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Characterization of antioxidant activity and analysis of phenolic acids and flavonoids in linden honey

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Abstract

Ten linden honey samples from China and Russia were analyzed in this study. Based on the determination of total polyphenol content (TPC) and total flavonoid content (TFC), the antioxidant activities of ten linden honey samples were analyzed. The results showed that the TPC and TFC in linden honey were ranged from 17.57 to 31.95 mg GAE 100 g^{-1} and 0.81 to 1.77 mg RE 100 g^{-1}. The TPC and TFC of linden honey were strongly correlated with the antioxidant capacity, and the pearson's correlation values ($p \leq 0.01$) were 0.828 and 0.770 respectively. In this work, the phenolic acids and flavonoids of linden honey were analyzed by high performance liquid chromatography (HPLC). It was found that there were a lot of lindenin and abscisic acid in linden honey. Principal component analysis (PCA) and differential characteristic analysis were used to analyze the composition differences among linden honey samples. The results obtained for TPC, TFC and antioxidant capacities of linden honeys indicate linden honey had good quality as a healthy food.

Keywords: linden honey; antioxidant activity; phenolic acids; HPLC.

Practical Application: The total content of polyphenol and flavonoid in linden honey was studied, and the relationship between the content and antioxidant activity of linden honey was explored. The composition of linden honey was quantitatively analyzed by high performance liquid chromatography, and the influence of physical property on linden honey was judged.

1 Introduction

Honey has been regarded as a natural food of high nutrition since ancient times (Martinello & Mutinelli, 2021). In addition to nutrition, the biological activity of honey has been extensively studied amid growing interest in the potential of naturally active substances to improve health and reduce the risk of disease (Hashim et al., 2021). A large number of studies have confirmed that the natural active substances in honey include organic acids, amino acids, proteins, polyphenols and minerals which make honey have physiological activities such as anti-oxidation, anti-bacteria and anti-inflammatory (Almasaudi, 2021; Chen et al., 2021; Elamine et al., 2021). Among on those physiological activities, the antioxidant activity of honey is closely related to phenolic acids and flavonoids in honey. The mechanism of this function is that honey polyphenols may reduce the production of reactive oxygen species, restore the activity of antioxidant enzymes, and improve the antioxidant and function of mitochondria (Battino et al., 2021). Since the composition and content of polyphenol in honey are determined by the nectar source plants, the antioxidant capacity of honey depends on its botanical origin and environmental conditions. In addition, honey polyphenols are derived from nectar plants, so some polyphenols are considered as markers for the origin of honey (Qiao et al., 2020; Schievano et al., 2010, 2013; Truchado et al., 2009). For example, some articles have reported on the characteristic substances of manuka honey (Gośliński et al., 2021; Oelschlaegel et al., 2012). The studies on the composition of linden honey polyphenols also pointed out that there may be a characteristic substance in linden honey (Frérot et al., 2006; Naef et al., 2004; Schievano et al., 2011).

At present, solid phase extraction (SPE) is considered to be a suitable way for honey sample pretreatment, while liquid chromatography with different detectors such as ultraviolet detector, electrochemical detector, fluorescence detector, diode array detector and mass spectrometry is considered to be the best form for qualitative and quantitative analysis of polyphenols in honey (Gao et al., 2020; Sergiel et al., 2014). In this research, the composition and content of polyphenols in ten samples of linden honey from northeast China and eastern Russia were determined by using solid-phase extraction method combined with HPLC-UV. The RP-C18 column was used for separation, and the contents of 13 substances separated by the column were focused. The contents of total polyphenol, total flavonoid and antioxidant activities of DPPH, ABTS and FRAP of ten linden honey species were also studied. Principal component analysis was then used to highlight differences in the composition and antioxidant activities of their samples related to honey samples.

