УДК 664/633.854.54

V. V. Titok, D. Sc. (Biology), Corresponding Member of Belarus NAS, director (CBG NAS of Belarus);
V. N. Leontiev, PhD (Chemistry), assistant professor, head of department (BSTU);
S. I. Vakula, junior researcher (IGC NAS of Belarus);
N. V. Anisimova, PhD (Biology), senior researcher (IGC NAS of Belarus);
T. V. Nikitinskaya, junior researcher (IGC NAS of Belarus);
V. G. Lugin, PhD (Chemistry), cheaf of CPCI (BSTU);
L. M. Shostak, junior researcher (BSTU); **A. V. Kilchevskiy**, D.Sc. (Biology),
Corresponding Member of Belarus NAS, chief scientific secretary NAS of Belarus;
L. V. Khotyleva, D. Sc. (Biology), Academician, senior researcher (IGC NAS of Belarus)

COMPLEX ANALYSIS OF FLAX SEEDS COMPOSITION FOR BREEDING PROGRAMS

Single plant selection using complex of seed quality traits requires development and optimization of the methods, which allow qualitative and quantitative analysis applying minimum quantity of seeds. The proposed evaluation scheme of seed composition enables optimal use of seed material for individual plant analysis: oil percentage (Rushkovsky's extraction method), fatty acid composition (vapor-liquid chromatography), protein and ash content (dynamic thermogravimetry), elemental composition (electron probe X-ray fluorescence method). Reproducibility of analytical methods used was assessed by the coefficient of variation, the reproducibility index (ARI) and the convergence of data on seasonal cultivation.

Introduction. Flaxseed or oil flax (*Linum usitatissimum* L.) is one of the oldest oil-bearing crops. Flaxseed is a source of ω-3 fatty acids, complete protein, dietary fiber, minerals and other micronutrients [1]. Unconformity in published data on composition of flax seeds can result from genetic/environmental variability or differences in analytic methods used by researchers [2].

The complexity of flaxseed structure and composition doesn't allow separating its components without the use of methods such as extraction, chromatography, thermogravimetry, X-ray fluorescence. In this case, all these methods should provide sufficient accuracy, maximum comparability and reproducibility of results. An important aspect of any analysis technique is availability, simplicity, time and seed saving in addition to accuracy and precision. These aspects are especially important in conducting individual selection with the assessment of seeds quality of a single plant.

The aim of our study was to develop and optimize the scheme of flax seed qualitative and quantitative analysis for individual plant breeding and complex trait selection.

Main part. Seeds of 25 flaxseed varieties were used for study. Plants were grown in standard field conditions. Threshing and cleaning of seeds were carried out manually.

The following economically valuable traits of oilseed flax were analyzed: oil content (percentage by weight of the seed); the fatty acid composition (content of α-linolenic, linoleic, oleic, stearic and palmitic fatty acids as a percentage of the total oil content); iodine value, ash content (percentage by weight of the seed); protein content (percentage by weight of the seed); elemental composition (in micrograms).

Flaxseed oil content was determined by dry residue mass after exhaustive extraction (S.V. Rushkovskiy's Soxhlet method) [3]. The air-dried material (50°C, silica gel, 72 hours) was frozen in liquid nitrogen (−195.75°C) and milled with porcelain mortar until homogenous powder. Seeds powder was filled in the extraction packets made of filter paper "white strip" (JSC "Himreaktivkomplekt", RF). Soxhlet extraction of samples was performed with hexane:isopropanol (1 : 1) for 24 hours. Completeness of oil extraction was tested by the ring test. Than extraction packets were dried (50°C, silica gel, 72 hours) and weighed. The seed oil content was calculated from the dry residue.

