

Development and validation of standardization methods of aqueous spropel extract

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Abstract

Methodological bases and uniform standardization criteria of humic compounds as substances for drug products have not been developed yet. This is due to the structural complexity of humic compounds, the variety of ways to extract them from natural objects, the impossibility of using many classical methods of analytical chemistry to identify and quantify humic substances (HS), the lack of standard samples. The identification of humic acids (HA) in the aqueous spropel extract (ASE) is identified after extracting from ASE by alkaline hydrolysis by the quantification method. After further precipitation with a concentrated sulfuric acid solution characteristic dark brown color is appeared. It was carried out the HA extraction from the sample of ASE, the precipitation of HA, the oxidation of HA and Mohr's salt titration in accordance with the methodology developed on the basis of SSTU 7083:2009. It was determined that the total mass fraction of HA in the ASE sample was 83.8 mg/g± 0.12%. The methods of identification and quantification of the total mass of HA in ASE have been developed and validated. The ASE has been standardized.

Keywords

ASE, HA, quantitative analysis, standardization, validation

Introduction

Spropel belongs, from the physicochemical point of view to the class of complex polydisperse macromolecular systems and contains a significant number of substances, the main group of which are humic substances, with not fully studied chemical structure (Yudina et al. 1998).

HS occupy a special space among the natural biologically active substances (BAS) and are complex systems of macromolecular organic compounds with aromatic, alicyclic, and heterocyclic moieties substituted with alkyl

chains and other different functional groups (Vašková et al. 2011). The complexity of the HS structure is caused by various factors and their formation conditions. The methods used to extract the HS from natural objects significantly affect both their composition and properties (Holland et al. 2014).

Unlike most BAS of natural origin, the biosynthesis of which is genetically determined and ordered, the formation of HA is chaotic. The organic bottom sediments decompose into simple compounds, from which the synthesis of complex organic substances takes place, which

is accompanied by condensation and polymerization of the starting compounds (Gomes de Melo et al. 2016). The reactions of synthesis and decomposition occur almost continuously. As a result, the most stable compounds accumulate (Kondratenko 2016; Klucakova and Veznikova 2017).

HA contain about 15 different types of functional groups in different proportions and configurations (Nebioso and Piccolo 2011), among which the most common in the HA structures are phenolic and carboxylic groups (Pidvalna and Poznyak 2004; Didkovska 2009; Chauke Tsakani Locrecia 2014) (Fig. 1).

The composition of functional groups and the structure of molecular fragments of HA depend on their source and extraction method (Tikhova et al. 2008). The question of the spatial arrangement of atoms in the HA molecules remains open.

The complexity of the HA structure, the presence of a large number of functional groups and the ability to form intermolecular and intramolecular bonds cause a wide range of interactions in which can enter HA. The presence of such groups as carboxyl, carbonyl, phenolic, and hydroxyl, in combination with aromatic structures, provides the ability of HA to ionic and donor-acceptor interactions. HA are involved in sorption and redox processes (Gomes de Melo et al. 2016; Savchenko et al. 2019). However, it is very difficult to identify the area or functional groups that determine a particular type of biological activity in the structure of the HA macromolecule.

The anti-inflammatory, antioxidant, hepatoprotective, antiviral activities in vitro and in vivo were studied and reported for HA (Buzlama and Chernov 2010; Aeschbacher et al. 2012; Van Rensburg 2015; Savchenko et al. 2019). It was found that HA promote wound healing and have antibacterial properties (Vašková et al. 2011; Van Rensburg 2015; Metin Çalışır et al. 2018).

At the same time, there is no reliable information on the mechanisms of the HA action on biological objects. In vivo and in vitro experiments have shown that humates exhibit antioxidant activity (Zhilyakova 1999; Pidvalna and Poznyak 2004; Perminova et al. 2007; Aeschbacher et al. 2012; Klucakova and Veznikova 2017). The anti-inflammatory activity of HA has been studied in the models of acute and chronic inflammation (Vašková et al. 2011; Van Rensburg 2015).