2 Materials and methods

2.1 Honey sample

Ten types of linden honey samples were obtained from northeast of China and east of Russia which were collected from June 2020 to February 2021. Three samples of linden honey are

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liquid honey (B, D, J) and the rest are crystallized honey (A, C, D, E, F, G, H, I). All honey samples were preserved at 4 °C before be used.

2.2 Chemicals and reagents

All chemicals and reagents used were of analytical grade. Folin-Ciocalteu reagent was purchased from Sigma-Aldrich (Shanghai, China). ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), DPPH (1,1-difhenyl-2-picril-hidrazyl) were acquired from Yuan Ye (Shanghai, China). Gallic acid, methyl Syringate, cinnamic acid, p-coumaric acid, cis, trans-abscisic acid, chrysin, p-hydroxybenzoic acid were purchased from Macklin (Shanghai, China). Rutin and vanillin were acquired from Aladdin (Shanghai, China).

2.3 Determination of total polyphenol content and total flavonoid content

In determination of total polyphenol content and total flavonoid content in honey samples, all spectrophotometric measurements were performed on a Micropore plate enzyme label instrument (Thermo Fisher Scientific Multiskan GO). The determination of total polyphenol content and total flavonoid content was a slight modify on the basis of previous research (Chang et al., 2011; Dezmirean et al., 2012; Gašić et al., 2014; Wabaidur et al., 2020).

For TPC assays, one gram of honey sample was dissolved in distilled water, constant volume to 5 mL. 0.5 mL this honey solution was mixed with 2.5 mL of Folin-Ciocalteu reagent solution (0.1 N) and 2.0 mL sodium carbonate solution (1 mol/L) was added to make the whole solution slightly alkaline. After reacting two hours in dark, the absorbance value of this solution was determined at 760 nm. Seven groups of different concentrations of gallic acid were used to construct the standard curve (R^2 = 0.9971). Total polyphenol content was expressed as mg gallic acid equivalent/100 g of honey sample.

For TFC assays, ten grams of honey sample was dissolved in 40 mL HCl (pH = 2), centrifuge at 8000 rpm for 15 min. Flavonoids in samples were purified by using Strata-X-A cartridge (Phenomenex, US). The eluent (methanol: formic acid = 95: 5) was used for constant volume to 2 mL. 1 mL this solution was mixed with 0.2 mL sodium nitrite (mass fraction = 5%), then added 0.2 mL aluminum nitrate (mass fraction = 10 wt.%) after 6 min, added 2 mL sodium hydroxide (mass fraction = 4 wt.%) after 6min, the volume was constant to 5 mL by deionized water. After reacting 15 min, the absorbance value of this solution was determined at 510 nm. Seven groups of different concentrations of rutin were used to construct the standard curve (R^2 = 0.9992). Total flavonoids content was expressed as mg rutin equivalent/100 g of honey sample.

2.4 Determination of antioxidant activities

For ABTS assay, 50 mL ABTS (7 mmol/L) reacted with the same concentration of 17.6 mL potassium persulfate solution to acquire the stable radical cation ABTS. The mixture was incubated at 20 °C for 12 h. The stock solution was diluted with ethanol until the absorbance at 734 nm was suitable for the concentration gradient of honey sample. 0.5 mL diluted honey sample of eight concentration gradient was mixed with 0.5 mL ABTS solution. After 15 min of incubation in dark, the absorbance was read at 734 nm against a contrast sample (honey solution/water = 1:1) and a blank sample (ABTS/water = 1:1) (Chang et al., 2011; Shen et al., 2018). The IC50 concentration of honey sample scavenge ABTS radical by comparing the clearance rate of vitamin C.

For DPPH assay, 0.02 g DPPH was dissolved in 100 mL ethanol. Full-wavelength scanning was performed to determine the maximum absorption wavelength of the DPPH experiment. After being diluted with ethanol until the absorbance at the maximum absorption wavelength (525 nm) got 0.8 - 0.9. 0.5 mL diluted honey sample of six concentration gradient was mixed with 0.5 mL DPPH solution. After 30 min of incubation in dark, the absorbance was read at 525 nm against a contrast sample (honey solution/ethanol = 1:1) and a blank sample (DPPH/water = 1:1) (Cetkovic et al., 2014; Kumazawa et al., 2012). The IC50 concentration of honey sample scavenge DPPH radical by comparing the clearance rate of vitamin C.