Extraction and determination of the fatty acids were made by the method of Welch [4] with modifications. Triacylglycerols were esterified to fatty acid methyl esters (FAME) by 2% solution of sulfuric acid in absolute methanol (conditions: 3 hours at (80 ± 1)°C, in an inert argon, internal standard is heptadekanoic acid C17 : 0, 0.27 mg/ml). The extraction was performed with hexane (0.50 ml) while stirring in a vortex mixer (5.0 s). FAMEs were separated by Hewlett-Packard 4890D gas chromatograph, equipped with a flame ionization detector and HP-Innowax capillary column (0.32 mm × 30 m in size, 0.50 μm). Helium flow rate was 26 cm/s, column temperature was 200.00°C. The temperature of injector and detector was 250.00°C. Injected sample volume was 1 ml. The individual fatty acids were identified by retention time of the separation of their standard mixtures

(Supelco Park, USA) and evaluated as a percentage of the total content by relative weight to the internal standard [5].

Thermogravimetric analysis of the flax seeds (5.00-5.10 mg) was performed on a TA-4000 thermal analyzer (TG-module 50) (Mettler Toledo STARe System, Switzerland) at a heating rate of 5°C/min and an air flow of 200 ml/min in the temperature range of 25–700°C. Mass loss curves were calculated using the STARe software [6].

Element composition of flaxseed was tested using scanning electron microscope JSM-5610 LV, equipped with chemical analysis system EDX JED-2201 JEOL (Japan). The backscattered electron detector of electronic microscope worked in a low vacuum mode [7].

Data analysis was processed using MS Excel and Statistica software. The convergence of data obtained with the use of various analytical methods (dynamic thermogravimetry/Kjeldahl method) and for successive generations of seed was evaluated using nonparametric correlation analysis [8]. For each option of the experiment (sample=variety×year) trials were performed triply. Analytical method reproducibility was estimated by standard deviation (SD) and coefficient of variation (CV) [9]. The average reproducibility index (ARI) was calculated for oil content, ash content and iodine value. This index characterizes the relevant variability of statistically stable process (method) to the width of the tolerance [10]. Total sample SD was adopted as a threshold (the width of the tolerance) for ARI calculation.

There are four main indicators of flaxseed quality: fatty acid composition, content of the oil, protein and ash. However, their simultaneous analysis is difficult and laborious. Therefore, we suggest a complex of methods allowing the optimal seeds usage, providing high reproducible data and possible for using in seed flax breeding programs.

Complex composition analysis allows using at about 60.00% of seeds from plant for physics-chemical tests and about 40.00% can be saved for breeding, hybridization and selection. We offer the following scheme of seeds consumption:

1) Oil content extraction analysis – 0.30 g of seeds (≈40 pcs.);

2) Gas chromatography of FAME for an oil formulation estimation – 0.03 g of seeds (6 pcs.);

3) Dynamic thermogravimetry (DTG) for the determination of proteins, nucleic acids, carbohydrates and ash content – 0.03 g of seeds (6 pcs.);

4) Multi-element analysis using energy-dispersive X-ray spectroscopy – ash residue from DTG.

With the average yield of flax of about 0.60 g (88 pcs) of seeds per plant [11], maximum intake for the component analysis is 0.45 g (approximately 60-65 pcs), thus 10-15 of seeds are conserved for the perspective samples propagation.

Optimization of the scheme included: 1) procedure error estimation (exhaustive extraction); 2) evaluation of data reproducibility (oil and ash content, iodine value); 3) adaptation of complex physicochemical methods (DTG, X-ray fluorescence analysis) to flax seed analysis.

Solvent extraction is a traditional method for oil content determination [12, 13]. Nuclear magnetic resonance and near infrared spectroscopy (indirect methods) require complete extraction tests calibration but are widespread in practical work [14]. Moreover, flaxseed breeders use inexpensive rapid methods of oil content determination: refractometry [15] and pycnometry [16, 17]. We used Soxlet extraction method with Rushkovskiy's modification [3]. Method is a highly productive (allowing simultaneous analysis of a large number of samples) and solvent saving. However, procedure error can arise from uneven grinding material, oil smearing, poor pre-drying of seeds and paper packets [18].

