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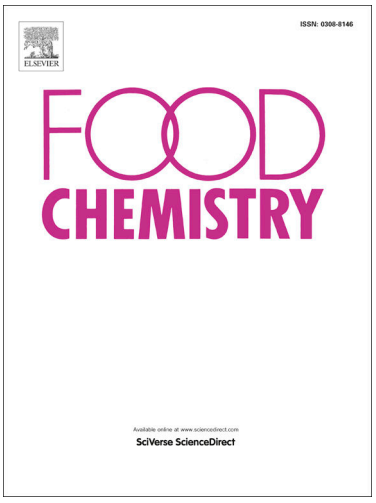

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Glutathionyl-S-chlorogenic acid is present in fruit of *Vaccinium* species, potato tubers and apple juice

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ABSTRACT

A hydroxycinnamate-like component was identified in highbush blueberry (*Vaccinium corymbosum*) fruit, which had identical UV and mass spectrometric properties to an S-linked glutathionyl conjugate of chlorogenic acid synthesized using a peroxidase-catalyzed reaction. The conjugate was present in fruits from all highbush blueberry genotypes grown in one season, reaching 7-20 % of the relative abundance of 5-caffeoylquininc acid. It was enriched, along with anthocyanins, by fractionation on solid phase cation-exchange units. Mining of pre-existing LC-MS data confirmed that this conjugate was ubiquitous in highbush blueberries, but also present in other Vaccinium species. Similar data mining identified this conjugate in potato tubers with enrichment in peel tissues. In addition, the conjugate was also present in commercial apple juice and was stable to pasteurization and storage. Although glutathionyl conjugates of hydroxycinnamic acids have been noted previously, this is the first report of glutathionyl conjugates of chlorogenic acids in commonly-eaten fruits and vegetables.

Keywords: Blueberry; potato; apple; polyphenols; glutathione; chlorogenic acid

1. INTRODUCTION

Blueberries are the second most purchased soft fruit in the UK after strawberries, with sales increasing year on year. Indeed, the UK blueberry market was worth ~ £ 140 M in 2013 and grew to £ 240 M in 2016 (https://www.kantarworldpanel.com/en). The UK production of blueberries has also grown ~ 8% over the period 2012-2016 but only 5% of total demand is currently met from UK production. The popularity of blueberries, partly driven by increased awareness of their potential health benefits and the economic potential for UK grown fruit to substitute for imported fruit, has encouraged the breeding of blueberry varieties that are more suited for growth and yield under UK conditions (e.g. https://www.producebusinessuk.com/insight/insight-stories/2017/08/09/blueberry-breeding-consortium-launched-to-discover-new-european-varieties) using the genetic and genomic resources developed at the James Hutton Institute (McCallum et al., 2016).

Blueberry components have been associated with varied potential health benefits including effects on cardiovascular disease (Rodriguez-Mateos et al., 2013), cancers (Stoner et al., 2010; Baba, Kowshik, Krishnaraj, Sophia, Dixit & Nagini, 2016), type 2 diabetes (Takikawa, Inoue, Horio, & Tsuda, 2010; Torronen, Sarkkinen, Tapola, Hautaniemi, Kilpi & Niskanen, 2010), obesity (Tsuda, 2008) and neuroprotective effects (Krikorian et al., 2010; Guo, Q., Kim & Lee, 2017). There has been a focus on their high polyphenol content and on their anthocyanin composition, the components which are responsible for their deep blue coloration. Indeed, blueberries have a distinct polyphenolic composition dominated by a diverse range of anthocyanins but also high amounts of chlorogenic acid (*5*-caffeoylquinic acid; Rothwell et al., 2013; Rodriguez-Mateos, Cifuentes-Gomez, Tabatabaee, Lecras, & Spencer, 2012). In highbush blueberry, *Vaccinium corymbosum*, the total amount of anthocyanins has been estimated at between 60 – 200 mg/ 100g FW but with chlorogenic acid content alone also estimated between 60 – 210 mg/ 100g fresh weight (Rothwell et al., 2013) with similar figures for lowbush blueberry varieties (Rodriguez-Mateos et al., 2012). Anthocyanins have often been specifically implicated in health beneficial effects (Takikawa et al., 2010; Tsuda, 2008; Norberto, Silva, Meireles, Faria, Pintado & Calhau, 2013; Prior, Wilkes, Rogers, Khanal, Wu & Howard, 2010) attributed to blueberries. However,

