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Comprehensive two-dimensional liquid chromatographic analysis of anthocyanins☆

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ABSTRACT

Anthocyanins are naturally occurring plant pigments whose accurate analysis is hampered by their complexity and unique chromatographic behaviour associated with on-column conversion reactions. This paper reports the evaluation of off-line comprehensive two-dimensional liquid chromatography (LC × LC) for the analysis of anthocyanins. Hydrophilic interaction chromatography (HILIC) was used in the first dimension in combination with reversed phase liquid chromatography (RP-LC) in the second dimension. For the selective detection of anthocyanins, diode array detection was used, while high resolution quadrupole-time-of-flight mass spectrometry (Q-TOF) was used for compound identification. As application, the HILIC × RP-LC separation of diverse anthocyanins in blueberries, red radish, black beans, red grape skins and red cabbage is demonstrated. Off-line HILIC × RP-LC revealed information which could not be obtained by one-dimensional HPLC methods, while the structured elution order for the anthocyanins simplifies compound identification and facilitates the comparison of anthocyanin content of natural products by means of contour plots.

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1. Introduction

Anthocyanins are a group of the flavonoid family of phenolic compounds comprising water-soluble pigments that are responsible for the red, blue and violet colours of many flowers, fruits, grains and vegetables [1]. These pigments are important to the food industry and have been recognised for their nutritional value, since they play a role in the reduced risk of a number of chronic and degradative diseases in humans [2,3]. Over the last two decades there has been a significant growth in anthocyanin research due to their potential use in applications varying from food colourants to supplements with potential health benefits [4].

Due to the complexity of many natural products the accurate qualitative and quantitative analysis of anthocyanins remains challenging. The most successful separation of anthocyanins is obtained by reversed phase liquid chromatography (RP-LC) [5]. Coupled to photodiode-array (PDA) detection, selective detection of anthocyanins is possible [6,7], since they absorb in the visible range around 500–550 nm in low pH mobile phase conditions. RP-LC hyphenated with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) provides increased sensitivity and structural elucidation capabilities [8]. Despite these advantages, however, RP-LC–MS also demonstrates some limitations for anthocyanin analysis. First, RP-LC separation of anthocyanins is characterised by extremely low optimal mobile phase velocity, and, when analysed under sub-optimal conditions, very low chromatographic efficiency [9,10]. This is a result of inter-conversion between different anthocyanin species in solution, which depends on the pH and anthocyanin structure [9–11]. Secondly, the chemical diversity and lack of commercially available standards hampers identification, whereas similar fragmentation patterns between different anthocyanin classes complicate structural elucidation. For these reasons, improved chromatographic separation of anthocyanins remains important.

The coupling of two independent LC separations in comprehensive two-dimensional liquid chromatography (LC × LC) offers a powerful approach for the separation of compounds in complex samples [12–15]. In LC × LC all sample components eluting from the first dimension are also subjected to separation in a second dimension [16], in this manner providing the advantages of increased

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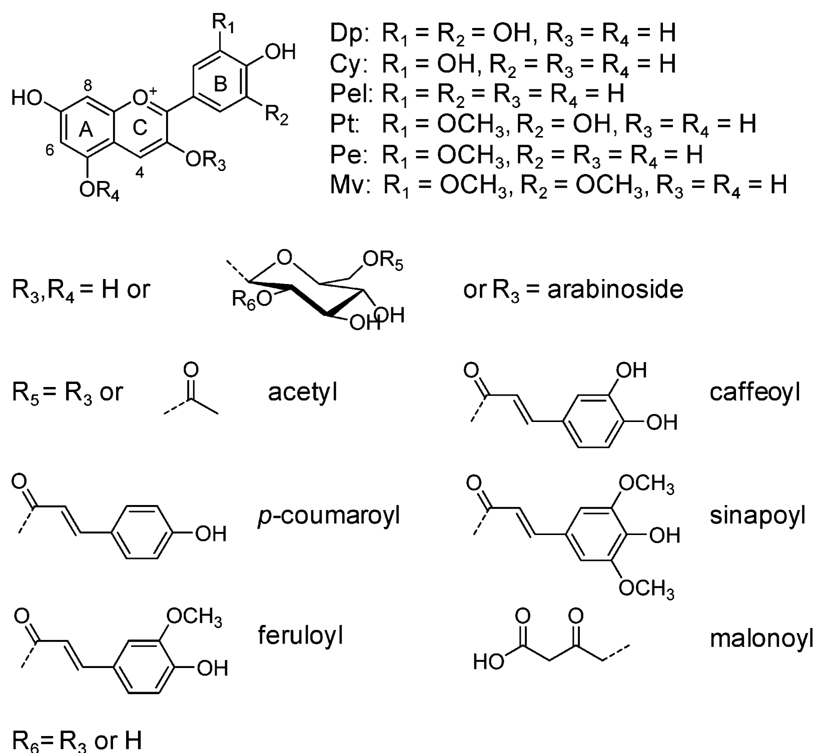

