Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

Analysis of Anthocyanin Metabolites in Strawberries Using UPLC-ESI-MS/MS

Hui Dong[1], Li Li[1], Li Yang[1], JingFang Fan[2], ChangLin Jin[2], Jia Feng[1], YaNan Li[1], ShiJia Song[3], Lei Yang[1*], JianJun Zhang[3*]

[1]Shijiazhuang Institute of Pomology, Hebei Academy of Agriculture and Forestry Sciences, National Pear Improvement Center, Shijiazhuang, China
[2]Hebei General Station of Plant Protection and Inspection, Shijiazhuang, China
[3]Hebei Academy of Agriculture and Forestry Sciences, Shijiazhuang, China
*Corresponding Author.

Abstract:

Strawberry is a delicious and nutritious berry with great demand, which is rich in active substances, such as ascorbic acid, polyphenols, and anthocyanins. Anthocyanins are a kind of water-soluble flavonoids with high nutritional values and officinal values, but in strawberries,its systematic profile is known a little. In the study, the anthocyanin in 5 strawberry varieties, including 'Hongxing' ('HX'), 'Shimei11' ('SM11'), 'Shimei12' ('SM12'), 'Darselect' ('DS'), and 'Sweet charlie' ('SC'), were analyzed using UPLC-ESI-MS/MS-based widely targeted metabolomes. The data showed that the total anthocyanin content of 'HX' was higher than other strawberrys. In addition, there were 27 anthocyanin metabolites to detect, which contain 18 differential, two of which were common. And 'HX' could be clearly differentiated from others by clustering analysis and Principal Component Analysis (PCA). Moreover, five up-regulated metabolites were identified as displaying important biological activities in HX, which were namely Cyanidin 3,5-O-diglucoside (Cyanin), Pelargonin, Cyanidin 3-O-glucoside (Kuromanin), Pelargonidin, and Cyanidin 3-O-rutinoside (Keracyanin). This work was helpful to a deeper understanding of different anthocyanin metabolites in HX and other four strawberries,which could be utilized for reference.

Keywords: *Strawberries, Cyanidin 3-O-rutinoside, Cyanidin 3,5-O-diglucoside, Pelargonidin, Pelargonin, Cyanidin 3-O-glucoside.*

I. INTRODUCTION

Strawberry *(Fragaria×ananassa Duch)*, belonging to Rosaceae, is one of the berries with the greatest demand. It is delicious and nutritious with an important role in fruit production. Strawberry is rich in ascorbic acid, polyphenols, anthocyanins, and other active substances, which has the functions of moistening lung, promoting salivation, invigorating spleen and stomach, benefiting heart, and strengthening brain [1,2].

Anthocyanins are water-soluble flavonoids, which are important bioactive compounds in berries, including strawberries. Anthocyanins, the main coloring substances in plants, are widely exist in the roots,

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

stems, leaves, flowers, and fruits of plants, making them appear different colors of red-blue-purple[3] . A lot of studies have revealed the biological functions of anthocyanins, including conferring stress resistance and attracting pollinators and seed dispersers [4]. In addition, anthocyanin has the ability to activate cell antioxidant protection, and protect against chronic diseases, such as cardiovascular diseases, cancer, depression[5] . It is essential to evaluate the metabolites of anthocyanins, which produced different phenolic compounds by intestinal microflora[6].

The strawberry variety 'Hongxing' ('HX') was bred by crossing 'Shisheng 553-2' (the superior line selected from the seed of 'Sugar Baby'; the female parent) with 'Sweet Charlie' (the male parent). The cultivation test was successfully conducted in Hebei Province of China. 'HX' has the characteristics of high quality, large size, high yield, disease resistance, good storage, and transportation tolerance[7]. However, the systematic analysis of the nutritional actives in 'HX' has not been performed.

Extensive targeted metabolomics of UPLC-ESI-MS/MS, which has many advantages has become very popular in recent years. It has been applied to metabolite analysis of corn, rice, potato, and citrus[8] . In this study, in order to better understand the anthocyanin profiling of 'HX' and clarify the differential anthocyanins, five important strawberry varieties, including 3 new varieties selected and bred in China 'Hongxing' ('HX') , 'Shimei11' ('SM11'), 'Shimei12' ('SM12') and 2 foreign varieties 'Darselect' ('DS'), 'Sweet charlie' ('SC'), were selected as experimental materials. The anthocyanin metabolites of these strawberrys were analyzed based on UPLC-ESI-MS/MS and the data could be utilized for reference.

II. MATERIALS AND METHODS

2.1. Materials and Preparation

Five varieties of strawberry were 'Hongxing' ('HX'), 'Shimei11' ('SM11'), 'shimei12' ('SM12'), 'Darselect' ('DS'), and 'Sweet charlie' ('SC'), respectively. The strawberry fruits were freeze-dried immediately and then were pretreated to extract. The method followed of Zheng et al[9].

2.2. Total Anthocyanin Content Detection

The total anthocyanin detection kit was purchased from Suzhou Comin Biotechnology. The detection methods were described as follows. Sample mass (g): the volume of extraction liquid (mL) was 1 : 5-10. After full homogenization, the extract was transferred to an EP tube, and the volume of the extract was fixed to 1 mL. After the cover was closed, ultrasonic extraction was conducted for 2 h. Centrifuged at room temperature for 10 min at 8000 g, the supernatant was taken for testing. Preheat the microplate detector for more than 30min; Preheat reagents 1 and 2 at 25ºC (room temperature) for more than 10 min. Take 20 μL supernatant and 180 μL reagent 1 in the water bath at 40ºC for 20 min, and the absorbance values at 530 nm and 700 nm were measured, denoted as A1 and A2, respectively. The absorbance values at 530nm and 700nm were measured, denoted as A3 and A4, respectively, the calculation method followed of Bai et al[10].