For FRAP assay, 0.067 mol/L K_2HPO_4 solution and KH_2PO_4 solution were mixed in a volume ratio of 4:6 to obtain a phosphate buffer with pH = 6.6. 0.5 mL of phosphoric acid buffer solution and 1% potassium ferricyanide were added into 0.2 mL six different concentrations honey solution or six different concentration vitamin C and mixed evenly. This solution was in water bath at 50 °C for 20 min. Then, 0.5 mL trifluoroacetic acid (mass fraction = 10 wt.%) was added into mixture solution. After that, the solution was centrifuged at 3000 rpm for 6-10 min. 0.5 mL of the supernatant was taken, added with 0.5 mL of deionized water and 0.5 mL ferric chloride (mass fraction = 0.1 wt.%) respectively. After standing for 10 min, the measurement was carried out at 700 nm (Chang et al., 2011; Kędzierska-Matysek et al., 2021). The IC50 concentration of honey sample scavenge FRAP radical by comparing the clearance rate of vitamin C.

2.5 Phenolic compounds extraction and HPLC analysis

For modern chemical analysis, sample preparation is the most important procedure. Solid-phase extraction (SPE) is the most popular pre-processing method due to its simplicity, efficiency and versatility (Arráez-Román et al., 2006; Michalkiewick et al., 2008; Sergiel et al., 2014). The SPE cartridges used was Strata-X-A (60 mg/3 mL) obtained from Phenomenex (Tianjin, China). Ten grams of each honey sample was added with 50 mL of hydrochloric acid (pH = 2) and stirred until the honey was dissolved completely. Then, the solution was centrifuged at 8000 rpm for 15 min. The SPE column was pretreated, activated with 3 mL methanol and balanced with 3 mL deionized water. After loading the Supernatant, washed with 3 mL deionized water and then used 3 mL formic acid/methanol (5:95) for elution. Blow the eluent with nitrogen until dry, and re-dissolved in 2 mL formic acid/methanol (2:98) and filtered through a 0.45 μm membrane filter before analyzed. Phenolic acids and flavonoids were analyzed by the high-performance liquid chromatography (HPLC) of Shimadzu LC-2010A (Kyoto, Japan), using a reverse

column Shim-Pack Rpep-ODS (250 mm × 4.6 mm, 5 μm) with an ultraviolet detector at 280 nm. The mobile phase consisted of a mixture of 0.1% aqueous formic acid (A) and methanol (B) with a flow rate of 0.7 mL/min. The injection volume was 20 μL. The gradient elution procedure was used 0-10 min from 10% to 15% (B), 10 - 18 min from 15% to 20% (B), 18 - 28 min from 20% to 22%(B), 28 - 34 min from 22% to 25%(B), 34 - 39 min from 25% to 30%(B), 39 - 45 min from 30% to 32%(B), 45 - 50 min from 32% to 33%(B), and 33% (B) for 10 min, 60 - 70 min from 33% to 37%(B), 75 - 80 min from 40% to 45%(B), 80 - 90 min from 45% to 52%(B), 90 - 96 min from 52% to 58%(B), 96 - 106 min from 58% to 65%(B), 106 - 120 min from 65% to 80%(B).