chlorogenic acids have also been associated with specific health benefits, although often from studies on chlorogenic acid-rich coffee intake (Tajik, Tajik, Mack & Enck, 2017; Santana-Gálvez, Cisneros-Zevallos & Jacobo-Velázquez, 2017).

Understanding which phenolic components are present in plant foods is crucial to understanding their possible bioactive effects. In this paper, we present evidence for the discovery of a glutathionyl conjugate of chlorogenic acid, which is ubiquitous in highbush blueberries. Mining pre-existing data from other studies, we also provide evidence that similar conjugates are present in fruit of other *Vaccinium* species, potato tubers and in apple juice, widely eaten foods in the UK and across the World.

2. MATERIALS AND METHODS

2.1. Reagents and Chemicals

All chemicals were Analar grade, or better, and where not indicated they were obtained from Sigma-Aldrich Chemical Company.

2.2. Blueberry material (Hutton)

Blueberry (*Vaccinium corymbosum*) cultivars Berkeley, Bluecrop, Elliott, Nui, Poppins, Reka, and genotypes D100, F100, RH38, RH48, RH52, ZDM005, ZDM035 and ZDM075 were grown at the James Hutton Institute, Dundee, Scotland, UK in 2012. Fruits were picked at full ripeness and frozen at -20°C directly after harvest and subsequently freeze-dried and then ground to a fine powder in a mortar and pestle. A bulk sample of ripe fruit from cultivar Berkeley plants (~ 2 kg) was picked in summer 2016 and frozen at – 20 ºC.

For each blueberry sample, freeze-dried berry powder (500 mg) was vortex mixed with ultra-pure water (UPW; resistivity 18.2 MΩ) (2 mL) and acetonitrile (ACN) containing 0.5% (v/v) formic acid (FA) (2 mL) in a 15 mL polypropylene centrifuge tube, then shaken at 180 rpm for 30 min at room

temperature on an orbital shaker. Tubes were centrifuged for 5 min at 2.500 g at 5 ºC and the supernatant transferred to another tube. The residue was re-extracted but with half the volume and the supernatants combined. After vortex mixing, the combined supernatant was transferred to 2 mL microfuge tubes and centrifuged at 10 000 g for 5 min at 5 ºC. The supernatants were recombined and aliquots (0.5 mL) and dried using centrifugal evaporation in a miVac Speed Vac (GeneVac Ltd, Ipswich, UK; max. temp. 45°C) and stored at -20°C.

2.3. Bulk extraction and ion exchange solid phase extraction (SPE) fractionation

After thawing on ice, berries of cultivar Berkeley harvested in 2016 (500 g) were added to 500 mL of ice-cold ACN containing 0.2 % (v/v) FA and homogenised using a Waring blender (5 X 30 s pulses). After filtration through muslin cloth, the extracts were concentrated using rotary evaporation to remove ACN and centrifuged at 2 500 g for 10 min at 5 ºC. The supernatant was retained.

