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DEVELOPMENT OF TECHNIQUES FOR QUANTITATIVE ANALYSIS OF LIME FLOWERS

Demyanenko D. V., Demyanenko V. G., Breusova S. V.

Department of Commodity Science, National University of Pharmacy

Introduction

Now one of the major problems in modern phytochemical production is standardization of herbal drugs and preparations thereof, but many species of medicinal plants (MP) and herbal substances obtained from them even nowadays have no corresponding pharmacopoeian monographs or their quality is tested under techniques which sometimes are not objective or rational enough [1, 2, 3, 4, 5]. Now quantity of monographs for MP in the State Pharmacopoeia of Ukraine (SPU) has reached 100 though it is less than those in the European and Belarus Pharmacopoeias [6, 7] (Table 1). The latter deserves especial attention because it includes unique monographs for approximately 50 species of MP which are absent either in SPU or in EP 7 and BP 2009 [6, 8, 9], moreover, there are species harvested in large-tonnage scale among them which are of interest for phytochemical industry: for example, immortelle flowers, alder leaves for production of Flamin and Altan respectively, pumpkin seeds, mountain ash fruits, beggars-ticks herb, birch buds, elecampane rhizome with roots, etc.

Table 1: Quantity of monographs for herbal drugs, substances and preparations in pharmacopoeias of different countries

Names of pharmacopoeias	Quantity of monographs				
	for herbal drugs	for tinctures, extracts and other dosage forms with herbal BAS	for essential oils	for resins, natural gums, mucilages, etc.	In total
SPU 1.4	76	12*	11	1	100
Pharmacopoeia of USSR XI, 1989 [10]	88	-	-	-	88
Pharmacopoeia of Belarus	136	-	9	1	146
JP XV, 2006 [11]	137	25	4	7	173
EP 7	145	43	31	12	231
BP 2009	157	48	36	14	255

* - *there are monographs only for tinctures*

The process of monographs' development and their introducing in pharmacopoeias meets with number of difficulties caused by significant variabilities in pharmacopoeian requirements of different countries to quality characteristics of MP, and also by expediency of adopting European monographs just translated into national language (without changes) for analysis of a certain MP species [1, 2, 4]. The latter problem, in its turn, is invoked by the following reasons:

- in many countries, in particular in Ukraine, both cultivated and wild-growing plants are harvested, unlike Europe where they are grown in simulated conditions. Some herbal drugs including lime flowers in most of countries are prepared only from wild plants [2];
- diversity and significant variability of qualitative and quantitative composition of biologically active substances (BAS) in herbal raw materials [12];
- quality characteristics of herbal drugs considerably depend on environmental conditions (soil and climate) which are variable even within Ukraine and especially differ from European ones, therefore many species of herbal drugs of domestic origin may be nonconformable to EP or BP monographs by some quality indexes or vice versa [5, 13, 14];
- some analytical procedures specified in EP 7 for quality evaluation of herbal drugs are not always objective and/or require hard-to-get, expensive reagents, chemicals, reference standards and so on [5, 14].

Hence, development of normative documentation for standardizing of herbal drugs and preparations on their basis remains an actual problem for today.

Now lime flowers (Tiliae flos) are one of six species of herbal drugs which have no corresponding paragraphs „Assay" in monographs of SPU, EP7, State Pharmacopoeia of the USSR, JP XV and BP 2009 [15]. Only in the State Pharmacopoeia of Belarus, p. 370 there is corresponding technique according to which lime flowers are standardized by flavonoid content: it should be not less than 0,5 % of dry herbal drug weight recalculated to quercetin.

Qualitative and quantitative composition of lipophilic substances, in particular essential oil, is not regulated at all in any monograph though these fractions of BAS have high anti-inflammatory and antimicrobial activity [16, 17].

