Journal Pre-proofs

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PII: S0308-8146(20)30377-0
DOI: https://doi.org/10.1016/j.foodchem.2020.126515
Reference: FOCH 126515

To appear in: *Food Chemistry*

Received Date: 30 October 2019
Revised Date: 24 February 2020
Accepted Date: 26 February 2020

Please cite this article as: Hong, H.T., Netzel, M.E., O'Hare, T.J., Optimisation of extraction procedure and development of LC–DAD–MS methodology for anthocyanin analysis in anthocyanin-pigmented corn kernels, *Food Chemistry* (2020), doi: https://doi.org/10.1016/j.foodchem.2020.126515

~~Optimization~~ Optimisation of extraction procedure and development of LC–DAD–MS methodology for anthocyanin analysis in anthocyanin-pigmented corn kernels

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Abstract: An ultra-~~high~~ high-performance liquid chromatography-~~-~~diode array detector-~~-~~mass spectrometry ~~(UHPLC-DAD-MS)~~ method was developed for characterisation and quantification of anthocyanin components in complex corn-kernel matrices. The anthocyanin profiles and total anthocyanin content (TAC) of mature seeds of five types of anthocyanin-pigmented corn were reported. Internal standards were used to validate the efficiency of extraction and optimise the liquid extraction procedure for anthocyanins. A total of eighteen anthocyanins were identified and quantified. Cyanidin-based glucosides were the major pigments of purple-pericarp sweetcorn (75.5% of ~~total anthocyanin content (~~TAC~~)~~) and blue-aleurone maize (91.6%), while pelargonidin-base glucosides composed the main anthocyanins of reddish-purple-pericarp sweetcorn (61.1%) and cherry-aleurone maize (74.6%). Importantly, previous studies ~~reporting~~ reported the presence of acetylated and succinylated anthocyanins in corn kernels; these compounds were found to be artefact pigments, generated during the extraction process. These crucial findings provide the correct anthocyanin profiles of pigmented corns, and ~~emphasize~~ emphasise the importance of using acidified solutions for the extraction of corn-based anthocyanins.

Keywords: Artefact pigments, extraction solution, esterification, analysis, anthocyanins, coloured corn, stability.

Introduction

Anthocyanins are natural purple, blue, and red pigments and are a major subclass of polyphenols/flavonoids (Yousuf, Gul, Wani, & Singh, 2016). These pigments are present in a wide range of flowers, fruits and vegetables, and have been reported to be associated with a range of human health benefits, including anti-inflammatory action (Blando, Calabriso, Berland, Maiorano, Gerardi, Carluccio, et al., 2018), antihypertensive activity (reduction of blood pressure) (Shindo, Kasai, Abe, & Kondo, 2007), and a slowing of age-related cognitive decline and memory loss (Lu, Wu, Zheng, Hu, Cheng, & Zhang, 2012).

Pigmented corns contain high concentrations of anthocyanins (up to 6.02 mg/g dry weight (DW)) (Yang, Chen, Yuan, Zhai, Piao, & Piao, 2009), with pericarp-pigmented corns normally having higher anthocyanin concentrations than aleurone-pigmented corns (Li, Zhang, Yang, Dong, Ren, Fan, et al., 2019). In general, the anthocyanin profile consists of cyanidin-, pelargonidin- and peonidin-based glucosides (Lao & Giusti, 2016; Nankar, Dungan, Paz, Sudasinghe, Schaub, Holguin, et al., 2016; Vayupharp & Laksanalamai, 2015). Because of the potentially high anthocyanin concentration in pigmented corn, and its association with various health benefits, several studies have attempted to extract and quantify the anthocyanins of pigmented corn (Lao & Giusti, 2016; Nankar, et al., 2016). Low stability of anthocyanins following extraction, and a strong tendency for anthocyanin to remain bound to the corn matrix, are still major issues to be addressed to improve the accuracy of anthocyanin quantification. In regard to extraction, although anthocyanins are water soluble, a combination of methanol (or ethanol) with water is required to optimise the efficiency of extraction solutions (Abdel-Aal, Hucl, & Rabalski, 2018; Downey & Rochfort, 2008; Vayupharp & Laksanalamai, 2015). This is principally because of interactions between anthocyanins and ionic carbohydrates (e.g., pectin) in sample matrices (Fernandes, Bras, Mateus, & de Freitas, 2014; Takahama, Yamauchi, & Hirota, 2013). Commonly, a range of 40% aqueous methanol to 100% methanol is used to extract anthocyanin from fruit and vegatable matrices (Chandrasekhar, Madhusudhan, & Raghavarao, 2012; Fredericks, Fanning, Gidley, Netzel, Zabaras, Herrington, et al., 2013). It is crucial to optimise the ratio of methanol to water in the extraction solvent, both to maximise the extraction capacity, and also to minimise extraction time. Secondly, prolonged extraction time (Zhang, Jordheim, Lewis, Arathoon, Andersen, & Davies, 2014) or high temperatures (Piyapanrungrueang, Chantrapornchai, Haruthaithanasan, Sukatta, & Aekatasanawan, 2016; Trikas, Papi, Kyriakidis, & Zachariadis, 2016) may increase the efficiency of anthocyanin extraction, but these conditions also reduce anthocyanin stability (Mori, Goto-Yamamoto, Kitayama, & Hashizume, 2007; Zhao, Corrales, Zhang, Hu, Ma, & Tauscher, 2008). Finally, because of the inherent poor stability of anthocyanins in non-acidified solvents (Aaby, Mazur, Nes, & Skrede, 2012; Chen, Inbaraj, & Chen, 2012), acidified extraction

