Analysis of Phenolic Compounds in *Nicotiana Tabacum* Based on Aqueous Two-Phase Extraction Coupled with Liquid Chromatography

ABSTRACT

Background: Phenolic compounds in *Nicotiana tabacum* L. have anti-tumor, antibacterial, anti-inflammatory, and other pharmacological effects. Therefore, either to optimize the utilization of plant resources or to reduce the physiological harm caused by cigarette, it is necessary to separate and analyze the phenolic compounds in *Nicotiana tabacum.*
Aim: To develop a protocol for extraction and analysis of phenolic compounds in *Nicotiana tabacum*. The method based on aqueous two-phase extraction (ATP) should provide a solid experimental basis for future large-scale medical industrial extraction.
Results: Five of phenolic compounds in *Nicotiana tabacum* including catechol, caffeic acid, coumarin, rutin, and quercetin were extracted with the developed ATP method and analyzed by the optimized liquid chromatography method simultaneously. In the concentration range of 10-200 μg/mL, the linear correlation coefficients of calibration curve for five compounds were all greater than 0.999. The developed ATP extraction method employed 70% acetonitrile water solution as the extractant. The ammonium sulphate was selected as the preferred phase separation salt. The extraction efficiencies of five phenolic compounds were all more than 90%. In concrete sample, the concentrations of five compounds were determined based on acetonitrile/salt ATPE system,
Conclusion: Analysis of phenolic compounds in *Nicotiana tabacum* was achieved based on acetonitrile/ammonium sulphate ATP extraction coupled with liquid chromatography. The developed ATP extraction protocol employed 70% acetonitrile water solution as the extractant. The ammonium sulphate was selected as the preferred phase separation salt. The accuracy and precision were satisfied. The protocol developed in this study is expected to be applied in medical industry.

Keywords: acetonitrile; ammonium sulphate; aqueous two-phase extraction; Nicotiana tabacum; phase separation salt; phenolic compounds.

1. INTRODUCTION

Nicotiana tabacum (tobacco) is a perennial herbaceous plant. It is widely used as a natural product [1] in addition to the principal source of nicotine. *Nicotiana tabacum* L. contains not only nicotine, which can treat muscle spasms and joint pain, but also phenolic compounds [2]. A variety of phenolic compounds were detected in *Nicotiana tabacum* L. and its smoke, such as chlorogenic acid, rutin, quercetin, coumarin, caffeic acid, etc. [3] In flue-cured tobacco, the content of chlorogenic acid and rutin accounted for 75-95 % of total phenols [4].

Studies have shown that chlorogenic acid and rutin have certain curative effects in anti-cancer and anti-HIV [5]. Other phenols such as quercetin [6], coumarin [7], caffeic acid [8] have been reported to have anticancer, antibacterial, anti-inflammatory, anti-tumor and other effects. Therefore, either to optimize the utilization of plant resources and flue-cured

tobacco, or to reduce the physiological harm caused by cigarette, it is necessary to separate and analyze the phenolic compounds in *Nicotiana tabacum*.

At present, many extraction methods of phenolic compounds in *Nicotiana tabacum* L. have been reported. Among them, the commonly used methods are microwave extraction technique and ultrasonic extraction technique [9,10]. However, these technologies are mainly used as pretreatment methods for the analysis of phenolic compounds, rather than for the industrial extraction of phenolic compounds. As for supercritical water or CO2 extraction technique for industrial extraction, although the extraction effect is good, it usually requires expensive special equipment [11]. The complex extraction process limits the large-scale application of supercritical fluid extraction.

In recent years, one of the industrial-scale extraction methods, aqueous two-phase (ATP) extraction is the increasing interest [12,13]. ATP system is an aqueous liquid–liquid system that results from the incompatibility between two aqueous solutions. Because of the different characteristics of the two phases, the concentration of the target substance in the upper and lower phases is also different. The different properties of different phase could be attributed to surface properties, charge effects and various forces such as hydrophobic bonds, hydrogen bonds and ionic bonds, etc. As the liquid-liquid two phases are separated, the target substance enriched in one phase. Thereby, the extraction is realized. ATP process shows great potential in the extraction or purification of biological compounds. [14].

