Contemporary analysis of the chemical composition and microbial contamination of RYO/MYO tobaccos from the Bulgarian market

Nikolay Nikolov[1], *Violeta* Nikolova[1], *Venelina* Popova[2*], *Todorka* Petrova[1], *Yovko* Dyulgerski[3], and *Radka* Bozhinova[3]

[1]Institute of Food Preservation and Quality, Agricultural Academy, 4003 Plovdiv, Bulgaria
[2]Department of Tobacco, Sugar, Vegetable and Essential Oils, University of Food Technologies, 4002 Plovdiv, Bulgaria
[3]Tobacco and Tobacco Products Institute, Agricultural Academy, 4108 Markovo, Bulgaria

Abstract. Fine-cut tobacco blends for handmade cigarettes (RYO/MYO) are gaining popularity not only because of their price advantage, but also because of the perceived possibility of "more controlled" use, which positions them as a preferred choice among certain consumer groups. In response to this demand, the modern production of RYO/MYO tobaccos relies on blends with controlled physical, chemical and microbiological characteristics, ensuring stable product quality. A study of seven commercial brands of RYO/MYO tobaccos distributed on the Bulgarian market was conducted. High content of nicotine (2.03-3.33%), reducing sugars (11.50-15.72%) and nitrogen (2.16-2.82%) was found, as well as the presence of glycerol and heavy metals, within the quantitative limits typical for the regarded tobacco product type. Despite the increased moisture content (14.16-21.60%), the low water activity gives the products relative microbiological stability. The analyses show low microbial contamination and a statistically significant negative correlation between microorganism abundance and water activity. The results obtained highlight the need for continued research on RYO/MYO tobaccos in view of their complex quality, safety and perceived lower risk compared to factory cigarettes. Such scientific evaluation is essential to better understand the actual characteristics and potential impact of these products on consumers, especially given their increasing market presence.

1 Introduction

The current popularity of fine-cut tobacco blends for handmade cigarettes (RYO/ MYO tobaccos) is due not only to economic factors (e.g., price advantage) but also to the public misperception that they are "more natural" and "less harmful" than manufactured cigarettes [1-3]. This creates a false sense of lower risk and promotes smoking, especially among younger people [4]. The perceived possibility of "more controlled" use is another factor, which positions them as a preferred choice among certain consumer groups. Scientific evidence shows that smokers of RYO tobacco tend to start smoking earlier, smoke more, and build higher nicotine dependence [5-6].

The chemical and technological differences between RYO/MYO tobaccos and factory-manufactured cigarettes are significant. Handmade cigarettes contain 0.48–1.15 g of tobacco [6-7] and are typically characterized by higher moisture, humectant and sugar content, which promotes the formation of a series of toxic compounds during cigarette combustion [1]. Flavoring additives also vary considerably, and some of them have well-documented adverse health effects [8]. The papers used for rand-rolled cigarettes typically have lower air permeability, leading to higher tar, nicotine and CO smoke emissions [9], and their variations can reach up to 60% depending on the materials chosen [10]. Numerous studies have shown that RYO/MYO cigarette smoke contains equal or substantially higher amounts of toxic substances compared to factory cigarettes – in some cases up to 85% more tar and nicotine [11-14].

In addition to organic toxins, tobacco contains heavy metals (As, Cd, Cr, Ni, Pb), known for their carcinogenic potential [15-16]. Their concentrations vary significantly depending on tobacco origin and the applied processing technologies [17-19].

At the same time, in recent years, the interest in the microbiome of different tobacco products has increased, as tobacco is a natural carrier of diverse microflora, including pathogenic and mycotoxin-producing species [20-22]. Metagenomic analyses have identified numerous bacterial and fungal genera in tobacco microbiota – *Bacillus, Pseudomonas, Staphylococcus, Aspergillus, Penicillium,* and *Candida* [23, 24]. Studies of illicit RYO/MYO tobaccos from the Bulgarian market have confirmed significant microbial contamination, including *Bacillus cereus* and aflatoxin-producing species of the genus *Aspergillus* [25].

All these considerations, even briefly presented, emphasize the need for in-depth studies on the chemical composition and microbiological safety of RYO/MYO tobaccos. Such studies are essential for the assessment of public health risks, as well as for the development of

* Corresponding author: vpopova2000@abv.bg

effective regulatory and educational strategies aimed at limiting their use and overcoming the misconception of their "lower harmfulness" [26, 27]. Therefore, the present study is aimed at the current physicochemical and microbiological characterization of RYO/MYO tobaccos offered on the Bulgarian market.

2 Materials and methods

2.1 Materials

The study was carried out with seven commercial brands of fine-cut tobacco blends for hand-rolled or hand-filled cigarettes (RYO/MYO tobaccos) distributed on the Bulgarian market. For the sake of the study the investigated brands were coded as samples with indices from FCT 8 to FCT 14.

