

Application of Scanning Electron Microscopy and X-Ray Evaluation of the Main Digestion Methods for Determination of Macroelements in Plant Tissue

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Abstract—Three commonly used digestion methods (dry ashing, acid digestion, and microwave digestion) in different variants were compared for digestion of tobacco leaves. Three main macroelements (K, Ca and Mg) were analysed using AAS Spectrometer Spectra AA 220, Varian, Australia. The accuracy and precision of the measurements were evaluated by using Polish reference material CTR-VTL-2 (Virginia tobacco leaves). To elucidate the problems with elemental recovery X-Ray and SEM-EDS analysis of all residues after digestion were performed. The X-ray investigation showed a formation of KClO_4 when HClO_4 was used as a part of the acids mixture. The use of HF at Ca and Mg determination led to the formation of CaF_2 and MgF_2 . The results were confirmed by energy dispersive X-ray microanalysis. SPSS program for Windows was used for statistical data processing.

Keywords—Digestion methods, determination of macroelements, plant tissue.

I. INTRODUCTION

SODIUM, calcium and magnesium are among the most important macronutrients defining the normal functioning of plants. Potassium is an essential nutrient and is very important for plant growth and yield. Although not included in the composition of organic compounds, it functions as an activator of many enzymes and plays a dominant role in the balance between cations and anions contained in the plant. Calcium plays an important role in maintaining the integrity of the cells and the permeability of the membranes, and magnesium participates in the construction of the chlorophyll molecule and is a cofactor, adapting a number of enzymes in an active state and supporting protein synthesis [1].

A lot of publications are dedicated to the evaluation of various mineralisation methods and measurement techniques for element analysis of different materials. Three decomposition methods have been compared by Curdova et al. [2] (dry ashing, microwave digestion and dry ashing in a mixture of oxidising gasses). Three measurement techniques (FAAS, ETAAS and ICP - MS) and six plant materials are used for the determination of Pb, Cd, Zn and Cu. No significant differences between the analytical methods have been found. It is concluded that the results obtained are affected to a greater extent by the decomposition procedure

than by measurement techniques. Analysing the results of the laboratories participating in Food Analysis Performance Assessment Scheme (FAPAS) in the period 1991-2000, Rose et al. [3] reported that the percentage of satisfactory results is in the range of 34 to 100 depending on the material, sample, and detection techniques. They compared five different methods for sample digestion and indicated that the pressure vessel can be associated with a good analytical performance. Poykio et al. [4] reach the same conclusion comparing digestion methods for multi-element analysis of several reference plant materials. According to these authors, microwave digestion, combined with ICP-AES or ICP-MS determination, is a very rapid and accurate method for analysing botanical materials.

It can be summarised that sample preparation is the crucial stage for obtaining correct results for heavy metals and nutrition elements content. This is especially important for the determination of plant materials because, as a rule, the plant material is not homogeneous and usually contains variable matrices. The current study is a part of a systematic research on the methods for plant sample preparation for analysis of the most important heavy metals and the basic nutrition elements. The purpose is to estimate the most widely spread standardised and non-standardized methods for sample preparation and to determine their limits of the application when solving the most significant ecological and agricultural problems.

II. EXPERIMENTS

A. Materials and Methods

The validity of the procedures was evaluated by analysing the certified reference material of Virginia tobacco leaves (a Polish reference material CTA-VTL-2) which contains $1.03 \pm 0.04\%$ K, $3.60 \pm 0.15\%$ Ca and $0.510 \pm 0.023\%$ Mg. Spectra AA-220 (Varian, Australia) atomic absorption spectrometer in flame mode was used for the determination of the elements investigated.

B. Reagents and Standards

The acids, including nitric acid, hydrochloric acid, perchloric acid, sulfuric and hydrofluoric acid, as well as H_2O_2 were obtained from E. Merck Company (Germany). Commercial individual standard solutions with concentration 1000 mg/l were used as a stock solution. The calibration was performed by using 5 aqueous standard solutions in 2% v/v HNO_3 .