Cryomilling (in liquid nitrogen) resolved a problem of non-uniform milling and oil loss by the smearing. Percentage of bound water was studied to assess systematic error caused by residual moisture. 25 previously air-dried samples (intact and milled seeds) were desiccated in oven to constant weight (about 2 hours at 105°C). Average humidity of the intact seeds was 6.34%, CV = 0.02, milled – 2.88%, CV = 0.08.

High variability of moisture content in ground samples probably resulted from milling fineness heterogeneity. The residual moisture may cause an overestimation of oil content of seeds up to 2.49 – 3.28%. This error is systematic and hence is not so important for flaxseed breeders. Nevertheless, overestimation of oil content data should be taken into account in the standardization and management evaluation of flaxseed.

Reproducibility of the Rushkovskiy's method was assessed using 300 sample dataset (25 varieties across 4 generations). Varietal averages (4 years) demonstrated significant correlation (r = 0.56). Stability of varieties rank during 4 seasons confirmed reasonability of the extraction method used. Individual samples CV was 0,002–0,080, while in 74% of cases the CV values did not exceed 0.05. Variety of CV data was used for estimation of the average reproducibility index ARI [9], which was 1.08 with the stated threshold of ± 2.39% (SD for hole 300 samples dataset).

Gas chromatography of FAME is a standard method to study the oil composition [20]. Flaxseed oil mainly consists of five fatty acids (FA): palmitic (≈5%), stearic (≈3%), oleic (≈18%), linoleic (≈14%) and α-linolenic acid (≈50%) [19].

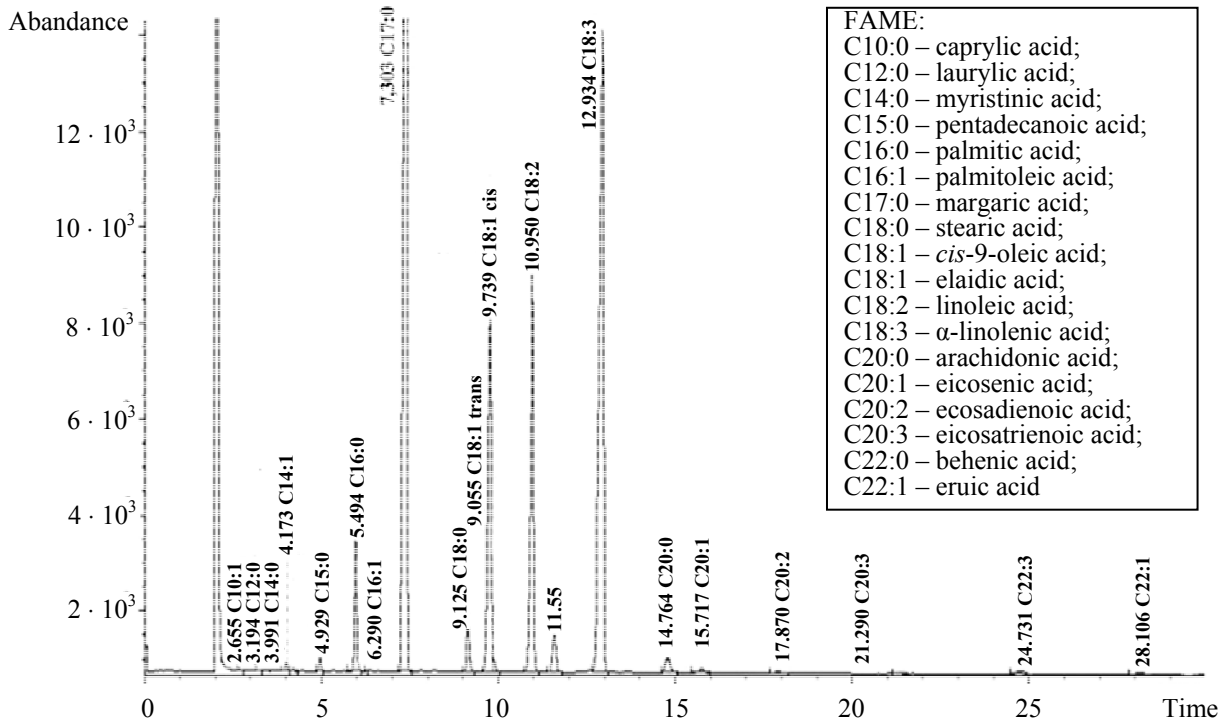

Fig. 1. FAME chromatogram (McGregor variety)

