UNIVERSITY OF PLOVDIV "PAISII HILENDARSKI"

FACULTY OF CHEMISTRY

DEPARTMENT OF CHEMICAL TECHNOLOGY

LILIYA STOYANOVA STOYANOVA

Impact of Organic Production on the Composition of Tobacco Seeds and the Potential Applications of Glyceride Oil

A B S T R A C T

of PhD Thesis

for awarding the educational and scientific degree "Doctor"

Higher education field - 4. Natural science, mathematics, and informatics;

Professional field - 4.2. Chemical sciences;

Doctoral Program - Technology of animal and vegetable fats, soaps, essential oils and perfumery - cosmetic preparations

Scientific supervisor: Assoc. Prof. Maria Angelova-Romova, PhD

PLOVDIV, 2024

The dissertation work contains 171 printed pages, 35 tables, 42 figures and 2 schemas. The bibliography contains 172 referances.

The dissertation work was discussed by the Extended Department Council at the Department of Chemical Technology, University of Plovdiv "Paisii Hilendarski" (Protocol №12/19.11.2024)

Scientific jury:

- Prof. Venelina Popova, DSc
- Prof. Maria Perifanova-Nemska, PhD
- Prof. Magdalen Zlatanov, PhD
- Prof. Ginka Antova, PhD
- Assoc. Prof. Zhana Petkova, PhD

The dissertation will be publicly defended on 24 February 2025 at 11:00 in Compas Hall.

The materials are available for those interested in the Central Library of University of Plovdiv "Paisii Hilendarski".

Acknowledgements:

I would like to express my sincere gratitude to my supervisor Assoc. Prof. Maria Angelova-Romova, PhD and the whole staff of the Department of Chemical Technology for the wonderful working atmosphere, experience, and support in the difficult moments of the dissertation realization.

Thanks to the management of the Institute of Tobacco and Tobacco Products - Markovo and my colleagues from the Department of Tobacco and Tobacco Smoke Chemistry, without whom I would not have been able to implement my ideas.

Last but not least, I thank my family for being there for me and sharing all the positives and negatives of my desire for personal and professional fulfillment.

I. INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is one of the most studied plants among the industrial crops in Bulgaria and the world. It is grown mainly for its foliage, which contains the alkaloid nicotine, for use in tobacco products for smoking, chewing, and sucking. Tobacco production in Bulgaria mainly involves the cultivation of small-leaved tobacco of the *Basmi* and *Kabakulak* variety groups and large-leaved tobacco of the *Virginia* and *Burley* variety groups. The *Basmi* variety group is typical for southern Bulgaria, with the *Krumovgrad* ecotype being of prominent commercial importance. The *Kabakulak* variety group is typical for cultivation mainly in Northern Bulgaria. Tobacco cultivation in Bulgaria is carried out according to two agrotechnical practices: conventional and organic. Since 2016 the Institute of Tobacco and Tobacco Products - Markovo has been growing tobacco for scientific purposes on a certified organic field at the Institute's experimental station in the town of Gotse Delchev. This industrial type of tobacco is distinguished by a completely organic production technology, without the use of modern plant protection products and soil fertilization.

In addition to the leaves of the tobacco plant, tobacco seeds, which are produced in extremely large quantities and are often a waste product of production, have attracted particular interest in recent years. Tobacco seeds are dark brown in color, small in size, egg-shaped, and convex on one side, ending in a protruding hilum at the small end of the seed. The tobacco inflorescence may contain between 3000 and 5000 seeds. If properly stored, they can be preserved for a long period and used both for cultivation and as raw material for processing. The chemical composition of tobacco seeds depends on the variety, the method of cultivation, and storage. Two potentially valuable products can be obtained by processing tobacco seeds: glyceride oil and expeller. Different tobacco varieties give good oil yields of up to 40 % of the total seed mass, the remainder consisting of crude fiber, protein, starch, and inorganic material. The oil contains no nicotine and is low in saturated fatty acids. Among the unsaturated fatty acids present in tobacco oil, linoleic acid is the most abundant. It is a n-6 essential acid with proven positive effects on human health and a sought-after additive in many pharmaceutical and cosmetic products. Along with fatty acids, tobacco oil also contains other biologically active substances beneficial to health such as tocopherols, phospholipids, sterols, and polyphenols.

Worldwide, science is turning its attention to the possible alternative uses of tobacco and the preservation of this industrial crop as an important economic raw material, and the importance of the flowers and seeds of this plant is increasingly being emphasized. The extended characterization of the chemical composition of tobacco seeds and glyceride oil, traced over time and under different production conditions, can guide the selection of varieties suitable for maximum utilization, as well as give new directions for closed-loop production with minimum waste products. Tobacco seed meal and glyceride oil are understudied in terms of their benefits to human health and hold potential as a food raw material and therapeutic oil with applicability in medicine and cosmetics.

II. CONCLUSION FROM THE LITERATURE REVIEW

Studied:

- mainly tobacco seeds from *Virginia*, *Burley* and *Meryland* varieties;
- the chemical composition of seeds and the lipid composition of the resulting oil from different varieties and different geographical areas;
- the fatty acid, sterol and tocopherol content of tobacco seed oil;
- the possibilities of using the oil for biodiesel production and work is underway to use tobacco seeds and their glyceride oil for applications in the pharmaceutical, cosmetic and food industries.

There is no evidance in the literature review for:

- the chemical and lipid composition of tobacco seeds grown under organic production conditions;
- the chemical and lipid composition of tobacco seeds of one variety group for two consecutive growing years;
- the chemical and lipid composition of waste tobacco seeds and options for their recovery;
- antioxidant activity of tobacco seed extracts, meal, and glyceride oil;
- incorporation of tobacco oil into cosmetic products - their stability, composition, and the possibility of microorganisms developing.

III. MATERIALS AND METHODS

Seeds of oriental tobacco, ecotype *Krumovgrad*, *Basmi* variety group, Bulgaria, were used. They were collected at the end of the plant`s vegetation period. Tobacco seeds vary in size and quality and are therefore subject to cleaning and fractionation. Seeds above 0,5 mm are considered to be germinable and used for cultivation and seed maintenance, while those below 0,5 mm are classified as unsuitable waste.

The Institute of Tobacco and Tobacco Products – Markovo grew and provided the tobacco plants, as follows:

- Seeds of oriental tobacco (fit for use), *Basmi* variety group, *Krumovgrad 58 (bio)* variety, grown on certified organic field in Gotse Delchev town with documentary evidence №0058291021, Cosmocert SA, in two consecutive years (2020 and 2021) – *Kr 58 (bio)*;

- Seeds (fit for use) of oriental tobacco, *Basmi* variety group, *Krumovgrad 58* variety, bred under conventional conditions at the experimental station, Kozarsko in two consecutive years (2020 and 2021) – *Kr 58 (conv.)*;

- Oriental tobacco seeds (fit for use), *Basmi* variety group, *Krumovgrad 90* variety, grown under conventional conditions at the experimental station, Kozarsko in two consecutive years (2020 and 2021) – *Kr 90 (conv.)*;

- Waste tobacco seeds (unusable) of different oriental tobacco varieties and different growing years;

- Cold pressed grape seed oil purchased from the commercial network (Mapa International LTD, Spain);

- Citronella essential oil (lemongrass), purchased from the commercial network (Bioherba R Ltd, Bulgaria).

The reagents used are of the required purity for analysis, provided by the companies *Sigma Aldrich, Merk, Honeywell, and Rai-Chem*.

Standardized, validated, and in-house laboratory methods of analysis are applied.

IV. AIM

The main goal of the research is to examine the influence of organic production on the composition of tobacco seeds and the glyceride oil extracted from them and to evaluate the possibilities for their application.

To achieve the goal set, it is necessary to solve the following tasks:

- Monitoring and comparison of the main chemical parameters of organically produced tobacco seeds of the same tobacco variety with those obtained from conventional (traditional) production.
- Determination of physical and chemical characteristics of tobacco oil obtained from seeds grown under organic and conventional conditions.
- Tracking changes in the chemical and lipid composition of seeds of two tobacco varieties from the same cultivar group over two growing seasons.
- Application and optimization of different techniques for extraction of glyceride oil from tobacco seeds.
- Determination of biologically active substances and their antioxidant activity in tobacco seeds, meal, and oil.
- Determination of the chemical and lipid composition of waste tobacco seeds and the resulting oil for their application.
- Investigating the possibilities of using organically produced tobacco seed oil for cosmetic purposes.

V. RESULTS AND DISCUSSION

1. Monitoring and comparison of the main chemical parameters of organically produced tobacco seeds from the same tobacco variety with those obtained from conventional (traditional) production

The oil content of the oriental tobacco varieties studied ranged between 36.50% and 42.32% (**Table 1**). In the case of *Kr 58 (conv.)* variety, a difference of 6% was recorded with respect to the seed oil content for two consecutive years. For the same variety *Kr 58 (bio)*, grown under organic production conditions, the difference was half - 3%. The results obtained for the organically produced variety are similar to the values obtained from seeds of variety *Kr 90 (conv.).*

Table 1. Total chemical composition of tobacco seeds of *Basmi* variety group in two consecutive years

Harvest / Indicators	Tobacco variety					
	Kr 58 (bio)		***Kr 58 (conv.)***		***Kr 90 (conv.)***	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**
Oil content,%	41.70±0.92	38.40±0.42	42.32±0.11	36.50±0.28	40.02±0.74	38.50±0.57
Proteins,%	19.14±0.66	20.10±0.14	21.03±0.48	18.65±0.21	19.90±0.30	19.88±2.00
- total nitrogen	3.06±0.11	3.22±0.02	3.37±0.08	2.98±0.03	3.18±0.05	3.18±0.32
Charbohydrates, % (by difference)	28.90±0.28	31.72±0.05	26.73±0.42	33.41±0.25	31.19±0.14	30.63±0.87
Ash, %	3.60±0.00	3.57±0.21	3.60±0.04	4.93±0.02	3.26±0.42	4.46±0.02
Moisture, %	6.66±0.05	6.21±0.01	6.32±0.03	6.51±0.00	5.63±0.01	6.53±0.02
Energy value, kcal/100 g	567.5±0.8	552.9±1.4	571.9±1.2	535.8±0.7	564.5±0.9	548.5±1.5

Mean ± SD, ($p < 0.05$), n = 3

The total nitrogen content of the tobacco seeds examined was about 3.5%. In seeds of variety *Kr 90 (conv.)* it was 3.18% for both harvests. Seeds of *Kr 58 (bio)* variety had a close average nitrogen content of 3.06% to 3.22%. The other conventionally bred *Kr 58 (conv.)* variety had the lowest reported total nitrogen value for the 2021 crop at 2.98%. The protein content is about 20% of their total chemical composition. The reported values are without significant differences (18.65% to 21.03%) between the different years of study and show stability with respect to this indicator. The total carbohydrate content of seeds of *Kr 90 (conv.)* was 30.63-31.19%. It was recorded with the lowest value in variety *Kr 58 (bio)* – 28.90% (2020) and the highest in variety *Kr 58 (conv.)* – 33.41% (2021). The data obtained for ash content of seeds produced in 2020 from both tobacco varieties, regardless of the cultivation method, were similar (3.26 - 3.60%). An increase in ash content of about 1% (4.46 - 4.93%) was observed when the ash content of conventionally produced seeds of *Kr 58 (conv.)* and *Kr 90 (conv.)* varieties from the 2021 harvest was examined. For the organically produced variety Kr 58 (organic), no significant difference in ash content was observed, averaging 3.58% for both years. Tobacco seeds of varieties *Kr 58* and *Kr 90* are characterized by a moisture content between 5.63% and 6.66%. The greatest difference was recorded in the values obtained for variety *Kr 90 (conv.)* - 1% between the first and second year. The differences in water content in *Kr 58 variety* did not exceed 0.5%, regardless of the cultivation method. The energy value of tobacco seeds is between 535.8 and 571.9 kcal/100g. Seeds from the 2020 harvest have a higher energy value (564.5 – 571.9 kcal/100g) than those from the 2021 harvest (535.8 – 552.9 kcal/100g), which is due to the higher fat content found in tobacco seeds over the same period.

Total and reducing sugars content in tobacco seeds was determined by continuous-flow analysis (**Table 2**). Total sugars did not exceed 3.0% and reducing sugars were about 0.5%.

Table 2. Content of total and reducing sugars determined by continuous-flow analysis

Harvest / Indicator	Tobacco variety					
	Kr 58 (bio)		***Kr 58 (konv.)***		***Kp 90 (konv.)***	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**
Total sugars, %	2.34±0.02	2.32±0.02	2.36±0.04	1.92±0.02	1.90±0.02	2.11±0.02
Redusing sugars, %	0.59±0.02	0.59±0.02	0.52±0.03	0.51±0.02	0.47±0.04	0.55±0.02

Mean ± SD, (p < 0.05), n = 3

The starch content in the studied tobacco seeds is presented in **Figure 1**. It is highest in seeds of the *Kr 58 (bio)* and *Kr 58 (conv.)* varieties - 6.18% and 6.32% in 2021. Seeds from the 2020 harvest of the same tobacco variety, under the two different cultivation methods, have a lower starch content - about 1% and 2%. The *Kr 90 (conv.)* variety recorded the lowest value for both consecutive years, as follows: 4.53% - 2020 and 4.10% - 2021.

The fiber content in the tobacco seed samples studied is high, between 18.75% for *Kr 58 (conv.)* – 2020 and 23.96% for *Kr 90 (conv.)* – 2020. In all samples, differences in fiber content between the two harvests within about 1% are observed. The results obtained show that fiber in tobacco seeds is in the highest percentage of total carbohydrates (64 - 77%) (**Figure 2**).