According to our patent [18] these groups of BAS are obtained during complex processing of lime

flowers by their extraction with liquefied difluorochloromethane (Freon R22). However application of this solvent in analytical practice requires special high pressure labware and qualified personnel. That's why for qualitative and quantitative analysis of lipophilic complex in lime flowers it's more expedient to use methylene chloride which is close to Freon R22 by its molecule structure, value of dielectric constant (ε=6,0-6,5) and, as a consequence, by dissolving power.

Thus, the aim of this article was to develop techniques for quantitative analysis of lime flowers taking into account various groups of BAS present in the herbal drug.

Materials and methods

Six batches of lime flowers harvested in 2008-2009 yrs. in Kharkiv (X0608, X0609 accordingly), Rivno (R0608, R0609 accordingly) and Zhitomir (Z0608, Z0609 accordingly) regions were the objects of our research.

Sampling and assay preparation of the herbal drug was carried out according to SPU 1.2 or EP7, 2.8.20 [6, 19].

Loss on drying or water content was determined by standard procedures of EP 7.

To develop the technique for determination of lipophilic substances content it was necessary to ground a sample weight of lime flowers and duration of analytical extraction for complete withdrawing of mentioned BAS.

As it has been noted above, for analytical purposes it's reasonable to use methylene chloride which can extract from lime flowers the complex of lipophilic substances similar by its composition to freon extract and has low boiling point (38-40°C).

To substantiate assay weight from the batch of lime flowers # R0608 comminuted to particle sizes not more than 0,355 mm we took samples 1, 5, 7 and 10 g in weight 5 items each and extracted with methylene chloride in Soxhlet apparatus at temperature 45-50°C during 4 hours, then the obtained extracts were cooled, filtrated through anhydrous sodium sulphate into preliminarily weighted vials. Solvent was evaporated under vacuum at ambient temperature until constant weight of a vial, and then it was weighed.

The content of lipophilic extractives taken with methylene chloride, *X, %* relatively to absolutely dry herbal drug was calculated under the formula (1):

$$X,\% = \frac{m_e \cdot 100}{m_H \cdot (100 - W)} \cdot 100,$$

where m_e – weight of the extractives obtained, g;
$m_н$ – weight of the herbal drug sample, g;
W – loss on drying of the herbal drug sample, %.

Content of essential oil in lime flowers was evaluated under the procedure of EP7, 2.8.12. Weight of the herbal drug sample was 200 g, distillation rate – 2,5-3,5 ml/min, volume of distillation liquid (water) – 500 ml, volume of xylene in the graduated tube – 0,50 ml.

Considering that after extracting of lipophilic complex the marc of lime flowers is used for producing of aqueous-alcoholic extract containing phenolic BAS, we also analyzed quantitatively total flavonoid content in experimental batches of the herbal drug under technique described in papers [20, 21].

All chemicals and reagents were of analytical grade.

Results and discussion

In our recent research [22, 23] it was found that yield of lipophilic BAS complex after complete extraction with liquefied difluorochloromethane (Freon R22) resulted in 1,66-2,78 % relatively to absolutely dry herbal drug depending on a batch. Extraction of lime flowers with liquefied tetrafluoroethane (Freon 134a) gave the volatile fraction with yield about 0,3% [22]. Authors [24] also obtained similar fraction with 0,13% yield from other linden species *Tilia tomentosa* mainly consisting of essential oil components.

Hence, in the monograph for lime flowers it's necessary to regulate the following quantity parameters (assay): "Lipophilic substances extracted with methylene chloride" and "Essential oil content".

Analyzing data of quantitative content of lipophilic extractives taken with methylene chloride from lime flowers, it was found (Table 2) that adequate accuracy of the analysis was provided if assay weight is not less than 7 g – relative error was less than 2 %.

The duration of analytical extraction needed for complete withdrawing of lipophilic extractives was determined by infusion of six 10 g assays of lime flowers during 1, 2, 3, 4, 5, 6 hours, then quantity of lipophilic extractives was revealed gravimetrically.