2.2 Methods

The determination of the physical and chemical indices of the regarded fine-cut tobacco blends was conducted using standardized and classical methods, as follows:

2.2.1 Physical indices

- *Moisture content, %* – according to BDS 8025:1984;
- *Cut tobacco particle size distribution, %* – according to BDS 8026:1988;
- *Tobacco cut width (strand width), mm* – according to BDS 12973:1975;
- *Water activity (aw)* – according to ISO 18787:2019. The analysis was carried out in order to select an appropriate standard for determining microbiological indicators. Triplicate measurements were made using a HygroPalm apparatus (HP23-AW-A, Rotronic, Switzerland) at 20°C.

2.2.2 Chemical indices

- *Total alkaloids (as nicotine), %* – according to ISO 15152:2003;
- *Reducing sugars, %* – according to ISO 15154:2003;
- *Total nitrogen, %* – according to BDS 15836:1988;
- *Mineral matter (ash), %* – according to BDS 15836:1999;
- *Chlorine content (as chlorides), %* – according to Analytical Method No G-267-01, rev. 4; SEAL Analytical, USA;
- *Glycerol, %.* The content was determined by extracting 4 g of ground tobacco with 50 mL of extraction solution containing methanol and internal standard 1,3-butanediol, on a laboratory shaker for 60 minutes. An aliquot was subjected to analysis with a gas chromatograph Agilent 7890 A with a flame ionization detector (FID) and an Innovax column (30 m, ID 0.320 mm; 0.25 μm) under the following chromatographic conditions: *detector* – flame ionization (FID), temperature 300°C, hydrogen flow 40 mL/min, air flow 400 mL/min, make up gas 20 mL/min; *injector* – temperature 250°C, injected sample volume 1 μL, split 5:1; *oven temperature regime* – 0 min to 6 min at 160°C, 6 min to 9 min at 230°C (step of 25°C), 9 min to 11.2 min at 230°C;
- *Macro- and microminerals.* Metal concentrations were determined after dry mineralization of the samples in a muffle furnace at 500°C for 5 hours and dissolution of the ash in 3M HCl. The concentration of the elements was measured using an atomic absorption spectrometer "SpectrAA 220" (Varian, Australia) at the following working wavelengths: K (%) - 766.5 nm, Pb (mg/kg) - 217.0 nm, Cd (mg/kg) - 228.8 nm, Ni (mg/kg) - 232.0 nm, Mn (mg/kg) - 279.5 nm, Zn (mg/kg) - 213.9 nm, and Cu (mg/kg) - 324.7 nm.

2.2.3 Microbiological indices

Standard methods, established in sanitary microbiology, were applied by preparing tenfold dilutions and plating the diluted suspensions on solid nutrient media. The following indices were determined:

- *Total microbial count, CFU/g* – according to ISO 4833-1:2013/A1:2022. From each sample (1 g of tobacco), 10-fold dilutions were prepared with 9 mL of buffered peptone water (up to 10^{-7}). Inoculations were made by pouring technique with Sabouraud Dextrose Agar, a non-selective medium containing tryptone, yeast extract and glucose, used for standardized count of bacteria in food and pharmaceutical products. Incubation was carried out at 30±2°C for 72 hours, and the results were registered as the number of colony forming units per gram of substrate (CFU/g).
- *Molds and yeasts, CFU/g* – according to ISO 21527-2:2011. The analysis was carried out by surface plating 0.1 mL of the diluted samples onto the same nutrient medium (Sabouraud Dextrose Agar). The medium is selective for the isolation of yeasts and xerophilic molds from products with low water activity. Incubation was performed at 25±2°C for 72 hours, with the results calculated as total mold and yeast counts (CFU/g).

2.2.4 Statistics

All analyses were conducted in two parallel replicates.

To assess the relationships between the determined microbial counts and the water activity (a_w) of fine-cut tobacco samples a correlation analysis was performed, based on the determination of Pearson's coefficient r and its significance α.

3 Results and discussion

3.1 Visual assessment – appearance and structural elements

In order to complete a more consistent characterization of the studied RYO/MYO tobaccos distributed on the national market, they were subjected to a preliminary visual assessment in terms of appearance and structure.

All of the samples (FCT 8 - FCT 14) were packaged in sealed polyethylene bags. The required information about the harmful effects of smoking, manufacturer and tobacco weight per item was available on the consumer packaging.

Each of the fine-cut tobacco blends was composed predominantly of tobacco fibers, cut unprocessed tobacco stems and cut reconstituted tobacco sheet. Samples FCT 11 – FCT 14 contained flavored mixtures, while samples FCT 8 – FCT 10 were without a noticeable dominating aroma.

3.2 Physical indicators

The results for the moisture content (%) in the studied RYO/MYO tobaccos are presented in Fig. 1.