methods have been developed with acetic acid (AA) (Downey & Rochfort, 2008), formic acid (FA) (Fredericks, et al., 2013), phosphoric acid (Trikas, Papi, Kyriakidis, & Zachariadis, 2016), or hydrochloric acid (HCl) (Abdel-Aal, Hucl, & Rabalski, 2018; Deineka, Sidorov, & Deineka, 2016; Vayupharp & Laksanalamai, 2015; Yang & Zhai, 2010b). The presence of a strong acid (HCl) in a methanol/water mix promotes ionisation of extraction solution, which assists with breaking the association between anthocyanins and pectin in plant matrices, allowing methanol and water to extract anthocyanins more efficiently, particularly from high starch samples. The combination of acidified methanol and water therefore provides a favourable environment for the extraction of both free and plant-cell-wall bound anthocyanins in corn (Lao & Giusti, 2016) and other matrices, such as purple wheat (Abdel-Aal, Hucl, & Rabalski, 2018). However, the stability of anthocyanins during extraction ~~procedure~~ at room temperature (rt) (Li, et al., 2019), 40 ºC (Abdel-Aal, Hucl, & Rabalski, 2018), or at 60–80 ºC (Piyapanrungrueang, Chantrapornchai, Haruthaithanasan, Sukatta, & Aekatasanawan, 2016) was not taken into account in these previous studies. In addition, the use of low acid concentrations of 0.1% HCl (Galvez Ranilla, Christopher, Sarkar, Shetty, Chirinos, & Campos, 2017) or 0.01% HCl (Vayupharp & Laksanalamai, 2015), or extraction with 100% methanol (Yang & Zhai, 2010a) in starchy matrices, have a low efficiency of extraction for anthocyanin.

Despite the benefits of extraction of an acidified methanol solution, anthocyanin stability can still be compromised. Downey et al. (2007) have previously reported that coumaroyl-, malonyl- and succinyl-based anthocyanins are unstable in highly acidified solutions. Ester bonds, such as those between the glucoside and coumaric acid, are hydrolysed by acid resulting in anthocyanin interconversion (Downey & Rochfort, 2008). The rate of this reaction can be slowed by using cooler extraction temperatures and lower acid concentrations, in contrast to the improved extraction efficiency provided at higher temperatures and higher acidity. Therefore, it is crucial to balance temperature, the composition of the extraction solution, and acidity to efficiently extract anthocyanins from the corn kernel matrix, while minimising subsequent degradation, or interconversion of the original anthocyanins. The current paper provides an optimised methodology for efficiently

extracting anthocyanins of kernels from a range of pigmented corn-types, without compromising the anthocyanin profile through degradation or anthocyanin interconversion. This methodology was further extended to take into account the properties of the acidic mobile phase used in HPLC analysis.

1. Materials and Methods

1.1. Materials

1.1.1. Plant materials

Mature kernels (approximately 10% moisture) of purple-pericarp sweetcorn (PPS), reddish-purple-pericarp sweetcorn (RPS), purple-pericarp maize (PPM), purple-pericarp-blue-aleurone maize (PP-BAM), blue-aleurone maize (BAM) and cherry aleurone maize (CAM) (Fig. S1) were harvested in autumn 2018 at the Gatton Research Facility, Gatton, QLD, Australia. A composite sample of PPS and white sweetcorn (anthocyanin-free) were also harvested at 26 days after pollination (DAP, 78% moisture), equivalent to the eating stage at which sweetcorn is consumed. Individual plants were self-pollinated by hand to exclude foreign pollen. Five cobs were harvested randomly, dehusked and immediately transported (1 h transit) to The University of Queensland, Health and Food Sciences Precinct at Coopers Plains, QLD, where they were stored at ~~–~~20 °C prior to analysis.

1.1.2. Chemicals

Cyanidin-3-glucoside (Cy3G), pelargonidin-3-glucoside (Pg3G), delphinidin-3-glucoside (Del3G) and peonidin-3-glucoside (Pn3G) standards were obtained from Sigma-Aldrich (Sydney, NSW, Australia) and Extrasynthèse (Genay, France). All other chemicals and solvents were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich. All chemicals were HPLC or analytical grade. ~~Deionized~~ Deionised water (Millipore Australia Pty Ltd, Kilsyth, VIC, Australia) was used throughout the study unless otherwise stated.

1.1.3. Solutions

A solvent combining methanol, deionized water and FA (80:19:1; solution A) was used as a matrix-free solution to dissolve 2 mg each of Cy3G, Pg3G and Pn3G in 10 mL to create master stock solutions of 200 mg/L. Solutions for nine calibration standard concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, and 50 mg/L of each external standard, together with 1 mg/L of Del3G (internal standard, IS) were prepared from the master stock solutions by diluting with a white (anthocyanin-free) sweetcorn matrix solution.

An extraction solution consisting of methanol, ~~deionized~~ deionised water (80:20) in 0.1M HCl (solution B) was prepared for sample extraction. IS (this anthocyanin has not been reported to exist in maize) was dissolved in the extraction solution to ~~have~~ give a final concentration of 100 mg/L. ~~The~~ ~~m~~Mobile phase A of the liquid chromatography (LC) system consisted of acetonitrile (ACN), deionized water, and FA (92:7: 1), v/v), and ~~the~~ mobile phase B consisted of 1% FA in ACN.