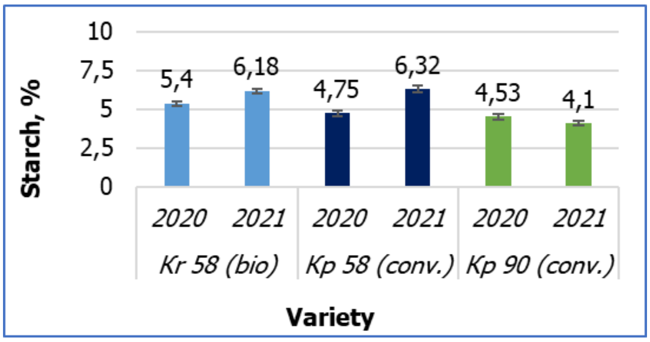


Figure 1. *Starch content in tobacco seeds*

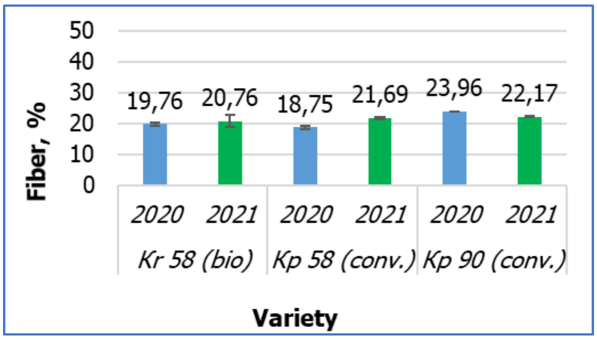


Figure 2. *Fiber content in tobacco seeds*

Macro- and microelements in tobacco seeds are represented by eight identified elements – potassium, sodium, calcium, magnesium, iron, manganese, copper, and zinc (**Table 3**). Of the macroelements, potassium (7294.0 – 8657.0 mg/kg) and magnesium (4021.6 – 4947.0 mg/kg) predominate, and of the microelements, iron (167.5 – 342.4 mg/kg). The sodium content in tobacco seeds of the *Kr 58* variety, regardless of the method of production, is lower (11.0 – 15.5 mg/kg) than the amount established in seeds of the *Kr 90 (conv.)* variety - 18.0 -18.9 mg/kg. In organically produced tobacco seeds, the calcium content is two times lower than in seeds of *Kr 90 (conv.)* and at the same time higher than in *Kr 58 (conv.)*. The magnesium content in *Kr 58 (bio)* seeds is higher than in conventionally produced seeds. Of the trace elements, iron is the predominant element. Seeds of the *Kr 90 (conv.)* variety are distinguished by almost two times higher iron content (340.5 - 342.4 mg/kg) compared to seeds of the *Kr 58* variety (167.5-196.5 mg/kg), regardless of the cultivation method. The content of the remaining elements in the studied tobacco seeds is in a similar order.

Table 3. Content of macro- and microelements in tobacco seeds

Elements mg/kg	**Tobacco variety**					
	Kr 58 (bio)		***Kr 58 (conv.)***		***Kr 90 (conv.)***	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**
K	8200.0±1.8	8000.0±1.6	7294.0±1.4	7545.0±1.5	8500.0±1.8	8657.0±1.6
Na	12.0±0.9	15.5±0.8	12.0±0.5	11.0±0.2	18.0±0.8	18.9±0.5
Ca	356.0±1.2	305.9±1.4	255.9±0.8	245.6±0.4	756.0±1.2	758.0±0.8
Mg	4848.5±1.4	4947.0±1.2	4197.2±1.5	4021.6±1.4	4598.0±1.6	4600.0±1.8
Fe	167.5±1.2	196.5±1.0	189.5±0.8	187.2±1.0	340.5±0.8	342.4±1.2
Mn	54.5±0.6	42.3±0.8	50.0±0.5	50.5±0.8	62.5±0.8	60.5±0.8
Cu	18.5±0.5	15.0±0.5	15.5±0.4	16.2±0.6	16.0±0.6	16.8±0.6
Zn	59.0±0.8	50.5±0.5	50.0±0.6	50.5±1.0	72.0±0.7	71.0±0.8

Mean ± SD, ($p < 0.05$), n = 3

The tobacco plant is a source of polyphenolic compounds – phenolic acids and flavonoids. In the seeds of tobacco of the *Kr 58 (bio)* variety, grown under organic production conditions, the amount of rutin and chlorogenic acid does not exceed 0.3 mg/g (**Table 4**).

It is impressive that in the same variety of seeds *Kr 58 (conv.)*, rutin and chlorogenic acid are not identified. In the seeds of tobacco from *Kr 90 (conv.)* variety, which is also grown according to standard practices, only rutin (0.20 mg/g) is found in close limits to the amounts determined in the organically produced *Kr 58 (bio)* variety – 0.25 - 0.27 mg/g.

Table 4. Polyphenol content in methanol extracts of tobacco seeds

Harvest / mg/g	Tobacco variety					
	Kr 58 (bio)		Kr 58 (conv.)		Kr 90 (conv.)	
	2020	2021	2020	2021	2020	2021
Rutin	0.25±0.05	0.27±0.02	N/D	N/D	0.20±0.02	N/D
Chlorogenic acid	0.20±0.02	0.23±0.04	N/D	N/D	N/D	N/D

Mean ± SD, ($p < 0.05$), n = 3; N/D – not detected

Conclusion:

- The chemical composition of seeds of *Kr 58* variety was without major deviations during the studied vegetation period compared to *Kr 90* variety, which indicates that the studied parameters can be considered typical for seeds of ecotype *Krumovgrad*, variety group *Basmi*.
- The studied tobacco seeds are characterized by high content of the main macronutrients - fats, proteins, and carbohydrates, regardless of the production method.
- The seeds of the tobacco varieties studied are low in total sugars but rich in fiber.
- Organic production does not significantly affect the composition of the main chemical components of the seeds.
- In the composition of organically produced tobacco seeds, although low levels of polyphenolic compounds are found - rutin and chlorogenic acid, which are absent in conventionally produced seeds of the same variety.

2. Determination of physical and chemical characteristics of tobacco oil obtained from seeds grown under organic and conventional conditions

The physical and chemical characteristics of tobacco seed glyceride oil produced under conventional and organic conditions are presented in **Table 5**. The refractive index is used as an indicator to determine the purity of the sample and to control the hydrogenation and isomerization processes that have occurred. Values in the range 1.4745 - 1.4761 were reported for the glyceride tobacco oils studied. The data obtained indicate a high unsaturation of the oil.

Table 5. Physicochemical indicators of tobacco seed oil

Harvest / Indicatos	**Tobacco variety**					
	Kr 58 (bio)		***Kr 58 (conv.)***		***Kr 90 (conv.)***	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**
Refractive index	1.4757 ±0.0001	1.4745 ±0.0001	1.4760 ±0.0002	1.4760 ±0.0001	1.4761 ±0.0002	1.4761 ±0.0001
Peroxide value, meqO_2/kg	2.41±0.03	1.89±0.07	2.70±0.02	2.20±0.04	2.40±0.05	2.00±0.06
Acid value, mgKOH/g	3.29±0.10	3.32±0.05	3.90±0.12	3.60±0.03	3.70±0.07	3.30±0.06
Free fatty acids, %	1.66±0.50	1.67±0.42	1.78±0.26	1.82±0.28	1.76±0.36	1.70±0.45
Iodine value,gI_2/100g	134±1	137±3	134±1	138±2	139±4	140±1

Mean ± SD, ($p < 0.05$), n = 3

The peroxide value for the examined tobacco oils is in the range 1.89 – 2.70 meqO_2/kg, without any significante diffrances between the two harvest years. The oil from seeds K*r 58 (bio)* has a peroxide value similar to those of oil from seeds *Kr 90 (conv.)*. The acid value of the ols is in the range of 3.29 – 3.90 mg KOH/g. The lowest acid value was recorded for seed oil of variety *Kr 58 (bio)* – 3.29 mg KOH/g and had similar values in both harvests. Conventionally produced seeds had higher values for acid value than organically produced seeds, with minimal difference observed between the two harvests. Seed oils from seeds *Kr 58 (bio)* for both harvest years had the lowest free fatty acids content compared to conventionally produced. The iodine value, as an indicator of the unsaturation of the fatty acid composition, was relatively high - 134 - 140 gI2 /100g and determined the tobacco oil to be of poor oxidant stability. The iodine number values for variety *Kr 90 (conv.)* were the highest of the three oil samples tested at 139 - 140 gI2 /100 g .

The oxidant stability results of the glyceride oils studied are presented in **Figure 3**. The reported induction period ranged from 6.2 to 19.8 h. The oils of variety *Kr 58 (bio)* had similar oxidant stability in both years, 12.2 h and 10.9 h, respectively. A large difference in stability was observed for oil extracted from conventionally produced seeds in the two consecutive years.

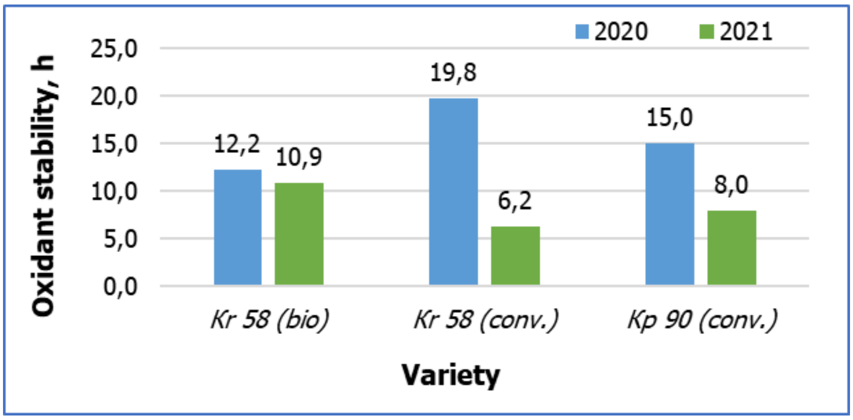


Figure 3. *Oxidant stability of tobacco oil*

Conclusion:

➢ Oil from organically produced seeds have better performance due to lower acid and peroxide values, as well as better repeatability in terms of oxidant stability compared to oil from conventionally produced seeds over the harvests monitored.

3. Monitoring changes in the chemical and lipid composition of seeds of two tobacco varieties from the same variety group over two growing seasons

3.1. Fatty acid composition of tobacco seed glyceride oil

Tobacco seeds of the Basmi variety group - variety *Kr 58 (bio)*, *Kr 58 (conv.)* and *Kr 90 (conv.)* have a high oil content - from 36.50 to 42.32%. Fourteen fatty acids were identified in the seed oil from both tobacco varieties. Results are presented in **Table 6**. Linoleic acid is predominated in the examined seed oils between 64.40% and 69.49%. The differences in linoleic acid content between the two successive years of cultivation per variety was between 0.5 - 3.5%. The second most abundant fatty acid (FA) in tobacco oil is oleic acid - 13.48% - 17.70%. In the seed oil of the *Kr 58 (bio)* variety, it is present in the largest amount of the samples tested - 17.3 - 17.7%. In the composition of tobacco oil, small amounts of linoleic acid ($C_{18:3}$) are also found: 0.50% - 0.68%. The highest content from saturated fatty acids (SFA) is reported for palmitic acid, between 10.98% and 14.20%.

It is in a higher amount in the tobacco seed oil from the Kr 58 (bio) variety (12.00 – 14.20%), compared to conventionally produced varieties (10.98 – 13.80%). In the fraction of saturated fatty acids stearic acid is found to be second (1.53 – 3.70%). Its quantity increases with decreasing oleic acid content in both varieties. The variation in the percentage of all identified fatty acids over the two harvests for the three oils studied was between 1 and 3%, indicating good crop stability.

Table 6. Fatty acid composition of tobacco seed oil

Fatty acids, %		**Tobacco variety**					
		Kr 58 (bio)		***Kr 58 (conv.)***		***Kr 90 (conv.)***	
		2020	**2021**	**2020**	**2021**	**2020**	**2021**
$C_{14:0}$	Myristic	0.10± 0.00	0.10± 0.00	0.10± 0.00	0.10± 0.00	N/D	0.11± 0.00
$C_{15:1}$	Pentadecanoic	0.10± 0.00	N/D	N/D	0.30± 0.00	N/D	0.21± 0.00
$C_{16:0}$	Palmitic	14.20± 0.5	12.00± 0.40	13.80± 0.60	11.20± 0.50	13.08± 0.40	10.98± 0.40
$C_{16:1}$	Palmitoleic	0.10± 0.02	0.20± 0.02	0.10± 0.01	0.20± 0.01	0.05± 0.00	0.20± 0.00
$C_{17:0}$	Margaric	0.10± 0.02	0.20± 0.02	0.20± 0.00	0.30± 0.01	0.14± 0.01	0.20± 0.01
$C_{17:1}$	Heptadecanoic	0.30± 0.02	0.40± 0.02	0.20± 0.01	0.30± 0.01	0.10± 0.00	0.41± 0.01
$C_{18:0}$	Staeric	2.30± 0.04	2.10± 0.05	2.90± 0.02	3.70± 0.02	1.53± 0.01	3.42± 0.02
$C_{18:1}$	Oleic	17.30± 0.50	17.70± 0.60	17.20± 0.80	14.40± 0.60	16.11± 0.60	13.48± 0.50
$C_{18:2}$	Linoleic	64.40± 0.80	65.90± 0.50	64.70± 0.50	68.20± 0.40	68.99± 0.80	69.49± 0.40
$C_{18:3}$	Linolenic	0.50± 0.02	0.60± 0.02	0.50± 0.02	0.60± 0.02	N/D	0.68± 0.04
$C_{20:0}$	Arahidic	0.10± 0.00	0.20± 0.01	N/D	0.20± 0.01	N/D	0.19± 0.02
$C_{20:1}$	Gadoleic	0.20± 0.01	0.10± 0.01	0.10± 0.00	0.10± 0.00	N/D	0.12± 0.00
$C_{22:0}$	Behenic	0.20± 0.01	0.40± 0.02	0.20± 0.01	0.30± 0.02	N/D	0.38± 0.02
$C_{22:1}$	Erucic	0.10± 0.00	0.10± 0.00	N/D	0.10± 0.00	N/D	0.13± 0.00

Mean ± SD, ($p < 0.05$), n = 3; N/D – not detected

Fatty acids are devited according to the number of doble bonds in their structure to saturated and unsaturated fatty acids – mono- (with one doble bond) and poly – (with more than one doble bond) in percentages presented in **Figure 4**. The highest content of polyunsaturated fatty acids (PUFAs) was found in all the samples analysed, which was due to the high amount of linoleic acid. The content of saturated (SFA) and monounsaturated (MUFA) fatty acids was in a similar range. The oil form seeds *Kr 58 (conv.)* variety has SFA and MUFA in ratio 1:1 for both harvests. Glyceride oil form seeds *Kr 90 (conv.)* variety has the lowest SFA content – 14.75 – 15.28%. No differences were found in terms of identified fatty acids in variety *Kr 58 (bio)* and *(conv.)*.

