

# PHYSICOCHEMICAL PROPERTIES OF SOME BIOLOGICALLY ACTIVE SUBSTANCES OBTAINED FROM VEGETABLE RAW MATERIAL THROUGH CO<sub>2</sub>-EXTRACTION

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## INTRODUCTION

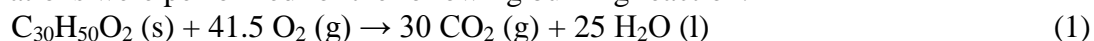
Extraction with the gases, which are in the supercritical state, has been successfully used lately as an ecology-friendly technique for producing valuable biologically active substances (BAS) [1–4]. This technique has all grounds to be called a “green chemistry” method [5]. Reliable data on physical-chemical properties of the BAS, obtained using the technique of supercritical fluid extraction with carbon dioxide, are required for BAS standartization [6]. Thus, using the technique of differential thermal analysis (DTA) within the range of 80–550 K, we investigated phase and physical transitions (melting, crystallization, evaporation, vitrification) in the samples of the CO<sub>2</sub>-extracts of pine shoots, weeping birch (*Betula pendula*) bark, holy thistle (Silybum) seed blending, and corcule of wheat and sea-buckthorn berries. The investigation was aimed at considering a possibility to use the temperatures of the aforementioned transitions as the reference ones when compiling different lots of the obtained products. In addition, qualitative and quantitative compositions of the CO<sub>2</sub>-extracts were studied using chromatographic and spectral methods. Radiation protecting, anti-atherogenic and anti-oxidative properties of CO<sub>2</sub>-pine extracts were investigated as well. Betulin (C<sub>30</sub>H<sub>50</sub>O<sub>2</sub>), it being a natural BAS which represents itself pentacyclic triterpenoid alcohol of lupane structure, was extracted from the CO<sub>2</sub>-weeping birch bark extract and purified up to the concentration of the ground substance of 99.5 mas.%. It exerts antiseptic, antiviral, choleric and gastro-hepatoprotective action. The elemental analysis of betulin revealed the following concentrations, mas.%: C – 81.67, H – 11.45, O – 6.88 (theory – 81.39; 11.38; 7.23, respectively). Its solubility at 296.35 K in chloroform (1.678 g of C<sub>30</sub>H<sub>50</sub>O<sub>2</sub> per 100 g of solvent), thermodynamic characteristics and phase transition temperatures were determined.

## MATERIALS AND METHODS

The differential thermal analysis (DTA) in the range of 80–650 K was performed in the helium atmosphere with the device, which was designed at the thermochemistry laboratory of the Research Institute of Chemistry with the Nizhny Novgorod State University [7]. Quartz served as a reference. The sample and the reference were 0.25–0.35 g in mass. The sample temperature and the temperature difference between the sample and the reference were

measured with a chromel–copel thermocouple, the measurement error being 0.5 K. The thermocouple was calibrated within the entire temperature range using a platinum resistance sensor and the reference substances. The heating rate in the experiments was 5 K/min. Deviation from linearity did not exceed 1%. To check the operation of the DTA device, the melting temperature of the reference n-heptane and the vitrification temperature of purified glycerine were determined. The obtained results coincided with corresponding reliable literary data for n-heptane [8] with the 0.2 K error, the latter being 1 K for glycerine [9].

Enthalpy of betulin burning was determined in the modified B–08MA calorimeter with a static calorimetric bomb [10]. The calorimetric system was calibrated using K-2–grade benzoic acid as a reference ( $\Delta U_c = -26460.0$  J/g when weighed in the air). The energy equivalent of the system was  $W = 14847 \pm 3$  J/K, the doubled square deviation from the mean being 0.02%. The betulin pellet-shaped samples were burned together with benzoic acid pellets at the oxygen pressure of  $3 \cdot 10^6$  Pa. Based on the chromatographic analysis data the used oxygen contained the following admixtures, mol.%:  $N_2 - 0.8$ ; CO and  $CO_2 - 0.002$ ; hydrocarbons – 0.001. The substance in a quartz crucible was ignited with a condenser discharge fed to the platinum wire, connected with the substance via a cotton thread. The temperature rise was measured with two platinum resistance sensors and the digital voltmeter, incorporated into the bridge circuit. The mass of the analyzed compound sample was determined based on the concentration of  $CO_2$ , formed during the sample burning. The calculations were performed for the following burning reaction:



The burning energy of the analyzed substance  $\Delta_c U$  was determined under calorimetric bomb conditions. Based on the average value of  $\Delta_c U$ , the molar mass (M), and taking into account the Washburn correction and the correction, attributed to variations in the number of gas gram-molecules, the standard values of  $\Delta_c U^\circ$  and  $\Delta_c H^\circ$  were calculated for the reaction of betulin burning. Based on the obtained  $\Delta_c H^\circ$  values the enthalpies of solid betulin formation  $\Delta_f H^\circ$  at 298.15 K were calculated using as follows:  $\Delta_f H^\circ(H_2O, l) = -285.830 \pm 0.042$  kJ/mol and  $\Delta_f H^\circ(CO_2, g) = -393.51 \pm 0.13$  kJ/mol [11].

The chromatographic-mass-spectral analysis was performed with the help of the Trace GC Ultra/DSQII chromatography-mass spectrometer. The capillary column TR 5 MS 60 m-long and 0.25 mm in diameter was used. The test sample in the amount of 1  $\mu$ l was syringed into the chromatograph injector, heated up to 583 K. The velocity of the gas-carrier flow (M 60-grade helium) was 1 ml/min. The column temperature varied from 473 to 585 K. The obtained mass-chromatograms were recorded within the 35–600 mass number range. The chromatogram recording began after 4 min (to cut off the solvent peak). The mixture components were identified using “NIST-2005” electron mass-spectra library.

Radiation protecting properties of the carbon-dioxide pine shoot extract were investigated using the model of the bone marrow form of radiation sickness. White nonlinear mice 20 g in mass were used. The animals were divided into four groups, each containing 20 species: 1st group – the animals fed only with vegetable oil; 2nd group – the animals fed only with  $CO_2$ –extract of pine shoots; 3rd group – the radiation-exposed animals; 4th group – the animals exposed to radiation against the background of the action of the  $CO_2$ –extract of pine shoots. The oil preparation of the  $CO_2$ –extract, the dose being 1500 mg/kg, was endogastrically introduced to the animals during seven days with the periodicity of 1 time a day. The reference animals were given vegetable oil. Three hours after the preparation introduction the animals were subject to the 3 Gy–dose of  $\gamma$ –radiation ( $Co^{60}$ ) at the therapeutic facility “Agat S”, that initiated development of the bone marrow form of radiation sickness.

The bone marrow and the blood to be analyzed were taken one day and seven days after irradiation. The bone marrow was taken from the femoral bone, whereas the blood was taken from the entire carcass after decapitation. As far as the bone marrow was concerned, the number of hemopoietic cells (myelocytes), the mitotic index and the number of chromatic aberrations were determined. As for the blood, the hemoglobin content, the number of leucocytes as well as the concentration of one of the secondary products of peroxide oxidation of lipids, namely malonic dialdehyde, were defined.

We also performed pre-clinical investigations and analyzed parameters of the anti-atherogenic and antioxidative action of the CO<sub>2</sub>-extract of pine shoots. The experiments were performed with pubertal Wistar male rats 280–300 g in mass. The experimental statistic groups consisted of 10 animals. The carbon-dioxide extract of pine shoots in the form of a 0.15% oil solution was endogastrically introduced to the male rats daily during 3 weeks against the background of experimental hyperlipidemia. Refined vegetable oil in corresponding amounts served as a reference substance in all experimental runs. The equitherapeutic daily dose of the preparation, given to the animals, was 10 mg/kg. Experimental atherosclerosis (hyperlipidemia) in the animals was brought up during 1 month with the help of an atherogenic diet: 3% of cholesterol, 0.25% of cholic acid, 0.3% of methyl uracil and ergocalciferol, the dose being 35000 Units/kg. These substances were included into a standard ration of the vivarium. In a month, against the background of the atherogenic diet, they began adding 2 ml of oil preparation of the pine shoots extract to the food of the animals from two test group. Then, after 10 and 30 days of feeding with the extract the animals (test groups comprising 10 rats) were slaughtered, and their blood and brain tissues were taken for biochemical analysis. The animals which were not given the extract of pine-tree shoots as a food addition (10 rats) served as a reference.