Table 2.- Quantitative content of lipophilic extractives taken from lime flowers with methylene chloride, depending on assay weight (herbal drug batch R0608, loss on drying 7,26 %)

Assay weight, g	1	5	7	10
Content of lipophilic extractives from lime flowers taken with methylene chloride in 5 samples, %	1,947	1,983	2,080	2,276
	1,857	1,934	2,233	2,221
	2,582	2,407	2,308	2,294
	2,768	2,371	2,186	2,265
	1,918	2,203	2,291	2,230
Statistics				
Average value	2,214	2,180	2,220	2,257
Mean-square error	0,191	0,097	0,041	0,014
Standard deviation	0,4269	0,2168	0,0919	0,0311
Sampling variance	0,1822	0,0470	0,0084	0,0010
Relative error, %	8,62	4,45	1,85	0,62

The results presented on Fig. 1 show that after 4 hours lipophilic BAS soluble in methylene chloride were taken almost completely.

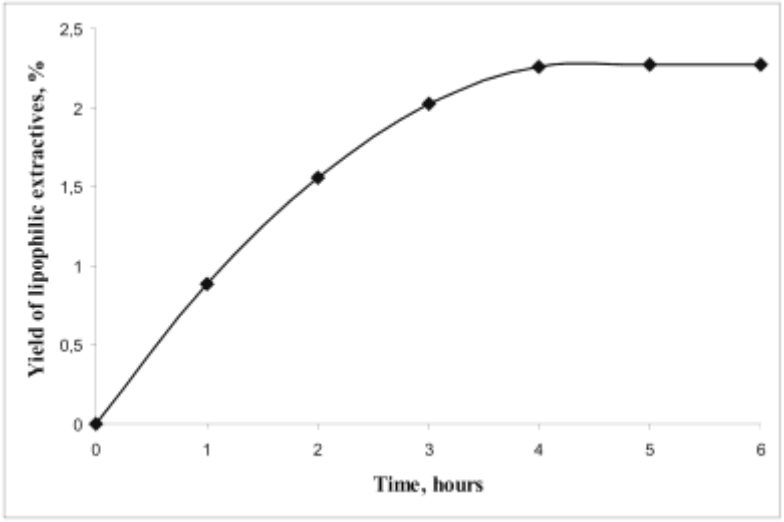

Fig. 1.- Yield of lipophilic BAS from lime flowers taken with methylene chloride depending on duration of analytical extraction

Further, under developed technique the following batches of lime flowers were evaluated: X0608, X0609, R0608, R0609, Z0608, Z0609.

It was found that content of the lipophilic extractives soluble in methylene chloride in the above-stated batches fluctuated within insignificant limits – 1,86-2,26 % (Table 3), that testifies onto rather stable accumulation of these BAS in lime flowers even under variable environmental conditions on the territory of Ukraine.

Table 3.- Quantitative content of lipophilic extractives soluble in methylene chloride in investigated batches of lime flowers

Batch #	X0608	X0609	R0608	R0609	Z0608	Z0609
Content of lipophilic extractives soluble in methylene chloride in 5 samples of each batch, %	2,103	1,998	2,276	1,864	1,925	1,839
	2,043	1,973	2,221	1,927	1,889	1,880
	2,070	1,990	2,294	1,941	1,916	1,873
	2,134	1,988	2,265	1,877	1,889	1,850
	2,103	2,043	2,230	1,877	1,927	1,888
Statistics						
Average value	2,0906	1,998	2,257	1,897	1,909	1,866
Mean-square error	0,016	0,012	0,014	0,015	0,008	0,009
Standard deviation	0,035	0,0263	0,0311	0,0343	0,0189	0,0207
Sampling variance	0,0012	0,0007	0,0010	0,0012	0,0004	0,0004
Relative error, %	0,75	0,59	0,62	0,81	0,44	0,50

From the results presented in Table 4 it's obvious that the essential oil content varied in rather wide limits – 0,34-0,55 ml/kg of herbal drug and considerably depended on harvest place: in the batches from Northwest of Ukraine this value was less than in the Eastern ones. Year of harvesting had less influence on quantity of essential oil in lime flowers.

