Yerba mate (Ilex paraguariensis A. St.-Hil.) is a plant species of the family Aquifoliaceae. I. paraguariensis A. St.-Hil. dried and minced leaves are present in a brewed tea, a beverage that has a social and almost ritualistic role in some South American modern societies. It is used both as a source of caffeine, in lieu or in parallel with tea and coffee, but also as a therapeutic agent for its alleged pharmacological properties. Yerba mate is known as a hypocholesterolemic, hepatoprotective, central nervous system stimulant, diuretic, and to benefit the cardiovascular system. It has been suggested for obesity management and is rapidly gaining attention at the world market, either as a tea itself or as an ingredient in formulated foods or dietary supplements [1,2].

In the last few years, compressed gases are commonly used for extraction of pharmacologically active substances from plant material because of the green procedure, absence of toxins and volatile organic solvents used for the extraction process [3-6]. The main advantage of supercritical fluid extraction (SFE), in comparison to other separation techniques (distillation, extraction with organic solvent) is the ability of extraction at moderate temperatures and possibility to obtain pure and sterile extract with no traces of solvent. This is very important for applications in the pharmaceutical, food and cosmetic industries. The most commonly used supercritical fluid for the extraction process is carbon dioxide, while other substances with easily accessible critical points, are too expensive (xenon), toxic (ammonia, nitrous oxide), flammable (ethane and pentane) or corrosive (ammonia). Low critical temperature (31°C), non-toxicity, and low cost have provided supercritical CO2 as a suitable solvent for food products [7]. Carbon dioxide is an attractive alternative to current liquid solvent
applications for the extraction of active components from natural products [8], such as yerba mate. Also, low viscosity, high diffusivity and low surface tension enable easy penetration of carbon dioxide throughout macro- and microporous materials. Also, the non-toxic and environmentally friendly nature of carbon dioxide makes it a clean technology. Lastly, it has a low enough critical point for the processing of thermo-labile materials [9-16]. Many factors, such as pressure, temperature and time may have a great impact on Yerba mate extraction. Most of the studies investigated the influence of only one factor of the extraction at the time. In this paper, the influence of extraction operating variables in a wide range (pressure: 100-400 bar; and time: 0.5-3.5 h) on the total yield and purine alkaloids content of I. paraguariensis A. St.-Hil. extracts was evaluated using the chemometric tools to provide the optimal extraction conditions.

Chemometric analysis today plays a very important role in a modern science, by describing and predicting new properties and indicating new patterns of interest in experimental life. This is irreplaceable for chemistry and biochemistry [17-22]. The evaluation of the obtained experimental results was performed by applying hierarchical cluster analysis (HCA) and principal component analysis (PCA), followed by analysis of variance (ANOVA). These chemometric methods are widely used in the differentiation of objects (i.e., chemical compounds, chromatographic parameters, molecular descriptors, food products, etc.) based on their physicochemical characteristics [23-26]. In the present study these methods were employed for classification of different extraction conditions in order to gain an overview of similarities among them and as a helpful tool for optimization of extraction parameters for obtaining extracts with the highest amount of bioactive compounds.

MATERIAL AND METHODS

Plant material

Yerba mate folium (Ilex paraguariensis A. St.-Hil., Aquifoliaceae) was purchased from Sinex Company, Niš, Serbia. The plant material is determined by the level of plant species and deposited in the Herbarium of the Laboratory of pharmacognosy, Department of Pharmacy, Faculty of Medicine, University of Novi Sad (Ip-31/08). The dried plant material was kept in a cardboard box in a dark, dry place until sample preparation. Prior to extraction, the leaves were ground in a mill until the particle size diameter reached 0.50 mm which was determined by sieving.

Chemicals

Standards of caffeine (ω > 0.99), theobromine (ω > 0.98), and theophylline (ω > 0.98) were purchased from Sigma-Aldrich (St. Louis, CA, USA). Commercial carbon dioxide (ω > 0.99) was obtained from Messer Tehnogas (Novi Sad, Serbia). Acetonitrile was purchased from Sigma (Deisenhofen, Germany). Ultrapure water was used for the preparation of all solutions (Milli-Q-quality). All solvents and reagents were of analytical grade unless indicated otherwise.