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C. Digestion Procedures

The most common methods for sample preparation were used as follows:

1. *Dry ashing* [5]: Procedure for dry ashing at 550, 500, 450 and 400°C in a muffle furnace, following Bulgarian Standard 11708-93, was used. 0.5 g of samples was weighed in 50 ml glass beakers, charged up to 350 °C for 4 hours on a hot plate with stepwise increasing temperature and finally ashed in a muffle furnace at 400, 450, 500 and 550 °C for 1 hour. After cooling, ashes were dissolved in 20 ml of 1.5% HNO₃.
2. *Wet mineralisation*: Several acid combinations for wet mineralisation were used as follows:
 - a) *HNO₃ + H₂O₂ digestion* [6]: One gram of oven-dried sample was transferred to a Teflon beaker to which 10 ml of concentrated nitric acid was added. The sample was then warmed on a hot plate to about 85-95 °C until the initial reaction had subsided. After cooling, 0.5 ml of 30% hydrogen peroxide was added dropwise, and the sample was then reheated. Stepwise additions of peroxide were repeated until the sample solution had clarified and no fats were visible. The sample was then diluted to 50 ml.
 - b) *Procedure for acid digestion* [7]: One gram of oven-dried sample was weighed into a Kjeldahl flask and 5 ml of 65% HNO₃ was added. The flask was placed on a preheated hot plate and heated until its content was evaporated to dryness. 14 ml of 72% HClO₄ was added to the sample and it was heated for 60 min. The digest was cooled, filtrated and diluted to 100 ml in a calibrated flask.
 - c) *ISO standard 5515-1979 (E)* [8]: A tobacco sample of 2.0 g, dried to constant weight, was put in a Kjeldahl flask of 100 ml and 30 cm³ HNO₃ and 4.0 cm³ H₂SO₄, as well as a few glass pearls, were added. The mixture was left to settle for 12 hours and after that, the flask was carefully heated in order to avoid the formation of foam. The heating of the liquid continued until the boiling point was reached and the process of boiling continued until the liquid acquired brown colour. After that, portions of 2.0 cm³ HNO₃ were added until the release of nitric oxides stopped and the release of white fumes began. The solution was cooled down, 15 cm³ nitric acid were added and the solution was heated up again until boiling with the release of white fumes began. The cooled solution was transferred into a measurement flask of 50 or 100 cm³ and was filled up to the marking.

The same standard was applied in the digestion procedure, including the use of HClO₄. 8.0 cm³ HNO₃ was added to the sample, it was left to settle for 12 hours and was brought to boiling without being evaporated to dryness. After cooling, an additional portion of 8.0 cm³ HNO₃ was added and the liquid was brought to boiling as the procedure was repeated once or twice more. After cooling, 8.0 cm³ HNO₃ and 4.0 cm³ H₂SO₄ were added. The decomposition began vigorously without heating. After the vigorous reaction stopped, the sample was cooled and 6.0 cm³ HNO₃ and 5.0 cm³ HClO₄ were added. The boiling continued until the solution became colourless.

Then it was cooled and brought to the volume of 50 or 100 cm³.

- d) *Procedure for acid digestion*: according to Bulgarian standard 17365-94 [9] for determination of heavy metals in tobacco and tobacco products: A tobacco sample of 2.0 g dried to constant weight was put into a Kjeldahl flask of 100 ml. 20 cm³ HNO₃ and 5.0 cm³ H₂SO₄ were added and the mixture was left to settle for 4 hours. After that, the flask was heated in a sand bath for 40 minutes at 80-90 °C. At the appearance of a dark yellow or brown colouring of the solution, the flask was cooled and 10 cm³ HNO₃ and 2 cm³ HClO₄ were added. The heating continued until the elimination of most of the acids. 10 cm³ of distilled water was added twice and the solution was heated until most of it evaporated. After cooling, the residue was treated twice with 4 cm³ 1 M HCl, and after that, it is filled up to 10 cm³ with 1 M HCl.
3. *Microwave digestion*: Procedure for microwave digestion with different acid mixtures, following EPA METHOD 3051 [10], suitable for determination of heavy metals by flame AAS, was used. A microwave digestion system (Milestone 1200 MEGA, Italy) with 10 MRD 300 rotor with 10 positions, max. pressure of 30 bars and max. power of 1000 W was used. 0.5 g homogenised dry sample was weighed into a Teflon vessel and 10 ml of concentrated nitric acid was added. Several additional alternative acid combinations including H₂O₂ and HF were used in accordance with EPA METHOD 3052 [11].

Nine blank samples (one for dry ashing and eight for wet and microwave mineralisation) were analysed in the same way as the main samples to be tested for contamination during the procedure.