Total flavonoid content in the analyzed samples was considerably variable (from 0,30 to 0,52 %) and depended mainly on place of harvesting, and lime flowers from the north-west of Ukraine (Rivno region) showed the best results (Table 5). Similar results have been obtained in the paper [21]. Besides, if consider the lower limit regulated by the monograph of Belarusian Pharmacopoeia, p. 370, then only one batch conformed to the norm (not less than 0,5 %). Therefore it's recommended to fix the lower limit 0,3 % for content of total flavonoids in the corresponding monograph draft.

Thus, after results of our research it's possible to make a conclusion that monographs for lime flowers existing in SPU, EP 7, BP 2009 are needed for the following amendments: "Assay" – lipophilic BAS extracted with methylene chloride - not less than 1,8 %, essential oil content - not less than 0,3 ml/kg, total flavonoids recalculated as quercetin - not less than 0,3 %.

Table 4.- Essential oil content in studied batches of lime flowers

Batch #	X0608	X0609	R0608	R0609	Z0608	Z0609
Essential oil content distilled with steam from the herbal drug, ml/kg	0,592	0,606	0,327	0,275	0,442	0,491
	0,535	0,496	0,381	0,384	0,439	0,382
	0,652	0,384	0,271	0,382	0,330	0,441
	0,479	0,437	0,376	0,327	0,333	0,443
	0,484	0,439	0,377	0,324	0,446	0,434
Statistics						
Average value	0,548	0,472	0,346	0,339	0,398	0,438
Mean-square error	0,033	0,038	0,021	0,020	0,027	0,017
Standard deviation	0,0736	0,0848	0,0477	0,0454	0,0609	0,0387
Sampling variance	0,0054	0,0072	0,0023	0,0021	0,0037	0,0015
Relative error, %	6,00	8,03	6,16	5,99	6,85	3,95

Table 5.- Quantitative content of total flavonoids in investigated batches of lime flowers

Batch #	X0608	X0609	R0608	R0609	Z0608	Z0609
Content of total flavonoids in the herbal drug recalculated as quercetin, %	0,363	0,323	0,516	0,454	0,297	0,324
	0,359	0,358	0,470	0,494	0,285	0,360
	0,381	0,366	0,521	0,459	0,306	0,350
	0,364	0,313	0,546	0,467	0,303	0,348
	0,389	0,358	0,533	0,475	0,311	0,341
Statistics						
Average value	0,371	0,344	0,517	0,470	0,300	0,345
Mean-square error	0,006	0,011	0,013	0,007	0,004	0,006
Standard deviation	0,0131	0,0238	0,0286	0,0157	0,0098	0,0135
Sampling variance	0,00017	0,00057	0,00082	0,00025	0,00009	0,00018
Relative error, %	1,58	3,10	2,48	1,49	1,46	1,75

Conclusions

Grounded and simple techniques for standardizing of raw lime flowers by their quantitative parameters have been developed. It was shown that existing pharmacopoeian monographs "Lime flower" are needed for amendments and changes. Six batches of lime flowers harvested in various regions of Ukraine in 2008 and 2009 yrs. were investigated. It was found that total content of lipophilic extractives taken with methylene chloride was stable in studied batches and slightly differed from those extracted with difluorochloromethane (Freon R22) being within 1,86-2,26 %. On the other hand, the essential oil content determined by hydrodistillation considerably fluctuated between investigated batches of raw lime flowers (0,34–0,55 ml/kg) and depended on region of their harvesting. Also it has been revealed notable differences between the batches in their flavonoid contents – 0,30-0,52 %.

Based on the results obtained it is offered to regulate the following quantity parameters in the amended monograph draft for lime flower: total extractives taken with methylene chloride - not less than 1,8 %, essential oil - not less than 0,3 ml/kg, total flavonoid content recalculated as quercetin - not less than 0,3 %.