Unique varietal FA ratio is preserved under varying environmental conditions. Thus, reliable reproducibility (in generations) of the seed FA composition could be an indirect confirmation of the reliability and reproducibility of the analytical method. In 2005–2009 years the correlations of α-linolenic (r = 0.92), linoleic (r = 0.99), stearic (r = 0.60) and palmitic acid (r = 0.51) content were statistically significant. The convergence of data annual variation of oleic acid (r = 0.51) was unreliable. This probably results from the flaxseed metabolism peculiarities, wherein oleic acid is a labile precursor for the biosynthesis of linoleic and α-linolenic acids [20].

Table 1

Data reproducibility (Rushkovskiy's extraction, gas-liquid chromatography of FAME, DTG methods)

Character	r	CV	ARI
Oil content, %	0.56*	0.03	1.08
Iodine value, g/100 ml	0.76*	0.06	1.40
Ash content, %	0.90*	0.03	3.45

Remarks: r – 2005–2009 years average; * – significant at $P \leq 0.05$; CV – coefficient of variation; ARI: <1 – low; >1.00 – average; > 1.33 –high).

Iodine value (IV) was calculated from fatty acid content data [21]. Basic reproducibility statistics for iodine value: r = 0.76, CV = 0.06, ARI = 1.40 (average SD ± 2.30 was used as a threshold). These results correspond to a high reproducibility of gas chromatography of FAME for flaxseed analysis.

Thermoanalytical methods are based on the study of sample properties at temperature change under specified conditions [22]. These methods allow obtaining valuable information about the structure, composition, and properties of biological tissues and organs [23]. Composition and thermochemical properties of intact flax seed were evaluated by the method of dynamic thermogravimetry (DTG).

Preliminary optimization of methods included experiments on the thermal analysis of individual tissues and components: intact and defatted cotyledons, seed coats, oil, storage proteins. Correlation analysis confirmed the high repeatability of protein and oil content measurements obtained respectively by DTG and classical Kjeldahl method (r = 0.80) [24, 25], DTG and exhaustive lipids extraction (r = 0.71).

Curve of mass loss from temperature (TG) is an indicator of the thermal stability of the sample (Fig. 2). Water (25–100°C), proteins (230–370°C), fatty acids (370–460°C), nucleic acids (460–530°C) and polysaccharides (530–700°C) [26] are removed one by one with the temperature rise. DTG mass loss of the flax seed is about 95.23–96.78% (Fig. 2), rest of 3.22–4.77% is an ash.

Total mass loss (measured as seed ash content) was used as an indicator of reproducibility of thermogravimetric method (other DTG characters, for example oil or protein content, was measured indirectly from mass loss curve). Correlation between ash content in 2005 and 2007 years was highly significant (r = 0.90). Average CV was 0.03, SD = ± 0.27 (threshold for ARI), ARI = 3.45. Thus, ash content is highly reproducible DTG factor.