2.6 Statistical analysis

All the analyses were repeat three times and the data are presented as mean. Pearson's correlation and LSD (Least Significant Difference) test were carried out by SPSS 26.0 (SPSS, Inc., Chicago, IL, USA) (An et al., 2020; Devi et al., 2018; Sarıdaş, 2021). Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed using SIMCA version 14.1 (Umetrics, Umeå, Sweden). The data were mean-centered and scaled to unit variance to ensure that all sample points were of the same importance in PCA and 0.95 confidence level on parameters and 0.05 significance level for DModX and Hotelling's T2 limits for outliers (Kečkeš et al., 2013). The date was also scaled to pareto to make sure that the data points that vary widely can be filtered in OPLS-DA and the superiority of the model had passed by 200 permutation tests (Shen et al., 2018).

3 Results and discussion

3.1 phenolic and flavonoid contents and antioxidant capacities in honey sample

Honey is thought to have antioxidant activity due to its ability to reduce tissue damage caused by free radicals in the body's biochemical reactions. The process of antioxidant involves many bioactive substances and different mechanisms of reducing free radicals, which leads to the lack of accurate results of a single antioxidant evaluation method. In addition, the chemical composition of the food, the type of active compounds and the interaction between active compounds will also lead to different evaluation methods to obtain different measurement results (Dulger Altiner et al., 2021; Karadag et al., 2009; Sariburun et al., 2010). Therefore, in this experiment, DPPH, ABTS and PRAP methods were used to evaluate the antioxidant capacities of honey, and the relationship between antioxidant capacities and TPC and TFC was analyzed. The experimental results are shown in Table 1. The TPC and TFC of the linden honey analyzed ranged from 17.57 to 31.95 mg GAE 100 g^{-1} and 0.81 to 1.77 mg RE 100 g^{-1}. The antioxidant capacities of linden honey presented a variation from 4.51 to 9.38 mg VC 100 g^{-1} in DPPH assays,10.89 to 34.83 mg VC 100 g^{-1} in ABTS assays and 30.88 to 51.73 mg VC 100 g^{-1} in FRAP assays. Compared to the other methods, the antioxidant value obtained by FRAP assays were the highest in the honey antioxidant capacities tests. It was also found in other studies that the reduction ability test of metal ions usually achieved better results in the antioxidant test of bee products compared with DPPH and ABTS (Chang et al., 2011; Dulger Altiner et al., 2021). This situation is due to the FRAP method is selective for both hydrophilic and lipophilic substances in honey. The pearson's correlation values ($p \leq 0.01$) between TPC and DPPH ($r = 0.828$), TFC and DPPH ($r = 0.770$), and correlation values ($p \leq 0.05$) between TPC and FRAP ($r=0.702$), indicated that phenolic content contributed to the radical scavenging activity of honeys.

3.2 Phenolic and flavonoids compounds in honey

Profiles of linden honey samples was illustrated in Figure 1. All the thirteen substances demarcated in Figure 1 were detected in ten linden honey samples. The quantitative standard curves of eight compounds under HPLC are shown in Table 2. Lindenin is the component with the largest response area in the chromatogram and is also the characteristic component of linden honey. Cis, trans-abscisic acid is the most abundant compound among the eight compounds in Table 3, the concentration of cis, trans-abscisic acid in this study was ranged from 137.19 to 402.83 μg/100 g honey, which is also a characteristic of linden honey as a forest honey species. Gallic acid and p-hydroxybenzoic acid were the other two components with high content in linden honey, and their maximum values are reached 314.09 and 296.98 μg/100 g honey, respectively. P-coumaric acid、methyl syringate、trans,

Table 1. Total polyphenol content and total flavonoid content and antioxidant activities of honey samples.

