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Fingerprint Chromatogram Identification of Agricultural Products Based on High Performance Liquid Chromatography

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Based on high performance liquid chromatography (HPLC), this paper studies the fingerprint chromatogram analysis and identification method of tea leaves and amanita phalloides toxins, by using chromatography and principal component analysis (PCA), it provides a new and effective method for tea leaves quality identification and mushroom toxin identification. The results show that the correlation coefficient, included angle cosine and overlap rate of PCA can be used to characterize the similarity of tea leaves fingerprint chromatogram, and the calculation of the fingerprint chromatogram using the included angle cosine is relatively more accurate. The main components of tea leaves are PC1-PC7, the cumulative contribution rate reaches 87.49%. The use of hierarchical clustering method and two-dimensional sorting method can distinguish different types of tea leaves, establish the accurate mass number and isotopic characteristics of four types of toxin molecules. At the same time, the corresponding secondary MS features and daughter ion information were established. The recovery rate of the four toxins ranged from 69.4% to 89.3% with standard deviations between 7.1% and 14.2 %. The method established in this paper has the advantage of rapid and accurate detection of toxins in poisonous mushrooms.

1. Introduction

Bioinformatics is a rapid developing life-research science in recent years, its analysis, identification and storage of biological information by computer and network are the major frontier subjects in life science today. The identification of agricultural products is one of the branches in bioinformatics (Bing et al., 2012; Carvalho et al., 2005). Its main methods include high performance liquid chromatography (HPLC), infrared spectroscopy (IR), gas chromatography (GC) and fingerprint chromatogram method (Chen et al., 2007; Cui et al., 2005; Elizarova et al., 2011), etc.

Based on high performance liquid chromatography (HPLC), this paper studies the fingerprint chromatogram analysis and identification method of tea leaves and amanita phalloides toxins, by using chromatography and principal component analysis (PCA), it provides a new and effective method for tea leaves quality identification and mushroom toxin identification (Das et al., 2017).

2. Experimental materials and methods

2.1 Equipment and reagents

Experimental equipment: Synapt HPLC-MS spectrometer; Masslynx software; UPLC column; HLB solid phase column; Solid Phase Extracter; UV detector.

Chromatography-MS conditions: column (tea): 4.5mm×250mm, column (poisonous mushroom): 55mm×2.2mm; mobile phase (tea): 0.03% TFA (trifluoroacetic acid)/10% Hexane nitrile;): 2mmo/L ammonium acetate solution/ammonium acetate - methanol solution; test wavelength 280nm; electrospray scan range: 200-1000m/z; desolvation temperature: 320□; extraction voltage: 4.5V; Next injection needs to be conducted 20min after current injection.

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2.2 Samples and pretreatment

The tea leaves are selected from Yunnan Province, China, according to different grades, the tea leaves are divided into 18 batches. In the test, take 1.5g tea leaves from each batch, and added them into 120mL75% methanol, filtered 3-5 times with test filter papers, wash the residue, and keep them for further use in 250ml constant volume. Five kinds of catechins such as catechin, caffeine and epigallocatechin (EGC) were selected as the control standard test solution. Each sample was injected 5 times repeatedly with the injection times set at 0, 3, 6, 9 and 12 hours respectively. Study the stability, repeatability and precision of the chromatographic method, and the relative area S_r of each peak in the chromatography can be expressed as:

$$S_r = \frac{RA_i}{ST} \times 100\% \tag{1}$$

RA_i is the relative area of the i-th peak spectrum; ST is the total area of the sample.
Toadstool (aka poisonous mushroom) samples were pretreated, dried at room temperature and grinded. Take 0.2g grinded powder in 10mL centrifuge tube, added 6mL methanol solution (containing 0.1% TFA), centrifuged for 15min and took the supernatant, placed the supernatant in another tube, again, added 6mL methanol solution to conduct extraction and obtained the final toxin solution. Then the extract was purified by HLB solid phase column, rinsed with chloroform and eluted with methanol.