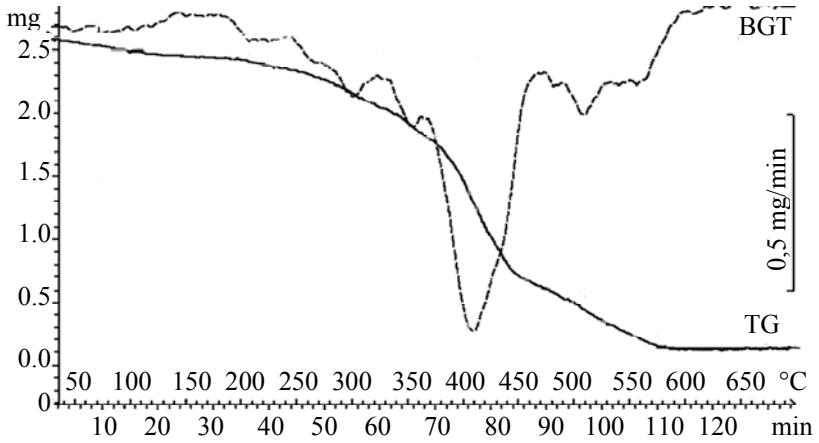

Fig. 2. DTG curve (Atalante variety).
Mass loss intervals, °C: 25–100 water; 100–230 low weight proteins; 230–370 storage proteins; 370–460 lipids; 460–530 nucleic acid; 530–700 polysaccharides

Seed's ash samples were scanned with EDX JED-2201 JEOL electron microscope (Fig. 3).

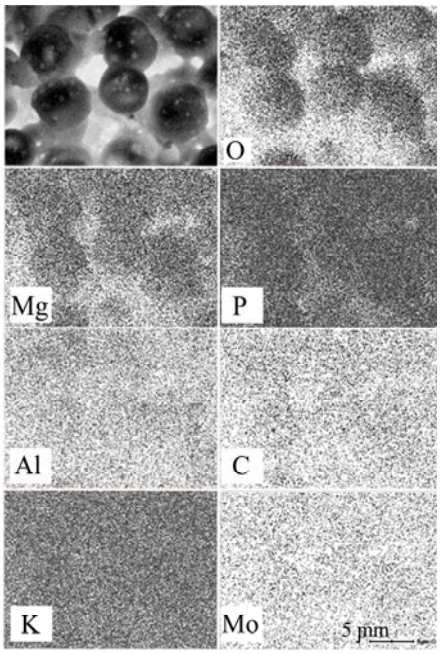

Fig. 3. XRF in scanning electron microscopy.
Analyzed sample – DTG ash residue of flax seed of Flanders variety.
Dark spots correspond to the quant emission of following elements: Oxygen – O; Magnesium – Mg; Phosphorus – P; Aluminum – Al; Carbon – C; Potassium – K; Molybdenum – Mo

After DTG flax seeds ash retains microgranular structure (Fig. 3) which is common for globoids (storage organelles of the seed).

Quantitative energy dispersive electron microprobe (X-ray fluorescence in scanning electron microscopy, XRF) analysis was used to estimate the elemental composition of the ash (Fig. 4). XRF analysis allows to determine the atomic structure of materials practically in the entire range of concentrations within the accuracy of 2%. It is based on the assumption that the intensity of characteristic radiation (Ia) emitted by atoms (A) is proportional to the concentration of the element (CA) [27]. According to ZAF-correction method, element content was estimated on the base of the characteristic spectral lines intensity measured (energy dispersive spectroscopy or EDX-spectrum) in the sample and reference standard (Fig. 4).

Unfortunately, the elemental composition was analyzed only on a single ash sample of each flaxseed variety. And so reproducibility of the data cannot be determined. However, received data corresponds to a common composition of flax seed's ash (in average: Na – 0.37 mg/g of seeds; Mg – 15.36 mg/g; Al – 0.42 mg/g; K – 21.57 mg/g; Ca – 3.72 mg/g; Fe – 0.39 mg/g; Zn – 1.46 mg/g).

CV and ARI statistics were calculated for seven elements: potassium, sodium, magnesium, phosphorus, iron, zinc and calcium (Table 2). EDX-data variation correlates with a total content of the element in the sample. For example, macronutrients (K, P, Mg) show spectral lines of high intensity (above 17 keV), which is consistent with a high content of elements in the ash, as well as to a low CV and high ARI.