Sample	TPC (mg GAE/100 g)	TFC (mg RE/100 g)	DPPH (mg Vc/100 g)	ABTS (mg Vc/100 g)	FRAP (mg Vc/100 g)
A	26.98 ± 1.57^{c}	1.15 ± 0.06^{c}	7.16 ± 0.09^{def}	31.38 ± 0.58^{b}	39.14 ± 4.71^{abc}
B	27.07 ± 0.26^{c}	1.23 ± 0.03^{c}	8.18 ± 0.17^{bcd}	29.25 ± 0.21^{c}	43.37 ± 5.90^{abc}
C	24.55 ± 0.17^{d}	0.93 ± 0.03^{d}	6.29 ± 0.22^{f}	34.83 ± 0.04^{a}	38.26 ± 4.05^{abc}
D	31.95 ± 0.49^{a}	1.47 ± 0.06^{b}	9.13 ± 0.11^{ab}	23.02 ± 0.78^{ef}	50.71 ± 5.27^{a}
E	26.53 ± 0.40^{c}	1.33 ± 0.11^{b}	8.35 ± 0.16^{abc}	23.46 ± 0.56^{e}	50.29 ± 8.32^{a}
F	17.57 ± 0.34^{g}	0.81 ± 0.02^{d}	4.51 ± 0.84^{g}	10.89 ± 0.19^{h}	30.88 ± 4.14^{c}
G	22.64 ± 0.34^{e}	0.62 ± 0.08^{e}	7.49 ± 1.18^{cde}	34.05 ± 0.35^{a}	51.73 ± 6.52^{a}
H	20.66 ± 0.30^{f}	0.91 ± 0.04^{d}	6.41 ± 0.59^{ef}	18.32 ± 0.31^{g}	32.89 ± 4.23^{bc}
I	29.25 ± 0.32^{b}	1.77 ± 0.15^{a}	9.38 ± 0.59^{a}	26.29 ± 0.39^{d}	48.21 ± 5.81^{ab}
J	21.56 ± 0.90^{ef}	1.48 ± 0.08^{b}	7.97 ± 0.52^{cd}	22.60 ± 0.23^{f}	39.85 ± 10.30^{abc}
mean	24.88	1.17	7.49	25.41	42.56

Mean values with different lowercase (A-G) in the same column are significantly different ($p \leq 0.05$) according to different linden honey samples.

trans-abscisic acid、pinobanksin、kaempferol were found in linden honey which is similar to previous studies.

3.3 Principal Component Analysis

Principal component analysis (PCA) was performed on the data for 18 variables (13 compounds, TPC, TFC, DPPH, ABTS and FRAP radical scavenging activities) in ten linden honey samples to investigate the distribution of honey samples from different forms of existence. The total four principle components can explain 88.2% of the total variance, each of four principle components represented 35.9%、27.7%、15.8%、8.8% of the variance, respectively. Honey samples were discriminated into different groups in the PCA score plot as shown in Figure 2. The distribution of crystallized honey was more dispersed, while the distribution of liquid honey was more concentrated which indicated that the composition of liquid honey was more different than that of crystallized honey.

The orthogonal partial least squares discriminant analysis (OPLS-DA) model was established by SIMCA to study the differences of specific variables among different forms of existence of linden honey. A permutation test with 200 cycles was used for cross validation to verity the degree of fit of OPLS-DA model. The results of permutation test usually require that there is a great