Aqueous two-phase extraction (ATPE) technique has been applied to the efficient crude separation of various natural products. For example, it was reported that the nicotine recovery rate reached 96.1% when an aqueous two-phase system was applied [15]. ATPE technique was also employed to separate β-glucuronidase (rGUS), an acidic recombinant protein from transgenic tobacco [16]. An aqueous two-phase partitioning system was developed for therapeutic antibodies and enzymes from tobacco extracts [17]. The ATPE was used to purify antibodies directly from cell culture medium [18] as well as protein, virus-like particles and plasmid DNA [19,20]. Based on these applications, ATPE is not only simple and environmentally friendly, but also can effectively improve the extraction rate of target compounds.

The development of aqueous two-phase system to extract polyphenols from *Nicotiana tabacum* can provide a technical basis for the comprehensive development of *Nicotiana tabacum*. Hitherto, few applications of ATPE technique for the extraction of phenolic substances in *Nicotiana tabacum* were reported. Further, to the best of our knowledge, there is not aqueous two-phase system has been applied to the extraction and separation of *Nicotiana tabacum* polyphenols.

In this paper, five kinds of easily available, high content and stable phenols, including catechol ($C_6H_6O_2$) [21,22], caffeic acid ($C_9H_8O_4$) [23], coumarin ($C_9H_6O_2$) [24], rutin ($C_{27}H_{30}O_{16}$) [25] and quercetin ($C_{15}H_{10}O_7$) [26] were selected as the target compounds. The extract efficient of them were studied. The results showed that the protocol developed in this study, based on acetonitrile/salt ATPE system, combined with high performance liquid chromatography, efficiently extracted and analyzed five target phenolic compounds in *Nicotiana tabacum* Leaves. In the aqueous two-phase system, factors such as salt type and acetonitrile concentration were systematically investigated. The analysis results of actual *Nicotiana tabacum* samples showed that the method developed in this study was accurate and reliable, which provided a solid experimental basis for future large-scale industrial extraction.

2. MATERIAL AND METHODS

2.1 Chemicals and reagents

Standards of caffeic acid (≥99.0%), rutin (≥99.5%), coumarin (≥99.6%), quercetin (≥95.0%), and catechol (≥97.5%) were purchased from China National Institutes for Food and Drug Control.

HPLC grade methanol and acetonitrile were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). Water was purified using a Milli-Q system (Molsheim, France). HPLC grade acetic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Analytical grade reagents including potassium chloride, sodium chloride, sodium dihydrogen phosphate, and ammonium sulfate were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The *Nicotiana tabacum* leaves were collected from Qingyu, Guizhou province, China.

2.2 Sample preparation

The stock solutions of caffeic acid, rutin, coumarin, quercetin, and catechol were prepared with acetonitrile at a series of concentrations of 1200 μg/mL, 800 μg/mL, 800 μg/mL, 660 μg/mL, and 800 μg/mL. The working solutions were then diluted from the stock solution with acetonitrile serially according to the experiment procedure.

For the linear range investigation, each stock solution was diluted to a series of working solutions at the concentration of 10 μg/mL, 50 μg/mL, 100 μg/mL, 150 μg/mL, and 200 μg/mL.

For concrete sample preparation, the *Nicotiana tabacum* leaves were dried and crushed. 1.2 g powder of leaves were then carefully weighted and transferred to a 50 mL centrifuge tube. 10 mL extractant (acetonitrile/water solution) was added. Further three drops of acetic acid were added to inhibit the dissociation of the phenolic compounds. After one-hour ultrasonic bath followed by centrifugation for 5 minutes, the supernatant was transferred to a 10 mL tube with plug. The supernatant was then extracted with aqueous two-phase procedure and introduced to the chromatographic system [27].