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DEVELOPMENT OF TECHNIQUES FOR QUANTITATIVE ANALYSIS OF LIME FLOWERS

Demyanenko D. V., Demyanenko V. G., Breusova S. V.

Introduction. The article is devoted to the development of techniques for quantitative analysis of lime flower in order to make amendments to existing pharmacopoeian monographs for this herbal drug. Lime inflorescences contain lipophilic biologically active substances (BAS) causing notable antimicrobial and anti-inflammatory effects and also more polar phenolic compounds with antiulcer activity. Considering this, it's necessary to regulate all these groups of BAS quantitatively. **Materials and methods.** For this study six batches of lime flowers harvested in 2008-2009 yrs. in Kharkiv, Rivno and Zhitomir regions were used as crude herbal drug. Loss on drying was determined by routine pharmacopoeian procedures. Total content of lipophilic substances was determined gravimetrically after Soxhlet extraction of samples 1, 5, 7 and 10 g in weight with methylene chloride, considering that by its extracting ability this solvent is close to liquefied difluorochloromethane (freon R22) used by us for obtaining of lipophilic complexes. The duration of complete analytical extraction was determined by infusion of six 10 g assays of lime flowers during 1, 2, 3, 4, 5, 6 hours, then quantity of lipophilic extractives was revealed gravimetrically. Quantity of essential oil in lime flowers was evaluated under the procedure of EP7, 2.8.12. Weight of the herbal drug sample was 200 g, distillation rate – 2,5- 3,5 ml/min, volume of distillation liquid (water) – 500 ml, volume of xylene in the graduated tube – 0,50 ml. Total flavonoid content recalculated to quercetin was determined after hydrolysis with acidified acetone, withdrawing of flavonoid aglycones with ethylacetate and by further spectrophotometry of their complexes with aluminium chloride. All quantitative determinations were replicated five times for each assay. All chemicals and reagents were of analytical grade. **Results and discussion.** It was found that adequate accuracy of the analysis of lipophilic extractives taken with methylene chloride was achieved if assay weight is not less than 7 g: relative error was less than 2 %. Study of extraction kinetics showed that after 4 hours lipophilic substances soluble in methylene chloride were taken almost completely. Analyzing 6 batches of lime flowers from different Ukrainian regions, it was found that yield of total extractives taken with methylene chloride did fall within insignificant limits – 1,86-2,26 %, that testifies onto rather stable accumulation of these biologically active substances in lime flowers even despite different climatic conditions. Essential oil content varied in rather wide limits – 0,34-0,55 ml/kg of herbal drug and considerably depended on harvest place: in the batches from Northwest of Ukraine this value was less than in the Eastern ones. Year of harvesting had less influence on quantity of essential oil in lime flowers. The relative error of the quantitative determinations was within 4–8 %. Total flavonoid content in the analyzed batches of lime flowers was considerably variable (from 0,30 to 0,52 %) and depended mainly on place

of harvesting, and this crude herbal drug from the north-west of Ukraine (Rivno region) showed the highest results among the analyzed samples. Besides, if consider the lower limit regulated by the monograph of Belarusian Pharmacopoeia, p. 370, then only one batch conformed to the norm (not less than 0,5 %). Therefore it's recommended to fix the lower limit 0,3 % for content of total flavonoids in the corresponding monograph draft or analytical normative documentation. The relative error of the quantitative determinations of flavonoids was within 1,5–3,1 %. **Conclusion.** Six batches of lime flowers harvested in various regions of Ukraine in 2008 and 2009 yrs. were investigated. Based on the results obtained it is offered to regulate the following quantity parameters in the amended monograph draft for lime flower: total extractives taken with methylene chloride – not less than 1,8 %, essential oil content – not less than 0,3 ml/kg, total flavonoid content recalculated as quercetin - not less than 0,3 %.

Keywords: Lime Flowers (Tilia cordata Mill.), Quantitative Analysis, Essential Oil, Flavonoids, Difluorochloromethane.