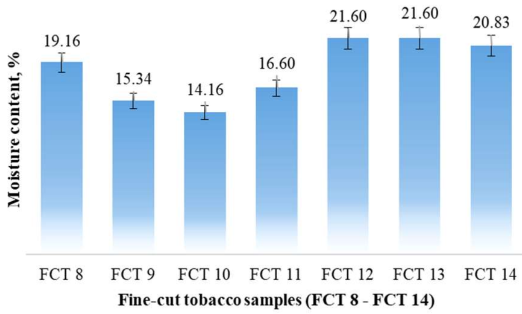


Fig. 1. Moisture content (%) in seven RYO/MYO tobaccos.

The moisture content of the analyzed RYO/MYO tobacco blends varied in a wide range, 14.16–21.60%. Significant differences were observed between two groups of samples; FCT 9 – FCT 11 (14.16–16.60%) and the remaining four samples (19.16–21.60%). The results showed that, with the exception of FCT 10, all of the RYO/MYO tobaccos studied had significantly higher moisture levels than those specified for factory cigarettes according to the standards (11.00–14.00%; BDS 866:1982). Those results were fully consistent with the observations of other authors, emphasizing that RYO/MYO tobaccos usually have a higher moisture content compared to factory products [1], as was, for instance, the case with fine-cut tobaccos from the Russian market (17.67–19.78%) [28]. Higher moisture content favors tobacco crush-resistance and the sensory perception of a “smoother” smoke, but it is also a major factor in smoke formation and composition, mainly through the impact of incomplete combustion and lower temperatures within the burning zone, which in turn modifies the pyrolytic processes during smoking. Cigarettes with higher moisture content (above 14%) have showed increased puff number values and higher levels of some smoke toxicants, such as CO, low-molecular weight carbonyls, certain phenols, higher-molecular weight semi-volatiles, etc. [29].

Fig. 2 presents the data from particle size determination (Fractions I – IV) in the studied RYO/MYO tobaccos.

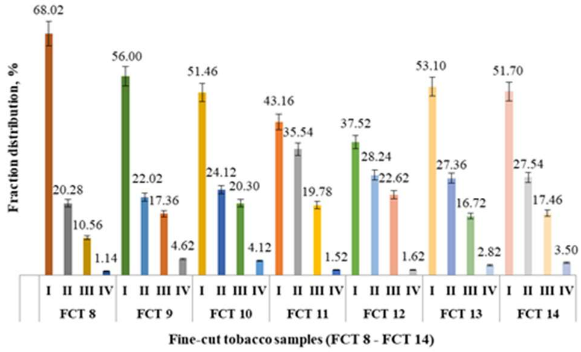


Fig. 2. Particle size distribution (Fractions I-IV, % of sample weight) in seven RYO/MYO tobaccos.

Fraction I – long fibers, above a 2 × 2 mm sieve; Fraction II – middle-sized fibers, above a 1 × 1 mm sieve; Fraction III – short fibers, above a 0.4 × 0.4 mm sieve; Fraction IV– dust/fines, below a 0.4 × 0.4 mm sieve.

The results of the sieve analysis of particle size distribution showed that Fraction I (long fibers, above a 2 × 2 mm sieve) prevailed in all of the studied RYO/MYO tobaccos, with a share varying from 37.52% (FCT 12) to 68.02% (FCT 8). The middle-sized fraction (above a 1 × 1 mm sieve) also constituted a significant part, 20.28–35.54%, and short fibers (above 0.4 × 0.4 mm) – between 10.56% and 22.62% of tobacco weight. The share of the dust/fines fraction was low – from 1.14% (FCT 8) to 4.62% (FCT 9), which indicated good technological control during tobacco cutting.

The measured width of the tobacco fibres (Fig. 3) confirmed that all samples were characterized by a very fine cut, varying between 0.28 mm (FCT 14) and 0.38 mm (FCT 8 and FCT 13). No significant differences were found either between the individual samples or compared to published data for other fine-cut tobacco brands on the Bulgarian market.

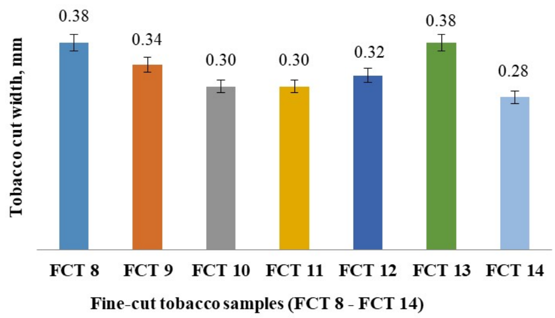
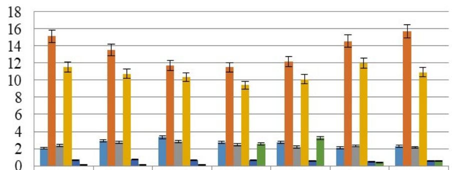


Fig. 3. Average tobacco cut width (mm) in seven RYO/MYO tobaccos.

3.3 *Chemical indicators*

The results for the determined indicators of the chemical composition of the studied RYO/MYO tobaccos are presented in Fig. 4.