Instrumentation and operational conditions

Plant material extraction was performed on a laboratory device, high pressure plant extraction (HPEP, NOVA-Swiss). The total weight of the plant material in the extractor was 60 g. During the 3.5 h extraction, samples were taken every half an hour for the total yield of extraction and purine alkaloids content determination. The extract was collected into a previously weighed glass vial and placed in the separator. The flow rate of 3.22×10⁻³ kg/min of carbon dioxide expressed under normal conditions was low enough to ensure the saturation of the supercritical carbon dioxide with solute. The temperature was kept constant at 40 °C and the extraction was carried out at different pressures: 100, 150, 200, 250, 300 and 400 bar.

Obtained extracts were dissolved in 10 mL of water pH 8 and solid-phase extraction (SPE) was used for the separation of purine alkaloids from other substances. The Supelclean™ LC-18 SPE cartridges 6 mL (0.5 g) used for SPE were obtained from Supelco, USA. The SPE was performed in a 12-position Vacuum Manifold, Supelco, USA. The Supelclean™ LC-18 SPE cartridges were conditioned with 2×6 mL of dichloromethane, chloroform, methanol and HPLC grade water. Purine alkaloids were eluted from the SPE cartridges with 2×10 mL of dichloromethane into an evaporating flask. The solution was evaporated to dryness under nitrogen. The residue of all samples was reconstituted in 2 mL of water pH 8.

In the obtained extracts, the content of purine alkaloids (caffeine and theobromine) was determined by applying the high-performance liquid chromatography (HPLC) method [27,28]. The results are expressed as mg/100 g of dry extract. The chromatography was performed using two-solvent isocratic elution. The HPLC-diode array detection (DAD) model Agilent HP 1100 system equipped with an autosampler ( Waldbronn, Germany) was used. The analytical column was the Zorbax Eclipse XDB-C8 (4.6 mm×150 mm, i.d., 5 µm particle size). The mobile
phase was water (pH 8) + acetonitrile (4:1) with a flow rate of 0.8 mL/min. The HPLC mobile phase was prepared fresh daily and filtered through a 0.45 µm nylon filter. Run time was 10 min and column temperature 25 °C. Injection volume was 15 µL and analytes were detected at 273 nm. All measurements were performed in triplicate.

Chemometric methods

HCA is a classification method which can be used for dividing a group of objects into classes (clusters) which contain similar objects [29]. In this study, the clustering of objects was achieved by applying Euclidean distances and single linkage algorithm. PCA is a technique for reducing the amount of data. PCA method calculates new (latent) variables combining the original variables and representing the multidimensional data structure in an optimal way [30]. In a multidimensional space, where the variables define the axes, the data are projected into a few principal components (PCs) that present linear combinations of the original variables. These PCs describe the maximum variation within the data, and they are characterized by scores and loadings. Loadings reflect the direction with respect to the original variables, while scores are the new coordinates of the projected objects. These PCs describe the maximum variation within the data, and they are characterized by scores and loadings. Loadings reflect the direction with respect to the original variables, while scores are the new coordinates of the projected objects. These PCs describe the maximum variation within the data, and they are characterized by scores and loadings. Loadings reflect the direction with respect to the original variables, while scores are the new coordinates of the projected objects.

Table 1. Influence of time and pressure of supercritical CO2 extraction on total extraction yield of yerba mate leaves (%)

<table>
<thead>
<tr>
<th>t / h</th>
<th>p / bar</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.1190±0.01</td>
</tr>
<tr>
<td>1.0</td>
<td>0.2395±0.01</td>
</tr>
<tr>
<td>1.5</td>
<td>0.3013±0.02</td>
</tr>
<tr>
<td>2.0</td>
<td>0.3675±0.01</td>
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<tr>
<td>2.5</td>
<td>0.4013±0.02</td>
</tr>
<tr>
<td>3.0</td>
<td>0.4353±0.02</td>
</tr>
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</table>

Results and discussion

Effects of time and pressure of extraction on total extraction yield

To determine the influence of time and pressure on total extraction yield, Yerba mate leaves were extracted during 0.5-3.5 h at different pressures 100-400 bar. The obtained results are presented in Table 1.

