

# Determination of Polyphenols in Cigar Tobacco by HPLC

Jing Zhang<sup>1,3</sup>, Ping Li<sup>1</sup>, Xiaolin Yue<sup>1</sup>, Ting Zhou<sup>2,3</sup>, Qi Chen<sup>2,3</sup>, Wenyuan Tan<sup>1,3,\*</sup>

<sup>1</sup> School of Chemical Engineering, Sichuan University of Science and Engineering, Zigong, Sichuan, 643000, China

<sup>2</sup> Sichuan Zhongyan Industry Co., Ltd. Great Wall Cigar Smoke Factory, Shifang, Sichuan, 618400, China

<sup>3</sup> Industrial Efficient Utilization of Domestic Cigar Tobacco Key Laboratory of Sichuan, Deyang, Sichuan, China

\* Corresponding author: Wenyuan Tan (Email: twyhyx@126.com)

**Abstract:** A high performance liquid chromatography (HPLC) method was developed for the determination of neochlorogenic acid, cryptochlorogenic acid and caffeic acid in cigar tobacco. Firstly, the extraction method, extraction liquid concentration and dosage, column temperature and mobile phase flow rate of polyphenols in cigar tobacco were discussed. Then the detection limit, recovery rate, precision and standard curve of three polyphenols were tested. Finally, tobacco samples were tested. The results showed that the optimal extraction conditions were: ultrasonic extraction, extraction time was 30 min, methanol volume concentration was 50%, dosage was 20 mL, the optimal detection conditions were: column temperature was 30 °C, flow rate was 1.0 mL/min, detection wavelength was 340 nm. The contents of neochlorogenic acid, cryptochlorogenic acid and caffeic acid were in the range of 0~0.202 mg/g, 0~0.331 mg/g and 0.050~0.073 mg/g, respectively, and the linear correlation coefficients of the three polyphenols were  $\geq 0.9991$ , indicating that the linear correlation of the method was good, and the detection limit was  $\leq 0.066$  mg/L. The precision was  $\leq 0.427\%$ , and the recovery was 95.13~96.39%. The results showed that the method had good precision, high accuracy, short analysis time and high detection efficiency, and could be used as an effective method for the detection of polyphenols in cigar tobacco leaves.

**Keywords:** Ultrasonic Wave Extraction; Cigar Tobacco; Polyphenols; High Performance Liquid Chromatography.

## 1. Introduction

The quality of tobacco leaves, which are the foundation of the tobacco industry, directly impacts the quality of cigar products. Polyphenolic compounds, as part of plant secondary metabolites, exist in tobacco leaves primarily as glucosides and esters, serving as important precursors of aroma. Differences in the content of these compounds are found throughout various parts of the tobacco plant, with the majority accumulating in the leaves and a smaller amount in the roots and stems [1-3]. They are categorized into tannins (chlorogenic acid), coumarins, flavonoids, and others [4]. Chlorogenic acid, the most abundant polyphenol in tobacco leaves, is formed through the dehydration condensation of quinic acid and caffeic acid. It is unstable under neutral and alkaline conditions, prone to isomerization reactions that produce cryptochlorogenic acid and neochlorogenic acid [5-6]. These compounds possess antioxidant properties and can eliminate free radicals in tobacco, thereby reducing oxidative stress damage to tobacco tissues [7-9]. A comprehensive analysis of the content of polyphenolic compounds in cigar tobacco leaves is essential for a deep understanding of their influence on the physiological characteristics and quality of tobacco leaves. It also allows for the targeted optimization of cultivation techniques and growth conditions for cigar tobacco leaves, effectively promoting an increase in tobacco leaf yield and an improvement in quality. This is of significant importance for the identification of polyphenolic compounds in Chinese cigar tobacco leaves and the quality control of tobacco products.