Biologically active substances from pine shoots, weeping birch (*Betula pendula*) bark and blending of holy thistle seeds, wheat corcule, and sea-buckthorn berries, were obtained by us using the technique of supercritical fluid extraction according to the patented technology [12] at 313 K and 15.0 mPa at the UEN facility, developed by the research-and-production company "Biofit" JSC (Nizhny Novgorod). As far as organoleptic factors are concerned, carbon-dioxide extracts from the pine shoots are a thick pasty composition at room temperature with a characteristic pine smell, its color being from yellow to dark-green. The density of carbon-dioxide extracts is 1.00–1.03 g/cm<sup>3</sup>. The kinematic viscosity, determined with a capillary viscosimeter at 323 K, is 900–1400 mm<sup>2</sup>/s.

The CO<sub>2</sub>-weeping birch bark extract is a white or slightly yellowish powder, containing 43 mas.% of the valuable substance betulin.

The blending was obtained from holy thistle seeds (50%), wheat corcules (30%), and schrot of sea-buckthorn berries (20%). The extract contains lecithin (2.4%) and carotinoids (39.3 mg%).

## RESULTS AND DISCUSSION

The investigations of the CO<sub>2</sub>-extracts from pine shoots using the IR-spectroscopy technique showed that the spectrum of the hydrocarbon fraction contains 2920, 2960, 2850, 1380 and 1460 cm<sup>-1</sup> absorption bands, characteristic for C–H oscillations. The IR-spectrum of carbonyl compounds contains 1730, 1250 cm<sup>-1</sup> absorption bands, typical for C=O groups. The absorption bands of the alcohol spectrum, namely 1730, 1250, and 1040 cm<sup>-1</sup>, are related to oscillations of C=O and O–H groups of alcohols and ether alcohols. The IR spectrum of polyfunctional compounds contains a whole set of bands, typical for different types of

oscillations. The analyzed test sample was pre-saponified to reveal the presence of combined acids (in the form of ethers). The elemental composition of the obtained acids after saponification was determined using gas-liquid chromatography (GLC), and neutral substances were separated in the column with aluminum oxide. The obtained results are listed in Table 1.

Table 1

Results of quantitative separation of carbon-dioxide extract of pine-tree shoots

Extracted fraction	Mass fraction, %	
	Before saponification	After saponification
<b>Neutral substances</b>	31.0	24.5
<i><b>Including</b></i>		
Hydrocarbons	1.21	1.22
Carbonyl-containing compounds (aldehydes, ketones, esters)	7.00	2.82
Alcohols	14.32	16.12
Polyfunctional compounds	3.41	2.21
<b>Free acids</b>	61.7	65.6
<i><b>Including</b></i>		
Higher fatty acids	6.74	10.74
Resin acids	51.86	46.06
Oxidized acids	3.10	8.80

The GLC technique allowed identifying 9 higher fatty and 10 resin acids in the carbon-dioxide extracts of pine shoots. The maximum concentration of vitamin F is 3.22%; that of vitamin E, 10–50 mg/100g. The content of monoterpene hydrocarbons and neutral compounds in the extracts from pine shoots was also determined. The extracts from pine shoots are characterized by high acid numbers (74–119 mg KOH/g) and low ether numbers (15–60 mg KOH/g).

We performed DTA within the range of 80–400 K for several CO<sub>2</sub>-extract samples. All the samples were pre-cooled at the rate of ~20 K/min from room temperature up to 80 K. Then, DTA curves were recorded during heating. The thermograms of all the samples revealed similar relaxation and phase transitions. Figure 1 exemplifies the thermograms of one of the studied CO<sub>2</sub>-extract samples from pine shoots.