To date, the antiviral activity of HA has been studied. The mechanism of the antiviral HA action is explained by the ability of their polymer molecules to inhibit the adsorption of the virus on the cell membrane (Orlov 1992). The data on the sorption properties of HA are known (Savchenko et al. 2019).

Today, considerable attention is paid to the HS, which can be explained by their antiviral, profibrinolytic, anti-inflammatory, and estrogenic properties (Aykaç et al. 2018). Experimental studies show that HA and FA, are effective in the treatment of cancer diseases using special methods of HS therapy (Jurcsik 1994; Bernacchi et al. 1996; Gomes de Melo et al. 2016; Aykaç et al. 2018; Kala et al. 2019).

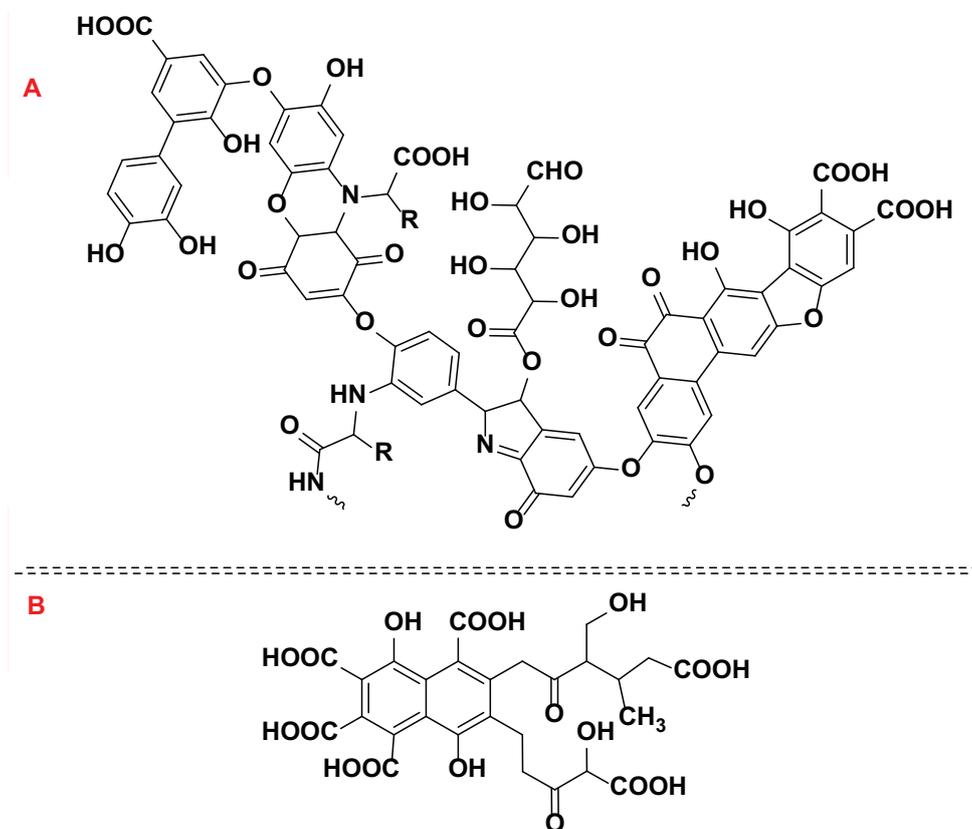


Figure 1. Structures of humic acid basic core (A) and fulvic acid (B). Source: (Wang and Mulligan 2018).

The mechanism of HS action can also contribute to the inhibition of both classical and alternative ways of complement activation, as well as phagocyte degranulation and production of inflammation-related cytokines such as IL-1 β , IL-6, IL-10, and TNF- α (Yudina and Tikhonova 2003; Jooné and Van Rensburg 2004; Junek et al. 2009; Van Rensburg and Naude 2009; Buzlama and Chernov 2010; Ji et al. 2016; Poluyanova 2017).