Numerous reports have been published both domestically and internationally on the extraction and qualitative and quantitative analysis of polyphenolic compounds. Extraction methods commonly used for polyphenols encompass microwave extraction, solid phase extraction, membrane technology extraction, and ultrasonic extraction [10-13], with

ultrasonic extraction being selected as the ideal method for extracting polyphenolic compounds in this experiment due to its efficiency, speed, and relatively low cost. Determination of polyphenols is primarily conducted using methods such as spectrophotometry, gas chromatography mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), and high-performance liquid chromatography-mass spectrometry (LC-MS) [14-17]. HPLC is considered the most suitable method for detecting polyphenolic substances because it allows for their detection at room temperature and offers advantages such as high efficiency, high selectivity, high sensitivity, and accuracy.

In this study, a high-performance liquid chromatography method for the simultaneous determination of chlorogenic acid, cryptochlorogenic acid, and neochlorogenic acid in cigar tobacco leaves was established through the employment of ultrasonic treatment. The method was utilized to measure the content of three polyphenolic compounds in six types of cigar tobacco leaves, with the differences in polyphenolic content among these leaves being analyzed. This offers significant technical reference for enhancing the quality and safety of cigar tobacco leaves.

## 2. Materials and Methods

### 2.1. Instruments and Reagents

1100 High performance liquid chromatograph (Agilent); AS7240AT Ultrasonic cleaning machine (Ottenes); LC-MSH-Pro-4B Magnetic stirrer (Richenbon); SHA-B Water bath thermostatic oscillator (Yi Neng experimental instrument factory).

Aesculin, Coumaric acid, scopolamine, scopolamine, coumarin, neochlorogenic acid (BZWZ), Cryptochlorogenic acid (PTTC), Caffeic acid, ferulic acid (Macklin), Carbinol (Titan), Glacial acetic acid (Knowles), Ascorbic acid (Cologne).

## 2.2. Chromatography Conditions

Eclipse Plus C18 Liquid chromatography column (4.6 mm×250 mm, 5 µm); Mobile phase A was water: carbinol: Acetic acid (88:10:2, v/v); Mobile phase B was water:

carbinol: Acetic acid (10:88:2, v/v); The column temperature was 30 °C and the flow rate was 1.0 mL/min. The amount of sample injection is 10 µL. The Detection wavelength was 340 nm. Table 1 shows the gradient time of mobile phase.

**Table 1.** The gradient time of mobile phase

| Time(min) | Mobile phase A% | Mobile phase B% | Flow rate(mL/min) | Maximum pressure(bar) |
|-----------|-----------------|-----------------|-------------------|-----------------------|
| 0.00      | 92.0            | 8.0             | 1.000             | 200                   |
| 10.00     | 92.0            | 8.0             | 1.000             | 200                   |
| 10.10     | 75.0            | 25.0            | 1.000             | 200                   |
| 15.00     | 75.0            | 25.0            | 1.000             | 200                   |
| 15.10     | 100.0           | 0.0             | 1.000             | 200                   |
| 22.00     | 100.0           | 0.0             | 1.000             | 200                   |
| 22.10     | 70.0            | 30.0            | 1.000             | 200                   |
| 27.00     | 70.0            | 30.0            | 1.000             | 200                   |
| 27.01     | 0.0             | 100.0           | 1.000             | 200                   |
| 30.00     | 0.0             | 100.0           | 1.000             | 200                   |
| 30.10     | 92.0            | 8.0             | 1.000             | 200                   |
| 35.00     | 92.0            | 8.0             | 1.000             | 200                   |

Standard solution configuration: Accurately weigh 20.00 mg of neochlorogenic acid, cryptochlorogenic acid and caffeic acid in 25 mL volumetric bottle, adjust the volume with 50% methanol solution to the scale, mix well, and store at 4 °C.

## 2.3. Sample Treatment

Accurately, 0.5000 g of samples were precisely weighed and placed in a 50 mL conical flask. Two drops of 0.5% ascorbic acid solution were added as a protective agent. Subsequently, 20 mL of 50% methanol extraction solution was introduced, and the samples were subjected to extraction in an ultrasonic oscillator for 30 minutes. Then, centrifugation was carried out at 8000 r/min for 5 minutes. After centrifugation, approximately 2 mL of the extracted liquid was filtered into a 5 mL centrifuge tube through a 0.45 µm needle organic phase filter membrane, and 10 µL of the extracted liquid was taken for analysis in high performance liquid chromatography.