D. Statistical Analysis

For evaluation of correctness of the results from the determination of K, Ca and Mg three generally accepted criteria were used:

1. $D = X - X_{CRM}$, where X_{CRM} is the certified value and X is the measured value. When D is in the frame of the borders of $\pm 2\sigma$, where σ is the standard deviation of the certified value, the result is considered to be good; when it is in the frame $-3\sigma \leq D \leq 3\sigma$ - satisfactory, and beyond these limits the result is unsatisfactory.
2. $D\% = D/X_{CRM} \cdot 100$ - percentage difference. When the rates of $D\%$ are in the limits $200\sigma / X_{CRM}$ the result is considered to be good, when the value is $-200\sigma / X_{CRM} < D < 300\sigma / X_{CRM}$ - satisfactory, and when it is $\pm 300\sigma / X_{CRM}$ the result is unsatisfactory.
3. $Z = (X - X_{CRM}) / \sigma$. When $Z \leq 2$ the result is considered as good when $2 \leq Z \leq 3$ - satisfactory when $Z > 3$ - unsatisfactory.

For evaluation of the effectiveness of the methods for sample preparation, we have used R criterion showing the extent of the extraction of elements in percent from the certified value. When the measured value X is within the borders of $X_{CRM} \pm U_{CRM}$ where U_{CRM} is the indefiniteness of the certified value, we accept a degree of extraction to be

100%. In all the remaining cases the percent of extraction is equal to $X / X_{CRM} \cdot 100$.

E. Samples Characterization

1. X-ray Powder Diffraction (XRD)

The XRD patterns were recorded on a Philips PW 1050 diffractometer, equipped with Cu K α tube and a scintillation detector. Data for cell refinements were collected in $\theta - 2\theta$, a step-scan mode in the angle interval from 10 to 90° (2 θ) at counting time of 3 s/step and steps of 0.03° (2 θ).

2. Scanning Electron Microscopy (SEM)

SEM images were recorded in a JSM 6390 electron microscope (Japan) in conjunction with energy dispersive X-ray spectroscopy (EDS, Oxford INCA Energy 350) equipped with ultrahigh resolution scanning system (ASID-3D) in a regime of secondary electron image (SEI) and backscattered electrons (BEC). The samples were coated with gold before imaging.

III. RESULTS

The most important elements for tobacco growth, yield and quality - K, Ca and Mg, were the subject of our investigation. At least duplicate analysis (decomposition and measurement) were accomplished for each sample. The results for potassium content are presented in Table I.

TABLE I
THE EFFICIENCY OF THE METHODS OF MINERALISATION IN THE DETERMINATION OF K IN SHEETS OF VIRGINIA (CTA-VTL-2), $X_{CRM} = 1.03 \pm 0.04\%$

Method	X, ppm	D	Z	R
Dry, 550°C	1.00±0.03	-0.03	-0.75**	97.1
Dry, 500°C	0.99±0.04	-0.04	1.00**	96.1
Dry, 450°C	1.07±0.04	0.04	1.00**	103.9
Dry, 400°C	0.95±0.03	-0.08	-2.00**	92.4
HNO ₃ + H ₂ O ₂	0.99±0.03	-0.04	-1.00**	96.1
HNO ₃ +HClO ₄	0.91±0.03	-0.12	-3.00*	88.3
HNO ₃ +H ₂ SO ₄ + HClO ₄ BG 17365-94	0.93±0.04	-0.10	-2.51*	90.3
HNO ₃ + H ₂ SO ₄ + HClO ₄ ISO 5515-1979	0.94±0.04	-0.09	-2.25*	91.5
HNO ₃ + H ₂ SO ₄ ISO 5515-1979	0.97±0.05	-0.06	-1.5**	94.2
HNO ₃ EPA 3051	0.99±0.03	-0.04	-1.00**	96.1
HNO ₃ + H ₂ O ₂ EPA 3051	0.99±0.03	-0.04	-1.00**	96.1
HNO ₃ + H ₂ O ₂ + HF EPA 3051	1.0±0.03	-0.03	-0.75**	97.1