These results showed that the yield of extraction rapidly increased with the time of extraction. One of the most important parameters in the SFE process is the extraction pressure, due to its strong influence on the extraction efficiency which is the main determinant of the solvent power of supercritical fluids. As can be seen from the parameters listed in Table 1, the extraction yield increased as the pressure increased.

By increasing the pressure from 100 to 400 bar at constant temperature (t = 40 °C), 9.21 times greater extraction yield was achieved. This is explained by the fact that increasing the pressure of SFE results in the higher density of the supercritical fluid, and subsequent increase in its solvating efficiency [13].

Effects of time and pressure of extraction on purine alkaloids content

HPLC analysis of Yerba mate extracts for purine alkaloids determination showed the presence of caffeine and theobromine, while theophylline was not present in any of the tested samples (Figure 1). This is not in line with Saldana et al, probably due to different origin of analyzed material [6].

The shorter retention time for theobromine can be explained with better interaction between the mobile phase and theobromine, which occurs as a result of the presence of hydrogen atom in the position 1 in the molecule, opposed to caffeine, which has a methyl group in this position [31].

Figure 2 clearly indicates that purine alkaloids (caffeine and theobromine) yield increased more or less linearly as the function of extraction time. Also, caffeine and theobromine content in Yerba mate extracts increased with the increase of pressure.
Extraction pressure of 400 bar gives 77.47 times greater yield of purine alkaloids than pressure of 100 bar. This is explained by the carbon dioxide increased power of dissolving with increased pressure. These results implied the significant role of both time and pressure in SFE. The values of the caffeine content in yerba mate were lower than the values obtained by HPLC analysis in a similar study which can be attributed to different levels of fragmentation and moisture content of the analyzed plant material [32]. The obtained results for theobromine are slightly lower than the results obtained in the same study conducted in Argentina [32]. This difference can be attributed to a higher level of fragmentation of the drug that was used in the mentioned study, which increased the specific surface area of the comminuted material, providing more intensive contact of the solvent and plant material.

**Chemometric analysis**

**HCA and PCA based on the extraction pressure**

The HCA was carried out on the set of the experimental data which included content of total active compounds, theobromine content and caffeine content in Yerba mate extracts obtained at different extraction pressures. The obtained dendrograms are presented in Figure 3.

Figure 3a shows that the extraction of total active compounds at 100 and 150 bar was significantly different compared to the extractions at 200–400 bar. Also, it can be seen that there was small difference between the extractions at 200 and 250 bar, since they were directly connected in the same cluster.
this case, the extraction efficacy significantly changed as the pressure increased from 150 to 200 bar. Clustering of the extraction conditions based on theobromine content (Figure 3b) showed that significant increase in its content was achieved when the pressure was changed from 300 to 400 bar. The pressure change from 200 to 250 bar did not cause any significant change in theobromine content. In the case of caffeine (Figure 3c), the significant increase of its content in the extracts was achieved when the pressure was changed from 100 to 150 bar. Also, in this case, the change of pressure from 200 to 250 bar leads to a slightly higher caffeine content.

PCA has some advantages in classification over HCA. It can show which variable mostly contributes to the distribution of the objects on the score plot. In this case, we applied PCA in order to confirm the grouping of the variables (objects) already obtained by HCA. The PCA results are presented in Figure 4.

PCA analysis resulted into two-component models which cover 99.8% of total variability (for content of total active compounds), 99.9% (for theobromine content) and 99.4% (for caffeine content).

In all three cases the grouping of variables (pressure values), already obtained by HCA, were confirmed by PCA. The distinguishing factor in all three score plots was principal component 1 (PC1), since it explained more than 97% of the data variation.

HCA and PCA based on the extraction time

The results of HCA based on the content of total active compounds, theobromine content and caffeine content in yerba mate extracts obtained at different extraction times are shown in Figure 5.

The obtained dendrograms showed similarities between the contents of total active compounds, theobromine and caffeine in extracts obtained after 2 and 2.5 h, as well as after 3 and 3.5 h. It implied that the extraction efficacy was not significantly different between 2 and 2.5 h, or 3 and 3.5 h, but it was significant between 1 and 1.5 h.