## 3. Results and Discussion

### 3.1. Pre-processing Condition Selection

#### 3.1.1. Selection of Extraction Method

Different extraction methods have different extraction effects on cigar tobacco. This study compared the extraction effects of ultrasonic, stirring and oscillation methods on the same cigar tobacco sample, as shown in Figure 1. The results showed that the extraction efficiency of neochlorogenic acid was similar between ultrasonic extraction and oscillation extraction, but the extraction efficiency of cryptochlorogenic acid and caffeic acid was higher than that of oscillation extraction, and the extraction effect was good. Ultrasonic extraction was the best way to extract polyphenols (neochlorogenic acid, cryptochlorogenic acid, caffeic acid) from tobacco leaves.

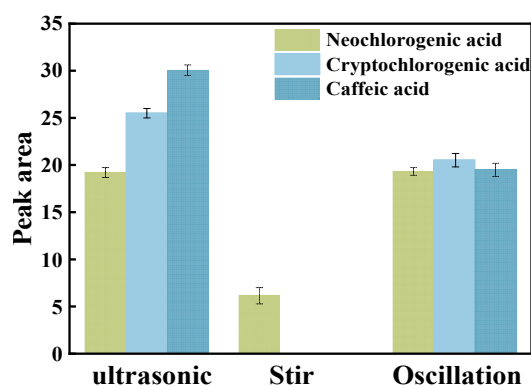


**Figure 1.** The extraction effect of different extraction methods

#### 3.1.2. Selection of Extraction Concentration (Volume Concentration)

In order to determine the optimal concentration of the extraction solution, this study investigated the influence of different extraction solution concentrations (30%, 40%, 50%, 60%, 70% methanol) on the extraction effect (Figure 2). The results indicated that under the identical volume, diverse concentrations of methanol demonstrated a specific tendency on the response values of neochlorogenic acid, cryptochlorogenic acid, and caffeic acid. The response values of the majority of substances reached the peak when the volume concentration of methanol was 50%, suggesting that the optimal dissolution and extraction efficiency might be attained under this condition. When the volume concentration of methanol is lower than 50%, the response value is relatively low. This might be attributed to the insufficient amount of methanol to fully dissolve the polyphenol substance in the sample. Once the volume concentration of methanol exceeds 50%, the response value commences to decline. This could be owing to the excessive methanol causing a dilution effect or influencing the retention and separation of substances within the column, thereby reducing the detection efficiency. This implies that under this experimental condition, the extraction solution with a volume concentration of 50% methanol constitutes an ideal option for achieving efficient analysis and optimizing the detection results.

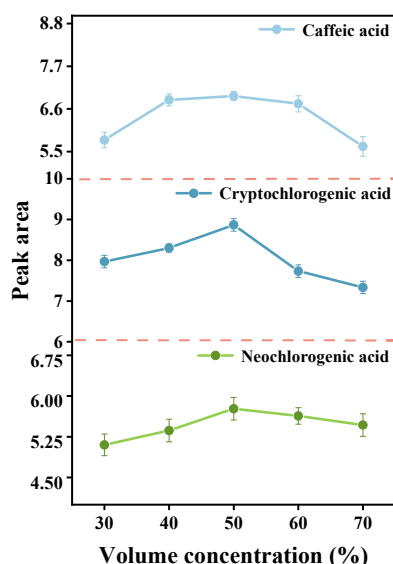


**Figure 2.** The extraction effect of different volume concentration

### 3.1.3. Selection of Extraction Liquid Volume

In order to select the appropriate amount of extracted liquid volume, this study investigated the influence of different extracted liquid volume on the extraction effect (Figure.3). With the change of methanol solution with volume concentration of 50%, the peak areas of neochlorogenic acid, cryptochlorogenic acid and caffeic acid reached the maximum value when the dosage was 20 mL. The results show that 50% methanol solution (volume concentration) can fully dissolve and extract these polyphenols, and achieve the best extraction efficiency. When the amount of 50% methanol solution (volume concentration) is less than 20 mL, the amount of solution is insufficient to fully extract substances, so the peak area is small. When the dosage exceeds 20 mL, too much solvent may lead to dilution or dispersion, but reduce the extraction effect of polyphenol substances. Therefore, 50% methanol solution (volume concentration) at 20 mL provides the optimal extraction conditions for these three polyphenols, ensuring maximum extraction efficiency. Beyond this volume, either increasing or decreasing the methanol solution volume will lead to a decrease in peak area, and these polyphenols cannot be adequately extracted. Therefore, when the dosage of 50% methanol solution (volume concentration) is 20 mL, it is determined to be the best extraction liquid dosage.