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

2.3. UPLC and ESI-Q TRAP-MS/MS

According to the method of LC-ESI-MS/MS system by Wang et al[11] and the method of Mass spectrometry by Chen et al[12-14] to analyze the extracts.

2.4. Analysis of Total Flavonoids Content

The analysis of total flavonoid content in strawberry fruit need about 2 g sample which should be freeze-dried and then ground into powder. The determination following the method of protocol of the Plant Flavonoids Test kit.

2.5. Qualitative and Quantitative Analysis

The abalysis was performed according to the method of Wang et al[15].

2.6. Statistical Analysis

In each experiment, there shoud be three biological replicates to perform. OPLS-DA, PCA, and Cluster analysis were performed by R (http://www.r-project.org/).

III. RESULTS

3.1. Analysis of Total Anthocyanin Content

The total anthocyanins content of ‘HX’, ‘SM11’, ‘SM12’, ‘DS’, and ‘SC’ were determined with the same method. The anthocyanins content of ‘HX’ was higher than that of ‘SM11’, ‘SM12’, and ‘DS’, reaching 238.1 μg/g The anthocyanins content of ‘SC’ ranked second at 214 μg/g, which was lower than that of ‘HX’, but the difference analysis was not significant FIGURE 1.

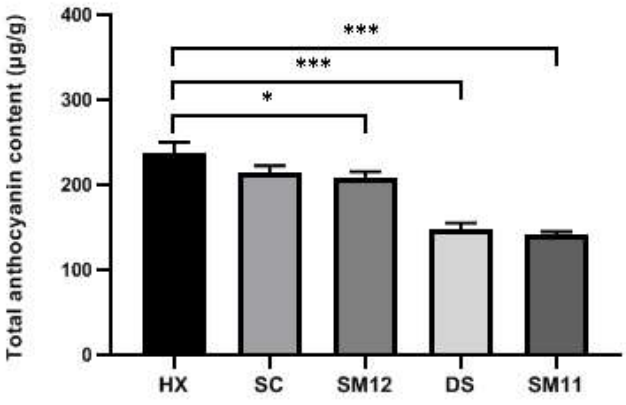


FIGURE 1: The total anthocyanins content of the strawberry varieties

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

3.2. Metabolic Profiling

The anthocyanin metabolites of 'HX' and others were performed by databases andUPLC-ESI-MS/MS. Twenty-seven anthocyanin metabolites were identified in this study, including 22 anthocyanins and 5 proanthocyanidins. The contents of anthocyanin metabolites in 'HX' contrasted with 'SM11', 'SM12', 'DS', and 'SC' varied greatly from the heatmap, while the contents of anthocyanin metabolites between 'SM11' and 'SC', and between 'SM12' and 'DS' were basically the same. The content of metabolites of 'HX', 'SM11', and 'SC' was quite different on the heatmap, although they were grouped into one category by clustering analysis. The results showed that 'HX' was clearly differentiated from others TABLE I, FIGURE 2.

TABLE I. The anthocyanin metabolites content of the strawberry varieties

	Index	Q1 (Da)	Q3 (Da)	Molecular Weight (Da)	Compounds	Class I
1	pma1590	463.10	301.40	463.12	Peonidin O-hexoside	Anthocyanins
2	pmb0541	697.10	696.90	697.10	Cyanidin 3-O-glucosyl-malonylglucoside	Anthocyanins
3	pmb0542	535.10	287.40	535.10	Cyanidin 3-O-malonylhexoside	Anthocyanins
4	pmb0547	551.10	303.70	551.10	Delphinidin O-malonylhexoside	Anthocyanins
5	pmb0550	449.10	287.30	449.10	Cyanidin 3-O-glucoside (Kuromanin)	Anthocyanins
6	pmb0554	519.10	271.40	519.10	Pelargonidin 3-O-malonylhexoside	Anthocyanins
7	pmb0563	301.10	273.60	301.10	Peonidin	Anthocyanins
8	pmb0837	577.10	425.40	576.10	Procyanidin A3	Proanthocyanidins
9	pmb2957	465.10	285.30	466.10	Cyanidin O-syringic acid	Anthocyanins
10	pmb2959	489.10	285.30	490.10	Cyanidin O-acetylhexoside	Anthocyanins
11	pmb2961	547.10	503.40	548.10	Peonidin O-malonylhexoside	Anthocyanins
12	pmb2962	473.10	269.30	474.10	Pelargonidin O-acetylhexoside	Anthocyanins
13	pmb2965	619.10	531.30	620.10	Cyanidin O-diacetyl-hexoside-O-glyceric acid	Anthocyanins
14	pme0430	575.00	285.30	576.13	Procyanidin A1	Proanthocyanidins
15	pme0433	577.00	425.90	576.13	Procyanidin A2	Proanthocyanidins
16	pme0435	579.10	127.30	578.14	Procyanidin B2	Proanthocyanidins
1	pme043	577.10	407.30	578.14	Procyanidin B3	Proanthocyanidi