3. Test results and analysis

3.1 Identification and Analysis of fingerprint chromatogram of Pu'er Qingmao Tea

The standard solution and the selected 18 varieties of samples were chromatographed to obtain a standard chromatogram as shown in Figure 1, the abscissa is the chromatographic peak appearance time, and the ordinate is the corresponding peak response value.

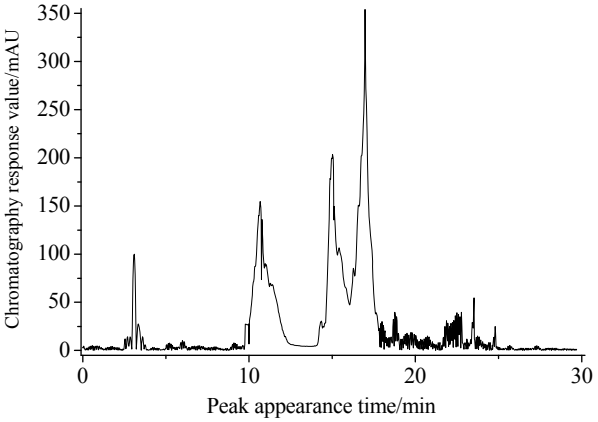

Figure 1: Standard fingerprint diagram of tea samples

Similarity analysis of extracted fingerprint chromatogram is conducted. The similarity calculation of medicines and health products mainly includes three indexes: correlation coefficient, included angle cosine and overlap rate of medicines. The expression of correlation coefficient ρ_r is

$$\rho_r = \frac{\sum_{i=1}^{n}\left(X_i - X_i^c\right)\left(Y_i - Y_i^c\right)}{\sqrt{\sum_{i=1}^{n}\left(\left(X_i - X_i^c\right)^2\left(Y_i - Y_i^c\right)^2\right)}} \tag{2}$$

n is the sample type; X_i^c and Y_i^c are the values of the variables in the control sample, respectively. ρ_r can be used to determine the relationship between the different attributes in the medicine. The vector included angle cosine is cos*(θ)*:

$$\cos(\theta) = \frac{\sum_{i=1}^{n} X_i Y_i}{\sqrt{\sum_{i=1}^{n} X_i^2} \sqrt{\sum_{i=1}^{n} Y_i^2}} \tag{3}$$

X_i is the chromatographic peak area or peak height of the i-th sample within the retention time; Y_i is the chromatographic average peak area or peak height within the retention time of the sample. cos*(θ)* can convert the data in fingerprint chromatogram into a multidimensional space problem, which is easier to calculate. The standard fingerprint chromatogram can choose the common mode for the selection and determination of the spectral data, that is to determine the average mass V_{mean} and the median vector V_{med}

$$V_{mean} = \sum \left[\left(X_{1j}, X_{2j}, ... X_{nj} \right) / n \right] \tag{4}$$

$$V_{med} = Median \left(X_{1j}, X_{2j}, ... X_{nj} \right) \tag{5}$$

The overlap rate of the fingerprint chromatogram is expressed by Formula 6 as:

$$Y_{or} = \frac{2 \times P_{common}}{P_{sam} + P_{sta}} \times 100\% \tag{6}$$

P_{common} is the common peak number of samples and standards; P_{sam} is the peak number of the sample to be measured; P_{sta} is the peak number of the standards. According to Formula 2-6, the ρ_r, cos*(θ)* and Y_{or} values of the 18 selected samples are shown in Table 1.