*satisfactory result; **good result

Results presented in Table I indicate that the dry ashing at temperatures above 400 °C allows the complete extraction of potassium from the tobacco material despite the insoluble residue remaining after completion of the procedure. This indicates that this element is not associated with the compounds insoluble under these conditions, containing mainly Si and Al. Acid decomposition in all variants leads to the formation of insoluble residue, probably due to incomplete dissolution of the matrix or the formation of poorly soluble compounds in the treatment process. The formation of a

precipitate was also observed by Walas et al. [12] during microwave mineralisation of the same reference material with nitric acid or various combinations of HNO₃, HF, HClO₄ and H₂O₂ in determining the total content of Zn, Fe, Mg, Cu and Ca. However, acid decomposition with HNO₃ and H₂O₂ also results in the complete extraction of potassium, as the mixture of HNO₃ and H₂SO₄ - 94% of the certified value. Considerably reduced, however, are the results obtained using the acid mixtures involving HClO₄ - 88.3-91.5%. The same conclusion was also reached by Poykio et al. [4] in the analysis of certified materials BRC CRM 101 (peach leaves) and BRC CRM 100 (spruce needles) and of pine leaves. In all three cases, in the use of a mixture of HNO₃ and HClO₄, the determined amount of potassium is about 10% lower than that in the use of other acid mixtures. The authors suggest that the reason for this is the formation of insoluble residue from KClO₄. The probable cause of reduced extraction in our research is the same. To test this assumption, we performed roentgen structure analysis of the sample of tobacco leaves obtained after digestion by method 6 (HNO₃ + HClO₄) and method 7 (HNO₃ + H₂SO₄ + HClO₄).

The diffraction pattern presented in Fig. 1 shows presence of high-intensity reflexes showing the formation of phases with well-formed crystallites. The analysis of the spectrum shows the presence of only one crystalline phase - KClO₄ (PDF - 70-0488).

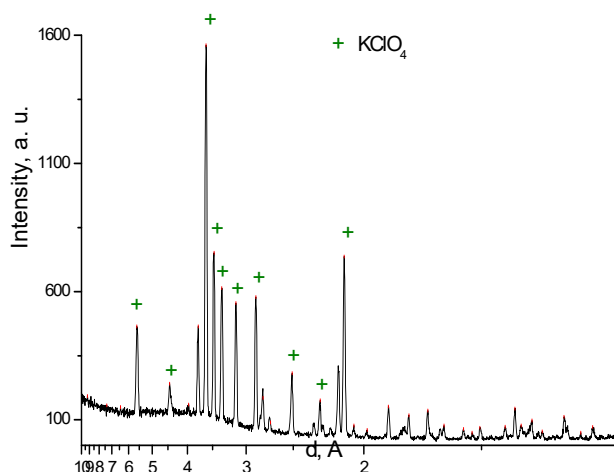


Fig. 1 X-ray pattern of the insoluble precipitate obtained by mineralizing the sample of tobacco by Method 6 (HNO₃ + HClO₄)

Fig. 2 shows the results of electron-microscopic testing of the same sample. The presence of well-formed crystals is noticeable, among which there are particles with a darker colour. The elemental analysis of the crystal phase shows the presence of only K, Cl, and O, apparently linked in the form of KClO₄. The integrated spectrum of the same sample shows the presence of carbon (residues of the organic matter), as well as of minor amounts of Al and Si.

Fig. 3 shows the results of X-ray analysis of the insoluble precipitate produced by mineralisation of a tobacco sample under Method 7 (HNO₃ + H₂SO₄ + HClO₄).

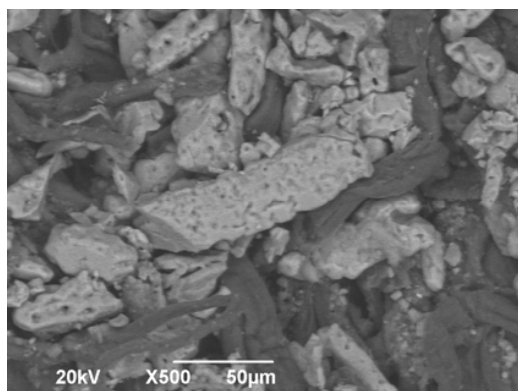


Fig. 2 SEM images of the insoluble precipitate obtained by mineralizing the sample of tobacco by Method 6 ($\text{HNO}_3 + \text{HClO}_4$). Scale bar 10 μm