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

7	6					ns
18	pme0442	303.00	149.30	303.24	Delphinidin	Anthocyanins
19	pme0444	493.20	331.60	493.20	Malvidin 3-O-glucoside (Oenin)	Anthocyanins
20	pme1397	271.00	215.10	271.24	Pelargonidin	Anthocyanins
21	pme1398	465.10	303.10	465.10	Delphinidin 3-O-glucoside (Mirtillin)	Anthocyanins
22	pme1773	595.00	287.90	595.00	Cyanidin 3-O-rutinoside (Keracyanin)	Anthocyanins
23	pme1777	611.00	287.70	611.00	Cyanidin 3,5-O-diglucoside (Cyanin)	Anthocyanins
24	pme1786	655.20	331.20	655.20	Malvidin 3,5-diglucoside (Malvin)	Anthocyanins
25	pme1793	595.00	271.80	595.00	Pelargonin	Anthocyanins
26	pme3391	479.00	317.00	479.00	Petunidin 3-O-glucoside	Anthocyanins
27	pme3392	433.10	271.00	433.10	Pelargonidin 3-O-beta-D-glucoside(Callistephin chloride)	Anthocyanins

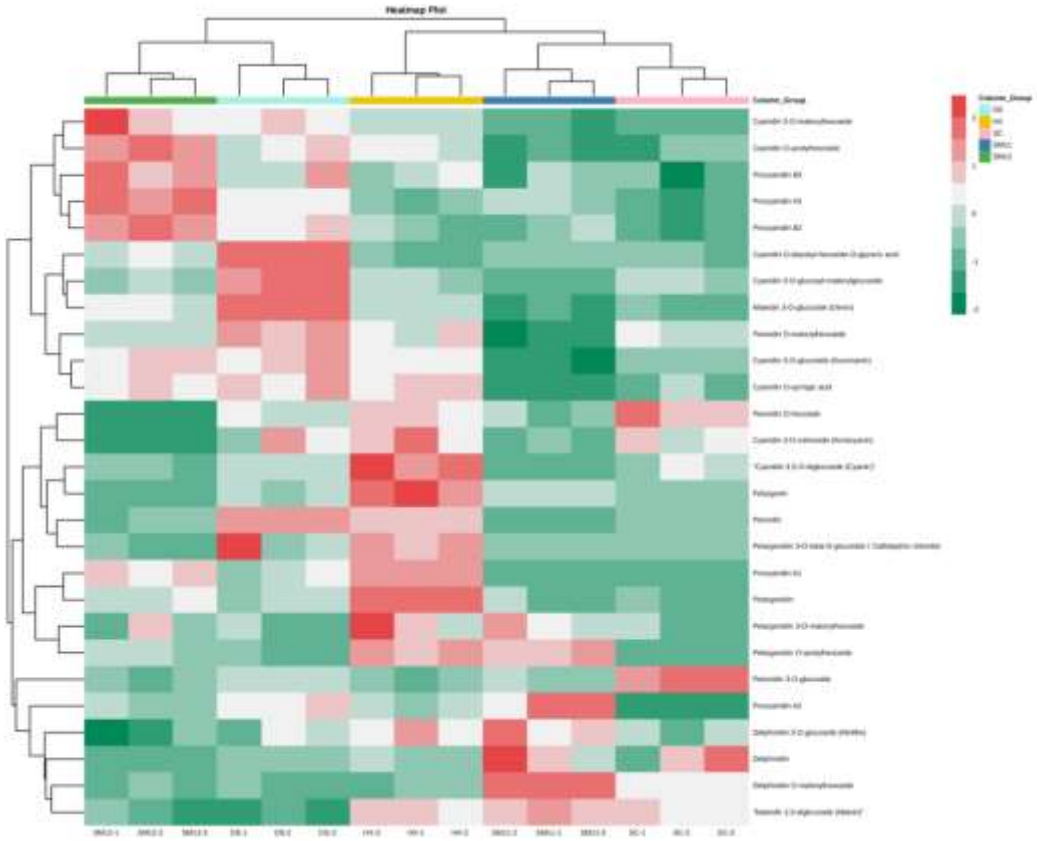


FIGURE 2: Clustering heat map of all anthocyanin metabolites

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

3.3. Differential Anthocyanin Metabolite Analysis Based on PCA

In this study, the extraction of PC1and PC2 were of 34.82% and 21.31%.The rate of cumulative contribution was 56.13%. In the score plot of PCA, 'HX', 'SM11', 'SM12', 'DS', and 'SC' were clearly differentiated. In the PCA 3D map, we could see that the clustering of samples was more intuitive. According to the PCA, we found that anthocyanin metabolic components of 'HX' were different from other strawberrys FIGURE 3(A-B).