Table 2

EDX-data reproducibility

Element	Mass (ash)	CV	ARI
K	32.75	0.02	5.83
Na	0.21	0.12	0.19
Mg	17.37	0.02	3.02
P	43.3	0.01	7.36
Fe	0.52	0.21	0.28
Zn	1.55	0.17	0.50
Ca	4.14	0.11	0.67

Remarks: ARI: <1.00 – low; >1.00 – average; >1.33 – high.

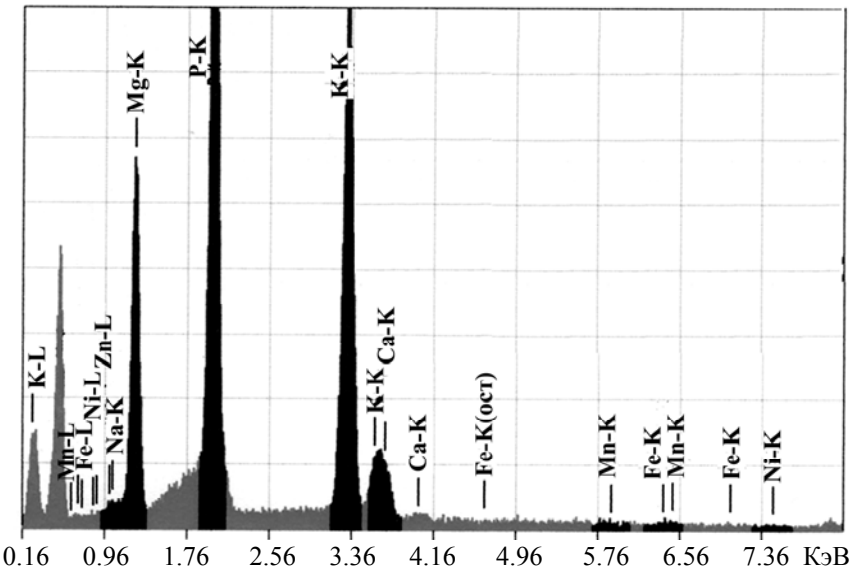

Fig. 4. EDX-spectrum of seed ash residue (Flanders variety). Na, Mg, P, K, Ca, Mn, Fe, Ni, Zn spectral lines are flagged

The correlation coefficient between the average content of the element and its CV was 0.85 (highly significant). Thus XRF analysis possesses a high potential for an assessment of the trace element content in seed ash.

Conclusion. Complex analysis of flax seeds composition enables estimation of varietal identity and breeding value of individual plants, as well as technological quality requirements for seeds and their derivatives.

Complex of methods for flaxseed composition analysis was used, however, complexity of structure and composition of flaxseed necessitated the preliminary adaptation and optimization of the approaches studied. Optimization of exhaustive extraction method (Rushkovskiy's method) includes cryomilling and data correction by the residual humidity. Intact seed thermogravimetry enablas the simultaneous analysis of the oil, protein, carbohydrate and ash content. Thermogravimetric analytical procedure was standardized for DTG data of individual seeds components (cotyledon, seed coat, oil, storage protein). Results of DTG correlate with the data of standard analytical methods (Kjeldahl, exhaustive extraction), that confirms accuracy and adequacy of this approach. As the X-rays penetration depth in ash particles is higher than in intact seed, the DTG ash residue was used to XRF analysis of the seed elemental composition.

Flaxseed composition data received with the methods of Rushkovsky's extraction, gas chromatography, DTG and XRF-analysis are characterized by a high reproducibility and low variability in repeated tests (according to ARI and CV statistics).Complex of analytical methods (see the proposed above scheme) allows assessment of flaxseed composition with minimum seed amount: 0.30 g for oil extraction analysis (Rushkovskiy's method), 0.03 g for gas chromatography, 0.03 g for dynamic thermogravimetry, ash residue from DTG is used for XRF-analysis. This is about 60.00% of seeds from plant for chemical analysis. So, 40.00% of seeds remains for breeding and individual plant selection.

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Recieved 28.02.2014