Fig. 1. General structures of black bean, blueberry, red grape skin, red cabbage and red radish anthocyanins. *Abbreviations*: Dp, delphinidin; Cy, cyanidin; Pel, pelaragonidin; Pt, petunidin; Pe, peonidin; Mv, malvidin.

resolution and selectivity in comparison to one-dimension (1-D) LC.

One of the principal requirements of an efficient LC × LC system is that the separation modes employed be orthogonal. This implies that the separation mechanisms to be used in each dimension be carefully selected in order to minimise the retention correlation between dimensions [17]. Hydrophilic interaction chromatography (HILIC) is receiving increasing attention as an alternative mode to RP-LC for the analysis of polar compounds. In fact, the combination of HILIC and RP-LC provides a high degree of orthogonality, and HILIC × RP-LC has successfully been applied to the analysis of a range of phenolic compounds [18–21]. However, to the best of our knowledge, LC × LC has to date not been applied to anthocyanin analysis.

The objective of this study was therefore to develop a HILILC × RP-LC method, employing the HILIC method recently developed for anthocyanins [22], for the separation of various anthocyanin-rich natural products. As application, several samples characterised by complex and diverse anthocyanin constituents were selected, including blueberries, grape skins, black beans, red cabbage and red radish (Fig. 1).

2. Experimental

2.1. Reagents and materials

Blueberries, black beans, grapes, red cabbage and red radish were purchased from a local supermarket. HPLC grade acetonitrile, methanol and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Steinheim, Germany) and formic acid from Merck (Darmstadt, Germany). Deionised water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). All mobile phases were filtered through 0.45 μm HLVP membrane filters (Millipore), and degassed in an ultrasonic bath. The OASIS HLB solid phase extraction (SPE) cartridges (6 mL, 500 mg) were from Waters (Milford, MA, USA).

2.2. Sample preparation

Each of the five samples were extracted in the same manner as reported previously [22]. The extracts were loaded on a preconditioned SPE cartridge, rinsed with acidified water and eluted with 3 mL × 1 mL methanol/formic acid (95/5, (*v*/*v*)) [22]. The methanolic fraction containing the anthocyanins was evaporated to dryness and re-dissolved in 0.5 mL methanol/formic acid (95/5, (*v*/*v*)) prior to HPLC analysis. No loss of anthocyanins was observed following SPE clean-up.

2.3. Instrumentation

One-dimensional HILIC-UV and RP-LC–UV analyses as well as off-line HILIC × RP-LC analyses were performed on a Waters Acquity UPLC system equipped with a binary pump, sample manager, column oven compartment, photodiode array (PDA) detector (500 nL flow cell, 10 mm path length) and controlled by Waters Empower software (Waters, Milford, MA, USA). UV–vis chromatograms were recorded at 500 nm using an acquisition rate of 10 Hz.

LC–MS and LC–MS^E analyses were performed on a Waters Acquity UPLC system equipped with a binary solvent manager, sample manager and column oven, and interfaced through an electrospray ionisation (ESI) source to a Waters Synapt G2 quadrupole time-of-flight (Q-TOF) mass spectrometer.

2.4. Chromatographic conditions

2.4.1. Off-line HILIC × RP-LC analyses

HILIC separation was performed on an XBridge BEH Amide column (150 mm × 4.6 mm i.d., 2.5 μm particles, Waters) with a

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