	FCT 8	FCT 9	FCT 10	FCT 11	FCT 12	FCT 13	FCT 14
Nicotine, %	2.03	2.93	3.33	2.69	2.72	2.11	2.30
Reducing sugars, %	15.12	13.52	11.70	11.50	12.14	14.53	15.72
Total nitrogen, %	2.36	2.74	2.82	2.48	2.18	2.32	2.16
Ash, %	11.52	10.75	10.33	9.42	10.12	12.01	10.94
Chlorine, %	0.70	0.75	0.63	0.67	0.54	0.52	0.58
Glycerol, %	0.16	0.17	0.18	2.58	3.24	0.43	0.54

Fig. 4. Chemical characteristics of the studied RYO/MYO tobaccos.

The seven RYO/MYO tobacco brands were characterized by high nicotine content, ranging from 2.03% (FCT 8) to 3.33% (FCT 10). In three other samples the nicotine content also was sufficiently close to the upper limit of the range – FCT 9 (2.93%), FCT 12 (2.72%) and FCT 11 (2.69%). The content of reducing sugars in the fine-cut tobacco blends was the highest in FCT 14 (15.72%) and the lowest in FCT 11 (11.50%) and FCT 10 (11.70%), thus confirming previous observations that RYO/MYO tobaccos generally contain more reducing sugars than factory-made cigarettes [1]. Sugars (naturally occurring and added) affect the physicochemical (e.g., pH, water retention capacity) and chemosensory (e.g., smoke smoothness and flavor) properties of cigarettes. They undergo multidimensional combustion, pyrolysis and pyrosynthetic processes in the burning cigarette, which produce a number of known smoke toxicants and carcinogens, such as aldehydes and ketones (formaldehyde, acrolein), furan derivatives, organic acids, volatile organic compounds, polycyclic aromatic hydrocarbons, etc. [30-32]. Total nitrogen varied within a narrow range (2.16–2.82%), with individual values being typical for cigarette tobacco blends. The total mineral matter (ash) content was low, corresponding to that found in quality bright tobaccos, varying between 9.42% (FCT 11) and 12.01% (FCT 13).

Chlorine has a negative impact on tobacco combustibility and smoking properties (aroma and taste). The highest chlorine content was recorded in sample FCT 9 (0.75%), and the lowest in FCT 13 and FCT 12 (0.52–0.54%). The data obtained corresponded to the recommended chlorine content threshold for high-quality tobaccos (below 1.50%) [33].

The content of glycerol, used as a humectant and a flavoring aid in tobacco processing (typically at levels of 1-5%), showed significant variation in the seven RYO/MYO tobaccos studied – from 0.16% (FCT 8) to 3.24% (FCT 12). That was most likely related to the need for better humidification of RYO/MYO tobaccos, whose packages are often not completely sealed after opening [34].

The obtained data on the chemical characteristics of the studied RYO/MYO tobaccos were comparable with previous studies of brands offered on the Bulgarian market, providing additional confirmation for the high variability of those indicators depending on tobacco blend and technological additives specifics.

The results for the macro- and microminerals' concentration in the analyzed RYO/MYO tobacco blends are presented in Table 1.

Table 1. Macro- and microminerals in seven RYO/MYO tobaccos.

Sample	K, %	Pb, mg/kg	Cd, mg/kg	Ni, mg/kg	Mn, mg/kg	Zn, mg/kg	Cu, mg/kg
FCT 8	2.87	1.00	0.70	2.80	169.80	30.30	13.00
FCT 9	2.70	1.00	0.10	3.70	145.70	25.90	12.10
FCT 10	2.79	nd	0.40	3.00	199.30	27.40	12.30
FCT 11	2.48	3.00	0.60	2.50	143.20	23.80	9.80
FCT 12	2.88	2.00	0.80	1.20	192.10	24.20	11.90
FCT 13	3.03	1.00	0.90	0.50	208.90	28.30	12.50
FCT 14	3.08	4.00	1.00	2.10	184.00	24.60	12.80
Average	*2.83*	*1.71*	*0.64*	*2.26*	*177.57*	*26.36*	*12.06*