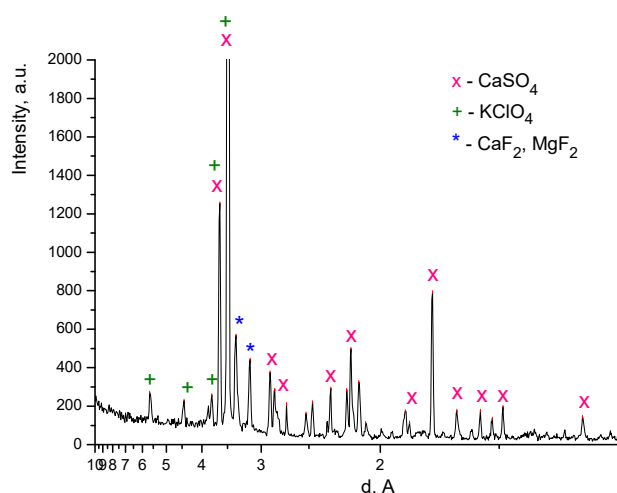


Fig. 3 X-ray pattern of the insoluble precipitate obtained by mineralizing the sample of tobacco by Method 7 ($\text{HNO}_3 + \text{H}_2\text{SO}_4 + \text{HClO}_4$)

High-intensity reflexes in the diffractogram show the presence of crystalline phases with well-formed crystallites. The mathematical treatment of the spectrum indicates the presence of KClO_4 (PDF - 70-0488) and CaSO_4 (PDF - 74-2421). This result confirms the assumption made that the use of HClO_4 leads to blocking of a portion of potassium in the form of insoluble KClO_4 and is not suitable oxidising agent in its determination in plant material.

The results of the SEM testing (Fig. 4) also lead to the same conclusion. Well-formed crystals with sizes from 1.0 to 20-30 μm are observed. The integrated EDS analysis shows the presence of the elements K, Ca, S, Cl, and O, apparently linked in the form of KClO_4 and CaSO_4 .

The detailed analysis (Fig. 5) shows that the massive crystalline phase (1) contains K, Cl, and O in an atomic ratio 1.0: 1.0: 4.0 (KClO_4), and the fine crystalline phase (2) - Ca, S and O in the same atomic ratio (CaSO_4).

The results of the X-ray analysis and the SEM-EDS testing confirm the assumption made that the use of HClO_4 leads to

blocking of a portion of potassium in the form of insoluble KClO_4 and is not suitable oxidising agent in its determination in plant material.

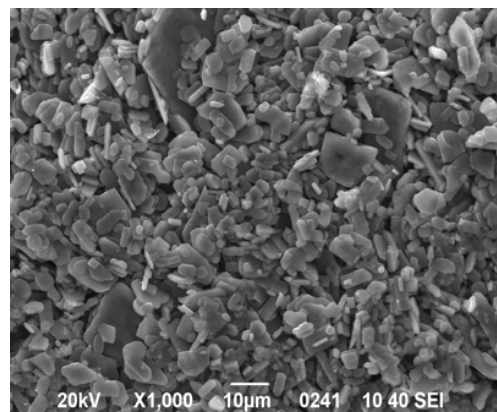


Fig. 4 SEM images of the insoluble precipitate obtained by mineralizing the sample of tobacco by Method 7 ($\text{HNO}_3 + \text{H}_2\text{SO}_4 + \text{HClO}_4$). Scale bar 10 μm

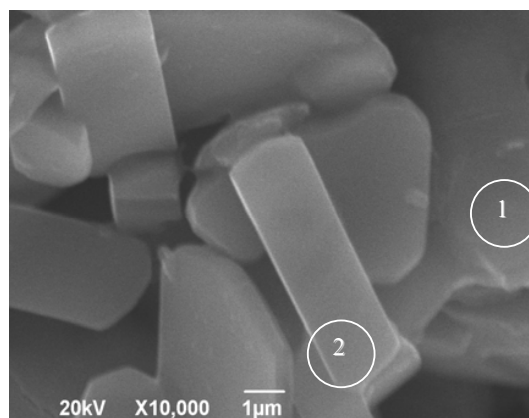


Fig. 5 SEM images the insoluble precipitate obtained by mineralizing the sample of tobacco by Method 7 ($\text{HNO}_3 + \text{H}_2\text{SO}_4 + \text{HClO}_4$). Scale bar 1.0 μm

The microwave mineralisation in all variants leads to a complete extraction of potassium and a suitable method for its determination.