2.3 Instruments

The HPLC system model in Shimadzu LC-10AD (Japan) consisted of a vacuum degasser, a binary gradient pump, an autosampler, a column thermostat, and an UV detector. Analytical balance (SartoriusBSA224S, China) and ultrasonic cleaner (KQ5200B, China) were used for sample preparation.

2.4 HPLC condition

Separation occurred on a Nova-Pak C18 column (3.9×150 mm, 5 μm). The acetic acid/water solution (0.5%, v/v) was used as mobile phase A for separation condition optimization. The mobile phase B was acetonitrile. The total flow rate was set at 1.0 mL/min. The sampling volume was 5.0 μL without further note. The ratio of phase A to phase B was adjusted according to the optimization progress. The column temperature was 35 °C. The wavelength of detector was set at 280 nm.

2.5 Optimization of ATP extract

To investigate the effect of phase separation salt, 5 model compounds were prepared, extracted and analyzed. For each compound, it was prepared at the concentration of 400 μg/mL. The solvent utilized was the acetonitrile/water (7:3, v/v) solution containing 1%(v/v) acetic acid. The ATP extraction was then carried out. 5 mL model solution was transferred to

a tube with plug. Excess sodium chloride was then added to conduct the phases separation. After phase separation, the volumes of upper and lower phases were measured. The upper acetonitrile phase was delivered to HPLC to get analysis. Then the ATP extraction process was repeated with salts of ammonium sulfate, potassium chloride, and sodium dihydrogen phosphate respectively. All experiments were performed in triplicate.

To investigate the effect of acetonitrile, the extractant containing 40%, 50%, 60%, 70%, and 80% acetonitrile were employed to prepare the sample solution respectively. For each model compound, the concentration in sample solution was 400 μg/mL. During investigation, the ammonium sulfate was utilized as the salt. The ATP extraction and HPLC analysis were conducted with the same procedure.

2.6 Standard addition recovery

The standards of caffeic acid, rutin, coumarin, quercetin, and catechol were added into 1.0 g powder of *Nicotiana tabacum* leave simultaneously. The amount of each standard added was 3 mg. The sample was introduced to 10 mL extractant (70% acetonitrile, 1% acetic acid) and then processed with 1 h ultrasonic bath and 5 min centrifugation. Afterward, the ATP extraction was carried out with the salt of ammonium sulfate. The HPLC analysis were conducted with the optimized method.

2.7 Extraction efficiency

The extraction efficiency (*E%*) of ATP extraction was calculated as follows

$$E\% = \frac{V_t C_t}{V_o C_o} \times 100\% \quad (1)$$

Where, V_t is the volume of upper phase (acetonitrile) in the proposed ATP system. C_t is the concentration of target compound in the upper phase. V_o is the volume of sample solution submitted to ATP extraction process. C_o is the concentration of target compound in the sample solution.

3. RESULTS AND DISCUSSION

3.1 Chromatography optimization

Phenolic compounds in *Nicotiana tabacum* were extracted simultaneously. Hence, their baseline separation on chromatography was necessary for quantification and qualification. The chromatography optimization of five model compounds was realized in two steps. The first step was the empirical method, and then the condition optimization was carried out according to the results. The second step was to verify the optimized method.

The empirical chromatography was carried out with a binary mobile phase at a flow rate of 1.0 mL/min. Eluent A was water containing 0.2% acetic acid (*v/v*). Eluent B was acetonitrile. The gradient program started at the eluent ratio of 30% B and maintained for 6 minutes. The ratio of elution B increased to 40% linearly within 6-10 min and then increased to 90% linearly during 10-14 min. Afterward, the ratio increased to 100% within 14-17 min and maintained until 20 min.

With this empirical method, the chromatographic spectrum of the mixed standard samples of five phenolic compounds is shown in Figure 1(A).