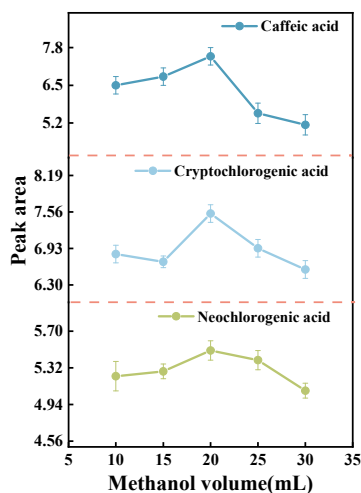


**Figure 3.** Effect of different methanol volumes on extraction efficiency

## 3.2. Selection of Detection Conditions

### 3.2.1. Selection of Column Temperature

Column temperature is an important factor affecting HPLC analysis. In this study, the effects of different column temperatures on polyphenols were investigated (Figure 4.). The results showed that the best response values were obtained when the column temperature was 30 °C, indicating that the temperature was very important for the analysis of these substances. The optimal column temperature reflects the equilibrium of the interaction between the substance and the column at 30 °C, which can not only improve the solubility and volatility of the substance, but also make the separation efficiency higher. This temperature helps maintain the stability and repeatability of the analysis, ensuring accurate and reliable results.

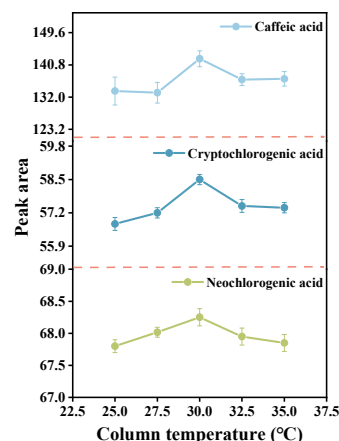


**Figure 4.** Effects of different column temperatures on polyphenols

### 3.2.2. Selection of Velocity

The change of flow velocity can significantly affect the separation degree. In this study, the influence of different flow velocity on the peak type of polyphenols was investigated (Fig. 5). From left to right, cryptochlorogenic acid, cryptochlorogenic acid and caffeic acid were shown in sequence. The results show that the optimal flow rate is 1.0 mL/min based on the separation time, peak location, separation effect and peak shape.

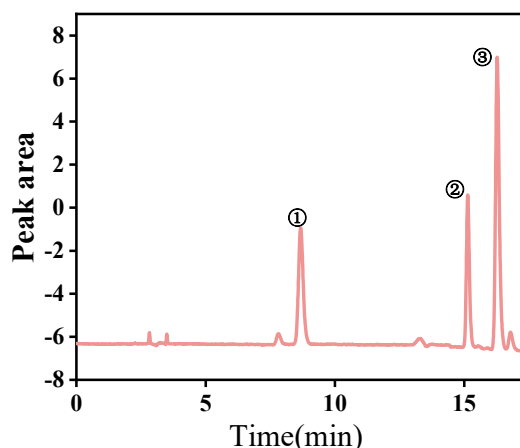


**Figure 5.** 1.0 mL/min mixed standard chromatogram

## 3.3. Methodological Investigation

### 3.3.1. Standard Curve and Detection Limit of the Method

By diluting the mixed standard solution, a series of standard working solutions were prepared, and the series standard solutions of 9 kinds of polyphenols were determined by using the selected conditions of high performance liquid

chromatography (except cryptochlorogenic acid, neochlorogenic acid and caffeic acid, other polyphenols were not detected in the sample), and the regression equation and correlation coefficient were obtained. According to the detection limits and quantitative limits of 3 times SNR and 10 times SNR (Table 2). As can be seen from Table 1, the linear

relationship between the levels of chlorogenic acid, neochlorogenic acid and caffeic acid was good in the range of 0.017~66.67, 0.066~66.67 and 0.033~66.67 mg/L, and the  $R^2$  values were all  $\geq 0.9991$ , indicating a good linear relationship, and the detection limit was 0.017~0.066 mg/L.