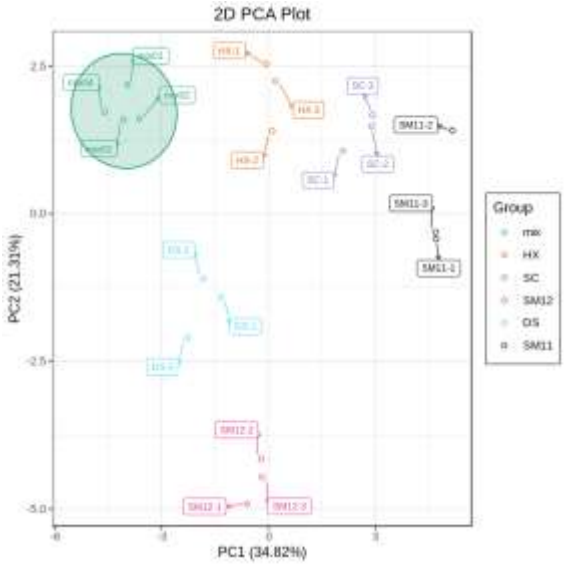


FIGURE 3(A): Differential Anthocyanin metabolite analysis of PCA.

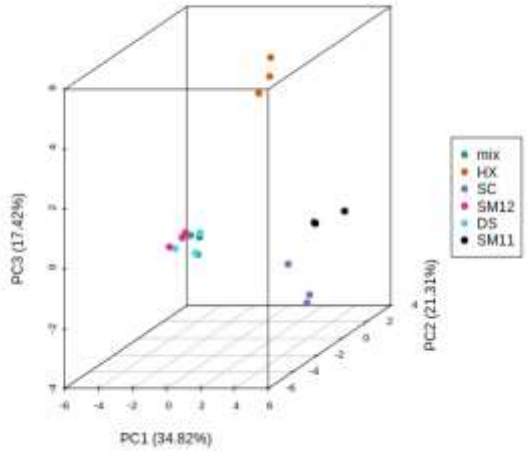
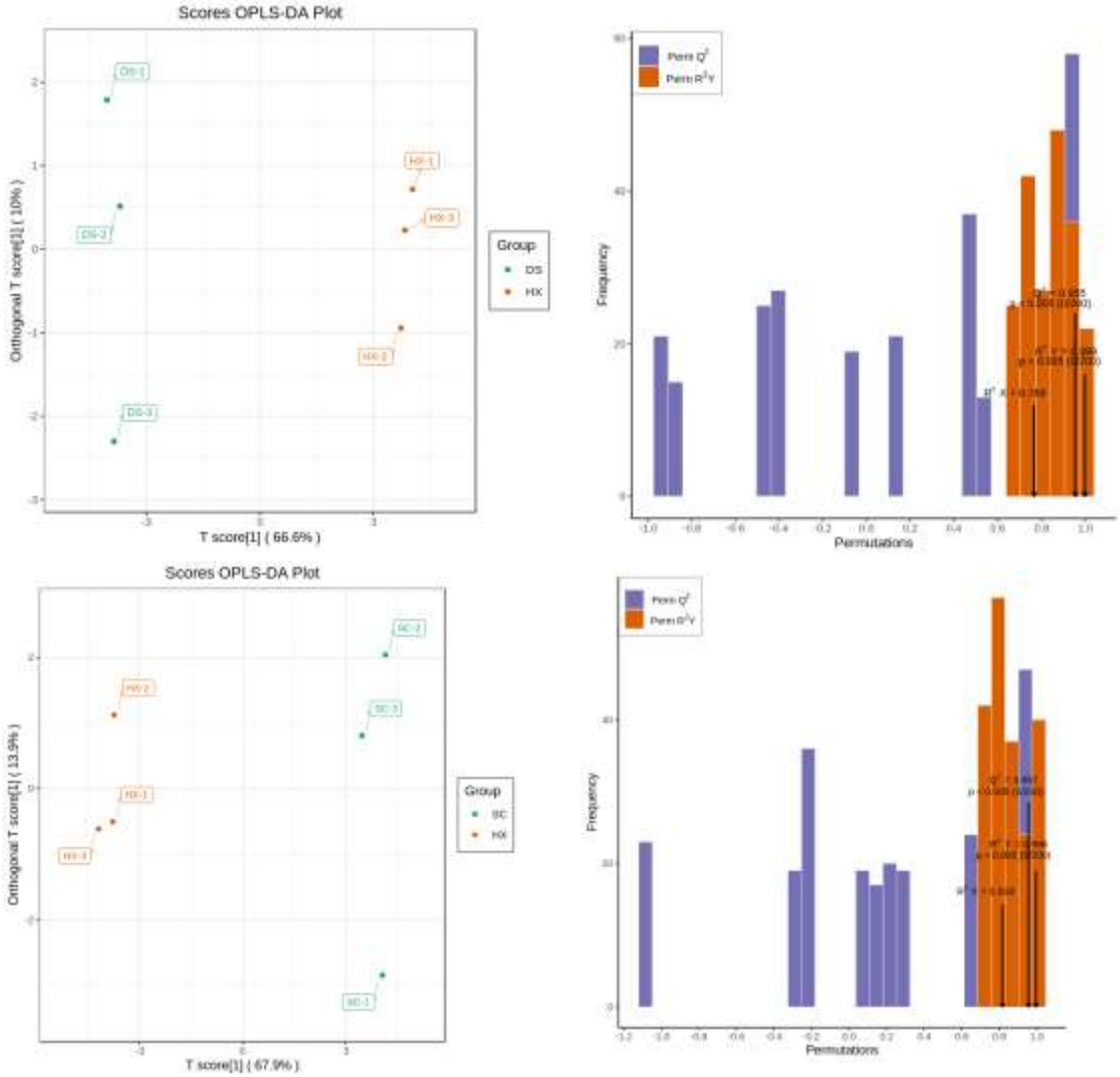


FIGURE 3(B): PCA 3D plot

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

3.4. Differential Anthocyanin Metabolite Analysis of OPLS-DA

In this study, using the model of OPLS-DA to compare the anthocyanin metabolite content of strawberrys in pairs to analyze the difference between 'HX' and 'DS' (R2X = 0.766, R2Y = 0.999, Q2= 0.955), 'HX' and 'SC' (R2X = 0.818, R2Y = 0.996, Q2= 0.957), 'HX' and 'SM11' (R2X = 0.801, R2Y = 1, Q2= 0.990), 'HX' and 'SM12' (R2X = 0.761, R2Y = 1, Q2= 0.981). The difference of metabolic between 'HX' and others could be further screened accroding the Q2 values which exceeded 0.9 FIGURE 4.



Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

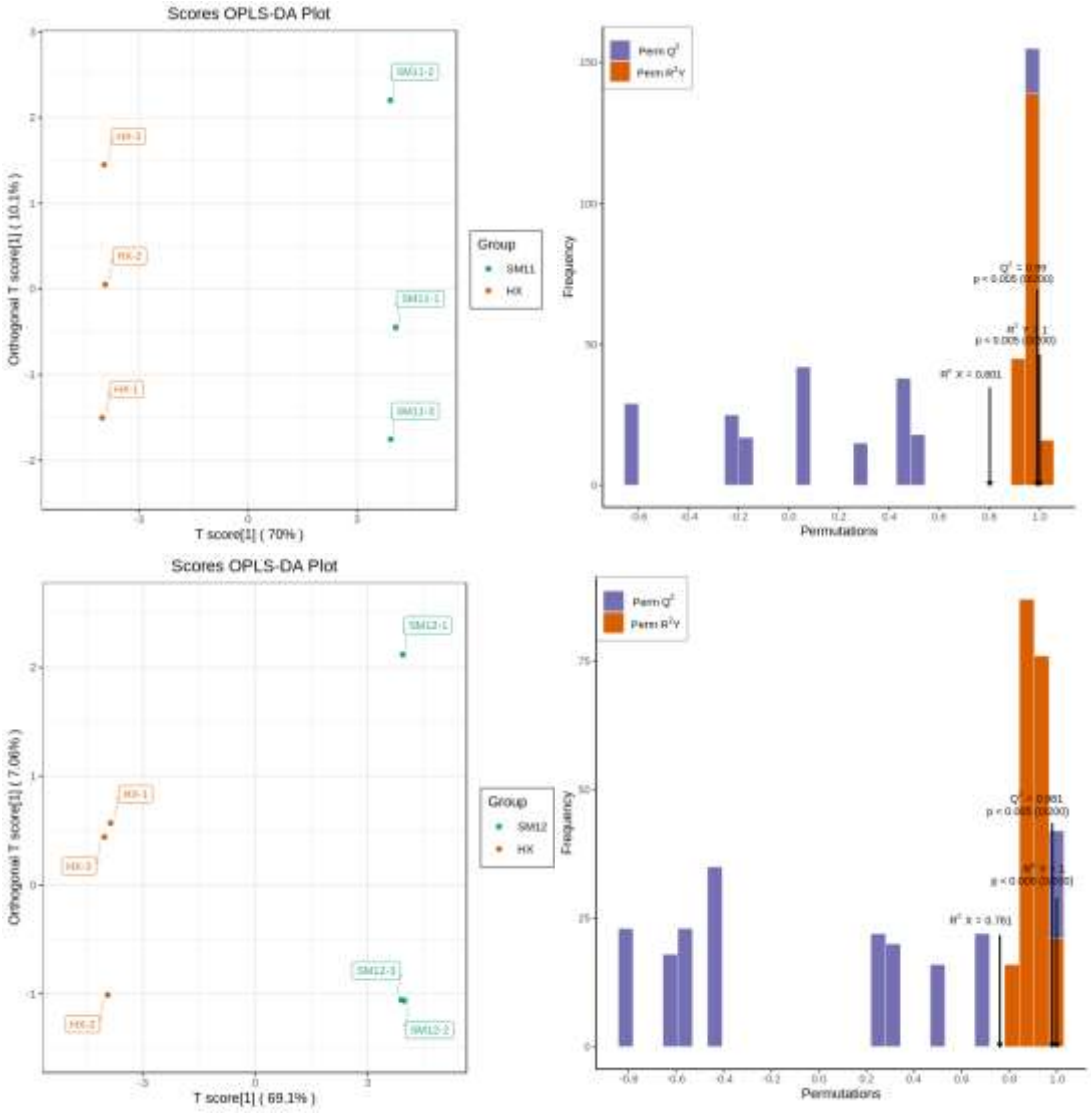


FIGURE 4: Analysis of the differential anthocyanin metabolite based on OPLS-DA.