The analysis of macro- and microelements in the studied RYO/MYO tobaccos showed single variations between the samples. Potassium (K) content was between 2.48% (FCT 11) and 3.08% (FCT 14), with sample FCT 13 having an identical value in the higher range (3.03%). Lead (Pb) was not detected in FCT 10, and in the remaining samples its concentration varied from 1.00 to 4.00 mg/kg, with an average content of 1.71 mg/kg. Those data were higher than some results previously reported – 0.27 µg/g [15] and 0.54 µg/g [18], but fully consistent with other data – 2.30 µg/g [16]. Cadmium (Cd; mean 0.64 mg/kg) was in the range of 0.60-1.00 mg/kg in most of the samples, while two of them showed distinctively lower levels, 0.10 mg/kg (FCT 9) and 0.40 mg/kg (FCT 10). The values obtained were below Cd levels reported for cigarette tobaccos from other countries [16-18] and comparable to data for cigarettes from Brazil [15]. Nickel (Ni) content ranged from 0.50 to 3.70 mg/kg, with an average of 2.26 mg/kg, which was compliant with that reported for tobaccos from the USA (2.21 µg/g). Manganese (Mn) also showed a wider range of variation, 143.20 – 208.90 mg/kg (average 177.57 mg/kg), thus exceeding data for tobaccos from Saudi Arabia [19] but staying lower than those reported for RYO and water pipe tobacco in Bulgaria. Zinc (Zn) ranged from 23.80 to 30.30 mg/kg (average 26.36 mg/kg), and copper (Cu) – between 9.80 and 13.00 mg/kg (average 12.06 mg/kg), which was fully consistent with previous results for RYO tobaccos in Bulgaria, but higher than other data [19].

In summary, the average concentrations of microelements in the analyzed tobaccos followed a descending order: Mn > Zn > Cu > Ni > Pb > Cd. The obtained values confirmed the presence of interregional differences related to the origin of the tobacco and the technological processes applied.

3.4 *Microbiological indicators*

3.4.1 Water activity (a_w)

The measured water activity (a_w) values for the analyzed RYO/MYO tobaccos (Fig. 5) showed that all samples had an a_w below 0.60, ranging from 0.494 (FCT 9) to 0.580 (FCT 13). Microorganisms are known to grow in the range of 0.62–0.99 a_w [35], and depending on the moisture content, three main groups are distinguished:

- xerophilic (a_w=0.62-0.75); fungi of the genus *Aspergillus*, osmotolerant yeasts, halotolerant bacteria;
- mesophilic (a_w=0.75-0.85); most of the fungi and bacteria;
- hydrophilic (a_w=0.85-0.99); mainly bacteria.

The results obtained indicated that the media provided by the studied tobaccos favored the development of mainly xerophilic microorganisms, while an activity of meso- and hydrophilic pathogens was unlikely. As stated above, all samples had an a_w below 0.60, which defines them as potentially microbiologically stable and safe with respect to the development of pathogenic microflora.

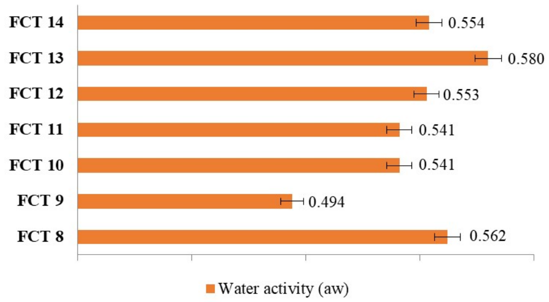


Fig. 5. Water activity (a_w) of the studied RYO/MYO tobaccos.

3.4.2 Tobacco microbiome

The determined values for the total microbial count (CFU/g) in the analyzed RYO/MYO tobaccos varied in a wide range – from 8.6 × 10^4 CFU/g in sample FCT 11 to 5.6 × 10^6 CFU/g in FCT 9 (Fig. 6). The highest microbial contamination was found in three of the samples (FCT 9, followed by FCT 10 and FCT 8), in which the number of microorganisms reached values of the order of millions. In the remaining four samples, the total microbial count was significantly lower – of the order of hundreds of thousands of CFU/g, which indicates substantial variability in the degree of microbial contamination between individual tobacco blends.

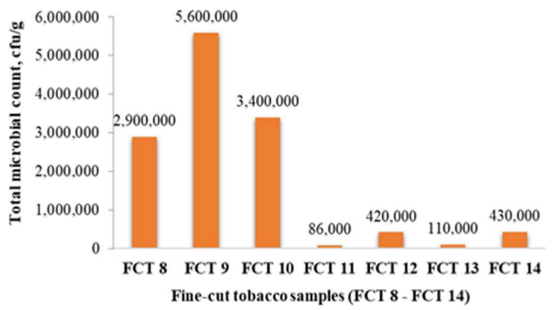


Fig. 6. Total microbial count (CFU/g) in the studied RYO/MYO tobaccos.

The total microbial count is a universal hygienic indicator reflecting the total amount of heterotrophic mesophilic bacteria with a similar type of metabolism. The results obtained were consistent with previous studies on unregulated tobacco for hand-rolled cigarettes, in which the count of bacteria in conditionally clean (uncontaminated) samples varied within the range of 1.36-2.34.10^2 CFU/g [25].

Fig. 7 presents the total amount of molds and yeasts (CFU/g) in the analyzed RYO/MYO tobaccos. Similarly to the results for the total microbial count, the highest values were found in samples FCT 9 and FCT 10 (3.6-2.5 × 10^6 CFU/g, respectively), while in the remaining samples the number of isolated micromycetes and yeasts was significantly lower – in the order of tens of thousands of CFU/g. The only exception from that unidirectional relationship between the total microbial and the mold and yeast count was sample FCT 8, which fell into the group with lower index values (4.4 × 10^4 CFU/g).