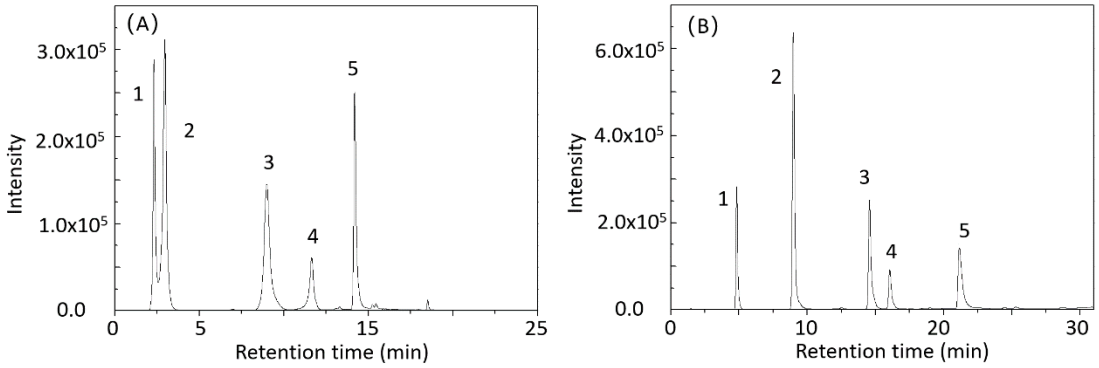

Fig. 1. The chromatogram of five standards with empirical LC method (A) and optimized LC method (B)

Peak 1: Catechol; 2: caffeic acid; 3: coumarin; 4: rutin; 5: quercetin

It can be found that two components of catechol (peak 1) and caffeic acid (peak 2) have not been separated completely. The peak of coumarin (peak 3) was broadened obviously. In order to solve the above problems, one means was to reduce the ratio of organic phase in the initial conditions of gradient program. The retention of caffeic acid could be enhanced with the lower organic mobile phase. Consequently, the separation of catechol and caffeic acid could be achieved.

The broaden of coumarin may attributed to non-specific interaction between coumarin molecule and silica gel of stationary phase in chromatography column. Base on this hypothesis, increasing the concentration of acetic acid in the mobile phase may reduce the peak broadening of coumarin. Acetic acid releases hydrogen ions to inhibit the dissociation of the phenolic compounds. Along with inhibition, the non-specific interaction of phenolic compounds and silica gel decreases.

Therefore, in the optimized method, the concentration of acetic acid in the eluent A was increased to 0.5%. The beginning ratio of eluent B was decreased to 15% and maintained for 2 minutes. Within 2-6 min, the ratio of B increased to 30% linearly. Afterward, the ratio increased linearly to 70% during 6-25 min and then to 90% during 25-28 min. Within 28-30 min, the ratio maintained.

With the optimized method, the mixed standard samples of five phenolic compounds were analyzed. The result chromatogram was shown in Figure 1(B). The five phenolic compounds were completely separated and each peak shape was symmetrical enough. The chromatographic analysis was completed within 30 min. The time consumption was relatively reasonable. All subsequent analyses were performed using the optimized mobile phase and gradient.

3.2 Method validation

The linear ranges of the method for five phenols were investigated. Each target compound was tested separately.

The relationship between the peak area and the sample concentration was calculated. The linear standard curves of the five substances were fitted. The respective linear regression equations are included in table 1. The results showed that under the optimized chromatographic conditions, the five substances all had good correlation in the proposed

concentration range10-200 μg/mL. The linear correlation coefficients (R^2) of each calibration curve were all greater than 0.999.