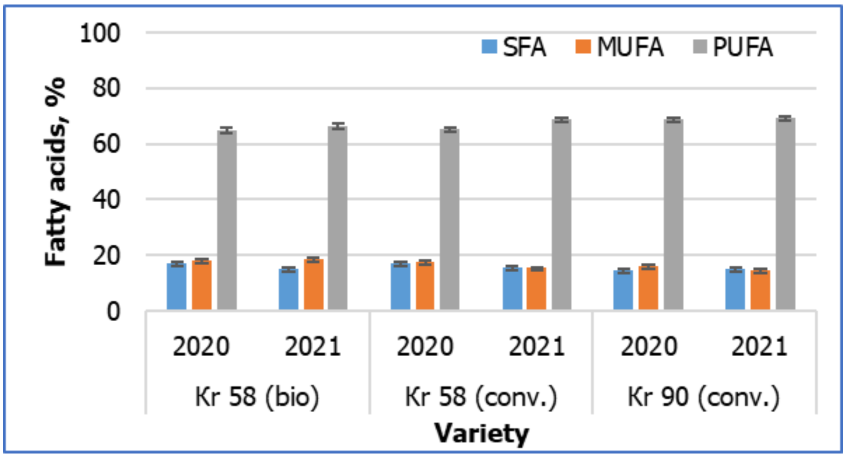


Figure 4. *Saturated and unsaturated (mono- and poly-) fatty acid content of tobacco oil in two consecutive years*

3.2. Functional properties of lipids - lipid indices

The functional properties of tobacco oil are determined on the basis of fatty acid composition by mathematically calculated lipid indices (**Table 7**).

Table 7. Lipid indices of tobacco seed oil

Lipid indices	**Tobacco variety**					
	Kr 58 (bio)		***Kr 58 (conv.)***		***Kr 90 (conv.)***	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**
Index of atherogenicity	0.18±0.02	0.15±0.01	0.17±0.01	0.14±0.01	0.15±0.01	0.14±0.01
Index of thrombogenicity	0.39±0.04	0.32±0.02	0.39±0.02	0.34±0.00	0.34±0.00	0.33±0.01
Hypocholesterolemic/hypercholesterolemic (HH)	5.75±0.05	6.96±0.02	5.93±0.01	7.36±0.01	6.51±0.02	7.54±0.02
Allylic Position equivalent (APE)	164.4±0.05	168.4±0.06	164.8±0.05	166.4±0.04	170.2±0.05	167.3±0.05
Bis-Allylic position equivalent (BAPE)	65.4±0.04	67.1±0.03	65.7±0.02	69.4±0.05	68.99±0.04	70.85±0.04
Oxidation stability Index (OSI)	0.97±0.01	0.89±0.01	0.95±0.01	0.79±0.01	0.81±0.01	0.72±0.01
Oxidability	0.66±0.01	0.67±0.01	0.66±0.01	0.70±0.01	0.69±0.00	0.71±0.01
The peroxidability Index (PI)	65.85±0.01	67.56±0.01	66.14±0.02	69.79±0.04	69.40±0.02	71.21±0.01

Mean ± SD, ($p < 0.05$), n = 3

The index of atherogeney of the oils is between 0.14 – 0.18, and the index of thrombogenicity is in the range of 0.32 to 0.39. These two indecis indicates oil contribution to the cardiovascular system when they are used for nutritional purposes. Values below 0.5 are considered to be a good low-risk indicator. The hypoholesterolemic/ hypercholesterolemic index ratio establish the effect of fatty acids on cholesterol content. The values for the h/H index are between 5.75 (*Kr (bio)*) and 7.54 (*Kr 90 (conv)*). h/H index of the 2021 harvest oil is higher in all samples tested. The oil from the seeds of organically grown variety had a lower h/H index then the conventionally produced ones. **Table 7** presents the allyl (APE) and bi-allyl (BAPE) position equivalents, which indicate the number of double bonds and their position in the fatty acid structure. High values of APE and Bape suggest rapid oxidation. The oxidant stability index for all oils is below 1. Oil from conventionally grown varieties is more prone to oxidation then that obtained from organically produces varieties. The peroxidation index showed lower values for the oil from variety *Kr 58 (bio)* compared to that from conventionally produced seeds. High peroxidation index values predict elevated levles of secondary metabolites such as malonic dialdehyde. In the initial stages of oxidation of the tobacco oil studied, the values obtained ranged from 65.85 to 71.21.

3.3. Phospholipid composition of tobacco oil

The total phospholipid content of the tobacco oils studied ranged from 1.4% to 1.8% (**Figure 5**), with higher phospholipid content reported in 2021, regardless of variety and seed cultivation method.

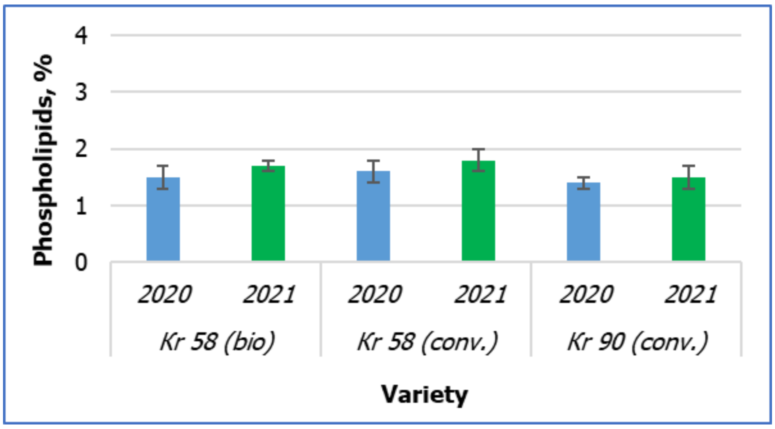


Figure 5. *Total phospholipid content in tobacco seed oil in two consecutive years*

The lowest content of phospholipids was found in seed oils of variety *Kr 90 (conv.)* in both years - 1.4% and 1.5%. The tobacco oils of variety *Kr 58 (conv.)* and variety *Kr 58 (bio)* did not differ significantly in phospholipid content. The individual phospholipid composition of the oil for both varieties includes eight isolated phospholipids: Phosphatidylcholine (PC); Phosphatidylinositol (PI); Phosphatidylethanolamine (PEA); Phosphatidic acids (PAs); Lysophosphatidylcholine (LPC); Phosphatidylserine (PS); Monophosphatidylglycerol (MPG); Diphosphatidylglycerol (DPG) (**Table 8**).

The highest content of PI, PC and PA range between 30.3% and 61.7%. The content of DPG was the lowest, between 1.0% and 4.0%. The highest PI content was found in the tobacco seed oil *Kr 58 (conv.)*, with no significant difference between the harvests, 61.7% (2020) and 61.0% (2021). The oil content from organically grown seeds *Kr (bio)* has lower phosphatidylinostiol content (38.0% - harvest 2020 and 36.0%- harvest 2021) compared to the same of conventionally produced variety. On the other hand, the content of PI in seed oil of varieties *Kr 58 (bio)* and *Kr 90 (conv.)* is similar. Phosphatidylcholine was isolated in the highest amount in tobacco oil of *Kr 58 (bio*) variety - 24.3% - 2020 and 23.2% - 2021.

Phosphatidic acids in the tobacco seed oil from seeds *Kr 58 (bio)* variety and *Kr 90 (conv.)* variety were in close limits between 12.0% and 14.0%, while lower values of about 4-5% were observed in the seed oil of *Kr 58 (conv.)* variety. A large difference in the content of MPG is reported in the seed oil of *Kr 58 (bio)* and *Kr 58 (conv.):* from 9.0 - 9.1% to 1.0-1.5%.

Table 8: Individual phospholipid composition of tobacco oil from two consecutive years

Phospholipids, %	**Tobacco variety**					
	Kr 58 (bio)		***Kr 58 (conv.)***		***Kr 90 (conv.)***	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**
PC	24.3±0.2	23.2±0.6	9.9±0.6	9.5±0.4	19.9±0.1	18.9±0.8
PI	38.0±0.6	36.0±0.8	61.7±0.2	61.0±0.7	30.3±0.8	31.0±0.4
PE	2.1±0.1	2.0±0.0	2.5±0.4	2.0±0.0	4.1±0.1	5.0±0.7
PA	12.3±0.4	12.0±0.1	8.6±0.3	8.8±0.1	12.8±0.1	14.0±0.7
LPCh	6.1±0.0	5.9±0.1	9.9±0.1	10.0±0.0	6.8±0.3	8.0±0.6
PS	4.1±0.2	3.9±0.1	4.9±0.4	4.5±0.1	11.6±0.4	12.0±0.7
MPG	9.1±0.3	9.0±0.2	1.5±0.2	1.0±0.1	8.8±0.6	7.5±0.4
DPG	4.0±0.1	4.0±0.1	1.0±0.0	1.0±0.0	2.2±0.1	1.8±01

Mean ± SD, ($p < 0.05$), n = 3

3.4. Unsaponifiables and sterols in tobacco oil

The total content of unsaponifiables in the glyceride oils of the studied tobacco seeds is on average about 3%. The results show that in the organically produced, *Kr 58 (bio)* variety difference of about 1% is reported between the two years, respectively 2.93% (2020) and 1.87% (2021). Tobacco seed oil *Kr 90 (conv.)* variety contains the highest amount of unsaponifiable matter, 3.37% (2020) and 2.89% (2021) (**Figure 6**). Sterols are a fraction of the unsaponifiable substances in seeds. An average sterol content of 1.07% was found in the tobacco seed oil of the studied seed varieties. Their total content in 2020 is lower in both varieties compared to the content in 2021. The total content in 2020 was lower in both varieties compared to the content in 2021. The organically and conventionally produced variety Kr 58 have similar values for both harvests (**Figure 7**).

The individual sterol composition of the tobacco oils was also determined. Eight components were identified in the oils (**Table 9**). The highest *β*-sitosterol content was recorded in tobacco seed oil variety *Kr 90 (conv.)* - (66.87% -2020 and 66.89% - 2021). Its presence in oil from conventionally and organically produced seeds of variety *Kr 58* is in the same order: 63.24 – 65.38% in variety *Kr 58 (bio)* and 63.44 – 65.15% in variety *Kr 58 (conv.*). Campesterol (17.25 - 19.52%) and stigmasterol (7.15 - 8.66%) were also found in larger quantities. The latter is in the highest quantity in organically produced variety *Kr 58 (bio)* 8.42% - 2020 and 8.66% - 2021.

From the data presented, the higher cholesterol content of 4.71% for *Kr 58 (conv.)* in 2021 to 7.69% in 2020 for *Kr 58 (bio)* is also noteworthy. The oil extracted in 2021 from variety *Kr 58 (conv.)* does not contain brassicasterol, which is present in minimal quantities in oils extracted from tobacco seeds of other harvests. Δ^5 - Avenasterol and Δ^7 - stigmasterol are also present in minimal amounts or absent in the oils during the indicated growing season.

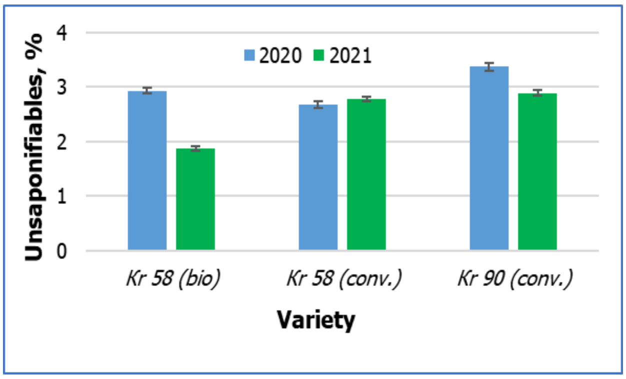


Figure 6. *Unsaponifiables in tobacco seed oil in two consecutive years*

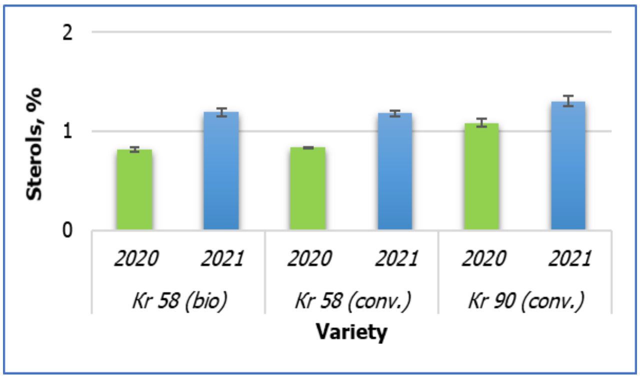


Figure 7. *Total sterol content of tobacco seed oil in two consecutive years*

Table 9. Individual sterol composition of tobacco seed oil in two consecutive years

Sterols, % \ Harvest	Tobacco variety					
	Kr 58 (bio)		***Kr 58 (conv.)***		***Kr 90 (conv.)***	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**
Cholesterol	7.69± 0.06	5.86± 0.05	5.99± 0.06	4.71± 0.04	5.89± 0.08	5.82± 0.05
Brasicasterol	0.04± 0.01	0.21± 0.04	0.12± 0.03	N/D	0.30± 0.10	0.17± 0.02
Campesterol	19.18± 0.48	18.92± 0.27	19.00± 0.05	19.52± 0.28	17.25± 0.05	18.36± 0.05
Stigmasterol	8.42± 0.05	8.66± 0.05	7.75± 0.05	7.15± 0.05	7.80± 0.25	8.00± 0.05
β-Sitosterol	63.24± 0.05	65.38± 0.06	65.15± 0.08	63.44± 0.05	66.87± 0.08	66.89± 0.08
Δ^5 - Avenasterol	0.13± 0.03	N/D	0.14± 0.01	0.50± 0.08	N/D	N/D
Δ^7 - Stigmasterol	0.04± 0.01	N/D	N/D	0.06± 0.01	0.05± 0.01	N/D
Δ^7 - Avenasterol	1.26± 0.05	0.97± 0.05	1.84± 0.05	4.61± 0.08	1.83± 0.05	0.76± 0.05

Mean ± SD, ($p < 0.05$), n = 3; N/D – not detected

3.5. Tocopherol composition of tobacco oil

Tobacco seed oil was examined for total tocopherol content and individual tocopherol composition, and the data are presented in **Table 10**. The content of tocopherols in tobacco oil is influenced by external environmental factors and the plant variety. The total tocopherol content in the tobacco seed oil ranged from 291 mg/kg (*Kr 58 (bio)*) to 355 mg/kg (*Kr 90 (conv.)*). In the second year it was significantly lower than in the first experimental year. The greatest difference was observed in the case of the variety *Kr 90 (conv.)*, where the tocopherol content in 2020 was 355 mg/kg, whereas in 2021 it was almost halved, at 124 mg/kg. The oil of the conventional variety *Kr 58 (conv.)* has similar values for total tocopherol content for both harvests.