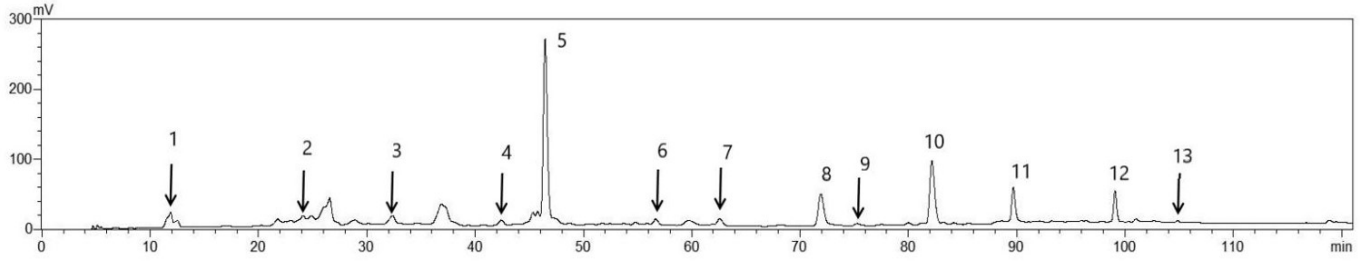

Figure 1. HPLC chromatogram of raw linden honey sample. (1: gallic acid, 2: p-hydroxybenzoic acid, 3: vanillin, 4: p-coumaric acid, 5: lindenin, 6: methyl syringate, 7: trans, trans-abscisic acid, 8: cis, trans-abscisic acid, 9: cinnamic acid, 10: pinobanksin 5-methyl ether, 11: pinobanksin, 12: kaempferol, 13: chrysin).

Table 2. Linear regression parameters of calibration curve, LOD and LOQ for the phenolic compounds.

Analytes	Linear range (1-40μg/mL)	Regression equation	R^2	LOD μg/mL	LOQ μg/mL	RSD (%)
Gallic acid	2-40	y = 55905.28x-13966.35	0.9991	1.36	4.07	8.01
p-Hydroxvbenzoic acid	2-40	y = 35142.12x-12175.30	0.9993	0.63	1.9	9.22
Vanillin	1-40	y = 113092.89x+31187.62	0.9984	1.21	3.62	5.47
p-Coumaric acid	1-40	y = 126460.21x-17227.72	0.9990	0.62	1.86	3.04
Methyl syringate	1-40	y = 74758.40x-26997.72	0.9993	0.25	0.75	1.56
Abscisic acid	2-40	y = 102705.98x-28053.21	0.9998	0.73	2.18	10.45
Cinnamic acid	2-40	y = 237545.03x+1775.78	1.0000	0.15	0.45	2.46
Chrysin	2-40	y = 130524.82x-86683.19	0.9995	0.14	0.43	2.28

The limit of quantification (LOQ) was determined as the lowest point in the range of calibration with sufficiently low relative standard deviation (RSD) value after seven replicate injections.

Table 3. Phenolic profile of ten linden honeys [μg 100 g^{-1}].

Analytes	Gallic acid	p-Hydroxvbenzoic acid	Vanillin	p-Coumaric acid	Methyl syringate	(+)-Abscisic acid	Cinnamic acid	Chrysin
A	138.45	225.19	30.18	35.85	96.24	264.67	13.75	21.39
B	209.72	296.98	70.27	42.98	162.16	352.28	12.11	26.23
C	70.89	215.84	41.46	35.49	88.41	157.40	7.44	21.85
D	314.09	150.09	43.51	36.16	164.45	172.06	10.97	27.34
E	255.14	99.12	10.35	16.90	66.85	168.33	13.41	33.20
F	74.05	171.26	33.86	19.53	125.29	137.19	1.78	20.92
G	22.13	181.53	46.28	13.21	133.22	153.05	12.91	20.82
H	100.82	266.39	54.11	15.76	171.35	149.04	9.67	20.52
I	69.31	81.11	23.15	46.50	48.13	163.10	23.17	20.91
J	37.06	170.49	33.15	45.40	70.07	402.83	15.87	46.82

Data are means (n=3).

difference between the predictive ability of model data and that of random scrambles. As shown in the Figure 3, the intercept of Q2 curve is -2.26 which means the results of permutation test was excellent. R2 (cum) parameters and Q2 (cum) parameters were 0.983 and 0.953, which showed excellent goodness of fit and strongly predictive capability for different forms of existence of linden honey samples, respectively. The variable importance in the projection (VIP) was carried out to evaluate the significance of variables. In general, variables with VIP values more than 1.5 were be considered as specific variables. The VIP values of all variables are shown in Figure 4, compared with crystallized linden honey, liquid linden honey had obvious advantages in phenolic acids content, mainly reflected in lindenin、pinobankin and cis, trans-abscisic acid, but there is no significant difference in TPC、TFC and antioxidant capacity.