Table 1. Linear range and correlation coefficient of five phenolic compounds

Compound	Regression equation	Linear range (μg/mL)	R^2
catechol	*y* =5150.2 *x* - 21139	10-200	0.9998
caffeic acid	*y* =136022 *x* - 285526	10-200	0.9991
coumarin	*y* =5738.5 *x* - 22251	10-200	0.9997
rutin	*y* =2506.5 *x* - 91321	10-200	0.9997
quercetin	*y* =6545.5 *x* - 184587	10-200	0.9992

3.3 ATP system optimization

Phase separation salt is one of the important factors due to salting out effect of ATPE. Naturally, in this acetonitrile-water aqueous two-phase system, different salts have different phase separation ability. It will eventually lead to the different extraction efficiency

The extraction efficiency of five target phenolic compounds were investigated with 4 kinds of salt. Four salts were ammonium sulfate, sodium dihydrogen phosphate, sodium chloride and potassium chloride respectively. The extractions were performed with extractant that containing 70% acetonitrile. All extracts were analyzed with the same chromatographic program. The results were compared in Figure 2.

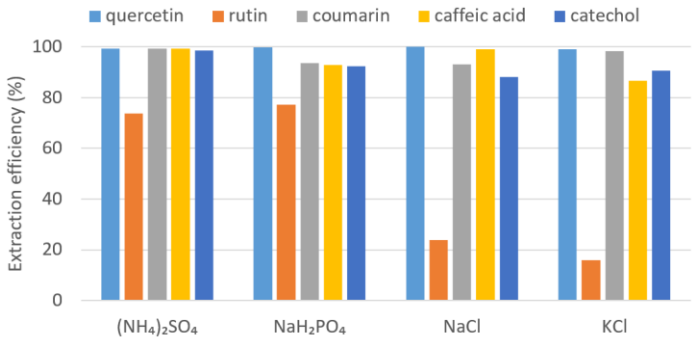

Fig. 2. Extraction efficiency with different salts.

The minimum extraction efficiency of rutin was observed when potassium chloride was applied. The maximum efficiency of rutin was achieved with the sodium dihydrogen phosphonate. The efficiency of caffeic acid was also the smallest when potassium chloride was applied. However, even with potassium chloride, the extraction efficiency of catechol, coumarin and quercetin were all more than 90%.

Among four salts, when ammonium sulfate or sodium dihydrogen phosphate was applied, the extraction efficiency of rutin was both greater than 70%. The extraction efficiency of other 4 target compounds were all greater than 90%. It inferred that the ammonium sulfate or sodium dihydrogen phosphate could be the preferred separation salt. The efficiency values of them are listed in table 2.

Table 2. Extraction efficiency (%) of ammonium sulfate and sodium dihydrogen phosphate

salt	catechol	caffeic acid	coumarin	rutin	quercetin
ammonium sulfate	98.61	99.22	99.4	73.81	99.36
sodium dihydrogen phosphate	92.37	92.75	93.68	77.34	99.72

It can be found that the effect of ammonium sulfate is better than that of sodium dihydrogen phosphate. In ammonium sulfate, the extraction efficiency of catechol, caffeic acid, coumarin and quercetin were 98.61 %, 99.22 %, 99.40 % and 99.36 %, respectively. The rutin extraction efficiency with sodium dihydrogen phosphate was greater than with ammonium sulfate. It is probably that sodium dihydrogen phosphate has a greater hydration capacity, which reduces the dissolution of rutin in salt water (lower phase of ATP system) and pushes rutin molecule into the organic phase. Nevertheless, the extraction efficiency of 73.81% with ammonium sulfate was satisfied. The following experiments were carried out with ammonium sulfate.

The effect of acetonitrile concentration in the extractant was investigated. The concentrations of five phenolic compounds in the upper phase (acetonitrile phase) were determined and the extraction efficiency were calculated accordingly. All results are listed in table 3.

Table 3. Extraction efficiency (*E%*) of five phenolic compounds from different acetonitrile concentration extractant

C_{ACN}	catechol	caffeic acid	coumarin	rutin	quercetin
40%	99.82	91.83	94.2	57.37	97.5
50%	97.95	98.34	98.33	69.98	95.19
60%	99.87	99.58	97.27	73.16	94.98
70%	98.61	99.22	99.40	73.81	99.36
80%	93.03	89.97	96.06	86.93	92.54

The concentration of acetonitrile showed little effect on catechol, caffeic acid, coumarin, and quercetin. The extraction efficiency of them kept at a high value and did not show an obvious trend. When the concentration of acetonitrile exceeds 70%, the extraction efficiency of them decreased obviously. The extraction efficiency of rutin was most affected by the variation of acetonitrile concentration. The value increased along with the increase of acetonitrile concentration continually.