Table II presents the results for Ca content in the certified material of tobacco leaves obtained under the above-described methods. In this case, the dry ashing allows the complete extraction of calcium from the tobacco material despite the insoluble residue remaining after completion of the procedure. This indicates that this element is not associated with the compounds containing mainly Si and Al, insoluble under these conditions. Acid decomposition with $\text{HNO}_3 + \text{H}_2\text{O}_2 + \text{HClO}_4$ also leads to its complete extraction, while decomposition with acid mixtures involving H_2SO_4 results in a significant lowering of the results. The reason for this is the formation of a poorly soluble precipitate from CaSO_4 as demonstrated by the results presented in Figs. 4 and 5.

TABLE II
THE EFFICIENCY OF THE METHODS OF MINERALISATION IN THE
DETERMINATION OF CA IN SHEETS OF VIRGINIA (CTA-VTL-2), $X_{CRM} = 3.60 \pm 0.15\%$

Method	X, ppm	D	Z	R
Dry, 550°C	3.73±0.11	0.13**	0.87**	103.6
Dry, 500°C	3.75±0.12	0.15**	1.00**	104.2
Dry, 450°C	3.54±0.10	-0.06**	-0.40**	98.3
Dry, 400°C	3.68±0.07	0.08**	0.53**	102.2
HNO ₃ + H ₂ O ₂	3.53±0.08	-0.11**	-0.47**	98.0
HNO ₃ + HClO ₄	3.46±0.07	-0.14**	-0.93**	96.1
HNO ₃ + H ₂ SO ₄ + HClO ₄ BG 17365-94	2.71±0.12	-0.89	-5.93	75.3
HNO ₃ + H ₂ SO ₄ + HClO ₄ ISO 5515-1979	2.72±0.11	-0.88	-5.94	79.6
HNO ₃ + H ₂ SO ₄ ISO 5515-1979	2.81±0.13	-0.79	-5.27	78.1
HNO ₃ EPA 3051	3.66±0.07	0.06**	0.40**	101.7
HNO ₃ + H ₂ O ₂ EPA 3051	3.61±0.06	0.01**	0.07**	100.3
HNO ₃ +H ₂ O ₂ +HF EPA 3051	3.23±0.10	0.37*	-2.46*	89.6

*satisfactory result; **good result

The use of an acidic mixture of HNO₃ and H₂SO₄ also leads to the formation of a crystalline precipitate from CaSO₄. Fig. 6 shows the results of X-ray analysis of the insoluble precipitate after completion of the procedure under Method 9 (HNO₃ + H₂SO₄).

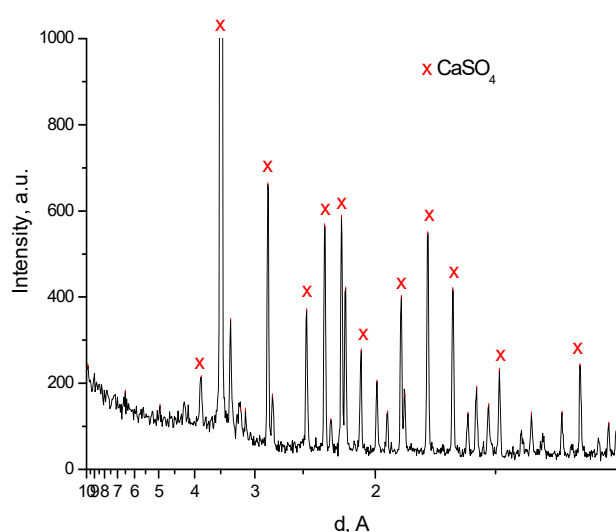


Fig. 6 X-ray pattern of the insoluble precipitate obtained by mineralizing the sample of tobacco by Method 9 (HNO₃ + H₂SO₄)

The lowering of the results by about 10% compared with the certified material is observed in the microwave mineralisation with a mixture of HNO₃, H₂O₂ and HF. The reason for this is the formation of an insoluble precipitate from CaF₂ which has already been observed in the analysis of soil samples. Similar results were obtained by Walas et al. [12] in the microwave mineralisation with a mixture of HNO₃ and HF of the same certified material.