The individual tocopherol composition of the studied tobacco seeds was represented by only two homologues- γ- (24.5 - 48.5%) and δ- tocopherol (51.5 - 75.5%), with the amount of the latter predominating. *Kr 58 (bio)* oil has a higher δ-tocopherol content than γ-tocopherol, with a ratio of 1:3 in 2020 and 1:2 in 2021. The oil from the conventional tobacco variety *Kr 58 (conv.)* is also dominated by δ-tocopherol, but the ratio for the two harvests is 1:2 in 2020 and 1:1 in 2021. This trend is also observed in the result for the oil of variety *Kr 90 (cont.)*, where the γ-tocopherol content is 24.5-35.1% and the δ-tocopherol 64.9-75.5%.

Table 10. Tocopherol content of tobacco oil - total and individual composition

Harvest / **Tocopherols**	**Tobacco variety**					
	Kr 58 (bio)		***Kr 58 (conv.)***		***Kr 90 (conv.)***	
	2020	**2021**	**2020**	**2021**	**2020**	**2021**
γ-tocopherol,%	27.3±0.3	33.1±0.2	32.8±0.1	48.5±0.2	24.5±0.5	35.1±0.1
δ-tocopherol,%	72.7±0.5	66.9±0.4	67.2±0.4	51.5±0.6	75.5±0.4	64.9±0.3
Total tocopherols, mg/kg	325±15	291±15	317±26	307±19	355±17	124±13

Mean ± SD, ($p < 0.05$), n = 3

Conclusion:

- A high linoleic acid content characterises tobacco seed oil.
- The total lipid composition of all the seeds examined showed little variation in the two successive harvests, making them suitable varieties for oil production with stable performance in terms of their fatty acid composition.
- Glyceride oil has good atherogenic and thrombogenic properties.
- The cholesterolemic index of the oil falls within the values of traditionally used vegetable oils, with oil from organically produced seeds having a lower index compared to oil from conventionally produced seeds.
- The oil from organically produced seeds has a higher phosphatidylcholine and tocopherol content than conventionally produced varieties.

4. Application and optimization of different techniques for extraction of glyceride oil from tobacco seeds

Oil extraction methods - maceration and ultrasonic extraction were applied to obtain glyceride oil from organically produced tobacco seeds variety *Kr 58 (bio)*. Three types of extractants were selected to extract the lipid fraction from the seeds, and their selection was based on the polar index (P`) as a measure of the degree of interaction. N-hexane (P`=0), n-hexane: acetone 1:1, (v/v) (P`=2.5), and ethyl acetate (P`=4.4) were used. The results of the analyses carried out showed different colors of the oil according to the type of solvent used (**Figure 8**). The glyceride oil extracted with n-hexane had the best visual qualities - pale yellow, clear, and without impurities. The oil extracted with ethyl acetate was darker while darkening and the presence of other fat-soluble components was observed when using the n-hexane: acetone combination. The oil content, irrespective of the extractant and extraction method used, ranged from 26.95% to 38.42% (**Figure 9**).

The highest oil content with n-hexane extractant (38.42%) and ethyl acetate (36.25%) was achieved by extraction with Soxhlet apparatus. When using n-hexane:acetone extractant, the highest yield was reported by the maceration method - 35.57%. Depending on the method of extraction using Soxhlet apparatus, the oil yield increases in the direction of non-polar extractant - n-hexane:acetone<ethyl acetate<n-hexane. In maceration the dependence is reversed - n-hexane<ethyl acetate<n-hexane:acetone. When extracting the

oil through ultrasonic extraction, there is no significant difference in oil yield compared to individual solvents.

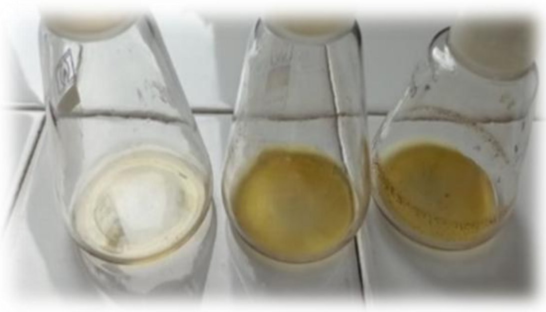


Figure 8. *Oil extracted with different polarity extractants (left, n-hexane; middle, ethyl acetate; right, n-hexane:acetone)*

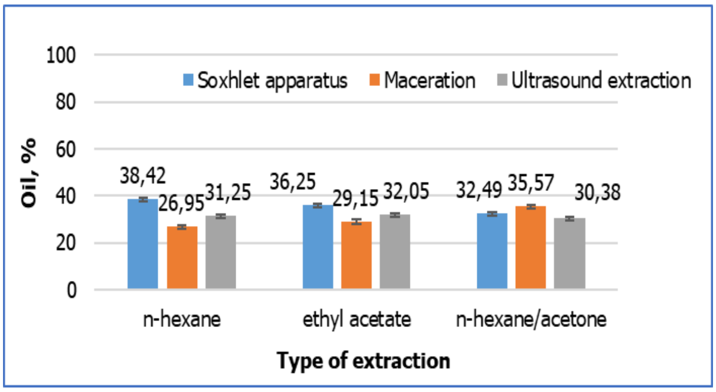


Figure 9. *Seed oil content under different extraction types and using different extractant, %*

The major fatty acid in all extracted glyceride oils, regardless of the type of extractant and method used, is linoleic $C_{18:2}$ (70.80 - 71.74%), and the oils are therefore designated as "linoleic type". The second most abundant fatty acid identified is oleic $C_{18:1}$ - 13.50 - 15.58%. In the SFA fraction, the highest content is palmitic acid $C_{16:0}$ - 9.73 - 10.03%, followed by stearic acid - 1.58 - 3.18%. All other fatty acids are below 1.0%. No significant difference in fatty acid composition was observed with respect to the influence of the extractant and extraction method used. Linoleic acid was extracted equally well with n-hexane, ethyl acetate, and n-hexane:acetone. The linoleic acid content was nearly 5.0% higher compared to the result obtained with Soxhlet apparatus extraction, 65.90% (see **Table 6**). The higher linoleic acid content was at the expense of the lower palmitic and oleic acid contents in the ultrasonic and maceration extraction. The percentages of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) in the oils obtained with different extractants and extraction methods were calculated. Tobacco seed oil, regardless of the extraction method, is characterised by a high PUFA content. In ultrasonic extraction of oils, SFA and MUFA are in comparable proportions, around 13.0%, and PUFA are over 70.0%, while in Soxhlet extraction, up to a 3.0% difference is observed between SFA and MUFA. The data in **Figure 10** show that in Soxhlet extraction with n-hexane, the proportion of SFA was 15.0%, MUFA accounted for 18.5% of the total fatty acid composition, and PUFA accounted for 66.5%. The lowest values of SFA were recorded in maceration with ethyl acetate - 11.0%.

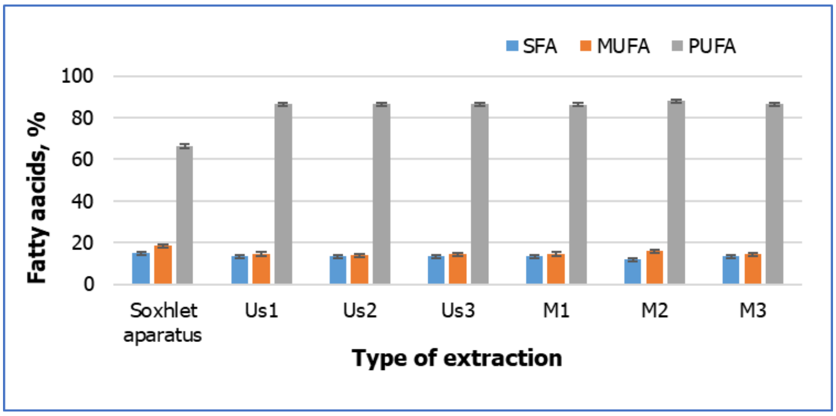


Figure 10. *Content of saturated, monounsaturated and polyunsaturated fatty acids in tobacco oil, under different extraction conditions - Ultrasonic extraction with: n-hexane (Us1), ethyl acetate (Us2), n-hexane:acetone (Us3); Maceration with: n-hexane (M1), ethyl acetate (M2) and n-hexane:acetone (M3)*

The total tocopherol content in the extracted oils was determined to be over 200 mg/kg, regardless of the extraction method and the extractant used (**Table 11**).

Table 11. Total and individual tocopherol composition of tobacco seed oil with different extractants and extraction methods

Toco-pherols (T)	***Soxhlet extractor***	***Ultrasound extraction***			***Maceration***		
	*n-**hexane***	*n-**hexane***	***ethyl acetate***	*n-**hexane: acetone***	*n-**hexane***	***ethyl acetate***	*n-**hexane: acetone***
γ-T,%	33.1±0.2	37.5±0.4	39.1±0.2	37.6±0.2	36.2±0.1	37.7±0.2	36.6±0.5
δ-T,%	66.9±0.4	62.5±0.6	60.9±0.5	62.4±0.3	63.8±0.3	62.3±0.4	63.4±0.5
Total, mg/kg	291±15	228±5	275±17	265±18	221±12	289±10	235±14

Mean ± SD, (p<0.05), n = 3

The tocopherol content was highest in oil extracted with n-hexane by Soxhlet apparatus (291 mg/kg) and lowest by maceration method with the same extractant (221 mg/kg). Ultrasonic extraction yielded the highest tocopherol content with ethyl acetate (275 mg/kg) and n-hexane: acetone (265 mg/kg), and the maceration method with ethyl acetate (289 mg/kg). The individual tocopherol composition is represented by two isomers, γ- and δ. The percentage of δ-tocopherol was higher compared to that of γ-tocopherol in all samples examined.

Conclusion:

- Glyceride oil from tobacco seeds can be successfully extracted, in addition to the classical method with a Soxhlet apparatus in the presence of the extractant n-hexane, also by applying a hexane: acetone maceration.
- The fatty acid composition of the oil is not affected by the type of extractant used and the extraction technique.
- The total tocopherol content in tobacco oil is highest when using a classical extraction method with a Soxhlet apparatus and a non-polar extractant n-hexane.
- Ultrasonic extraction or maceration with the polar extractant ethyl acetate can also successfully extract tobacco oil with a high tocopherol content.

5. Determination of biologically active substances and their antioxidant activity in tobacco seeds, oil, and meal

5.1. Total phenolic content and antioxidant activity of seeds

Tobacco seeds were used to prepare extracts with two extractants – 95% ethanol and 60% methanol. The total phenolic content of the tobacco seed extracts with 95% ethanol for both harvests did not exceed 1.12 mg GAE/g. Organically produced tobacco *Kr 58 (bio)* had the highest TPC for both years of vegetation, with values of 1.12 mg GAE/g in 2020 being higher than those in 2021 (0.80 mg GAE/g). Conventionally grown varieties *Kr 58 (conv.)* and *Kr 90 (conv.)* had similar values of 0.75 mg GAE/g and 0.70 mg GAE/g (**Table 12**). The total phenolic content of the extracts obtained with 60% methanol for both years was higher than the ethanol extracts for all types of seeds.

The antioxidant activity (AOA) of tobacco extracts was investigated by three methods (DPPH, FRAP and ABTS - methods) which belong to different mechanism of action. DPPH and ABTS methods belong to hydrogen atom transfer and FRAP method belongs to electron transfer.

Table 12. Total phenolic content in tobacco seed extracts for two consecutive years

Tobacco variety	Extractants			
	95% ethanol		60% methanol	
	mg GAE/g			
	2020	2021	2020	2021
Kr 58 (bio)	1.12±0.02c	0.80±0.04b	2.02±0.02d	1.60±0.04c
Kr 58 (conv.)	0.75±0.04a	0.70±0.02a	1.07±0.02a	1.35±0.05b
Kr 90 (conv.)	0.70±0.04a	0.80±0.02b	1.07±0.05a	1.41±0.03b

Mean ± SD, (p<0.05), n = 3

The antioxidant activity of tobacco seed ethanol extracts tested by all three methods was higher in 2020 compared to 2021. Tobacco seed ethanol extracts (2020 harvest) had the highest AOA tested by the FRAP method, 1.61-3.34 mM TE/g, followed by the ABTS method, 0.30-1.57 mM TE/g, and the DPPH method, 0.82 mM TE/g to 1.04 mM TE/g. Seed extracts of the Kr 58 (bio) variety had the highest activity by the DPPH and ABTS methods in both years. Seed extracts of *Kr 90 (conv.)* and *Kr 58 (conv.)* varieties have similar values for the DPPH and ABTS methods in 2021, while in 2020 the values differ.

It is noteworthy that the AOA determined by the FRAP method of the extracts of conventionally produced seeds, harvest 2020 is twice less than the reported activity for organically grown seeds. There was no difference in the AOA of the extracts tested by the FRAP method in 2021, ranging from 2.20 mM TE/g (*Kr 58 (conv.)*) to 2.39 mM TE/g (*Kr 58 (bio)*). The antioxidant activity data of extracts prepared with 95% ethanol are presented in **Table 13.**

Table 13. Antioxidant activity of tobacco seed extracts for two consecutive years

Method / Variety	Extractant– 95% ethanol					
	DPPH	ABTS	FRAP	DPPH	ABTS	FRAP
	mM TE/g					
	2020			2021		
Kr 58 (bio)	1.04± 0.02d	1.57± 0.02d	3.34± 0.05e	0.68± 0.01b	0.65± 0.02c	2.39± 0.02d
Kr 58 (conv.)	0.96± 0.02d	0.30± 0.00a	1.61± 0.04a	0.56± 0.00a	0.58± 0.01c	2.20± 0.04c
Kr 90 (conv.)	0.82± 0.01c	0.63± 0.02c	1.84± 0.04b	0.60± 0.00ab	0.45± 0.00b	2.31± 0.04d

Mean ± SD, (p < 0.05), n = 3

The results obtained for the antioxidant activity of the tobacco seed extracts tested with 60% methanol were higher than those obtained with the ethanol extracts - **Table 14**. The antioxidant activity determined by the DPPH method in 2020 was the highest for organically produced tobacco seed extracts at 1.19 mM TE/g. In 2021, a higher value was reported for all tobacco seed extracts, 0.45 to 1.53 mM TE/g. In contrast to 2020, when the highest activity is observed for organically produced seeds, in 2021 the conventionally produced seeds of Kr 90 (conv.) variety is characterised by the highest activity of 1.53 mM TE/g. Extracts of seeds Kr 58 (bio) had stable AOA values by this method for both harvests with a difference of less than 0.05 mM TE/g. The antioxidant activity of tobacco seeds, harvest 2020, determined by the ABTS method, ranged from 2.12 mM TE/g (*Kr 90 (conv.)*) to 2.80 mM TE/g (Kr 58 (bio)), and from harvest 2021, from 2.40 mM TE/g (*Kr 58 (conv.)*) to 3.89 mM TE/g (*Kr 90 (conv.)).* The data show that the AOA of the 2020 extracts is lower than that of 2021 for all seed extracts, similar to the results obtained with the DPPH method.