**Table 2.** The linear equation and detection limit of the method are presented

| Polyphenols            | Curve equation     | Correlation coefficient | LOD (mg/L) |
|------------------------|--------------------|-------------------------|------------|
| Neochlorogenic acid    | $y=12.744x-9.2358$ | 0.9992                  | 0.017      |
| Cryptochlorogenic acid | $y=8.4837x-2.6726$ | 0.9991                  | 0.066      |
| Caffeic acid           | $y=21.902x-12.316$ | 0.9993                  | 0.033      |

### 3.3.2. Relative Standard Deviation of the Method

To evaluate the reproducibility of the method, the same cigar sample was tested for 8 consecutive times (Table 3). It

can be seen from Tab.2 that the precision (RSD) of each polyphenol content is  $\leq 0.43\%$ . The results show that the precision of the method meets the requirements of analysis and is high.

**Table 3.** Relative standard deviation of the method

| Polyphenols            | Content (mg/L) |      |      |      |      |      |      |      | RSD (%) |
|------------------------|----------------|------|------|------|------|------|------|------|---------|
|                        | 1              | 2    | 3    | 4    | 5    | 6    | 7    | 8    |         |
| Neochlorogenic acid    | 3.26           | 3.26 | 3.24 | 3.22 | 3.26 | 3.24 | 3.24 | 3.25 | 0.43    |
| Cryptochlorogenic acid | 5.30           | 5.29 | 5.30 | 5.28 | 5.27 | 5.28 | 5.27 | 5.28 | 0.27    |
| Caffeic acid           | 1.95           | 1.95 | 1.94 | 1.96 | 1.95 | 1.95 | 1.95 | 1.95 | 0.32    |

### 3.3.3. Recovery of the Method

In order to evaluate the accuracy of the method, 0.5000 g cigar tobacco sample was taken, a certain amount of mixed standard solution was added, and measured in parallel for 3

times (Table 4). The results showed that the sample recovery was 95.13~96.39%. The results showed that the method was accurate and reliable, and could be used for the quantitative analysis of inorganic anions in cigar tobacco leaves.

**Table 4.** Recovery of the method

| Polyphenols            | Added (mg/L) | Detection concentration (mg/L) | Primary concentration (mg/L) | Recovery (%) |
|------------------------|--------------|--------------------------------|------------------------------|--------------|
| Neochlorogenic acid    | 4.641        | 7.842                          | 3.369                        | 96.39        |
| Cryptochlorogenic acid | 7.553        | 12.686                         | 5.598                        | 95.13        |
| Caffeic acid           | 3.131        | 4.660                          | 1.674                        | 95.38        |

## 3.4. Determination of Cigar Tobacco of Different Varieties

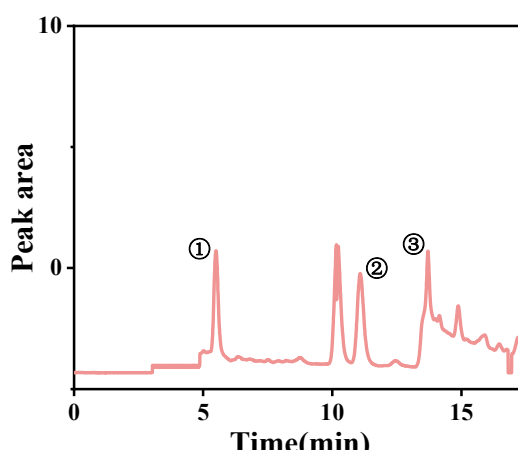


**Figure 6.** Chromatogram of cigar sample No. 4

**Table 5.** Content of polyphenols in different varieties of cigars

| cigars | Neochlorogenic acid<br>(mg/g) | Cryptochlorogenic acid<br>(mg/g) | Caffeic acid<br>(mg/g) |
|--------|-------------------------------|----------------------------------|------------------------|
| 1      | 0.122                         | 0.200                            | 0.073                  |
| 2      | 0.079                         | 0.118                            | 0.063                  |
| 3      | -                             | -                                | 0.069                  |
| 4      | 0.202                         | 0.331                            | 0.070                  |
| 5      | 0.057                         | 0.066                            | 0.050                  |
| 6      | -                             | 0.091                            | 0.069                  |