3.5. Differential Anthocyanin Metabolite Screening, Functional Annotation, and Enrichment Analysis

In the study, 5 obviously different anthocyanin metabolites between ‘HX’ and ‘DS’ (3 up-regulated and 2 down-regulated), 6 between ‘HX’ and ‘SC’ (4 up-regulated and 2 down-regulated), 13 between ‘HX’ and ‘SM11’ (12 up-regulated and 1 down-regulated), and 6 between ‘HX’ and ‘SM12’ (5 up-regulated and 1 down-regulated) were found. Compared with ‘SM11’ and ‘SM12’, most anthocyanin metabolites of ‘HX’ were up-regulated. And compared with ‘DS’ and ‘SC’, more than half of the anthocyanin metabolites of ‘HX’ were up-regulated. Further, there were 18 differential metabolites to observe from all the comparison groups and 5 important up-regulated metabolites in ‘HX’. They were Cyanidin 3,5-O-diglucoside (Cyanin), Pelargonin, Cyanidin 3-O-glucoside (Kuromanin), Pelargonidin, and Cyanidin 3-O-rutinoside (Keracyanin). In the Venn diagram, the metabolites of each comparison group are characteristic, which coud separate‘HX’from.others.

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

The results of KEGG indicated that 5 metabolites were taking part in the biosynthesis of anthocyanins. In addition, Pelargonidin was also involved in metabolic pathways, flavonoid biosynthesis, and biosynthesis of secondary metabolites FIGURE 5(A-D) FIGURE 6(A-E) TABLE II.

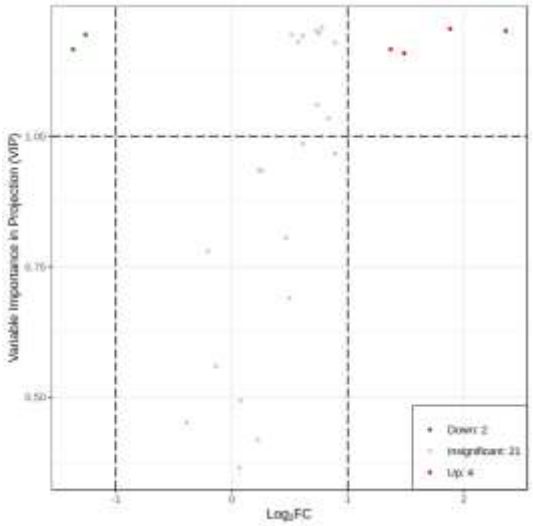


FIGURE 5(A): Volcanic plots of differential metabolites. 'DS' versus 'HX'.

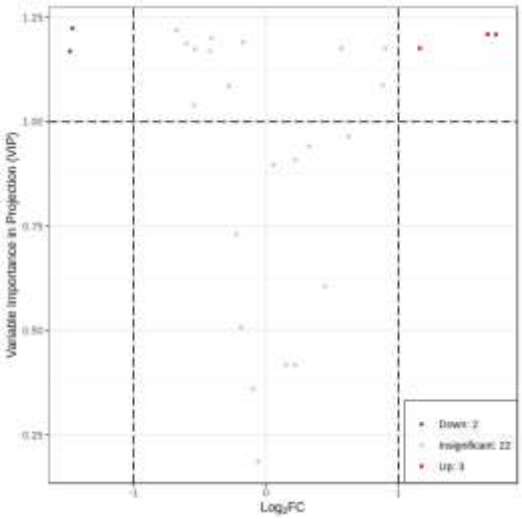


FIGURE 5(B):'SC' versus 'HX'.

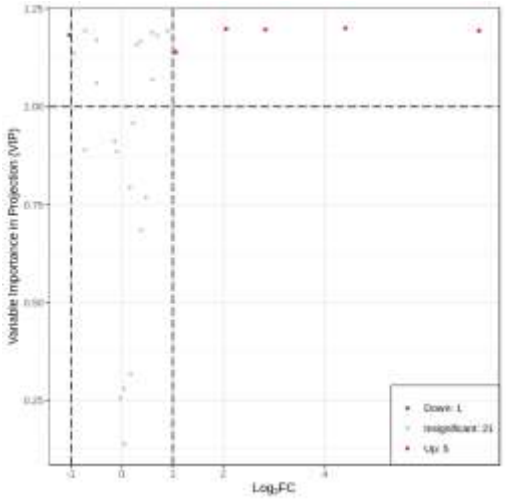


FIGURE 5(C):'SM11' versus 'HX'.

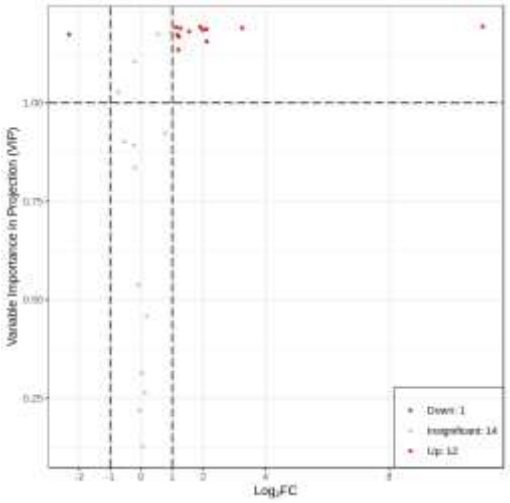


FIGURE 5(D): 'SM12' versus 'HX'.