As is the case with the other samples, an endothermal physical transition ( $T_{g1}$ ) appears in the given sample in the range of 240–255 K (Fig.1, curve 1) during its pre-heating. Then, at 273 K, there appear an endothermic peak of the water melting phase ( $T_m(H_2O)$ ) and one more physical transition ( $T_{g2}$ ) in the range of 300–320 K; after that, two endothermic peaks with the extrema at  $T_{vap1}=355$  K and  $T_{vap2}=380$  K are formed. At that, the sample mass decreased from 6 to 20 mas.%. It points to the fact that the last two peaks are attributed to evaporation of water and some volatile organic substances. This water, however, does not dissolve in the extract components. It seems that the extract elements do not dissolve in the water as well, since its melting temperature does not decrease.

The volatile components being evaporated, we again cooled the samples and took off the second thermogram during heating (Fig.1, curve 2). The thermograms of the secondary heating revealed the absence of water melting and evaporation peaks. The physical transitions in the ranges of 240–255 K and 300–320 K recurred and two more transitions ( $T_{g3}$  and  $T_{g4}$ ) with the average temperatures of 353 and 378 K were formed in the region of the evaporation

peaks. The recorded physical transition should be related to devitrification of the extract components.

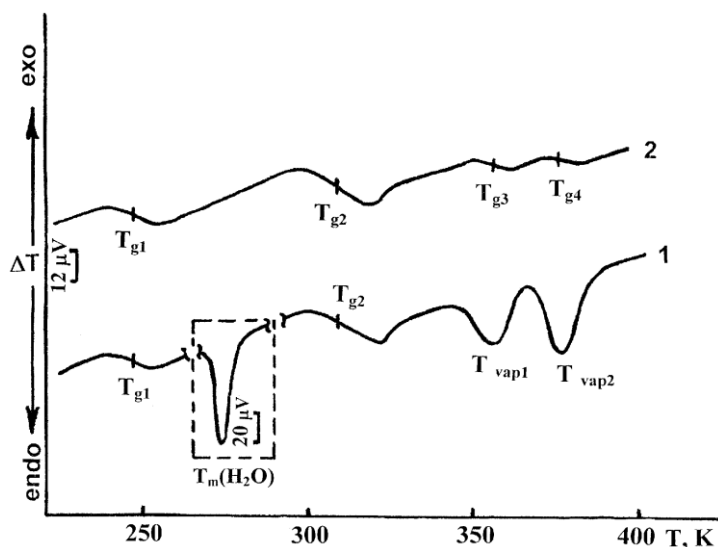


Fig.1. Thermograms of CO<sub>2</sub>-pine shoot extract [13] 1 – first heating; 2 – reheating after water evaporation

The analyzed samples of the pine shoot extracts demonstrated physical transitions ( $T_{g1}=247$  K and  $T_{g2}=309$  K), which recur and whose temperature does not depend on the water, present in the samples. They can be used for standartization of the given CO<sub>2</sub>-extract samples.

The investigations of the radiation protective properties of the preparation made of CO<sub>2</sub>-extract of pine shoots revealed, that after one day the 3-Gy dose irradiation yielded a substantial decrease in the number of leucocytes (leucopenia) as compared to the norm for both reference and test animals. After 7 days the number of leucocytes in the test group reached that inherent for the group of non-irradiated animals. The number of leucocytes in the reference group remained at nearly the same level (Table 2).

Table 2

Total content of leucocytes (thousand/1mcl) in the animal blood after the action of the pine shoot extract preparation <sup>1)</sup>

Animal groups	One day after preparation introduction	
	1	7
Reference (oil)	6150±589	7833±133
Reference (preparation)	4400±195	4400±241
Reference D=3 Gy	4050±781	3250±430*#
Experiment D=3 Gy + preparation	3617±298*\$	7025±419*\$

<sup>1)</sup>Authentic differences as compared to the reference: \* – oil

# – preparation

\$ – irradiation

The action of radiation was accompanied by substantial damage of the hemogenic system, namely the red bone marrow; however, the number of bone marrow cells of the test animal group was larger as compared to the reference one.

Reparative processes in this group were very intensive and after 7 days the number of hemopoietic cells reached the magnitude observed for intact animals (norm). As far as reference animals were concerned, the regeneration processes in the bone marrow were substantially slower than those in the test group. Seven day after the irradiation the number of the bone marrow cells was 2 times smaller as compared to the animals, which were fed with the pine shoot extract.