PCA based on extraction times resulted in two-component models which covered 99.8% of total variability (for content of total active compounds), 99.9% for theobromine content and 99.5% for caffeine content, and are shown in Figure 6.

The most significant principal component in these score plots was PC1 since it covered most of data variations (higher than 97%). Therefore, PC1 was selected as the axis on which the projections of the points can be discussed. According to PC1 axis, the grouping of the points (objects) was the same as in HCA. It can be seen that the distances between 0.5, 1 and 1.5 h were greatest, which implied sig-
nificant change in extraction efficacy between 0.5 and 1.5 h.

ANOVA

The results of ANOVA testing are presented in Table 2. These results indicate that the extracts obtained under the applied extraction conditions did not belong to the same population \((F >> F_{\text{crit}})\). This means that there was significant difference between the extracts regarding the contents of theobromine, caffeine and total active compounds.

The presented results of HCA, PCA and ANOVA methods indicated that changes in the applied extraction conditions had significant influence on the contents of bioactive compounds in yerba mate extracts. Specifically, the extraction of caffeine and theobromine should last at least 1.5 h at a pressure of 300 bar.

CONCLUSIONS

HPLC analysis of yerba mate leaves extracts showed that the total yield of extraction rapidly increased with the time and pressure of extraction. HCA revealed that the extraction yield at 100 and 150 bar was significantly different compared to the extractions at 200–400 bar. HPLC analysis of extracts confirmed the presence of theobromine and caffeine, while theophylline was not present in any of the tested samples. Purine alkaloids contents increased more or less linearly as the function of extraction time and pressure.

Clustering of the extraction conditions based on theobromine content indicated that significant increase in its content was achieved when the pressure was changed from 300 to 400 bar, and the significant increase in caffeine content in the extracts was achieved when the pressure was changed from 100 to 150 bar. The change of pressure from 200 to 250 bar did not cause the significant difference in both theobromine and caffeine content. According to HCA, the contents of active compounds, theobromine and caffeine, were not significantly different between 2 and 2.5 h, or 3 and 3.5 h, but they were significant between 1 and 1.5 h. Statistical results proved the great differences between the extracts regarding the contents of purine alkaloids and indicated that changes in the applied extraction conditions had significant influence on the contents of these bioactive compounds in Yerba mate extracts. Specifically, the extraction of caffeine and theobromine should last at least 1.5 h at pressure of 300 bar. It can be concluded that applied chemometric techniques could be a helpful tool for choosing the most promising extraction conditions that can provide Yerba mate extract with the highest content of target substances.

Acknowledgements

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Table 2. The results of ANOVA testing (SS - sum of squares; df - degrees of freedom; MS - mean square; F - Fisher’s value; p - probability level; \(F_{\text{crit}}\) - critical Fisher’s value)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p-value</th>
<th>(F_{\text{crit}})</th>
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<td>30</td>
<td>0.0307</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15.49</td>
<td>41</td>
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</tbody>
</table>
REFERENCES


B. TEOFILOVIĆ et al.: ANALYSIS OF OPERATING VARIABLES FOR YERBA MATE LIŠĆA UGLJEN-DIOKSIDOM

Yerba-mate je prirodni izvor purinskih alkaloida i drugih bioaktivnih jedinjenja sa istaknutom terapeutskom aktivnošću. U ovom radu ispitivan je uticaj operativnih varijabli na prinos purinskih alkaloida nakon ekstrakcije superkritičnim ugljen-dioksidom. Tečna hromatografija visokih performansi (HPLC) primenjena je za određivanje sadržaja kofeina i teobromina. Evaluacija rezultata eksperimenta izvršena je primenom hijerarhijske klaster analize (HCA) i analize glavnih komponenti (PCA), kao i analizom varijanse (ANOVA). HPLC analiza Yerba-mate ekstrakta pokazala je da su ukupni prinos i sadržaj teobromina i kofeina u uskoj vezi sa vremenom ekstrakcije i primenjenim pritiskom. Definisani optimalni uslovi za ekstrakciju kofeina i teobromina su 1,5 h pod pritiskom od 300 bar.

Kljucne reči: Yerba-mate, superkritična ekstrakcija, kofein, teobromin, hemometrika.