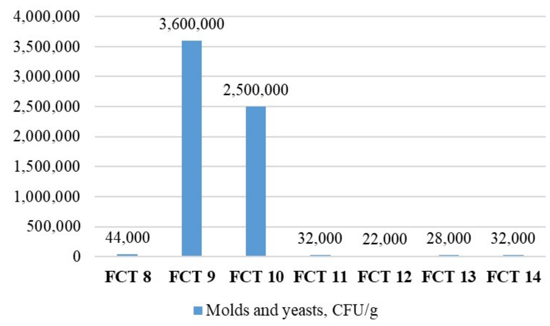


Fig. 7. Molds and yeasts count (CFU/g) in the studied RYO/MYO tobaccos.

The correlation analysis performed showed negative, medium-strength relationships between the amounts of microorganisms and the water activity of the products. The relationships were statistically reliable.

For the total microbial count, the relationship was proven at a significance level of 0.1 (r = -0.692 ≥ r_{crit} = 0.669, for α = 0.1 at f =5). For the molds and yeasts count, the relationship was proven at a significance level of 0.05 (r = -0.780 ≥ r_{crit} = 0.754, for α = 0.05 at f =5).

4 Conclusions

The RYO/MYO tobaccos from the Bulgarian market included in the study were characterized by physical parameters typical of fine-cut tobacco – a predominant share of long fibres and a cut width below 0.40 mm, without significant variation between the samples. The moisture content (14.16–21.60%) was higher than the regulatory limits for factory-produced cigarettes, suggesting potential differences in the combustion and pyrolysis processes during smoke formation, and in the composition and impact of RYO/MYO tobacco smoke, respectively.

In terms of chemical composition, the tobaccos studied were characterized by relatively high nicotine (2.03–3.33%), reducing sugars (11.50–15.72%) and total nitrogen (2.16–2.82%) contents, combined with lower levels of ash (9.42–12.01%) and chlorine (0.52–0.75%). Glycerol was detected in all samples analyzed, within the quantitative limits typical for cigarette tobacco.

New data on the content of macro- and microminerals in RYO/MYO tobaccos available on the national market have been obtained. The average K content was 2.83%, while the average concentrations of microelements followed a descending order of Mn > Zn > Cu > Ni > Pb > Cd, with the corresponding values being: Mn – 177.57 mg/kg; Zn – 26.36 mg/kg; Cu – 12.06 mg/kg; Ni – 2.26 mg/kg; Pb – 1.71 mg/kg; and Cd – 0.64 mg/kg.

The measured water activity values ($a_w < 0.60$) and the results of the microbiological analysis indicated a limited degree of microbial contamination and a low risk of pathogenic microflora development, which defines the tobacco blends as microbiologically stable and relatively safe. The observed negative, statistically significant correlations between the amount of microorganisms and water activity confirm the importance of moisture content for the microbiological status of the product.

The results obtained expand the knowledge about the characteristics of RYO/MYO tobaccos (physical, chemical, microbiological), in line with the need for ongoing research aimed at a more complete assessment of this product category, often perceived by consumers as a “less harmful” alternative to factory-produced cigarettes.