The investigations show, that the pine shoot extract possesses radiation protective properties. A proof of it is a larger number of bone marrow cells and leucocytes, as well as faster regeneration processes in the blood and hemopoietic system, observed in irradiated animals, which were fed with the pine shoot extract, as compared to the reference animals. Thus, it can play a role of a promising radioprotector.

Addition of the oil solution containing the pine shoot extract yielded a substantial reduction in the content of total cholesterol in plasma, an increase in the level of high-density antiatherogenic lipoproteins, and a plausible decrease in the atherogeneity index (Table 3). The observed variations in the analyzed parameters point to antiatherogenic properties, inherent to carbon dioxide pine shoot extracts.

Table 3

Effect of pine shoot extracts on the content of total cholesterol and high-density lipoprotein fractions in rat plasma

Parameter	Intact animals	Hypolipidemia (reference)	10 days after extract intake	30 days after extract intake
Total cholesterol, mmol/l	2.57±0.13*	7.92±0.58	5.56±0.3*	2.75±0.15*
HDLP**, mmol/l	1.38±0.09*	0.86±0.12	1.33±0.08*	1.29±0.075*
Atherogeneity coefficient	0.56±0.02	6.49±0.17	1.08±0.18*	0.47±0.015*

\*Plausible difference as compared to the reference,  $p < 0.05$

\*\*HDLP – high density lipoproteins

The results of DTA of the CO<sub>2</sub>-extract of weeping birch bark are shown in Fig.2. The experiment was carried out three times to verify reproducibility of physical and phase transitions in the sample. The sample was initially cooled with the rate of ~20 K/min from room temperature up to 80 K. Then, the DTA curves were recorded during its heating with the rate of 5 K/min. In experiments 1 and 2 (Fig.2, curves 1, 2) the heating was terminated at 400 K. Thermograms 1 and 2 revealed three successive endothermic physical transitions ( $T_{g1}$ ;  $T_{g2}$ ;  $T_{g3}$ ) in the range of 260–390 K with the average temperatures of  $T_{g1}=268$ , 269 K;  $T_{g2}=319$ , 318 K;  $T_{g3}=376$ , 373 K, respectively.

In experiment 3 (Fig. 2, curve 3) the heating was terminated at 510 K. Three physical transitions also appeared on thermogram 3 in the range of 275–390 K (Fig.2, curve 3). However, their temperatures were 3–10 K higher as compared to the first two experiments. It should be noted that after the first two runs the sample lost ~2% of its initial mass. It could be both adsorptive water and low-molecular volatile components. It is known [14, 15] that low-molecular substances (LMS) exert plasticizing action on polymers, thus lowering the

temperature of their physical transitions. It seems that an increase in the  $T_{g1}$ ,  $T_{g2}$  and  $T_{g3}$  temperatures in the third experiment can be explained by LMS evaporation from the CO<sub>2</sub>-weeping birch bark extract. In addition, the fourth physical transition ( $T_{g4}$ ) appeared on thermogram 3 in the range of 410–430 K (Fig.2, curve 3). Its amplitude was substantially larger than that of the first three transitions. After that, there appeared a high exothermal peak ( $T_{cr}=467$  K), which can be attributed to the crystallization process, and the endothermic peak ( $T_m=501$  K), which is likely to be related to melting (Fig.2, curve 3). The loss of the sample mass after the third heating was ~0.5 mas.%. It points to the absence of destructive processes in the sample. As it was noted above, the extract contains ~43 mas.% of betulin. Some observed transitions seem to be attributed to this very BAS.

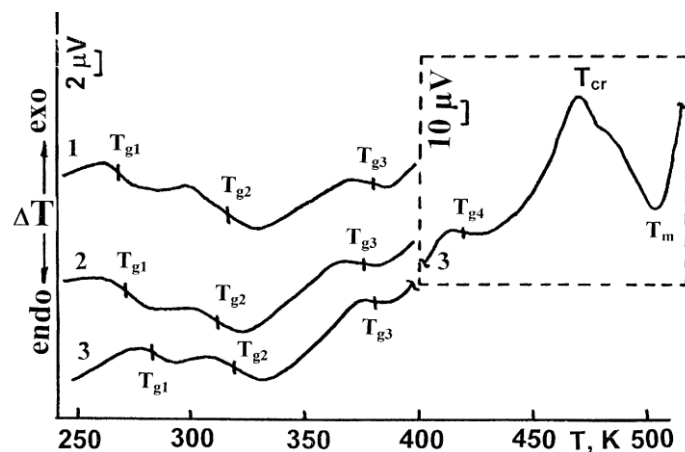


Fig.2. Thermograms of CO<sub>2</sub>-weeping birch (*Betula pendula*) bark extract:  
1 – first heating; 2 – second heating; 3 – third heating