Caption: “-” Indicates that it is not detected

For 6 kinds of cigar tobacco samples, 0.5000 g was accurately weighed and measured in parallel for 3 times, which was determined by high performance liquid chromatography (HPLC) (Table 5 and Figure 6). The results showed that aesculin, coumaric acid, scopolamine, scopolamine, coumarin and ferulic acid were not detected in 6 cigar leaves. By comparing the content of polyphenols in different cigar leaves, it was found that there were some differences among different cigar leaves. The contents of neochlorogenic acid, cryptochlorogenic acid and caffeic acid were higher in cigar tobacco sample No. 4, and relatively higher in cigar tobacco sample No. 1. No neochlorogenic acid and cryptochlorogenic acid were detected in tobacco leaf sample No. 3. It can be concluded that there are some differences in the content of polyphenols in cigar leaves, which may be related to the variety of tobacco leaves, growing environment and treatment methods.

#### 4. Conclusion

A method for simultaneous and rapid determination of polyphenols in cigar tobacco leaves was developed. The method used ultrasonic extraction to extract the target detection substance. Methanol solution with A volume fraction of 50% was used as the extraction liquid, and mobile phase A was water: methanol: acetic acid (88:10:2, v/v) and mobile phase B was water: methanol: acetic acid (10:88:2, v/v) were used for elution by high performance liquid chromatography. The sample volume was 10  $\mu$ L. At the same time, the sample extraction method, the concentration and dosage of the extraction liquid, the column temperature and the flow rate of the mobile phase were also discussed. The method can be used to quantitatively and qualitatively analyze three kinds of polyphenols in cigar leaves. The results showed that ultrasonic extraction was the best extraction method, extraction time was 30 min, methanol volume concentration of extraction solution was 50%, dosage was 20 mL, column temperature was 30  $^{\circ}$ C, flow rate was 1.0 mL/min, detection wavelength was 340 nm. The contents of neochlorogenic acid, cryptochlorogenic acid and caffeic acid were in the range of 0~0.202 mg/g, 0~0.331 mg/g and 0.050~0.073 mg/g, respectively. The linear relationship between chlorogenic acid, neochlorogenic acid and caffeic acid was good in the range of 0.017~66.67, 0.066~66.67 and 0.033~66.67 mg/L, with  $R^2 \geq 0.9991$ , indicating that the linear correlation of this method was good. The detection limits were 0.017 mg/L,

0.066 mg/L and 0.033 mg/L, respectively. The precision was  $\leq 0.43\%$ , and the recovery was in the range of 95.13%~96.39%. This method has few steps, simple principle, short detection time, accurate results and high sensitivity. It is suitable for simultaneous detection of polyphenols in cigar tobacco leaves, and has a good application prospect.