Considering all five phenolic compounds, when the acetonitrile concentration is 70%, each substance can obtain an extraction efficiency more than 90% except rutin. Therefore, 70% acetonitrile was used as the optimized extraction condition for the following experiments.

3.4 Concrete sample and Recovery

The chromatogram of concrete sample that extracted with the developed aqueous two-phase method is shown in Figure 3(A).

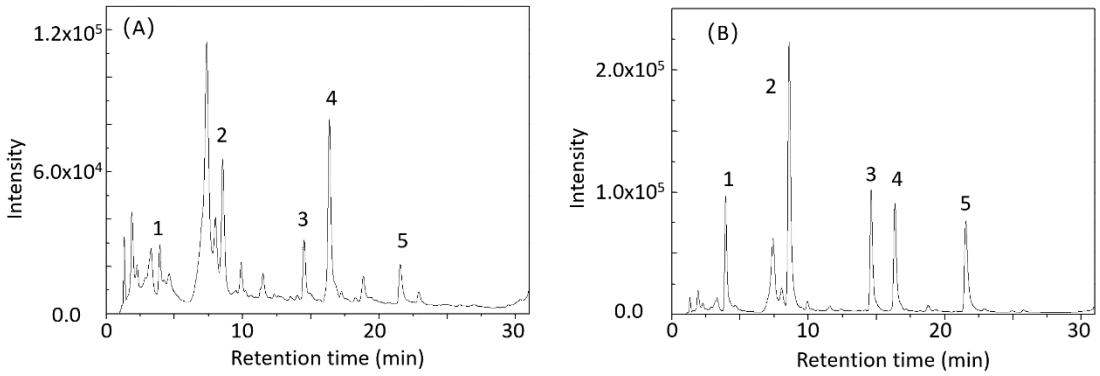

Fig. 3. Chromatogram of concrete sample(A) and standards added sample (B)
Peak 1: Catechol; 2: caffeic acid; 3: coumarin; 4: rutin; 5: quercetin

Five target compounds were detected and labeled as peaks 1, 2, 3, 4 and 5 respectively. The concentrations of catechol, caffeic acid, coumarin, rutin, and quercetin in the provided *Nicotiana tabacum* leaves were determined to be 0.54 mg/g, 1.1060 mg/g, 1.5720 mg/g, 4.540 mg/g, and 1.1876 mg/g, respectively.

The accuracy of the developed method was tested by the recovery of standard addition. 3 mg of each of the five reference materials were added to the concrete sample. The standard added samples were extracted with ATPE and analyzed by the optimized chromatographic method. The typical spectrum is shown in Figure 3(B). By comparing Figure 3(A) and Figure 3(B), the specificities of the five target phenolic compounds in the concrete sample were all confirmed. The calculated recoveries are listed in table 4.

Table 4. The recovery of the five substances with the ATP extraction

	catechol	caffeic acid	coumarin	rutin	quercetin
recovery	97.6%	98.8%	101.6%	97%	100.9%
RSD (%)	2.4	1.5	1.7	1.2	1.2

The good accuracy and precision were observed. The recovery of the five substances ranged from 97 to 101.6%. The relative standard deviation (RSD) was all less than 2.5% (n = 3).

