High-throughput HPLC analysis of anthocyanins and anthocyanidins in bilberry extract using a novel 2 μm polymeric-C18 column with high chemical stability and unique selectivity

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Introduction

Anthocyanin is a kind of flavonoid pigment found in various fruits and vegetables. Bilberry extract is a well-known source of anthocyanins and it contains 15 anthocyanins and their corresponding 5 aglycones (anthocyanidins). The bilberry extracts are commonly used in dietary supplements and pharmaceutical products to treat various eye disorders and to promote blood circulation. Anthocyanins are demonstrated to be responsible for the pharmacologically activity of bilberry extracts.

The content determination of individual anthocyanin and anthocyanidin is required for the rigorous quality control of bilberry extracts and products. Recently, a new RP-HPLC method has been developed to identify and quantify all the anthocyanins and anthocyanidins in bilberry extracts, and it has been adopted to the Italian Pharmacopeia[1), 2)]. This method uses the mobile phase containing 10% of formic acid, because the chemical structures and the colors of anthocyanins and anthocyanidins vary according to pH and the strongly acidic condition is required for reproducible and high-sensitive analysis. Also the method has a long analysis time (> 60 min/run) with a long length of C18 column (250 X 4.6 mm i. d., 5 μm) because it may be difficult to separate completely all the constituents with similar structure.

In this poster, we apply a polymeric-C18 column named YMC-Pack *Pro* C18 RS to analysis of anthocyanins with above described method. *Pro* C18 RS has an excellent stability even under strongly acidic condition (Fig. 1) and enhanced selectivity for compounds that differ slightly in structure or hydrophobicity (Fig. 2). Moreover, we show an improved method for high-throughput analysis with a novel 2 μm column (YMC-UltraHT *Pro* C18 RS, 100 X 3.0 mm i. d.). The combination of high durability, unique selectivity and excellent efficiency of UltraHT *Pro* C18 RS column enables the development of robust and high-throughput analysis method.

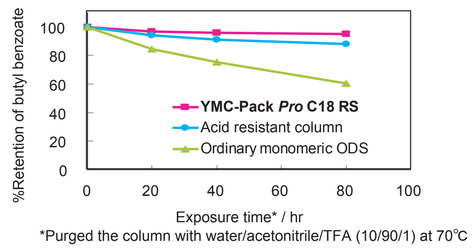


Fig. 1. Retention stability at pH 1, 70℃

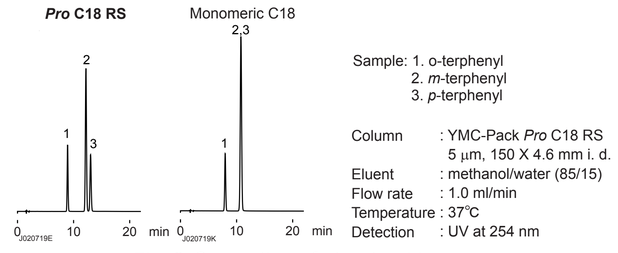
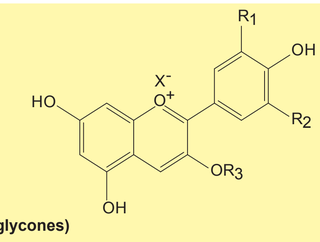


Fig. 2. Separation of structural isomers



Anthocyanidins (aglycones)

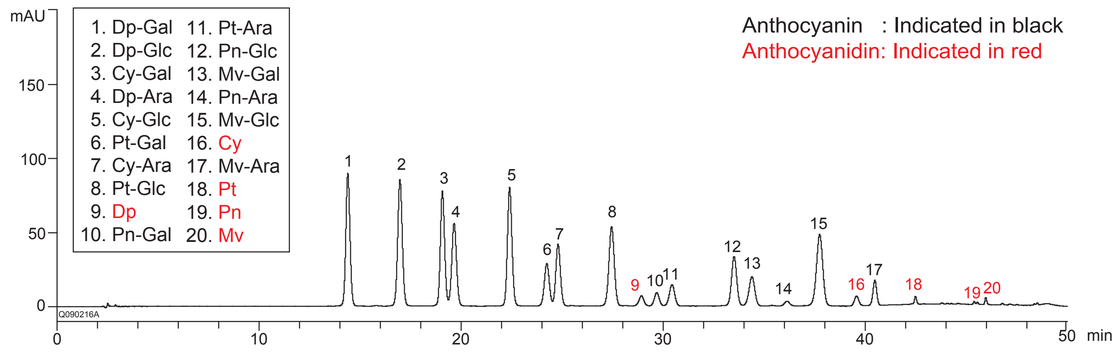
Compound	(abbr.)	R1	R2	R3
Delphinidin	(Dp)	OH	OH	H
Cyanidin	(Cy)	OH	H	H
Petunidin	(Pt)	OCH_3	OH	H
Peonidin	(Pn)	OCH_3	H	H
Malvidin	(Mv)	OCH_3	OCH_3	H