Table 14. Antioxidant activity of tobacco seed extracts with 60% methanol, for two consecutive harvests

Method / Variety	Extractant – 60% methanol					
	DPPH	ABTS	FRAP	DPPH	ABTS	FRAP
	mM TE/g					
	2020			2021		
Kr 58 (bio)	1.19± 0.02c	2.80± 0.00d	4.12± 0.04c	1.21± 0.01c	2.94± 0.02e	4.16± 0.04cd
Kr 58 (conv.)	0.32± 0.02a	2.27± 0.04b	2.86± 0.02a	0.45± 0.00b	2.40± 0.02c	3.14± 0.02b
Kr 90 (conv.)	0.34± 0.01ab	2.12± 0.04a	3.22± 0.02b	1.53± 0.01d	3.89± 0.04f	4.24± 0.04d

Mean ± SD, ($p < 0.05$), n = 3

The methanol seed extracts of *Kr 90 (conv.)* and *Kr 58 (bio)* varieties had higher activity compared to conventionally produced seed *Kr 58* tested by the FRAP method during both growing seasons. The antioxidant activity determined by the FRAP method of the extracts of seeds *Kr 58 (bio)* was not significantly different in the two consecutive years (4.12 mM TE/g for 2020 and 4.16 mM TE/g - 2021), while an increase was observed in the remaining samples in 2021. The activity reported by this method is the highest compared to the different methods applied for the AOA of the extracts. The antioxidant activity of tobacco seed extracts from organically produced *Kr 58 (bio)* variety was highest by the FRAP method. Mean AOA values of extracts were reported by the ABTS method and lowest by the DPPH method.

To estimate the total antioxidant activity of the extracts, the use of a mathematically calculated relative antioxidant capacity index (RACI) of the extracts is applied based on the methods for AOA - FRAP, ABTS, and DPPH methods. **Figure 11** presents the relative antioxidant capacity index of 60% methanolic and ethanolic tobacco seed extracts for 2020 and 2021.

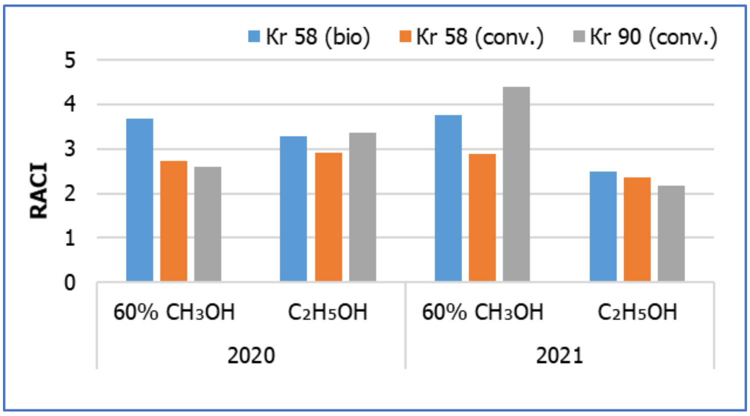
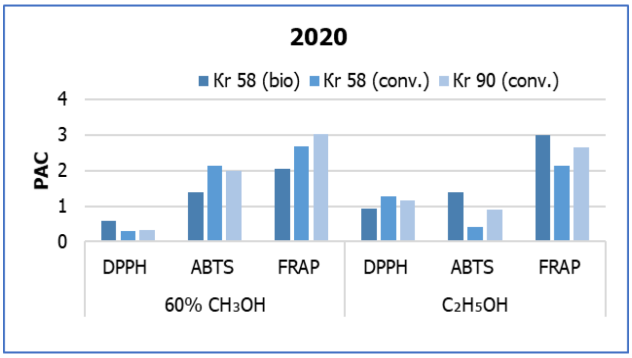


***Figure 11**. Relative antioxidant capacity index (RACI) of tobacco seed extracts obtained with 60% methanol and 95% ethanol extractant for two consecutive years*

The relative antioxidant capacity for the 2020 harvest was highest for the extracts with 60% methanol from seeds *Kr 58 (bio)* variety - 3.68, followed by *Kr 58 (conv.)* - 2.73 and *Kr 90 (conv.)* - 2.61. Ethanol extracts had antioxidant capacities between 2.92 (*Kr 58 (bio)*) and 3.35 (*Kr 90 (conv.)*). From the data in the figure, it can be seen that the ethanolic extracts of seed *Kr 58 (conv.)* and *Kr 90 (conv.)* varieties have higher AOA capacity compared to methanolic extracts in this year. The organically produced seed extracts had approximately the same relative antioxidant capacity for both extractants. In 2021, methanolic seed extracts of seeds *Kr 90 (conv.)* variety had the highest antioxidant capacity at 4.38, followed by organically grown seed extracts of *Kr 58 (bio)* variety - 3.74. The ethanolic extracts for all the seeds studied during this period had the lowest antioxidant capacity - below 2.49 *Kr 58 (bio)*.

The phenolic antioxidant coefficient (PAC) determines the antioxidant capacity of phenolic compounds. It represents the ratio between the total phenolic content and the specific antioxidant capacity, determined by the antioxidant activity method. The PAC data for tobacco seeds for both harvests are presented in **Figure 12.**



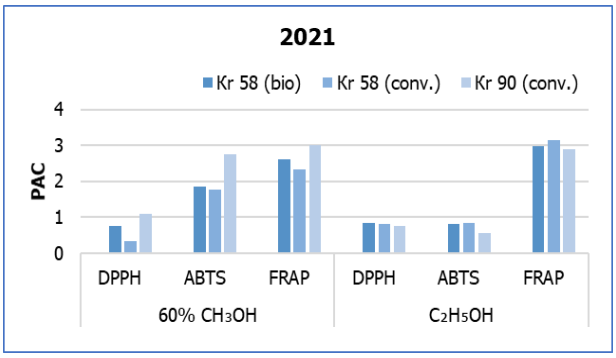


Figure 12. *Phenolic antioxidant coefficient (PAC) of tobacco seed extracts for 2020 and 2021*

From the data in the figures, it is evident that the highest correlation between phenolic compounds and the antioxidant capacity of the extracts was reported by the FRAP method for both the extracts with 60% methanol and the extracts with 95% ethanol. In 2020, the phenolic antioxidant coefficient for extracts with 60% methanol of seeds *Kr 58 (bio)* was the highest by the DPPH method - 0.59, by the ABTS method for conventionally produced seeds of the same variety - 2.12 and by the FRAP method for seeds of *Kr 90 (conv.)* variety- 3.01. For the extracts with 95% ethanol in 2020 by the DPPH method the highest phenolic antioxidant coefficient was reported for *Kr 58 (conv.)* variety - 1.28, and by ABTS method - 1.40 and FRAP method - 2.98 for *Kr 58 (bio)* variety. In 2021, extracts with 60% methanol had the highest phenolic antioxidant coefficient in seed variety *Kr 90 (conv.)* by all three methods: DPPH, 1.09; ABTS, 2.76 and FRAP, 3.01. Ethanol extracts in 2021 from all three seed types reported the best results by the FRAP method, from 2.89 to 3.14.

5.2. Total phenolic content and antioxidant activity of meal

After defatting the tobacco seeds, the meal was obtained, which was used to prepare extracts with three extractants - water, 95% ethanol, and 60% methanol. The data are presented in **Table 15**. The total phenolic content of tobacco seed meal extracts varied according to the extractant used as follows: with water, from 2.37 mg GAE/g to 3.98 mg GAE/g (*Kr 58 (conv.)*); with 95% ethanol – 1.15 mg GAE/g to 2.37 mg GAE/g (*Kr 58 (bio)*); with 60% methanol – 2.50 mg GAE/g (*Kr 90 (conv.)*) to 3.10 mg GAE/g (*Kr 58 (conv.)*).

The obtained results show that the highest content of total phenolic compounds is found in the water extracts of meal from seeds *Kr 58 (conv.)* variety in 2020 - 3.98 mg GAE/g, and in 2021 in *Kr 58 (bio)* variety- 3.66 mg GAE /g. The variety of *Kr 58 (bio)* had the highest TPC for extracts obtained with water in 2020, 3.11 mg GAE/g, and the lowest in 2021, 1.15 mg GAE/g, obtained with 95% ethanol. Extraction with 60% methanol yielded the best extraction of phenolic compounds from seed meal of *Kr 58 (conv.)* variety in 2020 and *Kr 90 (conv.)* variety in 2021. The efficiencies of the extractants relative to the obtained TPC were in the following order - water > 60% methanol > 95% ethanol.

Table 15. Total phenolic content of tobacco seed meal extracts for two consecutive years

Variety	Extractant					
	water	95% ethanol	60% methanol	water	95% ethanol	60% methanol
	mg GAE /g					
	2020			2021		
Kr 58 (bio)	3.11± 0.20bc	2.37± 0.19c	2.9± 0.10bc	3.66± 0.20de	1.15± 0.04a	2.6± 0.10a
Kr 58 (conv.)	3.98± 0.10e	1.94± 0.11bc	3.1± 0.00c	2.37± 0.20a	1.76± 0.15b	2.6± 0.10a
Kr 90 (conv.)	2.66± 0.10ab	1.70± 0.07b	2.5± 0.00a	3.45± 0.20cd	1.65± 0.45ab	2.8± 0.20ab

Mean ± SD, (p<0.05), n = 3

The antioxidant activity of tobacco seed meal extracts was analyzed by three methods. DPPH radical is soluble in various organic solvents but not in water. Therefore, the antioxidant activity (AOA) by the DPPH method was determined only for the tobacco seed meal extracts with 95% ethanol and 60% methanol. The results are presented in **Table 16.**

Table 16. Antioxidant activity of tobacco seed meal extracts from the 2020 and 2021 harvests determined by DPPH method

Variety	Extractant			
	95% ethanol	60% methanol	95% ethanol	60% methanol
	mM TE/g			
	2020		2021	
Kr 58 (bio)	1.97±0.04b	3.16±0.03bc	0.63±0.14a	3.18±0.04c
Kr 58 (conv.)	3.35±0.03c	3.06±0.01bc	1.93±0.11b	3.23±0.03c
Kr 90 (conv.)	2.25±0.10b	2.68±0.05a	0.67±0.14a	3.08±0.06b

Mean ± SD, (p<0.05), n = 3

The analyzed extracts had antioxidant activity by DPPH method from 0.63 mM TE/g to 3.35 mM TE/g. The obtained values of extracts with 60% methanol were higher than the ethanolic extracts. The highest activity was reported for variety *Kr 58 (conv.)* with ethanol - 3.35 mM TE/g in 2020 and with 60% methanol - 3.23 mM TE/g in 2021. The antioxidant activity of extracts with ethanol in 2021 was lower compared to 2020, which is consistent with the results obtained for the AOA of seed extracts (**Table 14**). Extracts with 60% methanol in 2021 had higher activity than the 2020 harvest. The AOA of the extracts with 60% methanol from organically grown seed meal had the least differences in values of the two harvests, 3.16 mM TE/g in 2020 and 3.18 mM TE/g in 2021.

The antioxidant activity of aqueous, ethanolic and 60% methanolic extracts of tobacco seed meal determined by ABTS method is shown in **Table 17**.

The value of aqueous extracts was between 9.20 mM TE/g and 12.90 mM TE/g, *Kr 58 (conv.)* variety. The antioxidant activity of the meal extracts from the 2021 harvest was higher than that of the extracts from the 2020 harvest. The lowest AOA was found for *Kr 58 (conv.)* variety, 9.20 mM TE/g in 2020, and *Kr 58 (bio)* variety - 11.90 in 2021. The highest AOA was recorded in 2021 for *Kr 58 (conv.)* variety – 12.90 mM TE/g. Ethanol extracts had an inverse relationship to water meal extracts, with higher AOA in 2020 than the results obtained in 2021. The antioxidant activity of ethanol-derived extracts ranged between 2.76 mM TE/g and 11.79 mM TE/g. A high value is reported for variety *Kr 58 (bio)* and *(conv.)* in 2020.

Table 17. Antioxidant activity of tobacco seed meal extracts from the 2020-2021 harvest determined by ABTS method

Variety	Extractant					
	water	95% ethanol	60% methanol	water	95% ethanol	60% methanol
	mM TE/g					
	2020			2021		
Kr 58 (bio)	10.90± 0.02c	10.95± 0.05e	14.70± 0.07e	11.90± 0.10d	2.76± 0.05a	10.10± 0.02a
Kr 58 (conv.)	9.20± 0.02a	11.79± 0.04f	14.20± 0.05de	12.90± 0.20f	4.94± 0.04c	11.10± 0.02c
Kr 90 (conv.)	10.60± 0.10b	9.63± 0.02d	10.90± 0.02b	12.30± 0.10e	3.42± 0.04b	14.70± 0.02e

Mean ± SD, ($p<0.05$), n = 3

The ABTS method results obtained with 60% methanol were the highest compared to the data obtained with the other extractants. The reported antioxidant activity was between 10.10 mM TE/g and 14.70 mM TE/g. The results obtained for the *Kr 58 (bio)* variety- 2020, *Kr 58 (conv.)*, and *Kr 90 (conv.)* varieties - 2021 are similar. The lowest value was reported with 60% methanol in the meal of variety *Kr 58 (bio)* - 2021.

The ability of tobacco seed meal extracts to reduce Fe^{3+} is presented in **Table 18**. The aqueous extracts of meal from *Kr 58 (bio)* and *(conv.)* varieties have similar values of 7.45 mM TE/g and 7.59 mM TE/g, and the meal extract of seed *Kr 90 (conv.)* variety has the highest activity in 2021 at 9.67 mM TE/g.

