73%
2	n-Hexadecanoic acid	12,75%
3	Linoleic alcohol	10,68%
4	1-Pentadecene	10,57%
5	Heneicosane	4,39%
6	Stearic acid	4,32%
7	Linoleic acid	4,11%
8	1,2-Epoxyoctadecane	2,99%
9	Neophytadiene	2,16%
10	Docosane	1,92%
11	Tetracosane	1,84%
12	Hexahydrofarnesyl acetate	1,62%
13	2,13-Octadecadien-1-ol	1,56%
14	9,12-Octadecadienoic acid	1,02%
	In total	**78,66 %**

Table 4. Substances with relative content more than 1 % in the extract from lime flowers of 2017

List №	Name of the substance	Percentage of the substances in the extract, %
1	Oleic acid	27,68%
2	Octadecanoic acid	15,02%
3	Hexadecanoic acid	12,91%
4	Eicosane	7,14%
5	Pentacosane	4,49%
6	E,E-10,12-Hexadecadien-1-ol acetate	3,40%
7	9,17-Octadecadienal	3,07%
8	14-Methyl-8-hexadecyn-1-ol	2,80%
9	Heneicosane	1,98%
10	9,12-Octadecadienoic acid	1,73%
11	i-Propyl 11,12-methylene-octadecanoate	1,43%
12	2-Methyl-Z,Z-3,13-octadecadienol	1,33%
13	7,11-Hexadecadien-1-yl acetate	1,15%
	In total	**84,14%**

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Considering the results presented in Table 3 and Table 4 it's necessary to notice that most of dominating substances in the studied extracts received from raw materials of 2016 and 2017 differ from each other quantitatively and qualitatively that is possible to explain by influence of weather conditions.

Conclusions

1. The comparative analysis of the freon extracts from lime flowers cropped in 2016 and 2017 years using method of gas-liquid chromatography has been carried out.

2. Basing on results of gas-liquid chromatography it has been established that the samples obtained from raw materials of 2016 and 2017 years considerably differ in their quantitative and qualitative characteristics of essential oil fractions: the studied samples contained 80 and 68 compounds accordingly. Total percentage of dominating substances was 78,66 % and 84,14 % of the assays weight respectively.

3. Thus, it's possible to make the general conclusion that weather conditions, which can change dramatically in spring from year to year in Ukraine, especially in its Northeast part, strongly influence accumulation of volatile compounds in the herbal raw material studied.

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Introduction. The article is devoted to gas-liquid-chromatography analysis of the freon extracts taken from lime flowers cropped in 2016 and 2017 years. It's well-known that quantitative content of a sum of extractives or active substances (and even their structure) can vary considerably in plants depending on harvesting place (and, hence, soils), harvest time, and, especially, weather conditions. This is the main reasons for difficulties in standardization of herbal drugs. Often they are standardized by total extractives, for some plant species there are no techniques of quantitative analysis at all in pharmacopoeias, such an example is lime flowers. Recently, in 2007, 2008, 2009 we studied lipophilic fractions from lime flowers and found slight differences in their quantitative and qualitative compositions. The main aim was to compare chemical composition of volatile compounds in lipophilic freon extracts from lime flowers considering that springs and beginning of summers in 2016 and 2017 was characterized by abnormal weather conditions. **Materials and methods.** The herbal raw materials were lime flowers cropped from linden Tilia cordata within Kharkiv region in 2016 and 2017, comminuted to particle size 0,5-2,0 mm. They were extracted with condensed difluorochloromethane (freon R22) in two parallel connected percolators of the experimental equipment. The extraction was two-stage at raw material : extracting solvent ratio 1:8 on each stage under constant circulation of a liquid phase. The minimum pressure and temperature were 12 atm and +30°C accordingly, maximum ones – 17 atm and +45°C. Total exposure of the process took about 2 hours on each stage. The extracts were dissolved in methylene chloride to make 1,00 % solution which was entered into the front injector of gas-chromato-mass-spectrograph «Agilent Technology 6890» (USA) including two consequently connected columns: Agilent 19091S-433HP-5MS, filled with adsorbed 5 % Phenyl-Methyl-Silox, and Agilent 165-6626 USB369911A, and also mass selective detector 5973N. The oven was programmed as follows: equilibration time – 0.5 min, temperature – from 50°C to 250 °C with rate 4°C/min, then 30°C/min to 300 °C, maximum temperature was 325°C, runtime – 51,667 min. Carrier gas was helium: in the first column the flow rate - 1 ml/min under 14,313 psi, and in the second one – 3,8 ml/min at initial pressure 3,8 psi, and in the outlet vacuum was provided. Transferring from gas-liquid chromatograph (GLC) to mass spectrometer occurred at 230°C. The detector and evaporator temperature was supported at 150°C. Electron ionization was spent at 70 eV in range of masses from m/z 29 to 450. Identification of compounds was carried out by comparison of the acquired mass spectra with the data of existing libraries (about 500000 substances). Pentadecane was used as the internal standard. Quantitative content of each substance expressed in percentage of total assay weight of the sample was determined by areas of corresponding peaks considering that 10^9 standard units of an area corresponded to 0,002 mg of a substance in the assay. Besides, relative percentage content of each substance in the assays was calculated. All solvents and chemicals were of analytical grade. Chromatographic experiments were reproduced in triplicate. **Results and discussion.** On the chromatograms of the difluorochloromethane extracts from lime flowers cropped in 2016 and 2017 on the territory of Kharkiv region 80 and 68 volatile substances were found accordingly. Most of them have retention times more than 35 minutes. Samples of 2017 contained more volatile substances with shorter retention times if compare with extracts obtained from 2016 crop. Total yield of the freon extract in 2016 was 2,75 %, and in 2017 – 1,42 % recalculated on dry weight of raw materials studied. Prevailing chemical groups of substances were aliphatic hydrocarbons and fatty acids, also there were alcohols and terpenoids in appreciable quantities. In the sample of 2016 total quantitative contents of aliphatic hydrocarbons, aromatic alcohols and terpenoids were approximately 3 times more in comparison with 2017. At the same time, fatty acids, especially unsaturated, and also aldehydes and ketones were found to be much less, and esters were absent at all in 2016 samples. The freon extract of lime flowers cropped in 2016 consisted of 14 substances which relative content exceeded 1 % with total contain 78,7 % of the whole assay, where the dominating ones were: hexatriacontane - 18,73 %, n-hexadecanoic acid - 12,75 %, linoleic alcohol - 10,68 %, 1-pentadecene - 10,57 %. The freon extract of the raw material of 2017 crop included 13 substances with total contain 84,14 % of the whole assay weight from which the prevailing ones are: oleic, octadecanoic, hexadecanoic acids with concentrations 27,68 %, 15,02 % and 12,91 % respectively. **Conclusions.** The comparative analysis of the freon extracts from lime flowers cropped in 2016 and 2017 years using method of gas-liquid chromatography has been carried out. Basing on results of gas-liquid chromatography it has been established that the samples obtained from raw materials of 2016 and 2017 years considerably differ in their quantitative and qualitative characteristics of essential oil fractions: the studied samples contained 80 and 68 compounds accordingly, total percentage of dominating substances was 78,66% and 84,14% of the assays weight respectively. Thus, weather conditions which can change dramatically in spring from year to year in Ukraine, especially in its Northeast part, strongly influence accumulation of volatile compounds in lime flower.

Keywords: difluorochoromethane, extraction, lime flower, gas-liquid chromatography.

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