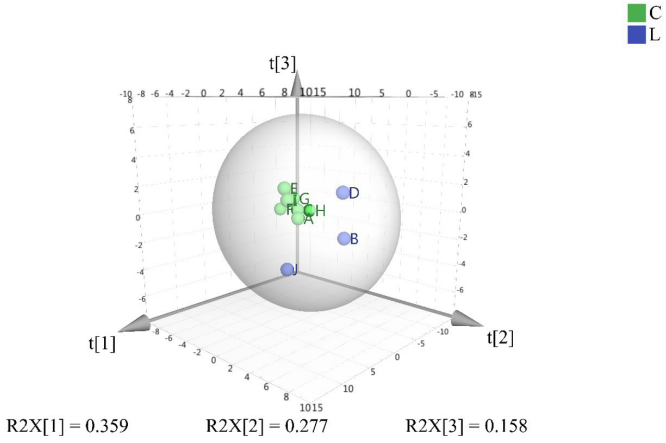

Figure 2. 3D PCA scores plots of honeys.

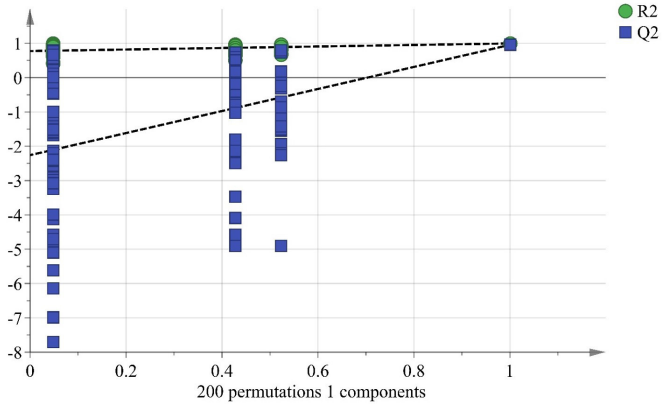

Figure 3. Permutation test (200 cycles, R2 = 0.983, Q2 = 0.953).

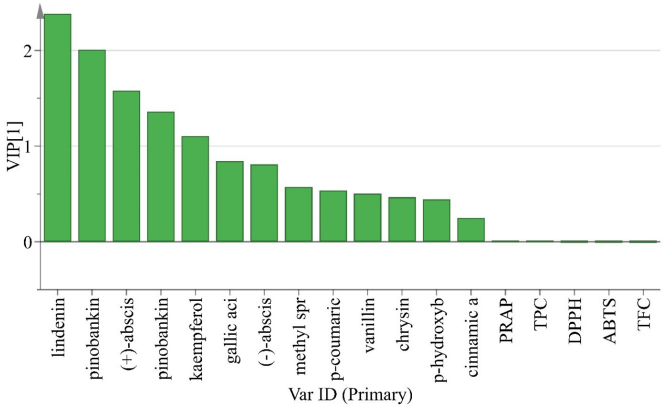

Figure 4. The VIP plot by OPLS-DA corresponding with discrimination between liquid linden honey and crystallized linden honey.

4 Conclusions

The proposed research indicated that the linden honey samples contained phenolic and flavonoids with the content ranged from 17.57 to 31.95 mg GAE 100 g^{-1} and 0.81 to 1.77 mg RE 100 g^{-1}. Based on the results of all antioxidant capacity tests in this work, the FRAP method had the highest value in the antioxidant activity test, and the antioxidant activity of DPPH method was confirmed to be strongly correlated with TPC and TFC. Studies on the phenolic components of linden honey showed that ten linden honey samples had similar chemical compositions and lindenin and abscisic acid are the highest content of substances. The physicochemical properties and phenolic composition of ten linden honey were investigated by PCA and OPLS-DA. The OPLS-DA results showed that there was a significance in the content of lindenin、pinobankin and cis, trans-abscisic acid between crystallized and liquid linden honey, but no significance difference in TPC, TFC and antioxidant activity.

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