4. CONCLUSION

The developed aqueous two-phase (ATP) extraction protocol employed 70% acetonitrile water solution as the extractant. The ammonium sulphate was selected as the preferred phase separation salt. Five phenolic compounds in Nicotiana tabacum leaves were extracted with the developed aqueous two-phase method and analyzed by the optimized LC method simultaneously. In LC process, the mobile phase modifier acetic acid was increased to 0.5% to inhibit the dissociation of the target phenolic compounds. Thereby, five phenolic

compounds were completely separated and each peak shape was symmetrical enough. The chromatographic analysis was completed within 30 min. The linear correlation coefficients (R^2) of calibration curve for 5 substances were all greater than 0.999 within the range of 10-200 μg/mL. In model concrete sample, the concentrations of catechol, caffeic acid, coumarin, rutin, and quercetin were determined to be 0.54 mg/g, 1.1060 mg/g, 1.5720 mg/g, 4.540 mg/g, and 1.1876 mg/g, respectively. The recovery of five substances ranged from 97 to 101.6%. The RSD of the developed method was less than 2.5% (n = 3). The accuracy and precision were satisfied.

ACKNOWLEDGEMENTS

All authors have made substantive contribution to this study and/or manuscript, and all have reviewed the final paper prior to its submission.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

Hua Wang: Conceptualization, methodology, resources, writing-original draft preparation, writing-reviewing and editing. Lina Zeng: Investigation, visualization, and resources. Hui Ding: Writing-reviewing, validation and supervision. All authors read and approved the final manuscript.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

REFERENCES

1. Hilly M, Adams ML, Nelson SC. A study of digit fusion in the mouse embryo. Clin Exp Allergy. 2002;32(4):489-98.
2. Schnablová R, Synková H, Vičánková A, Burketová L, Eder J, Cvikrová M, Transgenic ipt tobacco overproducing cytokinins overaccumulates phenolic compounds during in vitro growth. Plant Physiol. Biochem. 2006, 44, 526–534.
3. McGrath TE, Brown AP, Meruva, NK, Chan WG, Phenolic compound formation from the low temperature pyrolysis of tobacco. J. Anal. Appl. Pyrolysis 2009, 84, 170–178.
4. Hong QY, Ji HZ, Yan S, Yong PL, Ze L, Analysis on the Content of Chlorogenic Acid and Rutin in the Flue-cured Tobacco Leaves of Different Producing Areas. Nat. Prod. Res. Dev. 2006, 18, 670–673.
5. Huang S, Wang LL, Xue NN, Li C, Guo HH, Ren TK, Zhan Y, Li WB, Zhang J, Chen XG, Han YX, Zhang JL, Jiang JD, Chlorogenic acid effectively treats cancers through induction of cancer cell differentiation. Theranostics 2019, 9, 6745–6763.
6. Formica JV, Regelson W, Review of the biology of quercetin and related bioflavonoids. Food Chem. Toxicol. 1995, 33, 1061–1080.
7. Küpeli AE, Genç Y, Karpuz B, Sobarzo-Sánchez E, Capasso R, Coumarins and Coumarin-Related Compounds in Pharmacotherapy of Cancer. Cancers (Basel). 2020, 12, 1959.
8. Espíndola KMM, Ferreira RG, Narvaez LE. M, Silva RACR, da Silva AHM, Silva AGB, Vieira APO, Monteiro MC, Chemical and Pharmacological Aspects of Caffeic Acid and Its Activity in Hepatocarcinoma. Front. Oncol. 2019, 9, DOI: 10.3389/fonc.2019.00541.