Anthocyanins (glycosides)

Compound	(abbr.)	R1	R2	R3
Delphinidin-3-*O*-Galactoside	(Dp-Gal)	OH	OH	Galactose
Delphinidin-3-*O*-Glucoside	(Dp-Glc)	OH	OH	Glucose
Delphinidin-3-*O*-Arabinoside	(Dp-Ara)	OH	OH	Arabinose
Cyanidin-3-*O*-Galactoside	(Cy-Gal)	OH	H	Galactose
Cyanidin-3-*O*-Glucoside	(Cy-Glc)	OH	H	Glucose
Cyanidin-3-*O*-Arabinoside	(Cy-Ara)	OH	H	Arabinose
Petunidin-3-*O*-Galactoside	(Pt-Gal)	OCH_3	OH	Galactose
Petunidin-3-*O*-Glucoside	(Pt-Glc)	OCH_3	OH	Glucose
Petunidin-3-*O*-Arabinoside	(Pt-Ara)	OCH_3	OH	Arabinose
Peonidin-3-*O*-Galactoside	(Pn-Gal)	OCH_3	H	Galactose
Peonidin-3-*O*-Glucoside	(Pn-Glc)	OCH_3	H	Glucose
Peonidin-3-*O*-Arabinoside	(Pn-Ara)	OCH_3	H	Arabinose
Malvidin-3-*O*-Galactoside	(Mv-Gal)	OCH_3	OCH_3	Galactose
Malvidin-3-*O*-Glucoside	(Mv-Glc)	OCH_3	OCH_3	Glucose
Malvidin-3-*O*-Arabinoside	(Mv-Ara)	OCH_3	OCH_3	Arabinose

Fig. 3. Structures of 15 anthocyanins and 5 anthocyanidins in bilberry extract

Analysis of anthocyanins and anthocyanidins using 5 μm YMC-Pack *Pro* C18 RS



Column	: YMC-Pack *Pro* C18 RS (5 μm, 250 X 4.6 mm i. d.)
Eluent	: A) water/formic acid (90/10) B) acetonitrile/methanol/water/formic acid (22.5/22.5/40/10)
Gradient conditions	: 7-35%B (0-35 min), 35-65%B (35-45 min), 65-100%B (45-46 min), 100%B (46-50 min)
Flow rate	: 1.0 ml/min
Temperature	: 30℃
Detection	: VIS at 535 nm
Injection	: 10 μl
Sample	: Bilberry extract

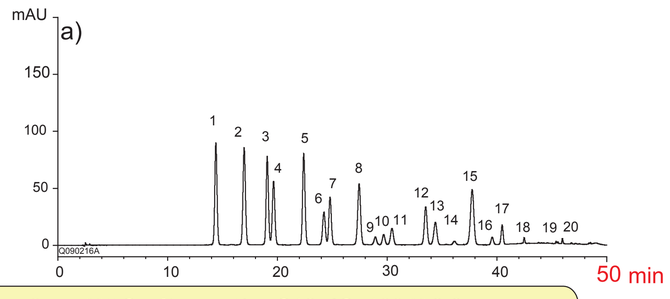
- The excellent and reproducible separation was obtained for all 15 anthocyanins and 5 anthocyanidins in bilberry extract (structure is shown in Fig. 3).
- High density polymeric-C18 phase of *Pro* C18 RS would be suitable for separation of bilberry extract under this strongly acidic condition (10% formic acid).

Fig. 4. Typical HPLC chromatogram of bilberry extract on *Pro* C18 RS with the official method described in the Italian Pharmacopeia[1)]

Method transfer from conventional LC with 5 μm to high-throughput LC with 2 μm

Conventional LC Method* : 5 μm YMC-Pack *Pro* C18 RS, 250 X 4.6 mm i. d.