Using dispersed sodium hydroxide to bring raw materials to a colloidal state and improve the interaction between the components is a rational strategy for the technology of humic preparations (Dragunov 1962; Didkovskaya 2009). Such approaches have been used to make an ASE with a high content of HA. The intensification of the classical method of obtaining HA with the additional use of dispersion (cavitation) increases the yield of HA compared to the classical method from 76.19% to 83.80%, which is almost 9% (Strus and Polovko 2016).

The introduction of drugs based on HS involves the study of their properties, the development of modification methods and selection of standardization criteria of both the raw material source of humic compounds as well the humic substances themselves.

Methodological bases and uniform standardization criteria of humic compounds as substances for drug products have not been developed yet. This is due to the many reasons such as the structural complexity of humic compounds, the variety of ways to extract them from natural objects, the impossibility of using many classical methods of analytical chemistry to identify and quantify humic substances, and the lack of standard samples.

The aim of our study was to develop standardization methods of ASE and to validate the developed methods.

Materials and methods

Materials

ASE was obtained by the following technology: 10 kg of sapropel from Prybych deposit, located in Volyn region, Ukraine (sapropel humidity 60%), pre-crushed and sifted through a sieve with a diameter of 1–5 mm, poured into a dispersant (DES 350/4000 (Ukraine)); added alkaline aqueous solutions 0.1 N and water (up to 100 l) in a ratio of 1:10 and dispersed, settled and filtered. The obtained extract was evaporated to 1:10 from the initial volume in a vacuum evaporator (at a temperature of 50–60 °C). The obtained extracts were drained and settled for 1 day. After settling, the liquid fraction was drained and the samples for experimental studies were taken (Strus and Polovko 2016).

The main group of biologically active substances in ASE are humic substances, which according to the research data have low toxicity, an expressed antiviral, wound healing and anti-inflammatory activities, suppress of the development of malignant tumors, etc. (Strus and Polovko 2020).

As a result of spectrometric micro- and macroelement analysis of sapropel (spectrometer “Elvax” “Elvatech” Ltd., Ukraine) it was found sulfur, sodium and calcium among the macronutrients. In the dry residue the sodium and calcium content are 81.273% and 17.73% respectively. Among the trace elements are Si, Cr, Fe, Co, the highest Fe content is 0.78%.

Reagents

The following reagents were used to develop the qualitative and quantitative analysis method for the determination of HA in ASE.

0.1 M alkaline solution of sodium diphosphate ($\text{Na}_4\text{P}_2\text{O}_7 \times 10\text{H}_2\text{O}$). Place 44.6 g of sodium diphosphate and 4.0 g of sodium hydroxide in a 1000 ml volumetric flask, dissolve a portion in 500 ml of purified water P and make up the solution with purified water P to the mark.

0.4 M solution of chromium mixture. Place 40.0 g of finely divided potassium dichromate in a 1000 ml volumetric flask, dissolve in 500 ml of purified water P and make up to the mark with purified water P. The solution is placed in a conical flask with a capacity of 3000 ml and sulfuric acid concentrated in an amount of 1000 ml is added in 100 ml portions carefully, with an interval of 10–15 min. After cooling, the mixture is poured into a dark glass vessel.

0.2 M solution of Mohr's salt ($(\text{NH}_4)_2\text{SO}_4 \times \text{FeSO}_4 \times 6\text{H}_2\text{O}$) 80.0 g of Mohr's salt is dissolved in 500 ml of purified water P, filtered into a volumetric flask with a capacity of 1000 ml, add 20 ml of concentrated sulfuric acid and make up purified water P to the mark.

Check of the concentration of Mohr's salt: in three conical flasks with a capacity of 100 ml place 10 ml of Mohr's salt solution, add 1 ml of concentrated sulfuric acid and titrate with 0.1 M potassium permanganate solution to a pale pink color that does not disappear within 1 min. Use the average of the three tests.

0.2% N-phenylanthranilic acid solution. 0.20 g of N-phenylanthranilic acid is triturated with a small amount of 0.2% sodium carbonate solution, transferred to a volumetric flask with a capacity of 100 ml, mixed and make up to the mark with 0.2% sodium bicarbonate solution.