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

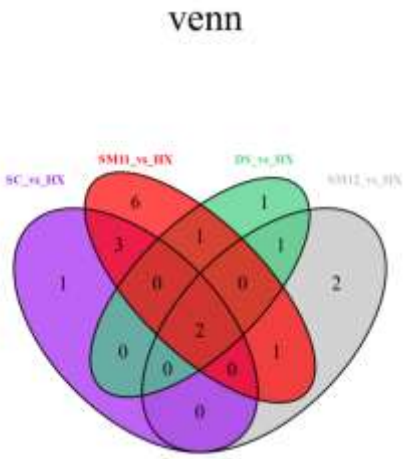


FIGURE 6(A): Venn diagram

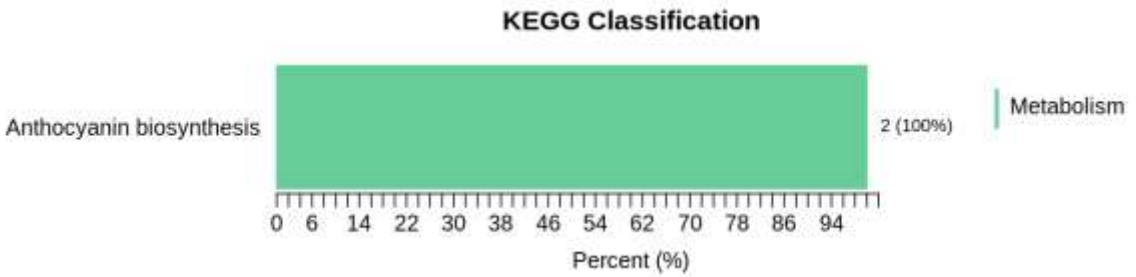


FIGURE 6(B): KEGG classification results. 'DS' versus 'HX'.

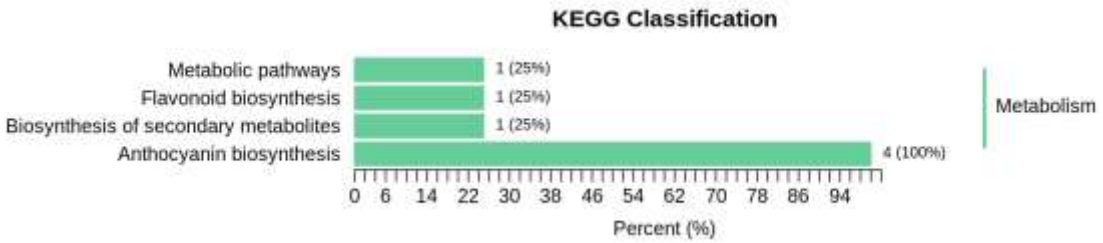


FIGURE 6(C): 'SC' versus 'HX'.

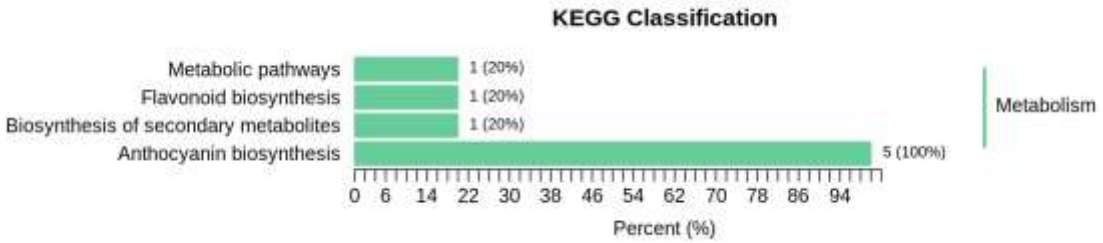


FIGURE 6(D): 'SM11' versus 'HX'.

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

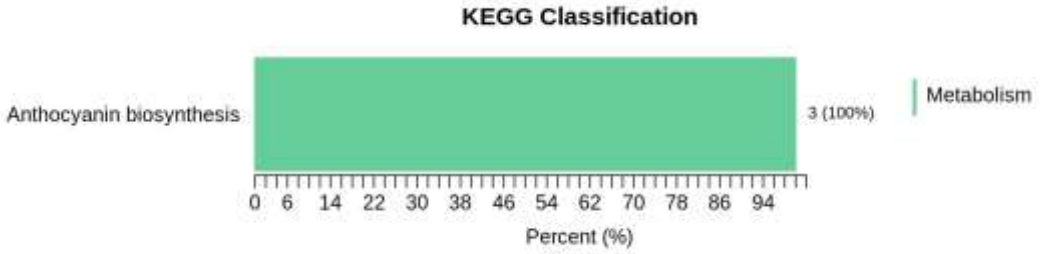


FIGURE 6(E): 'SM12' versus 'HX'.

TABLEII. Active up-regulated anthocyanin metabolites of HX.

Compounds	Class	HX-1	HX-2	HX-3
Cyanidin 3,5-O-diglucoside (Cyanin)	Anthocyanins	256000.00	302000.00	342000.00
Pelargonin	Anthocyanins	1470000.00	1130000.00	1210000.00
Pelargonidin	Anthocyanins	384000.00	390000.00	405000.00
Cyanidin 3-O-glucoside (Kuromanin)	Anthocyanins	9620000.00	8880000.00	9520000.00
Cyanidin 3-O-rutinoside (Keracyanin)	Anthocyanins	19500000.00	10600000.00	14000000.00

IV. CONCLUSION

In this study, the anthocyanin metabolites of 'HX' and others were systematically analyzed.In the comparison groups,there were 27 anthocyanin metabolites to detect, which contain 18 differential, two of which were common. Furthermore, the total anthocyanin content of 'HX' was higher than others, and 5 up-regulated differential metabolites were important, which could demonstrate the specificity of 'HX'

ACKNOWLEDGEMENTS

This research was supported by the Innovation Project of Hebei Academy of Agriculture and Forestry Sciences (2022KJCXZX-SGS-1), the Key Research and Development Program Project of Hebei (21326310D) ,the Scientific and Technological Innovation Talent Team Construction Project of Hebei Academy of Agriculture and Forestry Sciences (C21R0702), Modern Agricultural Industrial Technology System of Hebei (HBCT2021100213), the Innovative team project of Hebei Academy of Agriculture and Forestry Sciences (F21E06-2).