Flow rate: 1.0 ml/min
Injection: 10 μl
11.7-13.1 MPa (1710-1910 psi)



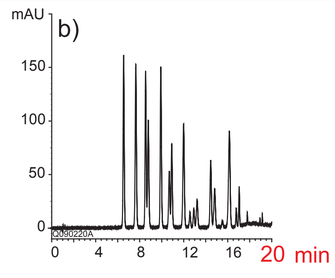
1. Dp-Gal 11. Pt-Ara
2. Dp-Glc 12. Pn-Glc
3. Cy-Gal 13. Mv-Gal
4. Dp-Ara 14. Pn-Ara
5. Cy-Glc 15. Mv-Glc
6. Pt-Gal 16. Cy
7. Cy-Ara 17. Mv-Ara
8. Pt-Glc 18. Pt
9. Dp 19. Pn
10. Pn-Gal 20. Mv

* Conditions are shown in Fig. 4.

High-throughput LC Method : 2 μm YMC-UltraHT *Pro* C18 RS, 100 X 3.0 mm i. d.

- Same linear velocity
- Column length 2/5X
- Gradient time 2/5X

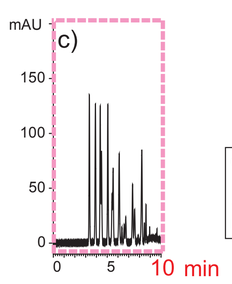
Flow rate: 0.43 ml/min
Injection: 4 μl
21.1-23.6 MPa (3080-3440 psi)



Gradient	: 7-35%B (0-14 min), 35-65%B (14-18 min), 65-100%B (18-18.4 min), 100%B (18.4-20 min)
Temperature	: 30℃
Detection	: VIS at 535 nm

- linear velocity 2X
- Gradient time 1/2X

Flow rate: 0.85 ml/min
Injection: 4 μl
39.9-44.5 MPa (5830-6500 psi)



Gradient	: 7-35%B (0-7 min), 35-65%B (7-9 min), 65-100%B (9-9.2 min), 100%B (9.2-10 min)
Temperature	: 30℃
Detection	: VIS at 535 nm

Comparing to conventional method
80% decrease in analysis time
85% decrease in solvent consumption

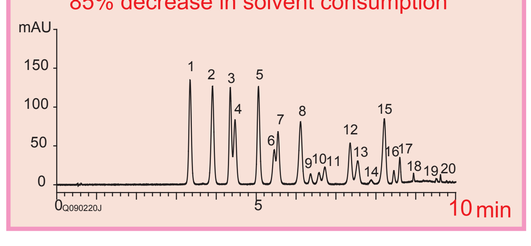
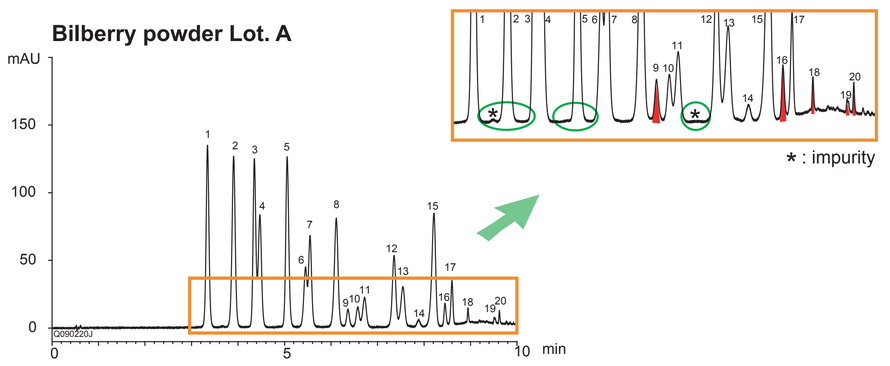
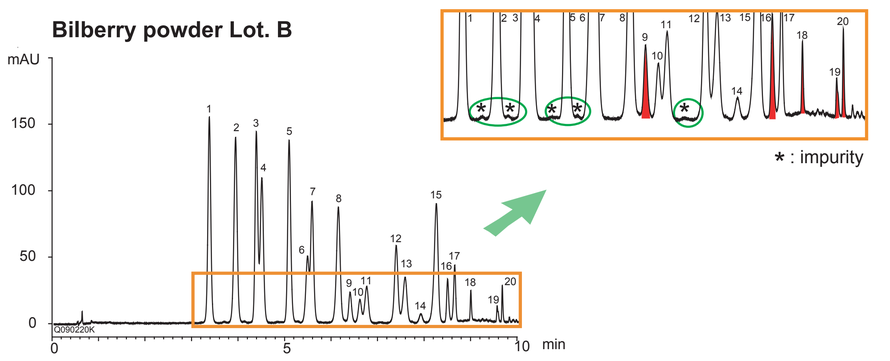