Methods

To standardize the developed ASE, the following indicators were selected: description, identification of HA, pH value, organic matter content, microbiological purity, quantitative determination of the total mass fraction of HA.

Description according to the requirements of the pharmacopoeia article of the European Pharmacopoeia (EPH) of the 10th edition “Extracts”. Odor 2.3.4. p. 133.

The pH value. Potentiometric determination of pH 2.2.3. p. 24 (EPH 2019).

Organic matter content. Organic matter content was determined using thermogravimetric method, which is based on determining the weight loss of the selected ASE

sample after calcination under 800 °C (Specification 20.1 – 2870305538 – 004:2016). Mass fraction of dry residue. The dry residue of ASE is determined by the method of Eph 10th ed., Vol.1, 2.8.16. p. 311.

Heavy metals

Determination is carried out according to the Eph 10th ed., Vol.1, 2.4.8(Method A) p. 139. The content of heavy metal should not exceed 0.001%. The standard is prepared using a reference solution of Pb.

Microbiological purity

Microbiological parameters of ASE were studied in accordance with the requirements of the Eph 10th ed., Vol.1, 2.6.12. p. 201, 2.6.13 p. 205.

Validation

The approach to the BAS validation in herbal preparations described in the literature was used to study the validation characteristics ASE (Grizodub et al. 2012). The specificity of this analytical procedure was proved by determining the number of milligrams in the HA sum in the studied ASE by volumetric titration. A control experiment was performed in parallel for increasing the specificity of the redox technique.

The method validation was done by evaluating linearity and precision.

Precision studies were performed on nine determinations in the concentration range of ASE from 80% to 120% according to the chosen method. For determining the linearity was performed the titration of each sample of ASE in a concentration from 80% to 120% of the selected at least three times.

Experimental part

The identification and quantification of HA were performed for standardization of the active substances in ASE.

The identification of HA in the ASE was performed after extracting from ASE by alkaline hydrolysis by the quantification method. The characteristic dark brown color is appeared after precipitation with a concentrated sulfuric acid solution.

Quantitative determination of the total mass fraction of HA in the ASE. The total mass fraction of HA was determined by the method described in the literature, after HA oxidation by I.V. Tyurin's method in B.A. Nikitin's modification and Mohr's salt titration (SSTU 7083: 2009, 2011).

Extract of HA from a portion of ASE. 2.0000 g of extract (exact portion) is placed in a conical flask with a capacity of 250 ml (flask A), add 100 ml of alkaline sodium diphosphate solution, and stir for 1 h on a shaker. The obtained suspension is transferred to a centrifuge tube and centrifuged for 15 min at 2000 rpm. The solution above the precipitate is decanted into a conical flask with a capacity of 1000 ml (flask B). The precipitate is

washed twice with 100 ml of 1% sodium hydroxide solution, each time centrifuged and the solution is poured into flask B.

To the precipitate in a centrifuge tube the 100 ml of 1% sodium hydroxide solution is added and transfer to flask A. The flask with the suspension is heated for 2 h in a boiling water bath and cooled to room temperature. The suspension is centrifuged and the solution is poured over the precipitate into flask B.

The remaining precipitate is washed twice with 100 ml of 1% sodium hydroxide solution and centrifuged, the solution is poured into flask B. The precipitate is washed three times with 100 ml of purified water P, centrifuged and the solution is poured into flask B. The content of flask B is filtered through a blue strip filter into a 1000.0 ml volumetric flask, made up to the mark with purified water P, and mixed (solution for determining the total mass fraction of HA).

Deposition of HA (HA identification). From a 1000 ml flask containing an alkaline HA solution, take 50.0 ml of the solution, transfer to a 100 ml conical flask and, while stirring, add dropwise concentrated sulfuric acid to a pH solution from 2.0 to 3.5. The pH solution is measured potentiometrically. The flask is heated on a boiling water bath for 30 min, cooled and left for 16 h for complete precipitation.