2.2. Methods

2.2.1. Anthocyanin analysis

Sample preparation

Sample preparation was followed (Hong, Netzel, & O'Hare, 2020), with modifications. Briefly, three rows of kernels were snap frozen by liquid nitrogen and cryo-milled using a ball mill (MM400 Retsch Mixer Mill, Haan, Germany) operated at 30 Hz for 60 s. The powdered sample (about 0.5 g) was transferred to a ~~15~~ 15-mL Falcon® tube, 120 µL of IS ~~was~~ were added and 3.88 mL of cold extraction solution B (4 °C) ~~was~~ were added. The mixture was sonicated for 10 min at 4 °C and then shaken on a horizontal reciprocating shaker (RP 1812; Paton Scientific, Victor Harbor, SA, Australia) at 250 rpm~~/min~~ for 10 min under dim light and cool temperature (4 °C) before being centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was removed and the pellet residue re-extracted twice using the same procedure with 4 mL of cold extraction solution B (4 °C). The combined supernatants were filtered through a 0.22 μm hydrophilic PTFE syringe filter into a UHPLC vial for analysis.

Instrumental conditions

Ultra-high-performance liquid chromatography–diode array detector–mass spectrometry (UHPLC–DAD–MS)

Anthocyanins were identified and quantified using a Q Exactive Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA; System 1), an Agilent 1290 Infinity UHPLC–DAD system (Agilent Technologies, USA; System 2), and a Shimadzu UHPLC–DAD–ESI-MS/MS system (Shimadzu, Kyoto, Japan; System 3) carried out on A Nexera X2 UHPLC system consisting of a system controller (CBM-30A), three pumps (LC-30AD), an autosampler (SIL-30AC), column heater (CTO-20AC), diode-array detectors (DAD) detector (SPD-M30A) and two degassers (DGU-$20A_{3R}$ and DGU-$20A_{5R}$). The Nexera X2 UHPLC system was coupled to an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) and the ESI source was operated with a nebulizer gas flow of 2 L/min, drying gas flow of 10 L/min, with a desolvation line (DL) temperature of 250 °C and heat block temperature of 400 °C. Selected ion monitoring (SIM) and product ion monitoring was operated at a collision energy of –20 V, and full MS scans in positive mode were in the range of *m/z* 100–1200. Labsolutions LCMS software Ver.5.85 (Shimadzu) was used for instrument control and data-processing.

Chromatographic separation was carried out on a reverse phase Acquity UPLC BEH C18 column (150 ×2.1 mm i.d., 1.7 µm particle size; Waters, Dublin, Ireland). Column temperature was maintained at 50 °C and at a flow rate of 0.25 mL/min. The DAD spectrum was scanned from 200 to 800 nm and monitored at 520 nm. The elution was programmed with 100% of mobile phase A as the initial isocratic eluant hold held for 1 min, followed by a linear gradient from 100% to 85% of mobile phase A for over 30 min, purging 3 min at 100% with mobile phase B, conditioning for 1 min, and re-equilibrating for 5 min.

Anthocyanin identification

Anthocyanins were detected by DAD at 520 nm and spiking external standards of Cy3G, Pg3G and Pn3G to determine standard elution times and to confirm molecular masses. The remaining compounds were determined by their unique absorption maximum on DAD detection (Fig. S3), and their mass-to-charge ratio and fragment pattern *via* mass spectrometry in positive ion mode (Fig. 2 and Table 3). Comparison of elution order to previously published literature (Deineka, Sidorov, & Deineka, 2016; Nankar, et al., 2016; Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2002; Paulsmeyer, Chatham, Becker, West, West, & Juvik, 2017; Zhao, Corrales, Zhang, Hu, Ma, & Tauscher, 2008) was additionally used to confirm anthocyanin identity.

Anthocyanin quantification

As more than 700 anthocyanins have been found (Wallace & Giusti, 2015), with only a few available as commercial standards, total anthocyanin concentration (TAC) was expressed as equivalents from one of the six basic anthocyanin standards, i.e., Cy3G, Pg3G, Del3G, petunidin-3-glucoside (Pt3G), Pn3G or malvidin-3-glucoside (Mal3G). In this study, the final concentration of individual anthocyanins was measured by comparison of the area of each peak from the DAD spectra to the external calibration curves of Cy3G, Pg3G and Pn3G. Equivalent concentrations were calculated for all anthocyanin malonyl-glucosides based upon their flavylium cations, due to differences in the maximum absorbance of Cy, Pg and Pn (Fig. S3). TAC was calculated as the sum of Cy3G, Pg3G, and Pn3G, and their respective malonated counterparts. Selected ~~Ion~~ ion ~~Monitoring~~ monitoring (SIM) ~~mode~~ in positive ion mode was scanned to calculate the relative contribution of the co-eluted compounds within each peak (Fig. S6). The percentage contribution was used to correct the peak area of DAD and to quantify the co-elut~~ed~~ing anthocyanin compounds.

2.2.2. Optimisation of the extraction procedure

A composite PPS sample was chosen as a representative of the pigmented corn samples for two reasons. Firstly, PPS contained a higher anthocyanin concentration compared to PP-BAMZ, BAM

and CAM (Hong, Netzel, & O'Hare, 2020; Nankar, et al., 2016). Secondly, PPS possesses a high sugar and starch content that have strong interactions with anthocyanins in their ~~matrices~~ matrices (Fernandes, Bras, Mateus, & de Freitas, 2014; Takahama, Yamauchi, & Hirota, 2013). Commonly used extraction solutions, including different methanol:water mixtures ranging from 0:100 (Jing & Giusti, 2007), 20:80 (Downey & Rochfort, 2008), 40:60 (Downey & Rochfort, 2008), 60:40 (Joshi, Rana, Kumar, Kumar, Padwad, Yadav, et al., 2017), 80:20 (Fredericks, et al., 2013), to 100:0 (Ma, Johnson, Liu, DaSilva, Meschwitz, Dain, et al., 2018) in 0.1M HCl were compared to optimise the solution for extraction of anthocyanins from a composite PPS sample. Five different solutions, including 1% FA (Fredericks, et al., 2013), 5% AA (Heffels, Weber, & Schieber, 2015), 0.1M HCl (Heffels, Buhrle, Schieber, & Weber, 2017), 0.05M HCl and 0.01M HCl (Fischer, Jaksch, Carle, & Kammerer, 2013) in 80% methanol were also used to extract anthocyanins from a composite ~~of~~ PPS sample to further optimise the efficiency of the extraction solution by acidification. Higher concentrations of HCl (>0.1M) in solution were avoided, due to the reported accelerated degradation of both anthocyanins and the UPLC column material (Downey & Rochfort, 2008).