Table 1: Similarity index of tea samples originated from different area

Sample number	cos(θ)	ρ_r	Y_{or}
1	0.994	0.991	1
2	0.993	0.989	1
3	0.995	0.982	1
4	0.983	0.983	1
5	0.996	0.997	1
6	0.992	1	1
7	0.990	0.993	1
8	0.982	0.972	1
9	0.991	0.983	1
10	0.993	0.980	1
11	0.990	0.980	1
12	0.990	0.995	1
13	0.994	0.988	1
14	0.997	0.991	1
15	0.987	0.986	1
16	0.962	0.967	1
17	0.789	0.802	0.68
18	0.817	0.885	0.63

As can be seen from Table 1, the obtained 18 cultivars of tea leaves based on evaluation indexes of ρr, cos(θ) and Yor can well express the similarities among different fingerprint chromatograms, and we can better distinguish the sun-dried Qingmao tea and green tea from the 18 cultivars of tea. Compared with the overlap rate, ρr and cos(θ) can better reflect the similarity between different peaks, the calculation results are more accurate, but Yor cannot measure the peak area of the chromatogram, so the calculation results are not representative.

The principal component analysis (PCA) of 18 varieties of tea is further carried out. The PCA maps the sample data in high-dimensional space to low-dimensional space and retains most of the original data. In the process of mapping, select proper comprehensive indicators to reflect the original information. Using DPS software to calculate the contribution rate and cumulative contribution rate of the samples of 18 tea varieties, the results are shown in Table 2. PC1-PC14 represents the main component of tea leaves.

Table 2: Contribution rate and cumulative contribution rate of tea samples

Principal component	Contribution rate/%	Cumulative Contribution rate/%	Principal component	Contribution rate/%	Cumulative Contribution rate/%
PC1	37.16	35.48	PC8	2.95	90.28
PC2	16.25	50.49	PC9	2.13	93.48
PC3	11.74	65.17	PC10	1.51	95.17
PC4	8.46	73.83	PC11	1.43	96.98
PC5	6.98	76.89	PC12	1.35	97.43
PC6	5.39	83.18	PC13	1.06	98.62
PC7	4.05	87.49	PC14	0.64	99.17

Because there are many components in the tea leaves, there are also many factors that affect the quality and grade of different tea cultivars. From the table we can see that, the contribution rate of components PC1 to PC7 are all more than 4%, they are respectively 37.16%, 16.25%, 11.74%, 8.46%, 6.98%, 5.39% and 4.05%, and the cumulative contribution rate reaches 87.49 %, they are the main component of the tea leaves. Therefore, when analyzing, we can get the information of each indicator by taking the main components PC1-PC7. The contribution rate of PC1 reaches 37.16%, which mainly reflectes the information of peak No.9, No.16, and No.17 of the original fingerprint chromatogram; and PC2 reflects the information of peak No. 5 and No.11.

3.2 Fingerprint chromatogram identification of four Amanita phalloides toxins in the toadstool

As the molecules of toadstool contain more hydroxy group and can be easily ionized, the experiment uses positive ion mode to conduct analysis on the optimal ionization efficiency of CH3OH, C2H3N, CH3COONH4 solution and water in different ratios, and determines 1.8mol/L CH3COONH4-H2O toxin as the injection reagent. By using different columns to conduct the test, HSS-T3 column was found to be more effective in the separation of toxins of toadstool. The results of the toxin separation of the three mobile phases were analyzed and we found that, when the C2H3N is not added into the mobile phase, the separation result is the best. Figure 3 shows the mobile phase combination of H2O-CH3OH-CH3COONH4. Under these conditions, the retention times of the four toxins in toadstool were: α-Amanitin: 1.46 min; β-Amanitin: 0.99 min; Phalloidin: 3.96 min; Phallacidin: 4.59 min, respectively.