Fig. 5. Method transfer from 5 μm 250 X 4.6 mm i. d. column to 2 μm 100 X 3.0 mm i. d. column

- Easy method transfer could be achieved from a conventional column (YMC-Pack *Pro* C18 RS, 5 μm, 250 X 4.6 mm i. d.) to a newly developed 2 μm column for high-throughput and high-resolution (YMC-UltraHT *Pro* C18 RS, 2 μm, 100 X 3.0 mm i. d.) without changing eluent condition.
- The analysis time could be reduced in 10 minutes using 2 μm column at a 2 times higher linear velocity maintaining excellent resolution of 20 constituents in bilberry extract.

High-throughput analysis of commercial bilberry extract powder



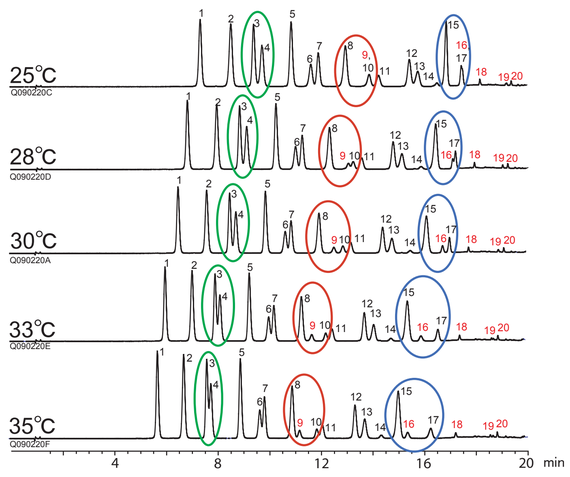


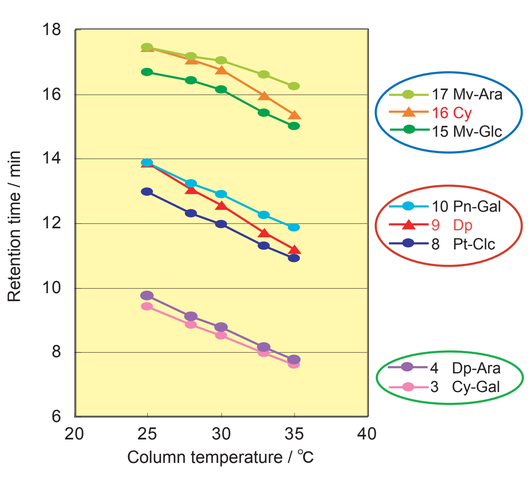
LC conditions are the same as Fig. 5c.

Fig. 6. HPLC profiles of two different lots of commercial bilberry powder

- Lot-to-lot variation is observed in the content of a small amount of impurities and anthocyanidins (red-colored peaks), which are markers of bilberry product quality.
- This high-throughput LC method would be suitable for quality control analysis of bilberry extract or its products, such as dietary supplements and pharmaceuticals.

Effect of column temperature on separation of anthocyanins and anthocyanidins





LC conditions are the same as Fig. 5b.

Fig. 7. Effect of column temperature on selectivity and elution order of anthocyanins and anthocyanidins.

- The slight change in column temperature (2-3℃) affected the retention of anthocyanins, and particularly anthocyanidins. As a result, the resolution varied more greatly between the peaks of anthocyanin and anthocyanidin (indicated with red and blue circle) than between the closely eluting peaks of anthocyanins (indicated with green circle).
- Precise temperature control was required to improve and maintain the resolution.

Conclusions

- YMC-Pack *Pro* C18 RS provides good and reproducible separation of anthocyanins and anthocyanidins under the strongly acidic condition.
- The conventional LC method using 5 μm 250 X 4.6 mm i. d. column can be easily transferred to the high-throughput LC method using 2 μm 100 X 3.0 mm i. d. column with maintaining resolution.
- The high-throughput LC method would be applicable to quality control analysis of various products made from bilberry.
- Column temperature would be important factor for optimizing separation of anthocyanins and anthocyanidins.

References

1) The Italian Republic Pharmacopeia, 11th revision.
2) Ciro Cassinese et al., Journal of AOAC international Vol. 90, No. 4, 911-919 (2007).