#### References

- [1] T. J. Zuo. Translated by Z. Q. Zhu, R. S. Sun, J. F. Ha. “Product, physiology and biochemistry of tobacco Shanghai.” Shanghai Far East Press. 1993.
- [2] H.C. Li, S.X. Wang, Tobacco, “Smoking Chemistry and Analysis, Henan Science and Technology Press, Zhengzhou, 1991.
- [3] Shaohao, L. Jianwen, Z. Zhe, Z. Qiu, Z. Ruina, Z. Mingqin, Z. “Effect of temperature on polyphenols metabolism of cigar tobacco leaf during air-curing process.” Journal of Northwest A & F University (Natural Science Edition) 2021, (43). pp. 1-9.
- [4] Marija Banožić, Jurislav Babić, and Stela Jokić. “Recent advances in extraction of bioactive compounds from tobacco industrial waste-a review.” Industrial Crops & Products. 2020 (144). pp. 112009-112009.
- [5] Meiling, X. Xiaohui, H. Yuchuan, S. Youyin, F. Yongpin, L. “Correlation analysis of routine chemical components, polyphenol and sensory quality of cigar germplasm resources.” J. Agric. Sci. Technol. 2019, (21). pp. 124-134.
- [6] Xinda Z, Amrit B, Abdur R, et al. “Screening of Polyphenols in Tobacco (Nicotiana tabacum) and Determination of Their Antioxidant Activity in Different Tobacco Varieties.” ACS omega, 2021, (39). pp. 25361-25371.
- [7] Xuezhi, Z. Chong, T. Nana, Z. Weiye, R. Yi, M. Xin, J. Ying, Z. Shibin, D. Chenjiang, Y. Xiaolei, Y. “Tea polyphenols alleviate high fat and high glucose-induced endothelial hyper-permeability by attenuating ROS production via NADPH oxidase pathway.” BMC Res. Notes. 2014, (7). pp. 1-17.
- [8] Septembre-Malaterre, A. Stanislas, G. Douraguia, E. Gonthier, M. P. “Evaluation of nutritional and antioxidant properties of the tropical fruits banana, litchi, mango, papaya, passion fruit and pineapple cultivated in Reunion French Island.” Food Chemistry. 2016, (212). pp. 225-233.
- [9] Yan, Z. Zhong, Y. Duan, Y. Chen, Q. Li, F. “Antioxidant mechanism of tea polyphenols and its impact on health benefits.” Anim. Nutr. 2020, (6). pp. 115-123.
- [10] Zhong Li, Lan Wang, Guangyu Yang, Honglin Shi, Ciqing Jiang, Wei Liu, Yunhuai Zhang. “Study on the Determination

- of Polyphenols in Tobacco by HPLC Coupled with ESI-MS After Solid-Phase Extraction.” *Journal of Chromatographic Science*, 2003, 41(01). pp. 36-40.
- [11] Xiang G ,Yang L ,Zhang X , et al. “A Comparison of Three Methods of Extraction for the Determination of Polyphenols and Organic Acids in Tobacco by UPLC–MS–MS.” *Chromatographia*, 2009, 70(5-6). pp. 1007-1010.
- [12] Sun, Y. Li, W. Wang, J. Bi, J. Su, S. “Determination of Rutin in Cigarette Tobacco, Filters, Mainstream Smoke and Burned Ash of Different Branded Cigarettes by High Performance Liquid Chromatography.” *Molecules* 2012, 17(4), pp. 3751-3760.
- [13] Xiang G, Yang H, Yang L, et al. “Multivariate statistical analysis of tobacco of different origin, grade and variety according to polyphenols and organic acids. ” *Microchemical Journal*, 2009, 95(2). pp. 198-206.
- [14] Li, F., Liu, Q., Cai, W, et al. “Analysis of Scopoletin and Caffeic Acid in Tobacco by GC–MS After a Rapid Derivatization Procedure. ” *Chromatographia*, 2009, 69(7-8). P p. 743-748.
- [15] Wang J, Lu D, Zhao H, et al. “Discrimination and classification of tobacco wastes by identification and quantification of polyphenols with LC–MS/MS.” *Journal of the Serbian Chemical Society*, 2010, 75(7). p. 875.
- [16] Liu, Hongxia, Guo, Xiaohua, Wu, Jingjie, Liu, Hui, Deng, Benliang and Zhao, Xusheng. "Determination of polyphenols in Chinese jujube using ultra-performance liquid chromatography–mass spectrometry" *Open Chemistry*, vol. 21, no. 1, 2023, pp. 20220305.
- [17] Kukhtenko H, Bevz N, Konechnyi Y, et al. “Spectrophotometric and Chromatographic Assessment of Total Polyphenol and Flavonoid Content in *Rhododendron tomentosum* Extracts and Their Antioxidant and Antimicrobial Activity. ” *Molecules*, 2024, 29(5). p. 1095.