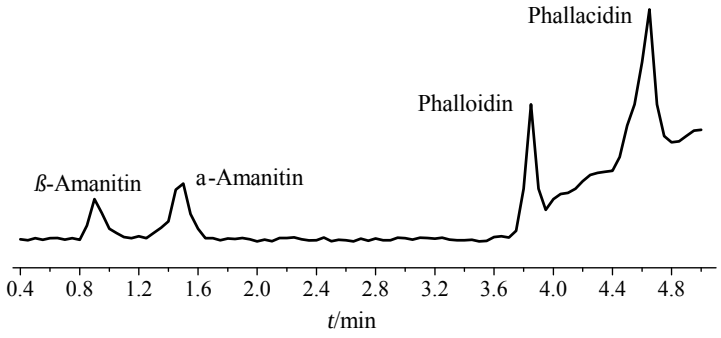

Figure 3: Effect of mobile phases separation with water-methanol-ammonium acetate

As mushroom samples contain many impurities, the samples were pretreated with CH3OH solution containing 0.1% TFA. After the treatment, the extracted toxins were prepared into 50-1000μg/L mixed solution of different concentrations, took the first MS as dose basis, extracted the parent ion as the quantitative ion to conduct calculation, then we can get the regression equation of the four toxins, set the regression equations of α-Amanitin, β-Amanitin, Phalloidin and Phallacidin as y_1—y_4, then they were: y_1=0.0238x-0.1372, y_2=0.0339x-0.5349, y_3=0.0495x-0.3342, y_4=0.0495x-0.2315, respectively. The correlation coefficient of the regression equation calculated by the four toxins all reached above 0.995, the regression effect was good, The detection limits were 10μg/L, the limit of quantification was 50μg/L.

Four kinds of toxins were prepared into standard solutions with different concentrations. The recoveries and standard deviations (RSDs) of different concentrations (400μg/L and 800μg/L) were calculated. The results are shown in Table 3.

Table 3 Similarity index of tea samples originated from different area

Compound	Added (μg/L)	Recovery (%)	*RSD* (%)
β-Amanitin	400	69.4	11.6
	800	75.2	14.2
α-Amanitin	400	78.7	7.8
	800	82.1	7.1
Phalloidin	400	73.5	10.4
	800	80.8	8.5
Phallacidin	400	82.7	8.8
	800	89.3	10.3

As can be seen from the table, the recoveries of the four toxins between the two concentrations ranged from 69.4% to 89.3% with a standard deviation of 7.1% to 14.2%. The recoveries of α-Amanitin were relatively low, with recoveries less than 76% for both concentrations, which may be related to the relatively small ionic strength of α-Amanitin, but the overall recovery and precision were satisfactory.

The test equipment needed to be adjusted before the sample tests, the required the mass measurement error was less than 3×10^{-6}. Table 4 shows the measured mass number, isotopic abundance ratios and their relative errors to the theoretical values for the four toxins in toadstool. Figure 4 shows the first-order MS fingerprint chromatogram of selected toxins, β-Amanitin and Phallacidin. From Table 3 and Figure 4, we can get the correlation errors of the determination results of the parent ions of four kinds of toxins. For example, the calculated absolute error of α-Amanitin was 1.0mDa and the relative error was 1.5×10^{-6}, the other three kinds of toxins were also calculated according to this method. Calculating the mass number of the toxin parent ions based on the TOF measurement is an important feature to verify the presence of the toxins in the sample. Similarly, the calculation of abundance and the abundance error can also be used as the basis for toxin determination. The abundance error calculated in this paper was within 2.5%, and the result is reliable.

Table 4 Accurate mass numbers and isotope abundance ratio of 4 toxin moleculars

Compound	Theoretical Calculated mass (m/z)	Actual measured mass (m/z)	Mass Difference (10^{-6})	Theoretical abundance ratio (%)	Actual abundance ratio (%)	Relative abundance Difference (10^{-6})
α-Amanitin	919.475	919.474	1.5	100	100	0
Isotope	920.182	920.189	1.7	49.3	50.5	1.5
β-Amanitin	920.392	920.389	1.0	100	100	0
Isotope	921.855	921.861	0.8	48.7	50.9	2.2
Phalloidin	790.388	790.383	2.4	100	100	0
Isotope	791.377	791.372	1.5	42.9	43.6	0.7
Phallacidin	848.270	848.274	2.0	100	100	0
Isotope	849.161	849.166	3.3	45.1	45.7	1.4