REFERENCES

[1]Afrin, S., M. Gasparrini, T. Y. Forbes-Hernandez, P. Reboredo-Rodriguez, B. Mezzetti, A. Varela-Lopez, F. Giampieri, and M. Battino. 2016. Promising Health Benefits of the Strawberry: A Focus on Clinical Studies. Journal of Agricultural and Food Chemistry 64 (22):4435-4449. doi:10.1021/acs.jafc.6b00857.

Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
May-June 2022 Page No. 2373-2384
Article History: Received: 24 February 2022, Revised: 05 April 2022, Accepted: 08 May 2022, Publication: 30 June 2022

[2] Guo, L., J. S. Kang, N. J. Kang, B. I. Je, Y. J. Lee, Y. H. Park, and Y. W. Choi. 2020. Pelargonidin suppresses adipogenesis in 3T3-L1 cells through inhibition of PPAR-gamma signaling pathway. Archives of Biochemistry and Biophysics 686. doi:ARTN 10836510.1016/j.abb.2020.108365.

[3]Fang, J. 2014. Bioavailability of anthocyanins. Drug Metabolism Reviews 46 (4):508-520. doi:10.3109/03602532.2014.978080.

[4]Khoo, H. E., A. Azlan, S. T. Tang, and S. M. Lim. 2017. Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food & Nutrition Research 61:1-21. doi:Artn 136177910.1080/16546628.2017.1361779.

[5]Mattioli, R., A. Francioso, L. Mosca, and P. Silva. 2020. Anthocyanins: A Comprehensive Review of Their Chemical Properties and Health Effects on Cardiovascular and Neurodegenerative Diseases. Molecules 25 (17). doi:ARTN 380910.3390/molecules25173809.

[6]Tena, N., J. Martin, and A. G. Asuero. 2020. State of the Art of Anthocyanins: Antioxidant Activity, Sources, Bioavailability, and Therapeutic Effect in Human Health. Antioxidants 9 (5). doi:ARTN 45110.3390/antiox9050451.

[7]YANG Li , Li Li , ZHANG Jianjun , DONG Hui , YANG Lei , YANG Qiuye, and and YAN Jin'e. 2017. A New Strawberry Cultivar 'Hongxing '. Acta Horticulturae Sinica 44 (S2):2667–2668.

[8]Wang, F., L. Chen, H. P. Chen, S. W. Chen, and Y. P. Liu. 2019. Analysis of Flavonoid Metabolites in Citrus Peels (Citrus reticulata "Dahongpao") Using UPLC-ESI-MS/MS. Molecules 24 (15). doi:ARTN 268010.3390/molecules24152680.

[9] Zheng Y , Sun X , Miao Y , et al. 2021.A systematic study on the chemical diversity and efficacy of the inflorescence and succulent stem of Cynomorium songaricum. Food & Function, 12.doi:10.1039/D1FO01275D

[10]Bai H,Song Z J,Zhang Y and Li D Y.2020.The bHLH transcription factor PPLS1 regulates the color of pulvinus and leaf sheath in foxtail millet (Setaria italica). Theoretical and applied genetics. 133(6):1911-1926. doi:10.1007/s00122-020-03566-4.

[11]Wang, A. M., R. S. Li, L. Ren, X. L. Gao, Y. G. Zhang, Z. M. Ma, D. F. Ma, and Y. H. Luo. 2018. A comparative metabolomics study of flavonoids in sweet potato with different flesh colors (Ipomoea batatas (L.) Lam). Food Chemistry 260:124-134. doi:10.1016/j.foodchem.

[12]Chen, P. N., S. C. Chu, H. L. Chiou, W. H. Kuo, C. L. Chiang, and Y. S. Hsieh. 2006. Mulberry anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside, exhibited an inhibitory effect on the migration and invasion of a human lung cancer cell line. Cancer Letters 235 (2):248-259. doi:10.1016/j.canlet.2005.04.033.

[13]Chen, Y. G., S. K. Wang, B. Geng, and Z. G. Yi. 2018. Pelargonidin induces antitumor effects in human osteosarcoma cells via autophagy induction, loss of mitochondrial membrane potential, G2/M cell cycle arrest and downregulation of PI3K/AKT signalling pathway. Journal of Buon 23 (3):735-740.

[14]Chen, Z. H., G. A. Nimmo, G. I. Jenkins, and H. G. Nimmo. 2007. BHLH32 modulates several biochemical and morphological processes that respond to P-i starvation in Arabidopsis. Biochemical Journal 405:191-198. doi:10.1042/Bj20070102.

[15]Wang, S. C., H. Tu, J. Wan, W. Chen, X. Q. Liu, J. Luo, J. Xu, and H. Y. Zhang. 2016. Spatio-temporal distribution and natural variation of metabolites in citrus fruits. Food Chemistry 199:8-17. doi:10.1016/j.foodchem.2015.11.113.