The HA are filtered through a blue strip filter. The precipitate on the filter and the flask in which the HA was precipitated were washed with 0.05 M sulfuric acid solution 3 times in 10 ml to give a clear filtrate. Obtained HA are dark brown, soluble in weak alkalis, slightly soluble in water.

The funnel with the precipitate on the filter is inserted into a volumetric flask with a capacity of 100 ml and dissolve the precipitate of HA with hot 0.05 M sodium hydroxide solution. Wash the filter with hot sodium hydroxide solution until the precipitate is completely dissolved and a clear filtrate is obtained. The obtained alkaline HA solution was cooled to room temperature and the volume of the flask was made up to 0.05 M with sodium hydroxide solution.

Oxidation of HA by I.V. Tyurin's method in B.A. Nikitin's modification and Mohr's salt titration. In a conical flask with a capacity of 100 ml is placed 20 ml of alkaline HA solution, evaporate the solution in a boiling water bath to dry sediment without overdrying it.

To a conical flask with dry sediment add 10 ml of 0.4 M solution of chromium mixture. The solution is added slowly, the walls of the flask are thoroughly washed off. The flask is closed with a funnel and placed in an oven for 20 min at a temperature of 150–160 °C. After cooling the flask, remove the funnel, wash with purified water, add 6 drops of 0.2% N-phenylanthranilic acid solution and titrate with 0.2 M Mohr's salt solution until the color changes from cherry red to green. In parallel make a control study.

1 ml of 0.2 M Mohr's salt solution corresponds to 0.001034 g of humic acids.

The total mass fraction of HA (X), in milligrams, in 1 g of ASE, is calculated by the formula:

$$X = \frac{(V_1 - V_0) \times K \times 1034}{m}$$

where: V_1 – the volume of Mohr's salt solution spent on titration of the control experiment, ml;

V_0 – the volume of Mohr's salt solution spent on titration of the analytical sample, ml;

K – the correction factor for the Mohr's salt solution;

m – the weight of the test portion, g.

Results and discussion

The HA extraction from the sample of ASE, the precipitation of HA, the oxidation of HA and Mohr's salt titration in accordance with the methodology developed on the basis of SSTU 7083:2009 were carried out. The amount of HA in 1 g of ASE should be at least 82.5 mg. It was determined that the total mass fraction of HA in the ASE sample was 83.8 mg/g \pm 0.12%.

The linear relationship between the concentration of the total mass fraction of HA from the mass of the ASE sample is the criteria for the acceptability of the proposed method for quantifying the total mass fraction of HA. The results are presented in Table 1 and Figure 2.

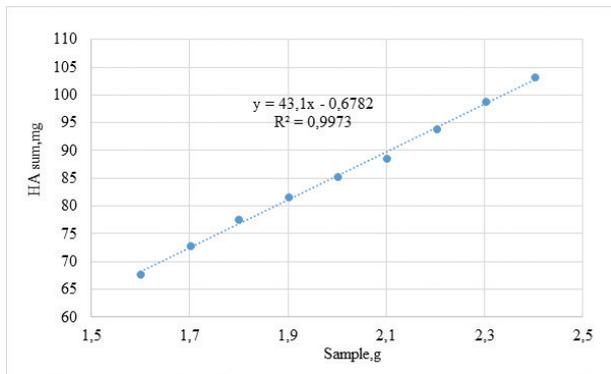


Figure 2. The dependence of the concentration of the total mass fraction of HA on the mass of the ASE sample.

Table 1. Linear dependence between the concentration of the total mass fraction of HA on the mass of the ASE sample.

Working concentration, %	ASE sample, g	Total mass fraction of HA, mg
80	1.6003	67.85
85	1.7011	72.94
90	1.7981	77.61
95	1.9008	81.60
100	2.0012	85.26
105	2.1001	88.62
110	2.2014	93.94
115	2.3019	98.82
120	2.4017	103.33

There is a linear dependence of the concentration of the total mass fraction of HA on the mass of the ASE sample with a correlation coefficient of 0.9986 (\geq 0.9981), the an-

gular coefficient of linear dependence (b) is 1.01, the free member of the linear dependence (a) - 0.80 \leq 2.60.