2.2.3. Method validation

Validation of the analytical procedure was performed according to the NATA (National Association of Testing Authorities, Australia) guidelines (NATA, 2018).

Principles of analytical calibration and anthocyanin recovery

Calibration standards of Cy3G, Pg3G and Pn3G were prepared by spiking the matrix-free and sweetcorn-matrix solution with appropriate volumes of anthocyanin stock solutions (200 µg/mL for each anthocyanin). Three calibration curves were established within the concentration range of 0.05–50 µg/mL for each external standard together with 1 µg/mL of internal standard. These calibration curves of concentration ratios were used to quantify anthocyanins in pigmented corn.

Additionally, the anthocyanin-free samples were spiked with three different concentrations (low– 0.1 µg/mL, medium– 10 µg/mL and high– 50 µg/mL; n = 9 for each concentration and anthocyanin) of Cy3G, Pg3G and Pn3G. The spiked samples were extracted and analysed, and recoveries calculated by comparing the ratio of detected and spiked concentrations of Cy3G, Pg3G and Pn3G.

Limit of detection (LOD) and quantification (LOQ)

LOD and LOQ were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively. The sweetcorn matrix was obtained from anthocyanin-free white sweetcorn at the same physiological maturity (26 DAP) as the analysed samples. LOD and LOQ were determined for the three predominant anthocyanins present in pigmented corn (Cy3G, Pg3G and Pn3G).

Matrix effect

Calibration standards of Cy3G, Pg3G and Pn3G within the concentration range of 0.05–50 µg/mL were prepared and analysed in matrix-free solution and sweetcorn matrix solution. The matrix effect was calculated by comparing the different slopes of the calibration curves in the matrix-free solution and sweetcorn matrix solution (S_s/S_m). The relative ratio of the two slopes was calculated to estimate the effect of matrix components on the signal strength of Cy3G, Pg3G, and Pn3G (NATA, 2018).

$$\%\text{ME} = \left(\frac{S_m}{S_s} - 1\right) * 100\%$$

Precision and accuracy

Intra- and inter-day precision of the method was assessed by degrees of freedom for repeatability (df = n– 1 = 8) within one day~~,~~ and repeated on three consecutive days. Samples were analysed on System– 3. The same extraction procedure was applied in the following week with a different ~~operator, and~~operator and analysed on System– 2.

$$\% \text{ accuracy} = \frac{measurement\ result}{true\ result} \times 100\%$$

2.2.4. Stability studies

Stability of individual anthocyanins in the extraction solution of PPS sample was investigated after storage at −20 °C, 4 °C and room temperature (rt, 23 °C) for 5 hours, 12 hours, 24 hours, 4 days and three weeks.

2.2.5. Statistical analysis

A one-way analysis of variance (AOVA), using Minitab 17 software for Windows (Minitab Inc., State College, PA, USA), was applied to assess variances of anthocyanin content in pigmented sweetcorn kernels and stored extraction solutions. Least significant differences ($p < 0.05$) were used to compare differences between means.

2. Results and discussion

2.1. Optimisation of the extraction solution

From the range of methanol:water ratios evaluated for anthocyanin extraction, 80% acidified methanol produced the highest anthocyanin extraction concentration for the PPS matrix, with a maximum TAC of 64.5 mg/100g fresh weight (FW) (Fig. 1A and Fig. 1B). Neat methanol has a slightly lower degree of extraction, potentially because it coagulates protein in plant cells instantly, forming a ring of coagulated protein around the cell walls, preventing further penetration of methanol (McDonnell, 2007). In contrast, 80% methanol solution penetrates the cell walls at a slower rate, and rate and allows continued extraction of anthocyanins. In addition, concentrated methanol tends to extract a large number of non-anthocyanin compounds from the pigmented corn matrix, causing a longer analysis time due to the requirement to elute all non-anthocyanin compounds from the LC-column. On the other hand, a high concentration of water in the extraction solution leads to a decrease in the degree of extraction efficiency of bound- anthocyanins. This is relevant since a substantial part of anthocyanins in the kernel matrix are bound to plant cells. As a result, a reasonably high methanol

level (80%) efficiently allowed the extraction solution to isolate anthocyanins from the complex matrix of the pigmented corn kernel.

Although, acid concentrations of 1% FA (Fredericks, et al., 2013), 5% AA (Tatsuzawa, Hosokawa, Saito, & Honda, 2012), or 0.01 M HCl (Brito, Areche, Sepulveda, Kennelly, & Simirgiotis, 2014) have been used in many previous studies to extract anthocyanin from different matrices, the present study observed that an extraction solution acidified with 0.1 M HCl produced a significantly ($Pp < 0.05$) higher TAC than either 1% FA, 5% AA or 0.01 and 0.05 M HCl (Fig. 1A)."