References

1. A. Haynes, W. Winnall, E. Brennan, K. Dunstone, N. Benowitz, D. Ashley, J. Samet, D. Hatsukami, M. Wakefield, Tobacco constituents, flavorants, and paper permeability of factory-made and roll-your-own cigarettes on the Australian market. Nic. Tob. Res. **26**(3), 289-297 (2024). https://doi.org/10.1093/ntr/ntad122
2. R. Edwards, Roll your own cigarettes are less natural and at least as harmful as factory rolled tobacco. BMJ **348**, f7616 (2014). https://doi.org/10.1136/bmj.f7616
3. E. Codinach-Danés, N. Obradors-Rial, J. Mendioroz-Peña, J. Villalbí, M. Bosque-Prous, A. Espelt, Future intentions and beliefs about roll-your-own cigarettes in adolescents. Atencion Primaria **53**(6), 102043 (2021). https://doi.org/10.1016/j.aprim.2021.102043
4. J. Martín-Álvarez, A. Almeida, A. Golpe, E. Asensio, Electronic device or regulated tobacco product? Learning from the diffusion of heated tobacco products in Spain. Pub. Health **219**, 61-66 (2023). https://doi.org/10.1016/j.puhe.2023.03.017
5. Y. Kochan, S. Rosenzweig, Unintended consequences of antismoking pricing policies: insights from smokers’ household expenditure on smoking behavior and public health. Sustainability **16**(1), 178 (2023). https://doi.org/10.3390/su16010178
6. S. Joseph, N. Krebs, J. Zhu, Y. Wert, R. Goel, S. Reilly, D. Sun, J. Richie, I. Nikiforov, P. Cheriyath, J. Muscat, Differences in nicotine dependence, smoke exposure and consumer characteristics between smokers of machine-injected roll-your-own cigarettes and factory-made cigarettes. Drug Alcohol Depend. **187**, 109–115 (2018). https://doi.org/10.1016/j.drugalcdep.2018.01.039
7. S. Gallus, A. Lugo, S. Ghislandi, C. La Vecchia, A. Gilmore, Roll-your-own cigarettes in Europe: use, weight and implications for fiscal policies. Eur. J. Cancer Prev. **23**(3), 186-192 (2014). https://doi.org/10.1097/cej.0000000000000010
8. L. van Nierop, J. Pennings, E. Schenk, A. Kienhuis, R. Talhout, Analysis of manufacturer's information on tobacco product additive use. Tob. Reg. Sci. **5**(2), 182-205 (2019). https://doi.org/10.18001/TRS.5.2.9
9. J. De Granda-Orive, C. Jimenez-Ruiz, Some thoughts on hand-rolled cigarette. Arch. Bronconeumol. **47**(9), 425-426 (2011). https://www.archbronconeumol.org/es-some-thoughts-on-hand-rolled-cigarette-articulo-S1579212911000759
10. T. Calduch, C. Jiménez, T. Segundo, M. Valle, A. Carlos-Roca, Tabaco de liar: una prioridad de salud pública y consumo. Gac. Sanit. **26**(3), 267-269 (2012). https://doi.org/10.1016/j.gaceta.2011.09.010
11. M. Kaiserman, W. Rickert, Handmade cigarettes: It's the tube that counts. Amer. J. Public Health **82**(1), 107-109 (1992). https://doi.org/10.2105/ajph.82.1.107
12. M. Laugesen, M. Epton, C. Frampton, M. Glover, R. Lea, Hand-rolled cigarette smoking patterns compared with factory-made cigarette smoking in New Zealand men. BMC Public Health **9**, 194-199 (2009). https://doi.org/10.1186/1471-2458-9-194
13. A. Marcilla, M. Beltran, A. Gomez-Siurana, D. Berenguer, I. Martinez-Castellanos, Comparison between the mainstream smoke of eleven RYO tobacco brands and the reference tobacco 3R4F.