Table III compares the effectiveness of the used methods of sample preparation in the determination of magnesium in the

leaves of Virginia tobacco (CTA-VTL-2). The results are similar to those for calcium. Also, in this case, the dry ashing and acid digestion with HNO₃ and H₂O₂ or HClO₄, as well as the microwave mineralisation in all variants lead to a complete extraction of magnesium. Acid mixtures involving H₂SO₄ are probably insufficiently effective due to the inclusion of a portion of magnesium in the residue from CaSO₄.

TABLE III
THE EFFICIENCY OF THE METHODS OF MINERALISATION IN THE
DETERMINATION OF MG IN SHEETS OF VIRGINIA (CTA-VTL-2), $X_{CRM} = 0.510 \pm 0.023\%$

Method	X, ppm	D	Z	R
Dry, 550°C	0.531±0.016	0.021	0.91**	104.1
Dry, 500°C	0.527±0.013	0.017	0.74**	103.3
Dry, 450°C	0.534±0.015	0.024	1.04**	104.7
Dry, 400°C	0.533±0.014	0.023	1.00**	104.5
HNO ₃ + H ₂ O ₂	0.498±0.011	0.012	-0.52**	97.6
HNO ₃ + HClO ₄	0.500±0.010	-0.010	-0.43**	98.0
HNO ₃ + H ₂ SO ₄ + HClO ₄ BG 17365-94	0.448±0.015	-0.062	-2.69*	87.8
HNO ₃ + H ₂ SO ₄ + HClO ₄ ISO 5515-1979	0.460±0.013	-0.050	-2.17*	90.2
HNO ₃ + H ₂ SO ₄ ISO 5515-1979	0.436±0.012	-0.074	-3.21	85.5
HNO ₃ EPA 3051	0.490±0.013	-0.020	-0.87**	96.1
HNO ₃ + H ₂ O ₂ EPA 3051	0.500±0.010	-0.010	-0.43**	98.0
HNO ₃ +H ₂ O ₂ +HF EPA 3051	0.531±0.013	0.021	0.91**	104.1

*satisfactory result; **good result

TABLE IV
DISPERSION ANALYSIS OF THE RESULTS OBTAINED BY DIFFERENT
MINERALISATION METHODS

Method	Element		
	K	Ca	Mg
Dry, 550°C	ns	ns	ns
Dry, 500°C	ns	ns	ns
Dry, 450°C	ns	ns	ns
Dry, 400°C	ns	ns	ns
HNO ₃ + H ₂ O ₂	ns	ns	ns
HNO ₃ + HClO ₄	●	ns	ns
HNO ₃ + H ₂ SO ₄ + HClO ₄ BG 17365-94	●	●	ns
HNO ₃ + H ₂ SO ₄ + HClO ₄ ISO 5515-1979	●	●	●
HNO ₃ + H ₂ SO ₄ ISO 5515-1979	●	●	●
HNO ₃ EPA 3051	ns	ns	ns
HNO ₃ + H ₂ O ₂ EPA 3051	ns	ns	ns
HNO ₃ + H ₂ O ₂ + HF EPA 3051	ns	●	ns

ns - No statistically significant difference is observed between the resulting value and the consensus value of the certified form; ● The results obtained are lower than the consensus ones.

Table IV presents the dispersion analysis of the results obtained by different mineralisation methods. No statistically significant difference is observed between our results for the content of K, Ca and Mg and the consensus values in dry mineralisation and acid digestion under methods 5, 10 and 11. The acidic mixtures involving H₂SO₄ lead to low results in the determination of Ca and Mg. HClO₄ interferes with the determination of potassium, and HF – of calcium.

IV. CONCLUSIONS

A comparative study of the most commonly used mineralisation methods of plant samples (dry ashing, acid decomposition and microwave mineralisation) was carried out to determine the total content of K, Ca and Mg on the basis of certified material from the leaves of Virginia tobacco CTA-VTL-2. It was found that:

- The degree of extraction of the tested elements is highly dependent on the mineralisation method of the samples. Dry ashing (450 - 550°C), acid decomposition with a mixture of HNO₃ and H₂O₂ and microwave mineralisation (HNO₃ + H₂O₂) are most suitable for the simultaneous determination of all the tested elements.
- The combination of nitric acid with H₂SO₄ and HClO₄, as well as the use of HF, leads to the formation of poorly soluble precipitates from KClO₄, CaSO₄ or CaF₂, making them unsuitable in the determination of K, Ca and Mg in the plant material.

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