Table 18. Antioxidant activity of tobacco seed meal extracts from 2020 and 2021 harvests determined by FRAP method

Variety	Extractant					
	water	95% ethanol	60% methanol	water	95% ethanol	60% methanol
	mM TE/g					
	2020			2021		
Kr 58 (bio)	7.45± 0.33ab	23.10± 0.55e	12.40± 0.06f	7.96± 0.33ab	4.20± 0.06a	7.43± 0.03a
Kr 58 (conv.)	7.59± 0.58ab	10.80± 0.11c	11.34± 0.06e	5.75± 0.36ab	8.40± 0.08b	7.85± 0.06b
Kr 90 (conv.)	5.02± 0.06a	16.80± 0.06d	8.36± 0.06c	9.67± 0.58b	4.30± 0.14a	8.91± 0.06d

Mean ± SD, ($p < 0.05$), n = 3

Ethanol extracts from the 2020 harvest have higher activity than those in 2021. The highest AOA by this method was recorded for the 2020 harvest, variety *Kr 58 (bio)* - 23.1 mM TE/g. In 2021, the FRAP method results for variety *Kr 58 (bio)* - 4.20 mM TE/g and variety *Kr 90 (conv.)* - 4.30 mM TE/g were similar. When 60% methanol extractant was used, a higher result was observed in 2020 with the highest value for *Kr 58 (bio)* variety - 12.40 mM TE/g and in 2021 the same variety showed the lowest value - 7.43 mM TE/g.

The relative antioxidant capacity index (RACI) of the studied extracts showed the highest activity for 2020 of the water extract of variety *Kr 58 (conv.)* - 7.4, followed by the ethanolic extract of the same variety - 3.7 (**Figure 13**).

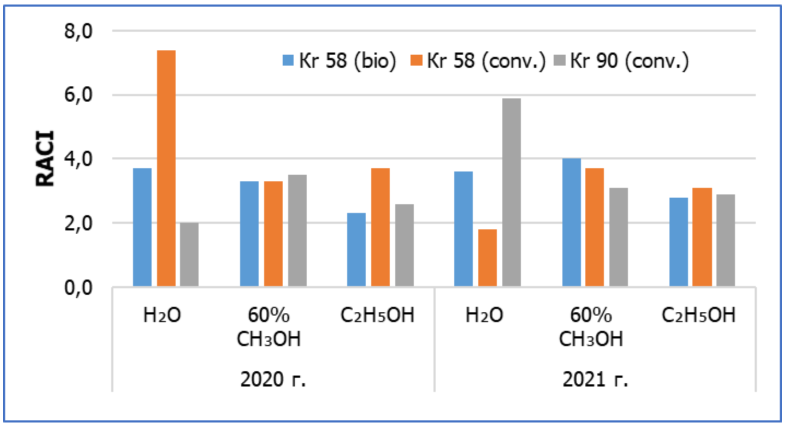


***Figure 13.** Relative antioxidant capacity (RAC) of tobacco seed meal extracts prepared with water, 95% ethanol and 60% methanol extractants for two consecutive years*

In 2021, the highest relative capacity was the aqueous extract of the *Kr 90* variety (conv.) - 5.9 and the methanol extracts of the variety *Kr 58 (bio)* and *(conv.)*. From the data in the figure, it can be seen that the RACI of the ethanolic extracts in the varieties in both years was in a close order (2.3 to 3.7). The aqueous extracts have an RACI from 1.8 to 7.4, and the methanol ones from 3.1 to 4.0, indicating that the relative antioxidant capacity varied widely in the former but was also highest in the latter. The higher RACI values for the aqueous extracts are because the coefficient was calculated at zero value for the DPPH method.

Figure 14 presents the phenolic antioxidant coefficient (PAC) of the meal extracts for the two years of monitoring. The aqueous extracts of the meal *Kr 90 (conv.)* by ABTS method (3.99) had the highest PAC in 2020, and in 2021 the meal extract of seeds *Kr 58 (conv.)* variety – 5.46. The phenolic antioxidant coefficient with 60% methanol was the highest under the ABTS method in all three varieties in both years (3.94-5.03). The FRAP method with 95% ethanol in 2020 had a higher phenolic antioxidant capacity compared to the other methods and compared to data in the following vintage. It was also the FRAP method that realised the highest capacity, 9.75 in variety *Kr 58 (bio)* and 9.83 in variety *Kr 90 (conv.)*.

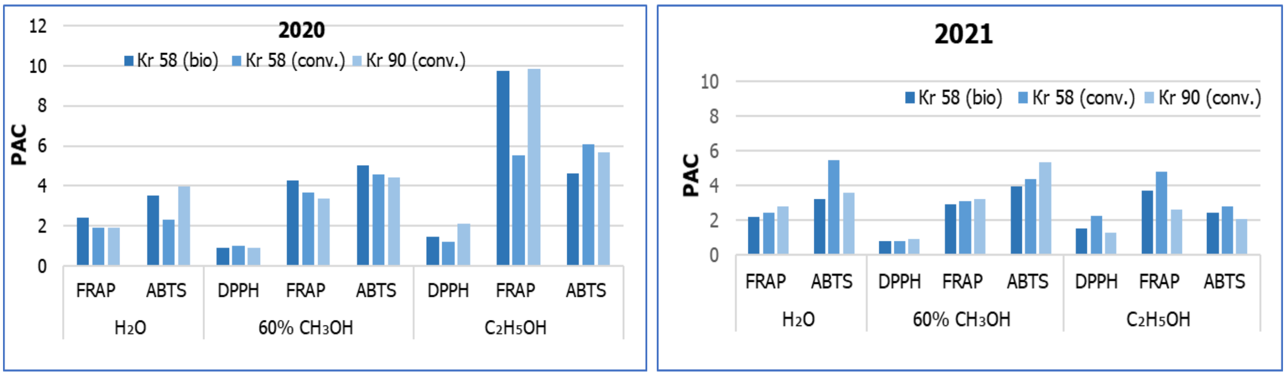


***Figure 14.** Phenolic antioxidant coefficient (PAC) of tobacco seed meal extracts for 2020 and 2021*

5.3. Total phenolic content and antioxidant activity of oil

The total phenolic content of the glyceride oil extracted from tobacco seeds is presented in **Table 19**. The extractants used were 80% ethanol and 80% methanol. The total phenolic content of the samples studied ranged between 0.29 mg GAE/g - variety *Kr 90 (conv.)* and 1.70 mg GAE/g - variety *Kr 58 (bio)*. The oil from the organically produced variety *Kr 58 (bio)* had the highest TPC, while the oil from variety *Kr 90 (conv.)* had the lowest phenolic content. Their total amount in oil from conventionally produced seeds is half that reported in seed extracts.

Table 19. Total phenolic content of tobacco seed oil

Variety	Extractant	
	80% ethanol	80% methanol
	mg GAE/g	
Kr 58 (bio)	1.44±0.06b	1.70±0.17b
Kr 58 (conv.)	0.32±0.01a	0.34±0.01a
Kr 90 (conv.)	0.30±0.01a	0.29±0.01a

Mean ± SD, (p<0.05), n = 3

The antioxidant activity (AOA) of tobacco oil (**Table 20**) showed no statistically significant differences between the activity of extracts by the DPPH method with both 80% ethanol and 80% methanol. The antioxidant activity for *Kr 58 (bio)* oil (0.92 - 1.10 mM TE/g), determined by the ABTS method, was half of the value for conventional varieties, regardless of the extractant used. The AOA of the oil by the FRAP method is similar. The lowest activity was recorded for variety *Kr 58 (bio)*, which did not exceed 1 mM TE/g. In the case of *Kr 58 (conv.)* and *Kr 90 (conv.)* oils, the data with 80% ethanol had higher values compared to those with 80% methanol. The highest activity by the FRAP method was exhibited by the oil of variety *Kr 58 (cont.)* - 2,71 mM TE/g. The antioxidant activity of tobacco oil was lower than that reported in seeds. This is because the oil represents the non-polar fraction in tobacco seeds extracted by an n-hexane extractant.

Table 20. Antioxidant activity of tobacco seed oil

Variety	Extractant					
	80% ethanol			80% methanol		
	DPPH	ABTS	FRAP	DPPH	ABTS	FRAP
	mM TE/g					
Kr 58 (bio)	0.52± 0.03b	1.10± 0.05a	0.85± 0.06a	0.45± 0.06b	0.92± 0.12a	0.93± 0.09a
Kr 58 (conv.)	0.44± 0.01a	2.70± 0.00b	2.71± 0.81b	0.44± 0.02b	2.70± 0.00b	2.40± 0.37b
Kr 90 (conv.)	0.47± 0.00ab	2.70± 0.00b	1.66± 0.50a	0.26± 0.01a	2.70± 0.00b	1.12± 0.12a

Mean ± SD, (p<0.05), n = 3

The total antioxidant capacity (**Figure 15**) of the oil showed the highest value in that of *Kr 58 (bio)* variety 5.59 - 5.66, both the extracts with methanol and ethanol. No difference was observed between the antioxidant capacity of the ethanol/methanol extracts for the oil of *Kr 58 (conv.)* - 2.98/3.01 and *Kr 58 (bio)* - 5.66/5.59. In variety *Kr 90 (conv.)* methanol extracts had lower antioxidant capacity – 2.20 than extracts obtained with ethanol – 2.89.

Figure 16 shows the phenolic antioxidant coefficient (PAC) of tobacco seed oil. The PAC shows better results when 80% ethanol is used (from 0.4 - DPPH method to 9.0 - ABTS method) than with 80% methanol (from 0.3 - DPPH method to 9.3 - ABTS method).

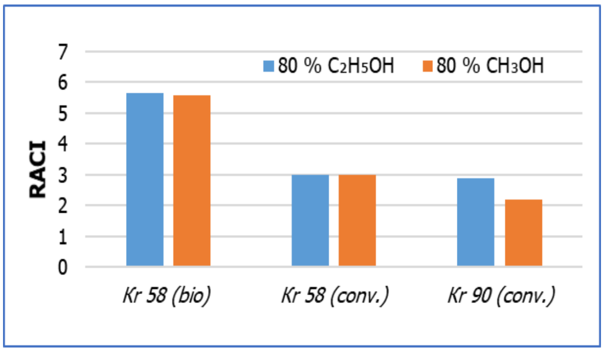


***Figure 15.** Relative antioxidant capacity index (RACI) of tobacco oil extracts*

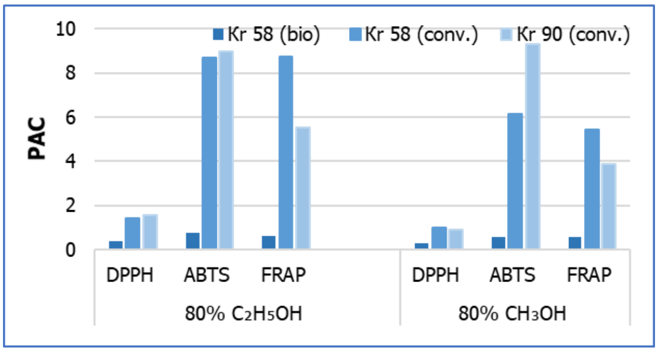


Figure 16. The phenolic antioxidant *coefficient* (PAC) of tobacco oil extracts

The phenolic antioxidant coefficient is higher in conventionally produced seed oil. The highest values were reported by the ABTS method for variety *Kr 90 (conv.)* with both methanol and ethanol. The data in the figure show that the phenolic antioxidant coefficient with ethanol and methanol has the same correlation on the results for oil from different varieties, namely - the coefficient is lowest for *Kr 58 (bio)* by all methods, with the highest coefficient by the ABTS method being *Kr 90 (conv.)*, and by FRAP - *Kr 58 (conv.*).

Conclusion:

- Organically produced seeds of variety *Kr 58 (bio)* had higher total phenolic content and consequently higher antioxidant activity compared to conventionally grown seeds in both vegetations.
- The extracts showed higher antioxidant activity against the FRAP electron transfer method (ability to reduce Fe^{3+} to Fe^{2+}), compared to the hydrogen atom transfer methods (ABTS method and DPPH method).
- The total phenolic content and antioxidant activity of meal extracts are higher than those of seeds.
- The results obtained for the AOA of the meal extracts with 60% methanol were higher compared to the activity with the other extractants, water, and 95% ethanol.
- The highest total phenolic content is reported in the oil from organically grown seeds of variety *Kr 58 (bio)* - on average 1.57 mg GAE /g.
- Oil from organically grown seeds had the highest antioxidant capacity, 5.66/5.59, but the lowest dependence in terms of phenolic antioxidant coefficient.
- Of the extractants used, better results were reported for seed and meal with 60% methanol, and for oil with 80% ethanol and 80% methanol.

6. Determination of the chemical and lipid composition of waste tobacco seeds and the resulting oil for their application.

Tobacco seeds may be considered waste in cases where they are not needed for cultivation or are compromised. Overproduction of seeds also leads to the generation of residues without use. Tobacco seeds are divided into small (below 0.5 mm) and large (above 0.5 mm). Those below 0.5 mm are considered unsuitable for sowing and are separated as waste. In order to determine their quality and potential use, analyses were carried out to determine the chemical and lipid composition of seeds of the pooled fraction of varieties

Krumovgrad 58 and *90* with a size below 0.5 mm, considered as waste seeds of the variety group *Basmi*.

6.1. Chemical composition

The oil extracted from waste tobacco seeds makes them a good source of fat. The oil content of the seeds is 32.1%. In addition to a good oil content, the waste seed is also characterized by a high protein content of 29.4 The total carbohydrate content (27.6 %) of the tobacco seed is typical for the seeds of this plant (up to 30 %). The high percentage of fiber (26.6 %) is also noteworthy, which makes the waste seed a good source of insoluble carbohydrates. The results for ash content, as an indicator of mineral elements, are high and coincide with data obtained for ash content of seeds with size above 0.5 mm. Based on the data obtained for carbohydrate, protein, and fat contents in the waste seeds, their energy value was calculated to be 517 kcal/100 g (**Table 21**).

Table 21. Chemical composition of tobacco waste seeds

Chemical composition	Waste seeds
Oil, %	32.1±1.0
Protein, %	29.4±1.4
Carbohydrates, %	27.6±0.5
Fibre,%	26.6±0.6
Ash, %	4.3±0.3
Moisture, %	6.6±0.03
Energy value, kcal/100 g	517±3

Mean ± SD, (p<0.05), n = 3

6.2. Lipid composition

Glyceride oil from waste tobacco seeds was analyzed for individual fatty acid composition (**Table 22**). Ten fatty acids were identified, with three of them predominating in the composition. Linoleic acid was present in the highest amount (71.94%), followed by oleic acid (13.70%) and palmitic acid (12.86%). The oil is rich in polyunsaturated fatty acids (71.94%) and is almost equal in saturated (14.30%) and monounsaturated (13.76%) fatty acids.