Toxicol. Rep. **1**, 122-136 (2014). https://doi.org/10.1016/j.toxrep.2014.05.004
14. A. Marcilla, M. Beltrán, A. Gómez-Siurana, I. Martínez-Castellanos, D. Berenguer, Comparative study of the composition of the mainstream smoke of five brands factory-made-cigarettes and their counterpart roll-your-own tobaccos. Univ. J. Manag. **6**(1), 12-23 (2018). https://doi.org/10.13189/ujm.2018.060102
15. G. Viana, K. Garcia, J. Menezes-Filho, Assessment of carcinogenic heavy metal levels in Brazilian cigarettes. Environ. Monit. Assess. **181**, 255-265 (2011). https://doi.org/10.1007/s10661-010-1827-3
16. Q. Dinh, R. Novirsa, H. Jeong, W. Nugraha, S. Addai-Arhin, P. Viet, N. Tominaga, Y. Ishibashi, K. Arizono, Mercury, cadmium, and lead in cigarettes from international markets: concentrations, distributions and absorption ability of filters. J. Toxicol. Sci. **46**(9), 401-411 (2021). https://doi.org/10.2131/jts.46.401
17. R. Caruso, R. O'Connor, W. Stephens, K. Cummings, G. Fong, Toxic metal concentrations in cigarettes obtained from U. S. smokers in 2009: Results from the International tobacco control (ITC) United States survey cohort. Int. J. Environ. Res. Public Health **11**(1), 202-217, 2014. https://doi.org/10.3390/ijerph110100202
18. E. Pinto, M. Cruz, P. Ramos, A. Santos, A. Almeida, Metals transfer from tobacco to cigarette smoke: evidences in smokers' lung tissue. J. Hazard. Mater. **325**, 31-35 (2017). https://doi.org/10.1016/j.jhazmat.2016.11.069
19. S. Dahlawi, A. Mulla, K. Salama, O. Labib, M. Aljassim, A. Akhtar, W. Asghar, T. Faraj, N. Khalid, Assessment of different heavy metals in cigarette filler and ash from multiple brands retailed in Saudi Arabia. J. King Saud Univ. Sci. **33**(6), 101521 (2021). https://doi.org/10.1016/j.jksus.2021.101521
20. L. Larsson, B. Szponar, B. Ridha, C. Pehrson, J. Dutkiewicz, E. Krysińska-Traczyk, J. Sitkowska, Identification of bacterial and fungal components in tobacco and tobacco smoke. Tob. Induced Dis. **4**(4), 1-8 (2008). https://doi.org/10.1186/1617-9625-4-4
21. A. Sapkota, S. Berger, T. Vogel, Human pathogens abundant in the bacterial metagenome of cigarettes. EHP **118**(3), 351-356 (2010). https://doi.org/10.1289/ehp.0901201
22. J. Pauly, G. Paszkiewicz, Cigarette smoke, bacteria, mold, microbial toxins, and chronic lung inflammation. J. Oncol. **2011**(1), 819129 (2011). https://doi.org/10.1155/2011/819129
23. S. Chattopadhyay, L. Malayil, E. Mongodin, A. Sapkota, A roadmap from unknowns to knowns: Advancing our understanding of the microbiomes of commercially available tobacco products. Appl. Microbiol. Biotechnol. **105**, 2633-2645 (2021). https://doi.org/10.1007/s00253-021-11183-4
24. J. Chopyk, S. Chattopadhyay, P. Kulkarni, E. Claye, K. Babik, M. Reid, E. Smyth, L. Hittle, J. Paulson, R. Cruz-Cano, M. Pop, S. Buehler, P. Clark, A. Sapkota, E. Mongodin, Mentholation affects the cigarette microbiota by selecting for bacteria resistant to harsh environmental conditions and selecting against potential bacterial pathogens. Microbiome **5**, 22, 1-13 (2017). https://doi.org/10.1186/s40168-017-0235-0
25. D. Young, H. Yong, R. Borland, L. Shahab, D. Hammond, M. Cummings, N. Wilson, Trends in roll-your-own smoking: Findings from the ITC four-country survey (2002-2008). J. Environ. Public Health **2012**(1), 406283 (2012). https://doi.org/10.1155/2012/406283
26. A. Rivera, R. Tyx, Microbiology of the American smokeless tobacco. Appl. Microbiol. Biotechnol. **105**(12), 4843-4853 (2021). https://doi.org/10.1007/s00253-021-11382-z
27. E. Smirnova, E. Gnuchikh, E. Bubnov, A. Slavyansky, Studying factors affecting yields of toxic compounds in smoke of products made of smoking fine cut tobacco. Proceedings of the Voronezh State University of Engineering Technologies **86**(1), 196 (2024). https://doi.org/10.20914/2310-1202-2024-1-196-200
28. O. Zha, S.C. Moldoveanu, The influence of cigarette moisture to the chemistry of particulate phase smoke of a common commercial cigarette. Beitr. Tabakforsch. Intern. **21**(3), 184-191 (2004). https://doi.org/10.2478/cttr-2013-0779
29. N. Nikolov, V. Nikolova, S. Peeva, V. Popova, Tobacco derivatives and smoking products illicitly traded in Bulgaria - technological assessment and analysis. AIP Conf. Proc. **2889**, 080006 (2023). https://doi.org/10.1063/5.0172807
30. I. Stepanov, M. Berman, M.C. Brinkman, A. Carll, V. Exil, E.G. Hansen, A. El Hellani, S.V. Jabba, N.O.F. Kassem, M. Rezk-Hanna, R. Talhout, A.M. Stroup, Sugars in tobacco products: toxicity research and implications for tobacco product regulation. Chem. Res. Toxicol. **38**(5), 747-758 (2025). https://doi.org/10.1021/acs.chemrestox.4c00550
31. J.L.A. Pennings, J.W.J.M. Cremers, M.J.A. Becker, W.N.M. Klerx, R. Talhout, Aldehyde and volatile organic compound yields in commercial cigarette mainstream smoke are mutually related and depend on the sugar and humectant content in tobacco, Nic. Tob. Res. **22**(10), 1748-1756 (2020). https://doi.org/10.1093/ntr/ntz203
32. M. Banožić, S. Jokić, Đ. Ačkar, M. Blažić, D. Šubarić, Carbohydrates - key players in tobacco aroma formation and quality determination. Molecules **25**(7), 1734 (2020). https://doi.org/10.3390/molecules25071734
33. S. Reddy, S. Krishna, D. Reddy, C. Rao, K. Rao, Productivity, leaf quality and nutrient-use efficiency of FCV tobacco (*Nicotiana tabacum*) genotypes to levels of N and K application under

irrigated Alfisols of Andhra Pradesh. Indian J. Agron. **62**(4), 510-518 (2017). https://doi.org/10.59797/ija.v62i4.5560

34. J. Zhang, Y. Wang, Y. Zhu, M. Su, Y. Xie, Y. Zheng, H. Zhu, M. Liang, R. Li, X. Zheng, Pyrolysis characteristics of different types of tobacco and its correlation with the released aroma components under heating condition. J. Anal. Appl. Pyrolysis **181**, 106646 (2024). https://doi.org/10.1016/j.jaap.2024.106646
35. P. Kanekar, S. Kanekar, Xerophilic and Xerotolerant microorganisms. In: Diversity and biotechnology of extremophilic microorganisms from India. Microorganisms for Sustainability. Springer, Singapore, 281-288 (2022). https://doi.org/10.1007/978-981-19-1573-4_10