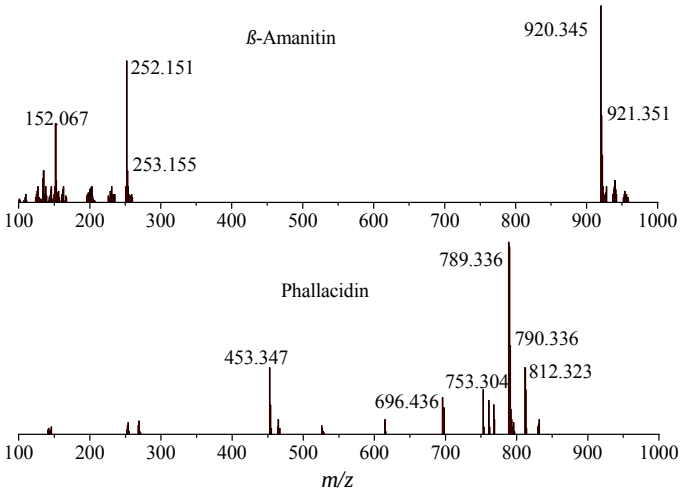

Figure 4: Mass spectrum fingerprint chromatogram of 4 kinds of toxin molecular

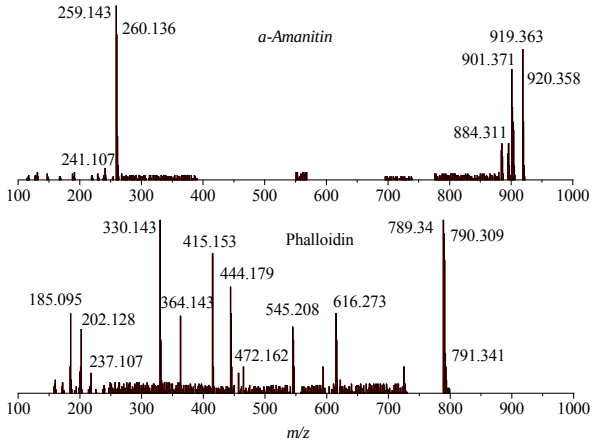

Figure 5: MS/MS2 spectrum fingerprint chromatogram of 4 kinds of toxin molecules obtained by Q-TOF MS^2

Figure 5 shows the secondary MS fingerprint chromatogram of α-Amanitin and Phallacidin. Based on the Mass Fragement software, the fingerprint chromatogram was analyzed, and the relationship between the toxic parent ions and the daughter ions was reported. From the report, select structural formulas with less mass errors and better fragmentation patterns, the Superscript Numbers of each structural formula are the mass number and relative error of the secondary MS fingerprint chromatogram, also, the fragmentation patterns of secondary MS provide a basis for the identification of toxins.

4. Conclusion

Based on HPLC, this paper studies the fingerprint chromatogram analysis and identification method of tea leaves and amanita phalloides toxins, by using chromatography and PCA, it provides a new and effective method for tea leaves quality identification and mushroom toxin identification. The conclusions are as follows:

(1) The analysis results of tea leaves show that the correlation coefficient, included angle cosine and overlap rate of PCA can be used to characterize the similarity of tea fingerprint chromatogram, and the calculation of the fingerprint chromatogram using the included angle cosine is more accurate relatively. The main components of tea leaves are PC1-PC7, the cumulative contribution rate reached 87.49%. The use of hierarchical clustering method and two-dimensional sorting method can distinguish different types of tea leaves.

(2) During the analysis of the mushroom samples, the paper establishes the accurate mass number and isotopic characteristics of four types of toxin molecules. At the same time, the corresponding secondary MS features and daughter ion information were established. The recovery rate of the four toxins ranged from 69.4% to 89.3% with standard deviations between 7.1% and 14.2 %. The method established in this paper has the advantage of rapid and accurate detection of toxins in poisonous mushrooms.

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