3.2. Stability

The stability of the six major anthocyanins in PPS extracted with solution B was tested under three different temperatures: 23 °C, 4 °C and –20 °C. The results (Fig. S2) showed that the concentration of Cy3G, Pg3G and Pn3G increased, with a concurrent decline in the concentration of their malonated counterparts over the storage period at 23 °C. These findings are in agreement with previous observations (Downey & Rochfort, 2008) of the stability of anthocyanins in grape skin held in acidified solutions for 24 hours at rt. The main reason for the observed increase and decline, respectively, is that strongly acidified solutions (e.g. 0.1 M HCl) hydrolyse the ester linkages between malonic acid and Cy3G, Pg3G and Pn3G in the malonated anthocyanins, resulting in an increase of Cy3G, Pg3G and Pn3G. This reaction, however, is slowed down at lower temperatures (–(–20 °C, 4 °C), as seen in Fig. S2. All anthocyanins were stable for up to 24 hours without any significant ($p < 0.05$) change in TAC ($p < 0.05$) at 4 °C. In fact, the extraction solution of purple-pericarp sweetcorn can be stored for up to 3 weeks at –20 °C, or for 24 hours in a refrigerator or LC-autosampler at 4 °C, without any significant ($p < 0.05$) degradation. The findings also confirm that low temperature maintenance during the extraction procedure is crucial to minimising the degradation or alteration of anthocyanin components.

Esterification products of anthocyanins

Apart from the findings above that malonyl moieties of Cy3G, Pg3G and Pn3G can be hydrolysed in acidified extraction solution to ~~result in~~give a decrease in the concentration of malonyl anthocyanins and an increase in free anthocyanins, the malonyl moieties of Cy3G, Pg3G and Pn3G could also be esterified in acidified methanol to create methyl-malonate esters (Fig. 2A and Table 1). The products of this esterification reaction appeared after the PPS extract~~ion~~ was stored at 23 °C for 5 hours and were quantifiable by DAD and MS/MS after 4 days at this temperature (Fig. 2 and Fig. S4). These esterification products were also found by Vayupharp and Laksanalamai (2015), comparing the anthocyanin profile of a Thai waxy purple corn cob extracted in acidified water and in acidified ethanol. Vayupharp and Laksanalamai (2015) reported these compounds as natural anthocyanins, although it is more likely they were the esterification products formed from ethanol and anthocyanins.

Identification of the esterification products of anthocyanins

The MS/MS fragmentation pattern of compound N3 including *m/z* 603 = *m/z* $[M+H-31]^+$, *m/z* 575 = *m/z* $[M+H-59]^+$ and *m/z* 535 = *m/z* $[M+H-100]^+$ indicated the removal of a $-OCH_3$, $-COOCH_3$ and methyl malonate group (Fig. 2B and Table 1). Therefore, N3 is Cy-3-(methylmalonatemalonylglucoside). Compound N1 (*m/z* 549 $[M]^+$ and MS/MS fragmentation of *m/z* 517 = *m/z* $[M+H-31]^+$, *m/z* 490 = *m/z* $[M+H-59]^+$, *m/z* 449 = *m/z* $[M+H-100]^+$ are methyl malonate moieties of Cy3G (Fig. 2C and Table 1). Likewise, nine new esterification products in Table 1 and Fig. S4 were identified from their MS/MS fragmentation patterns.

Six of the esterified compounds listed in Table 1 above have been previously reported in purple-pericarp maize and blue-aleurone maize as Cy-3-(6''-succinylglucoside (*m/z* 549, N1), Cy-3-(6''-malonylsuccinylglucoside) (*m/z* 635, N2 and N3), Pn-3-(6''-succinylglucoside) (*m/z* 563, N4), Pg-3-(malonylsuccinylglucoside) (*m/z* 619, N5 and N6) and Cy-3-disuccinylglucoside (*m/z* 649, N7, N8 and N9) (Lao & Giusti, 2016; Nankar, et al., 2016). In the current study, these compounds could not be detected either by DAD at 520 nm or by MS in fresh samples, unless the samples were stored at 23 °C for at least 5 hours.

Therefore, the succinyl and ethylmalonyl anthocyanins previously reported in acidified purple maize extracts (methanolic or ethanolic) (Nankar, Dungan et al. 2016; Pascual-Teresa, Santos-Buelga et al. 2002; Vayupharp and Laksanalamai 2015) are highly likely not to be endogenous pigmented maize anthocyanins, but esterification products formed between the malonyl component and the acidified extraction solvents.

2.2. Method validation

LOD, LOQ and matrix effect

The current study shows Cy3G had a slightly higher LOD and LOQ than Pg3G and Pn3G (Table S1) due to a broadening of its chromatographic peak. These findings are in agreement with reported LODs and LOQs for anthocyanin compounds in a berry matrix (Brito, Areche, Sepulveda, Kennelly, & Simirgiotis, 2014). The effect of the kernel matrix was determined by comparing the standard curves in the matrix-free extract solution with that of the sweetcorn matrix solution. Calibration curves of the standards were linear with a high coefficient of determination (~~R^2~~ r^2 > 0.999), with the matrix effect for all anthocyanin standards being lower than 8.8%. The method also showed a high recovery for low, medium and high concentration levels of the external standards (93.4~~–~~–109.2%), indicating a satisfactory accuracy of the current extraction method (Table S1).

Precision and accuracy

The inter-day and intra-day accuracy was assessed by determining the relative changes of external standards at three different concentrations (9 replicates per concentration per standard) over three consecutive days. The results of percent relative standard deviation (RSD %) in Table S2 indicated an acceptable range, with 0.9 to 3.4% for intra-day variation, and 1.7 to 3.7% for inter-day variation. Although there was an increase in the highest RSD % between different analytical instruments from 3.7 to 5.7%, the inter-instrument range together with the relative accuracy still indicated the present UHPLC~~–~~–~~PDA~~DAD~~–~~–MS/MS method was reliable, accurate and reproducible (Table S2).