Таблица 22. Fatty acid composition of tobacco waste seed oil

Fatty acids, %	**Tobacco waste seed oil**
$C_{8:0}$ Caprylic	0.05±0.01
$C_{11:0}$ Undecylic	0.04±0.01
$C_{12:0}$ Lauric	0.06±0.01
$C_{15:0}$ Pentadecanoic	0.02±0.01
$C_{16:0}$ Palmitic	12.86±0.05
$C_{17:0}$ Margaric	0.14±0.01
$C_{17:1}$ Heptadecanoic	0.06±0.01
$C_{18:0}$ Stearic	1.13±0.01
$C_{18:1}$ Oleic	13.70±0.10
$C_{18:2}$ Linoleic	71.94±1.05
SFA	14.30±0.02
MUFA	13.76±0.11
PUFA	71.94±1.05

Mean ± SD, (p<0.05), n = 3

The lipid indices of glyceride oil from waste tobacco seeds are presented in **Table 23**. The atherogenic and thrombogenic index of waste seed oil does not exceed 1.0. The values are low, indicating good atherogenic and thrombogenic properties. The hypocholesterolemic/hypercholesterolemic index of waste tobacco seed oil is 7.0. It is recommended that this index has values above 1.0, which makes the oil suitable for the prevention of cardiovascular diseases. The peroxidation index of tobacco seed oil is relatively high (72.0), indicating a tendency to oxidation of the oil. The prediction of the shelf life of an oil can be assessed by the oxidant stability index. The values for APE (171,0) and BAPE (72.0) are correlated with the high percentage of unsaturated fatty acids in the oil composition. The oxidant stability index is 1.0. This indicates a tendency of the oil to oxidation and determines a shorter shelf life.

Table 23. Lipid indices of glyceride oil from waste tobacco seeds

Lipid indecies	Tobacco waste seed oil
Index of atherogenicity (IA)	0.2±0.0
Index of thrombogenicity (IT)	0.3±0.0
Hypocholesterolemic/hypercholesterolemic (h/H)	7.0±0.1
The peroxidability index (PI)	72.0±1.0
Allylic Position equivalent (APE)	171.0±1.9
Bis-Allylic position equivalent (BAPE)	72.0±1.0
Oxidation Stability Index	1.0±0.1

Mean ± SD, (p<0.05), n = 3

Biologically active components in the lipid fraction of tobacco seed waste, as with all plant seeds, consist of phospholipids, unsaponifiable substances, sterols and tocopherols. Data on their total content in tobacco waste seeds and glyceride oil are presented in **Table 24**. Waste tobacco seeds were analyzed for total phospholipid content, 0.2% in seed and 1.5% in oil. The unsaponifiable matter in the waste seed was 0.7% and in the seed oil 3.4%. The total sterol content of the seed (0.2%) and oil (0.7%) is low. The total tocopherol content of the waste seeds examined was 4.0 mg/kg and of the glyceride oil 144 mg/kg. Both γ-tocopherol and δ-tocopherol were identified, with γ-tocopherol being present in higher amounts (81.5 mg/kg).

Table24. Lipid composition of tobacco waste seeds

Biologically active components	Content in waste seeds	Content in waste seed oil
Phospholipids, %	0.2±0.06	1.5±0.2
Unsaponifiable matter, %	0.7±0.1	3.4±0.9
Sterols, %	0.2±0.1	0.7±0.4
Tocopherols, mg/kg	4.0±1.0	144.0±6.0
γ-tocopherol, mg/kg	2.3±0.2	81.5±2.1
δ-tocopherol, mg/kg	1.7±0.7	62.5±2.4

Mean ± SD, (p<0.05), n = 3

The individual phospholipid composition of waste tobacco seed oil is presented in **Figure 17**. The highest content of phosphatidylcholine is 35.7%. Phosphatidylinositol (23.8%) and lysophosphatidylinositol (11.9%) were also found in higher amount compared to other identified phospholipids.

The main plant sterols are β-sitosterol and campesterol, and these are also identified in the highest amount in tobacco seed oil. Nine types of sterols have been identified in glyceride oil and their individual composition is characteristic of tobacco seed - **Figure 18**. The highest content was *β*-sitosterol (61.2%), followed by stigmasterol (15.2%) and campesterol (10.2%). The cholesterol content of tobacco seed waste oil was 4.5%.

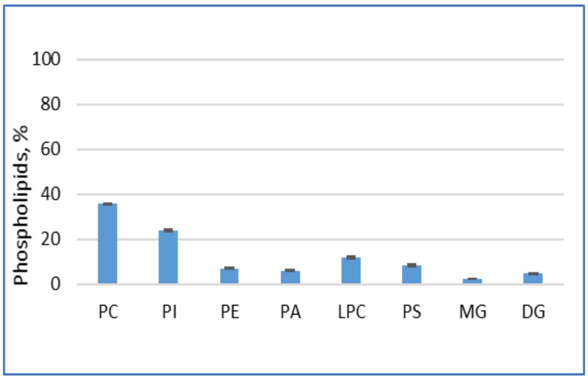


***Figure 17**. Individual phospholipid composition of waste tobacco seed oil*

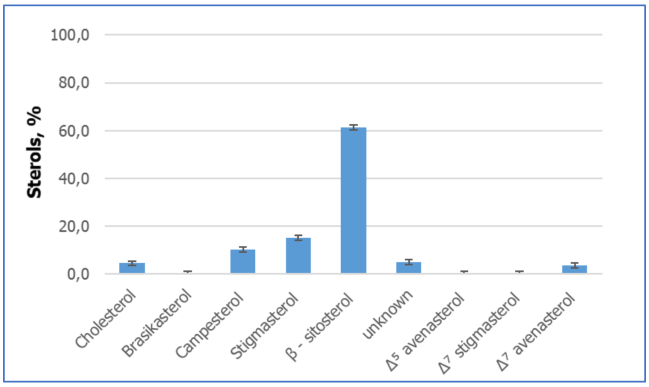


***Figure 18.** Individual sterol composition of waste tobacco seed oil*

PC - phosphatidylcholine, PI - phosphatidylinositol; PE - phosphatidylethanolamine; PA - phosphatidic acids; LPC - lysophosphatidylcholine; PS - phosphatidylserine MG - monoglyceride; DG - diglyceride

6.3. Antioxidant activity of tobacco waste seeds extracts

Seed waste extracts were analyzed for total phenolic content and antioxidant activity. The extractants used were water, 60% methanol, and 95% ethanol. The results showed that the total phenolic content (TPC) of the seeds ranged from 0.89 mg GAE/g to 2.34 mg GAE/g, with the highest TPC extracted with 60% methanol, 2.34 mg GAE/g, and the lowest with ethanol, 0.89 mg GAE/g, depending on the extractant used.

The antioxidant activity of waste seed extracts with 60% methanol by ABTS and FRAP methods was higher compared to the results obtained for the other two extractants, water, and ethanol. The relative antioxidant capacity index (RACI) of the water extracts was 1.41, while it was the same for the extracts with 60% methanol and 95% ethanol at 3.32. The results are presented in **Table 25.**

Table 25. Total phenolic content (TPC) and antioxidant activity (AOA) of waste seed extracts

Methods					
Extractant	**TPC, mg GAE /g**	**DPPH, mM TE/g**	**ABTS, mM TE/g**	**FRAP, mM TE/g**	**RACI**
water	1.46±0.43	-*	3.82±2.85	3.14±0.25	1.41
60% methanol	2.34±0.28	8.71 ±0.53	5.97±0.42	7.53±1.65	3.32
95% ethanol	0.89±0.08	8.96±1.29	0.20±0.32	2.41±0.22	3.32

*Mean ± SD, n = 3 * - no test conducted*

Conclusion:

- Waste tobacco seeds can be used as a good natural source of fat, protein, and fiber.
- The waste seeds do not differ in chemical and lipid composition from cultivable seeds above 0.5 mm.

➢ Glyceride oil from waste tobacco seeds can be used for therapeutic and cosmetic purposes.

7. Exploring the potential of organically produced tobacco seed oil for cosmetic applications

Two approaches have been used to evaluate tobacco oil as a base oil for cosmetic purposes:

- analysis of glyceride tobacco oil and comparison with a well-known cosmetic oil of similar composition - grape seed oil;
- development of emulsion cream with tobacco and grape seed oil, analysis and comparison.

Tobacco seed oil variety *Kr 58 (bio)* and cold-pressed grape seed oil were examined for major physical components, and the results are presented in **Table 26.**

Table 26. Physical parameters of tobacco and grape seed oil

Physical parameters	Oil	
	Tobacco	Grape
Peroxide value, meqO_2/kg	2.15±0.37	3.45±0.07
Oxidant stability (100°C), h	12.20±0.10	9.20±0.10
Acid value, mg KOH/g	3.26±0.04	0.18±0.01
Refractive index	1.476±0.000	1.473±0.000
Iodine value, g I_2/100 g	136.85±0.20	134.58±0.24

Mean ± SD, (p<0.05), n = 3

The data obtained for both oils are similar. Tobacco oil has a lower peroxide value (2.15 meqO_2/kg) than grape seed oil (3.45 meqO_2/kg). The result obtained is in the range between 1 - 3 meqO2/kg, considered advisable for oils used in cosmetic products. The slow oxidation of tobacco oil is also proven by its higher oxidative stability. In addition to oxygen, the oil remains stable for 12.2 hours at 100°C, while grape seed oil shows a lower stability - 9.20 hours at 100°C. On the other hand, tobacco seed oil has a higher acid value (3.26 mg KOH/g) in the direction of grape seed oil (0.18 mg KOH/g). Tobacco and grape seed oils have a similar refractive index above 1.470. The iodine value of the oils (136.85 and 134.58 gI2/100 g) corresponds to their fatty acid composition, as shown in **Table 27.** Tobacco and grape seed oil were studied in terms of fatty acid composition, the result showed that both oils can be considered unsaturated with high levels of linoleic acid (tobacco oil - 65.90% and grape oil - 60.16%). Tobacco oil has a higher content of palmitic acid ($C_{16:0}$ - 12%) and lower oleic acid ($C1_{8:1}$ - 17.70%) in the direction of grape oil, where ($C_{16:0}$ - 7.98% and $C_{18:1}$ - 27.11%).

Ideal lipids have a 1:1:1 ratio of SFA, MUFA and PUFA, respectively. For tobacco oil this ratio is 1:1:4 and for grapeseed oil 1:2:4. Tobacco oil has a ratio closer to the ideal lipid compared to that of grape seed oil, and it is notable that both oils have a high result for PUFA due to the high percentage of linoleic acid.

Table 27. Fatty acid composition of tobacco and grape seed oil

Fatty acids, %		Oil	
		Tobacco	Grape
$C_{14:0}$	Myristic	0.10±0.00	0.04±0.01
$C_{16:0}$	Palmitic	12.00±0.50	7.98±0.21
$C_{16:1}$	Palmitoleic	0.20±0.05	0.12±0.01
$C_{17:0}$	Margaric	0.20±0.03	0.07±0.02
$C_{17:1}$	Heptadecanoic	0.40±0.05	0.05±0.01
$C_{18:0}$	Stearic	2.10±0.01	3.14±0.55
$C_{18:1}$	Oleic	17.70±0.20	27.11±0.30
$C_{18:2}$	Linoleic	65.90±0.80	60.16±0.20
$C_{18:3\ n-6}$	Linolenic	0.60±0.20	0.04±0.02
$C_{18:3\ n-3}$	Linolenic	N/D	0.43±0.03
$C_{20:0}$	Arachidic	0.20±0.05	0.25±0.05
$C_{20:1}$	Gadoleic	0.10±0.04	0.24±0.05
$C_{22:0}$	Behenic	0.40±0.05	0.30±0.05
$C_{22:1}$	Erucic	0.10±0.00	0.03±0.00

Mean ± SD, ($p<0.05$), n = 3; N/D – not detected

Analysis for tocopherol content shows that both oils are rich in these compounds. The tocopherol content is represented by α-, γ- and δ-tocopherols and two tocotrienols, α- and γ-. The total tocopherol content of tobacco oil is 175.0 mg/kg, which is half the amount found in grape seed oil (498.0 mg/kg). δ-Tocopherol dominates in tobacco oil (68.4%), whereas this isomer is not detected in grape seed oil. Grape seed oil is rich in α-tocopherol (52.4 %). α- and γ-Tocotrienol were identified only in grape seed oil. The content of γ-tocopherol in both oils was almost equal The results are presented in **Table 28.**

Table 28. Tocopherol composition of tobacco and grape seed oil

Tocopherols	Oil	
	Tobacco	Grape
α- tocopherol, %	7.1±0.1	52.4±1.7
α- tocopherol, %	N/D*	9.2±0.4
γ- tocopherol, %	24.5±1.1	25.2±0.6
γ- tocotrienol, %	N/D	13.2±0.8
δ- tocopherol, %	68.4±0.6	N/D
Total, mg/kg	175.0±2.1	498.0±4.2

Mean ± SD, ($p<0.05$), n = 3; N/D – not detected

Analyses on the possible use of tobacco oil in cosmetics continue with its incorporation in emulsion cream, and the same approach has been applied to grapeseed oil. An oil-in-water (O/W) type emulsion was chosen for the purpose of the experiment as it allows an emollient content of up to 80%, has a cooling and matting effect on the skin. This type of base emulsion helps to introduce hydrophilic molecules. The disadvantage is that these emulsions are thermally unstable and are a good medium for the growth of microorganisms and also foam when emulsified. In order to reduce these effects, dimethicone, an essential oil with antimicrobial activity and a proven function as a natural preservative, lemongrass essential oil, have been added as an antifoaming agent. The lowest possible concentration of essential oil (0.5%) was chosen as it can cause sensitivity at higher concentrations. Lemongrass has proven uses as an insect repellent, muscle pain relief, antifungal, and antimicrobial properties, making it a suitable choice when formulating an emulsion-type moisturizer (e.g. body milk).

The tobacco oil emulsion base was created by selecting ingredients with the idea of an organic, natural product. Tobacco oil, grown under organic production conditions, on a certified biofield, was used as the main emollient in the formula. The remaining ingredients are water and glycerol (humectants), glyceryl stearate SA (emulsifier), cetearyl alcohol (viscosity regulator), beeswax (co-emulsifier), and dimethicone (emollient). Only dimethicone is a synthetic product, although according to various laws, the use of certain ingredients within regulated limits is permissible.