9. Banožić M, Banjari I, Jakovljević M, Šubarić D, Tomas S, Babić J, Jokić S, Optimization of Ultrasound-Assisted Extraction of Some Bioactive Compounds from Tobacco Waste. Molecules 2019, 24, 1611.
10. Banožić M, Babić J, Jokić S, Recent advances in extraction of bioactive compounds from tobacco industrial waste-a review. Ind. Crops Prod. 2020, 144, 112009.
11. Jokić S, Gagić T, Knez Ž, Banožić M, Škerget M, Separation of active compounds from tobacco waste using subcritical water extraction. J. Supercrit. Fluids 2019, 153, 104593.
12. Wang B, Ezejias T, Feng H, Blaschek H, Sugaring-out: A novel phase separation and extraction system. Chem. Eng. Sci. 2008, 63, 2595–2600.
13. Yin Y, Jia J, Wang T, Wang C, Optimization of natural anthocyanin efficient extracting from purple sweet potato for silk fabric dyeing. J. Clean. Prod. 2017, 149, 673–679.
14. Aguilar O, Rito-Palomares M, Aqueous two-phase systems strategies for the recovery and characterization of biological products from plants. J. Sci. Food Agric. 2010, 90, 1385–1392.
15. Xing J, Li F, Ping J, Recovery and Purification of Nicotine from Waste Tobacco by Aqueous Two-Phase System/Reverse Extraction. Nat. Prod. Commun. 2009, 4, 1934578X0900400.
16. Ross KC, Zhang C, Separation of recombinant β-glucuronidase from transgenic tobacco by aqueous two-phase extraction. Biochem. Eng. J. 2010, 49, 343–350.
17. Platis D, Labrou NE, Development of an aqueous two-phase partitioning system for fractionating therapeutic proteins from tobacco extract. J. Chromatogr. A 2006, 1128, 114–124.
18. Silva MFF, Fernandes PA, Aires BMR, Azevedo AM, Integrated purification of monoclonal antibodies directly from cell culture medium with aqueous two-phase systems. Sep. Purif. Technol. 2014, 132, 330–335.
19. Nascimento KS, Rosa PAJ, Nascimento KS, Cavada BS, Azevedo AM, Aires BMR, Partitioning and recovery of Canavalia brasiliensis lectin by aqueous two-phase systems using design of experiments methodology. Sep. Purif. Technol. 2010, 75, 48–54.
20. Azevedo AM, Rosa PAJ, Ferreira IF, Pisco AMMO, de Vries J, Korporaal R, Visser TJ, Aires BMR, Affinity-enhanced purification of human antibodies by aqueous two-phase extraction. Sep. Purif. Technol. 2009, 65, 31–39.
21. REN Z, AI X, HUANG Z, ZHANG Y, WANG Z, ZHANG F, Determination of catechol in tobacco by high performance liquid chromatography-tandem mass spectrometry. Chinese J. Chromatogr. 2015, 33, 541.
22. Wornat MJ, Ledesma EB, Marsh ND, Polycyclic aromatic hydrocarbons from the pyrolysis of catechol (ortho-dihydroxybenzene), a model fuel representative of entities in tobacco, coal, and lignin. Fuel 2001, 80, 1711–1726.
23. Li S, Pi J, Zhu H, Yang L, Zhang X, Ding W, Caffeic Acid in Tobacco Root Exudate Defends Tobacco Plants From Infection by Ralstonia solanacearum. Front. Plant Sci. 2021, 12, DOI: 10.3389/fpls.2021.690586.
24. McAdam K, Enos T, Goss C, Kimpton H, Faizi A, Edwards S, Wright C, Porter A, Rodu B, Analysis of coumarin and angelica lactones in smokeless tobacco products. Chem. Cent. J. 2018, 12, 142.
25. Gullón B, Lú CTA, Moreira MT, Lema JM, Eibes G, Rutin: A review on extraction, identification and purification methods, biological activities and approaches to enhance its bioavailability. Trends Food Sci. Technol. 2017, 67, 220–235.
26. da Silva ANP, de Matos NA, Leticia AMS, Farias de SAB, Dantas CS, Cunha A, Silva BF, Quercetin Attenuates Acute Lung Injury Caused by Cigarette Smoke Both In Vitro and In Vivo. COPD J. Chronic Obstr. Pulm. Dis. 2020, 17, 205–214.
27. Božinović M, Vičević R, Zekić N, Šalić A, Jurinjak TA, Zelić B, Intensification of endo-1,4-Xylanase Extraction by Coupling Microextractors and Aqueous Two-Phase System, Processes. 2023, 11, 447.