The obtained results of precision studies are presented in Table 2 and show that the method is precise, because the value of the relative confidence interval is less than the critical value for the result convergence: $\Delta\% = 1.41 \leq 1.60$ and the insignificance criterion of systematic error $\delta = 0.51$ (Table 2).

Table 2. The study results of the precision of the total mass fraction of HA quantitative determination method.

Parameters	Value	Standard 1	Standard 2	Conclusion
Precision ΔZ	1.41	≤ 1.60	-	Sustained by the first standard

The experimental research results of the total mass fraction of ASE quantitative determination in 6 series of ASE and metrological characteristics of the average result are presented in Table 3.

Table 3. Metrological characteristics of the total mass fraction of HA in ASE average quantitative determination result.

No.	The content of the total mass fraction of humic acids, mg	S ²	S	S \bar{x}	Δx	$\Delta \bar{x}$	$\bar{\epsilon}, \%$	$\epsilon, \%$
1	89.25	3.9061	1.9764	0.8069	5.0805	2.0741	3.36	5.79
2	89.24							
3	85.8	3.9061	1.9764	0.8069	5.0805	2.0741	3.36	5.79
4	85.16							
5	87.37							
6	89.85							

The study results of the microbiological indicators are presented in Table 4.

Table 4. Microbiological indicators of ASE.

Name of the indicator	SPhU norm	Analysis results
Quantity of MAFAM*, CFU in 1 cm ³ of production, no more than	100	80
Bacteria genus. <i>Enterobacteriaceae</i> in 1 cm ³	No	No
<i>Staphylococcus aureus</i>	No	No
<i>Pseudomonas aeruginosa</i>	No	No
The number of yeasts and molds, CFU in 1 cm ³ , not more than	10	5

Note: * – MAFAM – mesophilic aerobic and facultative aerobic microorganisms.

The specification and study results are presented in Table 5.

The therapeutic effect of sapropels and their extracts is determined by the qualitative and quantitative composition of minerals and organic substances. The content of organic substances and their component composition in sapropels of different deposits widely varies depending on the conditions of their formation (geological, hydrogeological, climatic, physicochemical and biological factors), namely 10–95% in terms of dry matter.

Our experimental studies showed that the amount of organic matter and dry residue in the studied samples of ASE were 40.2 \pm 0.41% and 10.14 \pm 0.23%, respectively. In the specification in permissible norms of organic matter and dry residue we put quantitative indicators taking into account the

Table 5. Specification and research results of quality ASE indicators.

Indicator	Permissible norms	Analysis results
Description	Homogeneous liquid, dark brown in color, with a faint specific odor characteristic of raw materials.	Meets
Identification of the HA	After acidification of the solution with sulfuric acid to the pH value 2–3, a dark brown precipitate of HA is formed (precipitation of HA)	Meets
Hydrogen index (pH)	7–11	10 ± 0.2
The content of organic matter in terms of dry matter, %	Not less than 20	40.2 ± 0.41
Mass fraction of dry residue, %	Not less than 5	10.14 ± 0.23
Microbiological purity	In 1 cm ³ of ASE no more than 100 CFU MAFAM * is allowed; not more than 10 CFUs of yeasts and molds, the absence of bacteria of the families <i>Enterobacteriaceae</i> , <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i>	Meets
Quantitative definition The total content of HA, mg / g	82.5–85.0	83.8 ± 0.12
Heavy metals, %	Not more than 0.001	Meets

Note: * MAFAM – mesophilic aerobic and facultative aerobic microorganisms.

sapropel composition variations of different deposits. Permissible norms for the pH value are set in the range of 7–11 due to the pH value of various sapropel deposits, as well as the extraction conditions of humic acids (alkaline pH value).

Conclusion

The methods of identification and quantification of total mass of HA in ASE have been developed and validated.

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The ASE has been standardized according to the following indicators: description, identification of humic acids, pH value, organic matter content, microbiological purity, quantitative determination of total mass fraction of HA.

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