Thus, the temperatures of the first four physical transitions ( $T_{g1}=272$  K,  $T_{g2}=319.5$  K,  $T_{g3}=378$  K, and  $T_{g4}=418$  K), which are well-reproduced during the sample heating up to the temperature not higher than 430 K, can be recommended for standartization of the CO<sub>2</sub>-weeping birch bark extract.

Using the technique of chromatographic mass-spectral analysis it was found that the content of admixtures in the betulin, purified by a three-fold recrystallization from the solution in chloroform, does not exceed 0.5 mas.% (Fig.3). It is apparent from Fig.3, that only the peak, corresponding to betulin, is observed on the mass-chromatogram.

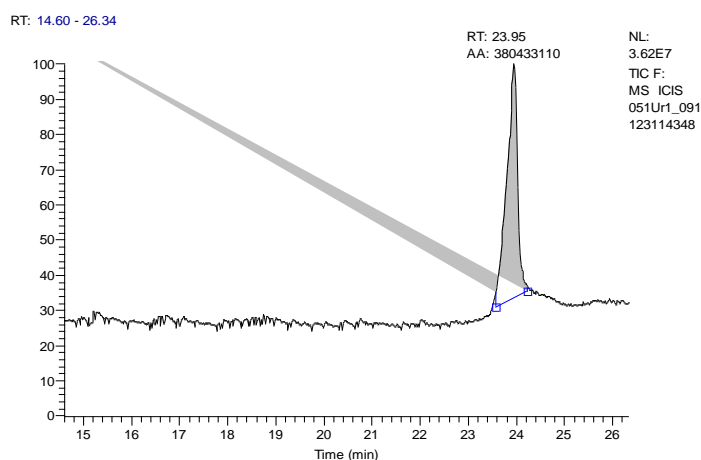


Fig.3. Mass-chromatogram of betulin

The temperatures of its relaxation and phase transitions were determined using the DTA technique (Fig. 4). An endothermic transition, namely a comparatively sharp deviation of the base line from zero, attributed to an increase in the sample heat capacity, appeared on the betulin thermogram in the range of 220–260 K. According to the classification of Westrum–McCullough [16], such transitions in crystalline substances belong to glassy ones (G-type). The average temperature of the interval was  $T_G=246$  K. Then the exothermic peak of crystallization ( $T_{cr}=433$  K), i.e. transition into the crystals, which are stable within the given temperature range, and the endothermic peak of their melting at  $T_m=524$  K.

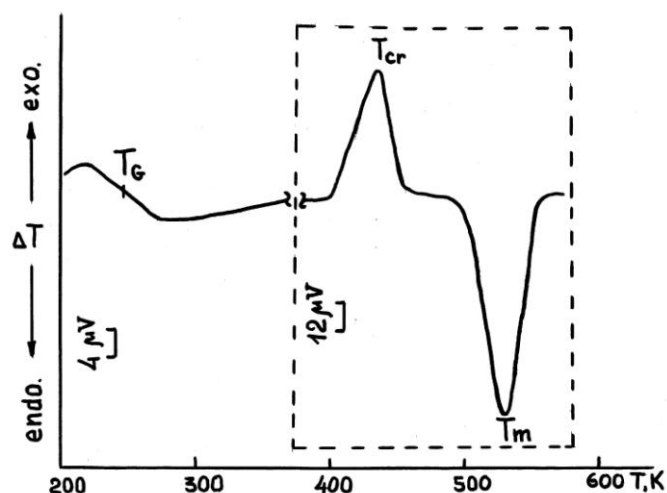


Fig.4. Thermogram of betulin

The standard enthalpy of betulin burning, determined by us, was  $-3163.3 \pm 4.2$  kJ/mol, and the standard enthalpy, calculated based on it, was  $-769.8 \pm 4.2$  kJ/mol.