3.4. Identification of anthocyanins in pigmented corn

Based on the unique absorption maxim~~ums~~a of anthocyanins in pigmented corn, they can be classified into three anthocyanin groups; group 1: Cy-based glucosides, λ_{max} 514 nm including peak 1, 3^1, 3^2, 3^3, 3^4, 6^1, 6^2, 6^3; group 2: Pg-based glucosides, λ_{max} 504 nm including peak 2, 5^1, 5^2, 8^1, 8^2, and; group 3: Pn-based glucosides, λ_{max} 517 nm including peak 4, 7^1, 7^2, 7^3, 9 (Fig. 3 and Fig. S3). Samples were spiked with Cy3G, Pg3G and Pn3G standards at different concentrations to identify retention times, molecular masses and their fragment ions of the anthocyanins in pigmented corn. Other anthocyanins without commercially available standards were identified based on their molecular masses and specific fragmentation patterns (Fig. S5 and Table 3), determined by System 3 and System 1 in positive mode, and comparing the elution- order to previous reports (Deineka, Sidorov, & Deineka, 2016; Nankar, et al., 2016; Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2002; Paulsmeyer, Chatham, Becker, West, West, & Juvik, 2017; Zhao, Corrales, Zhang, Hu, Ma, & Tauscher, 2008).

Next, anthocyanins were tentatively identified based on their respective MS/MS fragmentation pattern. For example, cyanidin-3-(dimalonylglucoside) (Cy3DMG) (*m/z* 621, Fig. S5) was identified by the molecular ion at *m/z* 621 and the formation of the fragment ion at *m/z* 577 $[M+H-44]^+$ corresponding to the loss of CO_2 from the carboxyl group. The fragment ion *m/z* 535 $[M+H-87]^+$ was formed from the fragmentation of Cy3DMG (*m/z* 621) due to the loss of a malonyl group at *m/z* 87 $[C_3H_2O_3]$~~,~~, and the key fragment ion at *m/z* 287 $[M+2H-87-87-162]^+$ is a predominant fragment of Cy3DMG after the loss of two malonyl groups (~~2x87~~ 2 × 87 Da) and one glucose (162 Da) (Fig. S5). Likewise, peonidin-3-(dimalonylglucoside) (Pn3DMG) (*m/z* 635) was confirmed by the detection of its daughter ion of *m/z* 591 $[M+H-44]^+$, representing the loss of CO_2 from the carboxyl group (44 Da) of its fatty acid moiety. The fragment ion of *m/z* 549 $[M+H-87]^+$ corresponded to peonidin-3-(malonylglucoside) Pn3MG after elimination of a malonyl group (87 Da). The MS^4 fragment at *m/z* 301 corresponded to the flavylium cation of peonidin, formed from the loss of a sugar and two malonyl moiety units. Likewise, eighteen different anthocyanins and isomers

in pigmented corn kernels were identified and quantified in the present study: Cy3G, four isomers of cyanidin-3-(malonylglucoside) (Cy3MG), three isomers of Cy3DMG, Pg3G, two isomers of pelargonidin-3-(malonylglucoside) (Pg3MG), two isomers of pelargonidin-3-(dimalonylglucoside) (Pg3DMG), Pn3G, and three isomers of Pn3MG and Pn3DMG (Table S2, Fig. 3 and Fig. S5).

3.5. Quantification of anthocyanins in pigmented corns

In the present study, the anthocyanin profile, together with the proportions of individual anthocyanin components of pigmented corn was basically in agreement with other studies analysing anthocyanins in purple-pericarp, cherry-aleurone and blue-aleurone maize (Nankar, et al., 2016; Vayupharp & Laksanalamai, 2015; Yang & Zhai, 2010b; Zhao, Corrales, Zhang, Hu, Ma, & Tauscher, 2008). Anthocyanin succinyl glucoside and ethylmalonylglucoside ~~were~~ reported in previous studies (Nankar, et al., 2016; Vayupharp & Laksanalamai, 2015) were absent in all pigmented corn kernels (Table 2). TAC of PPS (from 288.6 to 593.3 mg/100g DW) was almost the same concentration as that of PPM (from 438.5 to 667.2 mg/100g DW). TAC of these two purple cultivars was significantly higher than those of PP-BAM, RPS, BAM and CAM. These findings are in agreement with previous studies of anthocyanin content in pigmented corn kernels (Nankar, et al., 2016; Vayupharp & Laksanalamai, 2015). While cyanidin-based glucosides was the main pigment in PPS, PPM, PP-BAM (71.6 to 82.9%) and BAM (91.6%), pelargonidin-based glucosides accounted for up to 65.0% of anthocyanins in the RPS accession and almost 75% in CAM. Interestingly, peonidin-based glucosides ~~was~~ were not detectable in BAM and CAM, whereas it was a major pigment in PPS and PP-BAM (Table 2). Anthocyanin malonyl-glucosides and dimalonyl-glucosides were significantly higher than non-malonylated glucosides in all pigmented corn kernels. Both ~~of~~ the malonylated and dimalonylated glucosides were observed to have low stability in acidified solutions (Fig. S2). Therefore, both temperature and the concentration of acid need to be considered prior to extraction of pigmented corn samples.

Optimised method for System 3 analysis

In the current study, four isomers of Cy3MG could be detected by MS in PPS, PPM, PP-BAM and RPS, with a main isomer eluting at 12.97 min and three minor isomers at 9.88, 10.75 min and 11.31 min, respectively. However, the isomer at 11.31 min of Cy3MG co-eluted with Pn3G. In addition, an isomer of Pn3MG co-eluted with Pg3MG and another isomer of Cy3DMG at 15.25 min. These co-eluted compounds were impossible to quantify from the area of those peaks on the DAD chromatogram without information about the percentage contribution of individual compound in each peak. Consequently, selected ~~Ion~~ ion ~~Monitoring~~ monitoring (SIM) in positive ion mode was employed to determine the ratio of each co-eluted compound (Fig. S6) from total area of the peaks ~~on~~ in the DAD chromatogram at 520 nm.