Emulsion bases were prepared with the presented ingredients, both tobacco oil-based and grapeseed oil-based. The resulting emulsion bases E1 (with tobacco oil) and E2 (with grapeseed oil) are white in color, have a homogeneous appearance, are lump-free, do not delaminate in storage, and are easily washed off with water. The emulsion bases are odorless, and those with 0.5% lemongrass essential oil added have a faint, fresh citric odor (**Figure 19**).

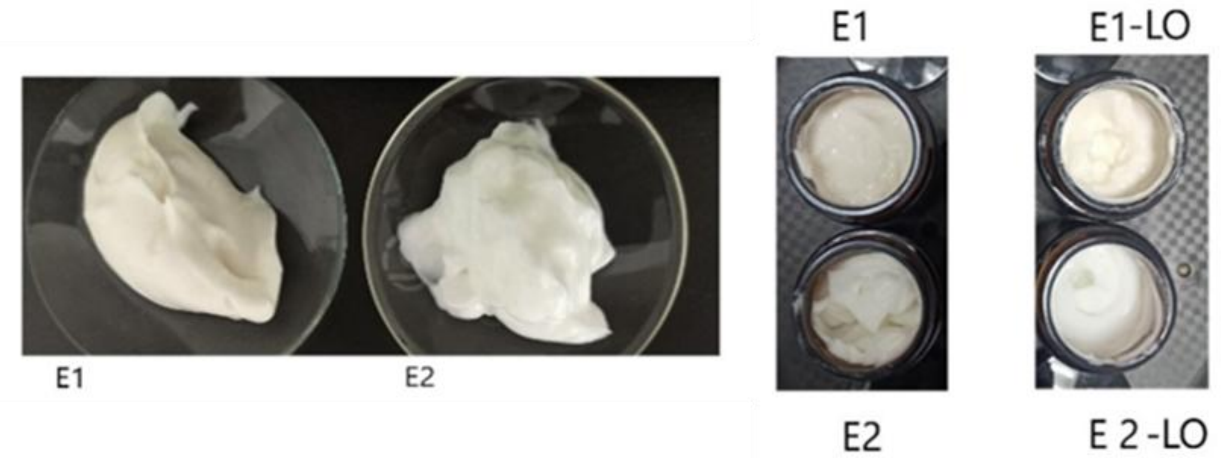


Figure 19. *Emulsion creams:*

E1 – emulsion base with tobacco oil, E1 – LO - emulsion base with tobacco oil + 0.5 % essential oil of lemongrass, E2 – emulsion base with grape seed oil, E2 – LO - emulsion base with grape seed oil + 0.5 % essential oil of lemongrass

Some physical and chemical parameters of the prepared emulsions were determined and the results are presented in **Table 29**. The moisture content of all samples was above 80%. The refractive index for all emulsions was above 1.600, which compared to the index of the incorporated oils, is high. The emulsions with essential oil had higher index compared to the base ones, the lowest refractive index was recorded for E2 and the highest for E2-LO. For E1 and E1-LO the values for this index are similar. The emulsion creams were also tested for oxidant stability. The E1 base had slightly lower oxidant stability compared to E2, with values within one order of magnitude. The addied 0.5% lemongrass essential oil increased the oxidant stability in E1-LO.

Table 29. Physical parameters of emulsion creams with tobacco and grape oil

Parameters	Base		Base with 0.5% lemongrass essential oil	
	E1	**E2**	**E1 - LO**	**E2 - LO**
Moisture, %	82.2±0.8	85.1±0.7	81.1±0.5	84.0±0.7
Refractive index	1.644±0.000	1.624±0.000	1.651±0.000	1.675±0.000
Oxidant stability (110ºC), h	2.60±0.01	2.77±0.01	3.85±0.02	2.68±0.02

Mean ± SD, (p<0.05), n = 3

Tobacco oil emulsion (E1) has a lower pH than grape oil-based E2 emulsion (Figure 20). The pH of the emulsions, measured immediately after preparation and cooling, indicates their alkaline character – pH > 7. After 48 days, the pH decreases, with an E1 pH of 6.64, and this value is maintained for up to 90 days after preparation and storage. Emulsion cream E1-LO and E2 have a neutral character after 48 days of storage. Emulsion E2-LO retains its alkaline character until day 48 and then acquires a neutral pH like other emulsions. The neutral pH of the emulsion is due to the high water content and the lack of an added pH regulator.

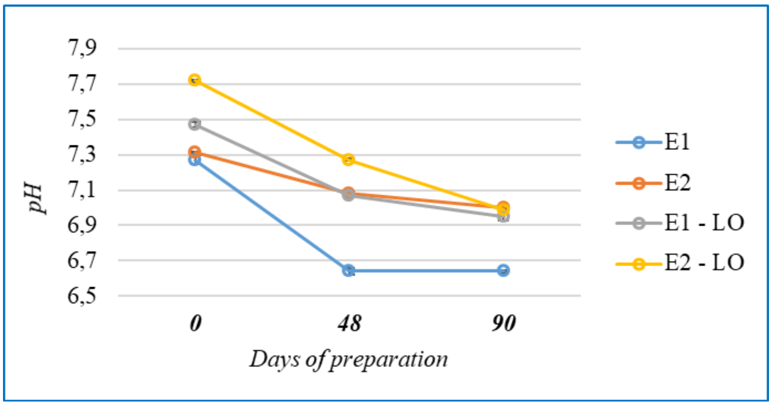


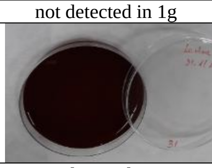
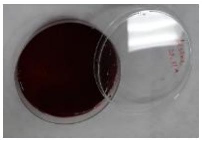
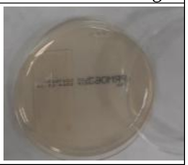
***Figure 20.** pH of the emulsions during storage*

Emulsion creams with high water content imply easier contamination with mold fungi and yeasts, as well as lower antimicrobial activity. Therefore, an assay was conducted for the total number of microorganisms, molds, and yeasts, and antimicrobial activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*). The results obtained are presented in **Table 30**. The data showed that the total number of mesophilic microorganisms isolated in three of the emulsions was less than 10 cfu/g. Only emulsion E2 had a total plate count (TPC) of 1.1.104 ±6.4.103 cfu/g. Molds and yeasts for all emulsions were below 10 cfu/g. Gram-positive and Gram-negative bacteria were not isolated. Despite their high water content, the emulsions had good antimicrobial properties, with E1 having better TPC compared to E2. Lemongrass essential oil has a positive effect in E2-LO suppressing the development of colonies of microorganisms.

Conclusion:

- Tobacco oil can be successfully applied as a cosmetic base oil and incorporated into cosmetic formulations with good physical and chemical characteristics.
- Emulsion cream based on tobacco oil can be defined as a moisturizer.
- The developed cosmetic preparation based on tobacco oil can be defined as close to natural and at the same time affordable based on the ingredients incorporated in it.

Table 30. Microbiological analysis of emulsion creams with tobacco and grape seed oil

Emulsion	Total number of aerobic mesophilic microorganisms, cfu/g	Molds and yeasts, cfu/g	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Candida albicans*
E2	$1.1.10^{4} \pm 6.4.10^{3}$	<10	not detected in 1g	not detected in 1g	not detected in 1g	not detected in 1g
						
E2-LO	<10	<10	not detected in 1g	not detected in 1g	not detected in 1g	not detected in 1g
						
E1	<10	<10	not detected in 1g	not detected in 1g	not detected in 1g	not detected in 1g
						
E1-LO	<10	<10	not detected in 1g	not detected in 1g	not detected in 1g	not detected in 1g
						

VI. FINDINGS

The research carried out and the results obtained allow us to summarize the following conclusions:

- A detailed characterization of the chemical and lipid composition of two varieties of Bulgarian tobacco seeds - *Krumovgrad 58* and *Krumovgrad 90* grown under organic and conventional production is presented. It was found that organically produced tobacco seeds are a rich source of macronutrients with high energy value. Tobacco seeds are a natural source of linoleic acid - 65%, regardless of the method of cultivation of the tobacco plant.
- It has been found that organically produced tobacco seeds and the glyceride oil obtained from them have a higher content of biologically active substances - phospholipids, tocopherols, and polyphenolic compounds. The glyceride oil obtained from them also has good oxidant stability and good atherogenic and thrombogenic properties.
- The study of the chemical composition of two varieties of tobacco seeds of the *Basmi* variety group, in two consecutive growing seasons, identifies them as a resistant crop irrespective of the method of production. Tobacco seeds of *Krumovgrad 58* and K*rumovgrad 90* can be successfully used as a source of fiber and raw material for glyceride oil production.
- The extraction method and the extractant used affect the yield of glyceride oil and its tocopherol composition. The fatty acid composition of the oil remains unsaturated regardless of the extraction/extraction method used.
- Seed extracts, meal, and oil may be a source of polyphenolic compounds. The most suitable extractant for polyphenolic compounds from tobacco seeds and tobacco meal is 60% methanol, and for extracting them from tobacco oil, 80% ethanol and 80% methanol.

Seed extracts showed higher antioxidant activity by FRAP electron transfer method (ability to reduce Fe^{3+} to Fe^{2+}) compared to hydrogen atom transfer methods (ABTS method and DPPH method). The antioxidant activity of meal extracts was found to be higher than that of seed and glyceride oil. The highest relative antioxidant capacity was obtained from the tobacco oil extracts studied. Extracts from organically produced tobacco seeds, meal, and oil had better total phenolic content and higher relative antioxidant capacity than conventionally produced ones.

- Waste, unsuitable for sowing, tobacco seeds are a valuable source of glyceride oil and energy. They have an identical chemical and lipid composition to usable tobacco seeds and can also be successfully used as raw material.
- Tobacco oil is a suitable ingredient for the preparation of moisturizing cosmetic products. A recipe has been developed for the preparation of emulsion cream based on tobacco oil.

VII. SCIENTIFIC CONTRIBUTIONS

- For the first time, the chemical composition of seeds from a Bulgarian variety of tobacco, grown in organic production conditions, was investigated.
- For the first time, a study has been conducted on the total content of phenolic compounds and antioxidant activity of seed extracts, meal, and oil from organically and conventionally produced Bulgarian tobacco varieties.
- For the first time, the chemical and lipid composition of waste tobacco seeds unfit for sowing has been studied in detail. The possibility of fully utilizing waste tobacco seeds for the production of glyceride oil, fiber, and natural antioxidants has been proven.

VIII. APPLIED CONTRIBUTIONS

- Various techniques have been used for the extraction of glyceride oil from tobacco seeds. It has been found that oil extraction by maceration and ultrasound with an extractant n-hexane: acetone are fast and affordable methods for oil extraction.
- A method for extracting glyceride tobacco oil with a high content of tocopherols has been proposed – maceration and ultrasound extraction with an extractant ethyl acetate.
- A recipe has been developed for the preparation of an emulsion cream based on natural ingredients, containing tobacco oil and lemongrass essential oil as a natural preservative.

IX. PUBLICATIONS

1. Stoyanova, L., Romova, M. (2024). Bioactive compounds and nutritive Composition of Waste seeds from *Nicotiana tabacum* L. (Solanaceae). *Current Research in Nutrition and Food Science Journal*, 12(1). doi: http://dx.doi.org/10.12944/CRNFSJ.12.1.30; Referenced and indexed in WoS and Scopus (Q3)

2. Stoyanova, L., Angelova-Romova, M., Docheva, M., Kirkova, D. (2024). Total phenolic content and antioxidant activity of extracts obtained from tobacco waste seeds, grown under

organic production. *International Journal of Secondary Metabolite*, *11*(3), 408-420. https://doi.org/10.21448/ijsm.1370869; Referenced and indexed in Scopus (Q4)

3. Stoyanova, L., Angelova-Romova, M. (2024). Chemical composition of seeds from organically grown tobacco plants. *Bulgarian Chemical Communications*, 56 (D2) 49-54, DOI:10.34049/bcc.56.D.S2P34; Referenced and indexed in WoS (Q4)

4. Stoyanova, L., Angelova Romova, M., Docheva, M., Kirkova, D., Dureva, V. (2023). Determination of polyphenols in oriental tobacco seeds of Krumovgrad ecotype grown conventionally and under bio production conditions. *Ecology and Health Conferen*ce, 2367-9530 Plovdiv 2023, 66-70, http://hst.bg/bulgarian/conference.htm

X. CONFERENCES

1. ACM2 Seminar - Instrumental Techniques for Chemical Analysis - 2 June 2022, Plovdiv

Poster - *"Lipid composition of seeds from oriental tobacco grown in a bio-field*" - L. Stoyanova, M. Angelova-Romova

2. Conference with international participation "ECOLOGY AND HEALTH" 2023 - autumn

Report - *"Determination of polyphenols in seeds of oriental tobacco of Krumovgrad ecotype, grown conventionally and under conditions of organic production"* - L. Stoyanova, M. Angelova-Romova, M. D. Kirkova, V. Dureva

3. 12th Scientific Conference on Chemistry with International Participation of the Faculty of Chemistry at Paisii Hilendarski University - October 2023

Poster - "*Chemical composition of seeds from organically grown tobacco plants*" - L. Stoyanova, M. Angelova-Romova

4. Xth International conference of young scientists - Plovdiv 2024

Poster - *"Influence of the extraction method on tocopherol content in tobacco seed oil*" - L. Stoyanova, M. Angelova-Romova, D. Kirkova, M. Docheva, V. Dureva

5. Workshop ACM2 - Instrumental techniques and methods for chemical analysis - challenges and new solutions - 5 June 2024, Plovdiv

Poster - *"Formulation of Oil-in-Water Emulsion Utilizing Tobacco Seed Oil*" - L. Stoyanova, M. Angelova-Romova

XI. CITATIONS

1. Stoyanova, L., Romova, M.. (2024). Bioactive compounds and nutritive Composition of Waste seeds from *Nicotiana tabacum* L. (Solanaceae). *Current Research in Nutrition and Food Science Journal*, 12(1):

- Abd El-Baset, Walid S., Rania IM Almoselhy, and Susan MM Abd-Elmageed. (2024). Physicochemical characteristics and nutritional value of safflower oil: A potential sustainable crop for Egypt. *North African Journal of Food and Nutrition Research*, 8(18) 140-153.