The DTA results for the  $\text{CO}_2$ -blending extract are shown in Fig. 5. The sample was initially cooled with the rate of  $\sim 20$  K/min from room temperature up to 80 K. The DTA curves were recorded during its heating with the rate of 5 K/min. Figure 5 (curve 1) demonstrates the thermograms of the  $\text{CO}_2$ -blending extract. Two physical transitions ( $T_{g1}=175$  K and  $T_{g2}=200$  K) appeared for this sample; then, a large exothermic peak ( $T_{cr}=212$  K), related to crystallization, and four endothermic peaks ( $T_{m1}$ ,  $T_{m2}$ ,  $T_{m3}$  and  $T_{m4}$  at the temperatures of 233, 250, 273, and 285 K, respectively) were observed. The peaks  $T_{m1}$ ,  $T_{m2}$  and  $T_{m4}$  are likely to be attributed to melting of the extract components, whereas the peak  $T_{m3}=273$  K should be related to melting of the water contained in the  $\text{CO}_2$ -blending extract (Fig.5, curve 1).

After the first heating the sample in the crucible separated into three layers and lost 2.6% of its initial mass. The upper layer is orange and transparent and is likely to contain carotinoids; the middle layer is orange with suspended particles consists of carotinoids and sterols, and the lower layer is water. The loss of the mass seems to be attributed to evaporation of volatile components. After two next heatings the sample lost another 7 mas.% of its mass, that points to continuing evaporation of volatile components or to existing destructive processes. Physical and phase transitions appeared in the  $\text{CO}_2$ -blending extract at the temperatures of  $T_{g1}=174, 179$  K;  $T_{cr}=218$  K;  $T_{m1}=234.5, 234$  K;  $T_{m2}=247, 248$  K;  $T_{m3}=268, 269$  K, respectively (Fig.5, curves 2, 3). In addition, the fourth endothermic peak ( $T_{m4}=276$  K) appeared in the middle layer. These transitions belong to devitrification, crystallization and melting of the  $\text{CO}_2$ -blending extract component.



Thus, the physical and phase transitions, namely  $T_{g1}=175$  K,  $T_{cr}=212$  K,  $T_{m1}=234.5$  K,  $T_{m2}=249.5$  K, and  $T_{m3}=268$  K, which are well reproduced for different extract lots, can be recommended for standartization of the CO<sub>2</sub>-blending extract.

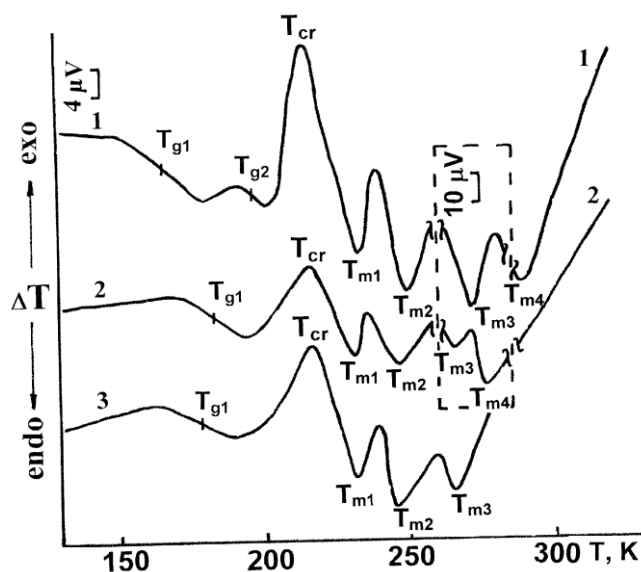


Fig.5. Thermograms of the CO<sub>2</sub>-blending extract:  
1 – entire extract, 2 – middle layer, 3 – upper layer

## CONCLUSIONS

The investigations on physical and chemical properties of the BAS, obtained from the vegetable raw using the technique of CO<sub>2</sub>-extraction, showed that a diversity of relaxation and phase transitions is observed in them. However, it is possible to distinguish some of them which are well reproduced from lot to lot. These transitions can be recommended as reference ones for BAS standartization.

## ACKNOWLEDGMENTS

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