Generally, the TAC in the pericarp-pigmented accessions (222.5–667.2 mg/100 g FW) was significantly higher than the aleurone-pigmented accessions (10.5–40.8 mg/100 g FW). Anthocyanin content in the PPM was higher than that reported in Chinese purple maize (304.5 mg Cy3G equivalents/100 g FW) (Zhao, Corrales, Zhang, Hu, Ma, & Tauscher, 2008), but similar to that of Thai waxy purple corn (421–636 mg/100 g at ~~the~~ a similar moisture content of 12%; ~~(~~Vayupharp & Laksanalamai, 2015). The blue aleurone accession, BAM, displayed a similar TAC to some blue corn varieties from the United States and Mexico, such as ‘Navajo Blue’ (41 mg/100 g FW), ‘Santa Clara Blue’ (58 mg/100 g FW), ‘Hopi Blue’ (56 mg/100 g FW), ‘Ohio Blue’ (54 mg/100 g FW), while the cherry aleurone, CAM, displayed a similar TAC to the reddish-purple variety, ‘Flor Del Rio’ (11 mg/100 g FW) (Nankar, et al., 2016).

3. Conclusions

A fully validated method with low LOQ and high accuracy to quantify anthocyanins, including their isomers, in pigmented corn was developed and validated. The method is applicable to a range of corn types, including pericarp-pigmented or aleurone-pigmented sweetcorn or maize and potentially other anthocyanin-containing commodities. A total of eighteen different anthocyanin compounds, consisting of cyanidin-, peonidin-, and pelargonidin-glycosides, were identified and quantified from

a range of pigmented sweetcorn and maize. Coloration of pigmented kernels was directly influenced by the proportion of individual anthocyanin components present. While the predominant anthocyanins in blue-aleurone and purple-pericarp corn were cyanidin-based glucosides (91.7% and 76.5%, respectively), pelargonidin-based glucosides accounted for over 60% in reddish-purple-pericarp and 75% in cherry-aleurone accessions.

For the first time, the present study found that although acidified extraction solutions stabilise the flavylium cation of anthocyanins, they were also the main cause of hydrolysis and esterification reactions affecting the malonyl and carboxyl moieties in the endogenous extraction. Consequently, nine 'artefact' anthocyanins were identified by LC-DAD-ESI-MS. The creation of artefact anthocyanins can be reduced by the use of low temperatures during extraction, ~~instrumental analytical operation~~analysis~~,~~ and storage of extracts.

Acknowledgements: This study was funded in part by 'Naturally Nutritious' (Horticulture Innovation Ltd., project HN15001).

Conflicts of Interest: The authors declare no conflict of interest.

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Table 1: Esterification products of purple corn anthocyanins in acidified methanol at 23 °C identified by System 3.

Anthocyanins	~~RT(~~RT (min)	λmax (nm)	*m/z*	MS/MS
Cy-3-(methylmalonateglucoside) (N1)	19.6	514	549	517, 449, 287
Cy-3-(methylmalonatemalonylglucoside) (N2)	22.5	514	635	603, 575, 535, 287
Cy-3-(methylmalonatemalonylglucoside) (N3)	23.6	514	635	603, 575, 535, 287
Pn-3-(methylmalonateglucoside) (N4)	24.1	520	563	531, 517, 301
Pg-3-(methylmalonatemalonylglucoside) (N5)	25.9	500	619	271
Pg-3-(methylmalonatemalonylglucoside) (N6)	27.2	500	619	271
Pn-3-(methylmalonatemalonylglucoside) (N7)	27.6	514	649	617, 605, 563, 301
Cy-3-(dimethyldimalonateglucoside) (N8)	28.1	520	649	605, 549, 287
Pn-3-(methylmalonatemalonylglucoside) (N9)	28.7	520	649	617, 573, 549, 301

RT: Retention Time; N1 to N9 are esterification products producing after 5-hour storage of PPS extraction at rt.

Table 2: Individual anthocyanin concentrations and total anthocyanin content (TAC) in mature seeds of pigmented corn accessions determined by LC–_System 3 at 520 nm. PPS: purple-pericarp sweetcorn; RPS: reddish-purple-pericarp sweetcorn; PPM: purple-pericarp maize; PP-BAM: purple-pericarp-blue-aleurone maize; BAM: blue-aleurone maize and CAM: cherry- aleurone maize; TPg; total pelargonidin content; TPn: total peonidin content; TCy: total cyanidin content.

Antho-cyanins	**TAC in pigmented corn (mg/100 g DW)**					
	PPS[a]	**RPS[b]**	**PPM[a]**	**PP-BAM[a]**	**BAM[b]**	**CAM[b]**
Cy3G	72.4–_137.1	21.78 ± 0.2	127.5–_260.1	72.2–_101.2	4.2 ± 0.0	0.6 ± 0.0
Pg3G	5.4–_19.6	41.1± 0.4	9.8–_15.8	4.9–_14.7	1.1 ± 0.0	1.2 ± 0.0
Pn3G	14.0–_26.2	5.2± 0.1	15.8–_25.1	11.6–_21.3	0.0	0.0
Cy3MG*	116.4–_246.9	34.4± 0.1	212.1–_246.5	125.6–_134.6	16.5 ± 0.2	1.1 ± 0.1
Cy3DMG*	22.0–_52.8	7.3± 0.2	49.6–_72.3	31.3–_45.1	16.8 ± 0.1	1 ± 0.0
Pg3MG*	16.2–_43.7	73.3 ± 0.6	23.8–_35.9	14.6–_35.1	1.1 ± 0.0	3.5 ± 0.1
Pg3DMG*	4.6–_11.4	21.6 ± 0.1	5.7–_12.1	3.8–_9.8	1.2 ± 0.0	3.2 ± 0.1
Pn3MG*	26.0–_57.5	13.5 ± 0.1	4.8–_25.5	12.5–_34.0	0.0	0.0

Pn3DMG*	10.3–22.3	4.5 ± 0.1	3.1–13.2	5.7–15.2	0.0	0.0
TPg	24.3–73.9	136 ± 1.1	39.5–64.1	11.4–59.6	3.4 ± 0.0	7.8 ± 0.2
TPn	49.8–106.0	23.1 ± 0.3	23.7–59.4	29.8–70.5	0.0	0.0
TCy	210.9–418.7	63.3 ± 0.3	363.5–555.7	185.2–258.0	37.4 ± 0.1	2.7 ± 0.1
TAC	288.6–593.3	222.5 ± 1.7	438.5–667.2	244.3–351.7	40.8 ± 0.1	10.5 ± 0.2

[a] experiments were performed in biological replicates (n = 6–12) for the purple accessions.

[b] a composite sample for each of the BAM, CAM and RPS accessions.

* anthocyanins having multiple isomers.

Table 3: Characterisation of anthocyanins detected in pigmented corn by System 3 and System 1 in positive ion mode.

Anthocyanins	Elution time (min)[a]	λ_{max} (nm)	m/z[a]	MS/MS[a]	Precursor ions (*m/z*)[b]		Molecular formula[b]	Fragments[b]
					Observed	Theoretical		
Cy3G	6.41	514	449	318, 287, 163	449.1075	449.1078	$C_{21}H_{21}O_{11}^+$	287.0547
Pg3G	9.88	504	433	323, 271	433.1127	433.1129	$C_{21}H_{21}O_{10}^+$	271.0600
Pn3G	11.31	517	463	322, 301, 286	463.1232	463.1235	$C_{22}H_{23}O_{11}^+$	301.0705
Cy3MG*	9.88, 10.75, 11.31, 12.97	514	535	517, 491, 449, 287, 163	535.1078	535.1082	$C_{24}H_{23}O_{14}^+$	287.0547
Pg3MG*	13.74, 15.25	504	519	475, 433,385, 271, 158	519.1134	519.1133	$C_{24}H_{23}O_{13}^+$	271.0600
Cy3DMG*	15.25, 15.78, 16.50	514	621	577, 535, 491, 287, 169	621.1089	621.1086	$C_{27}H_{25}O_{17}^+$	287.0547
Pn3MG*	14.85, 15.25, 16.87	517	549	531, 505, 463, 301, 286	549.124	549.1239	$C_{25}H_{25}O_{14}^+$	301.0705
Pg3DMG*	18.66, 19.46	504	605	587, 561, 518, 522, 271	605.1136	605.1137	$C_{27}H_{25}O_{16}^+$	271.0600
Pn3DMG*	21.17	517	635	617, 591, 549, 301, 287	635.1242	635.1243	$C_{28}H_{27}O_{17}^+$	301.0705

[a]: samples were tested by System 3, [b]: System 1.
*: anthocyanins ~~have~~ with multiple isomers.

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Fig. 1: TAC (+/- SE (standard error)) in a composite PPS sample at 26 DAP; A: anthocyanin extraction by a combination of methanol/water (8:2) acidified with different acids (HCl, FA and AA); B: anthocyanins extraction by different methanol/water mixtures acidified with 0.1M HCl. All extractions were carried out in triplicate and % the standard error of the mean (SEM) was <7%.

Fig. 2: (A) an example of the esterification and hydrolysis reaction of Cy3MG in acidified methanol at 23 °C. (B) mass spectrum of new esterification products of Cy-3-(6''-methylmalonate-3''-malonylglucoside (N3) and (C) diagrams of fragmentations of (N3) and Cy-3-(6''-methylmalonateglucoside) (N1) by System 3.

Fig. 3. Anthocyanin profiles of different pigmented corn samples analysed by System 3 at 520 nm. Peaks: (1) Cy3G; (2) Pg3G; (3^1); (3^2), (3^3) and (3^4) Cy3MG isomers; (4) Pn3G; (5^1) and (5^2) Pg3MG isomers; (6^1), (6^2) and (6^3) Cy3DMG isomers; (7^1) and (7^2) Pn3MG isomers; (8^1) and (8^2) Pg3DMG isomers; (9) Pn3DMG; IS: internal standard of Del3G (1 µg/L); PPS: purple-pericarp sweetcorn; RPS reddish-purple-pericarp sweetcorn; PPM: purple-pericarp maize; PP-BAM: purple-pericarp-blue-aleurone maize; BAM: blue-aleurone maize and CAM: cherry aleurone maize.

Highlights:

- A fully optimised extraction procedure for anthocyanin-pigmented corn was developed
- Eighteen genuine anthocyanins were identified in pigmented corns
- Nine 'artefact' anthocyanins were identified during the extraction procedure

- Highest anthocyanin content was found in purple-pericarp corn kernels
- The use of acidified solutions for extraction is essential for accurate assessment

CRediT author statement

Author Contributions: Conceptualization, H.H. and T.O.; methodology, H.H.., M.N.; software, H.H.; formal analysis, H.H., and T.O.; investigation, H.H.; resource, T.O.; data curation, H.H. and T.O.; writing—original draft preparation, H.H.; writing—review and editing, H.H., M.N., and T.O; supervision, T.O.; project administration, T.O.; funding acquisition, T.O.

Sincerely

Dr Hung Trieu Hong

(on behalf of the authors)

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The authors declare no conflict of interest.