The ion exchange solid phase extraction procedure was adapted from a previous method (McDougall et al., 2017) based on He & Guisti (2011). Briefly, a Strata X-C cation mixed mode SPE unit (200 mg/ 6 mL capacity; Phenomenex Ltd, Macclesfield, UK) was pre-equilibrated with 2 X 5 mL methanol and washed with 2 X 5 mL of UPW. All steps were carried out using ice-chilled eluents and with the collection tubes on ice. The bulk blueberry extract (5 mL) was applied to the SPE unit, the unbound material was collected then the wash fraction collected after addition of 2 X 5 mL of UPW. The unit was then washed with 2 X 5 mL methanol and the yellowish methanol fraction was collected. The units were red as the anthocyanins remained bound. The units were then washed with 2 X 5 mL of methanol containing 0.5 % (v/v) NH_4OH to elute the bound fraction that was immediately adjusted to pH < 2 using 20 % FA on ice. Finally, the unit was washed with 2 X 5 mL of 0.5 % (v/v) NH_4OH in 60 % methanol/40 % UPW and this fraction was also adjusted to pH < 2 using 20 % FA.

The bulk extract and the various fractions were assessed for total phenol content using the Folin method and for anthocyanin content using the pH differential method (Deighton, Brennan, Finn & Davies, 2000). Aliquots of the fractions containing equivalent phenol content (i.e. 500 μg gallic acid equivalents) were dried in a Speed-Vac prior to further analysis.

2.4. Liquid chromatography-mass spectrometry (LC-MS^n) of blueberry extracts

Dried extracts were dissolved in 30% aqueous ACN containing 0.5% (v/v) FA (475 μL) and 25 μl morin (25 mg in 100 mL methanol) was added as an internal standard. The dried aliquots from the SPE procedure were dissolved in 500 μL of 10 % ACN containing 0.2% (v/v) FA. After centrifugation at 10 000 g for 5 min at 5 ºC, the extracts were filtered in 0.45 μm filter vials (Thomson Instrument Co., London, UK). Extracts (20 μL) were analysed on an LTQ Orbitrap XL ion trap mass spectrometer system (Thermo Scientific, Hemel Hempstead, UK) with accurate mass capabilities with autosampler, photodiode array detector (PDAD), and Accela pump, controlled by Xcalibur™ software using the method detailed in McDougall et al., (2017). ~~The autosampler was set at 10°C and the column at 30°C. The column was a Synergi 4 u Hydro-RP 80A (with polar end capping, 150 mm x 2 mm, 4 μm, Phenomenex Ltd., Macclesfield, UK) column with a 4 × 2 mm C18 SecurityGuard™ cartridge (Phenomenex Ltd) and the mobile phase was composed of 0.1% (v/v) aqueous FA (A) and 0.1% (v/v) FA in ACN (B) and the flow rate was 300 μL min^{-1}. Samples were eluted with 2.5% B for 4 min, 2.5 to 25% B over 24 min, 25 % to 50% B over 4 min and held at 50% B for 2 min, from 50 % to 100% B over 4 min and held at 100% B for 3 min, then 100 to 2.5% B over 1 min and held at 2.5% B for 9 min. The PDAD scanned discrete channels at 280 nm, 365 nm and 520 nm and continually scanned across 200-700 nm. The mass spectrometer used an electrospray ionization (ESI) interface and analysed samples in both positive and negative modes with full scan from 120-2000 amu and data-dependent MS^2 scanning of the 3 most intense ions. The capillary temperature was 380°C with sheath and auxiliary gases at 40 psi and 15 psi, respectively.~~

The peak areas of components with specific *m/z* values were estimated using the resident Xcalibur™ software and data is presented as averages ± standard error of triplicate sample injections. Significant differences were assessed using paired T-test using Microsoft Excel. Under the chromatographic conditions used, galactosides and glucosides of anthocyanidins were not separated so these are estimated combined as hexosides. Pentosides were assumed to be arabinosides (Wu & Prior, 2005). The ratio of peak areas for selected components to the internal standard morin was also calculated.

2.5. Extraction and LC-Time-of-Flight mass spectrometric analysis of fruit from various *Vaccinium* species

Ripe fruit from *V. vitis-idaea, V. uliginosum and V. angustifolium* (cv. Blomidon) [grown in Tahe, Heilongjian Province, China] and *V. corymbosum* (cv. Bluecrop), *V. darrowii* (cv. Misty), *V. virgatum* (cv. Climax) [grown in Dalian, Liaoning province, China] were harvested in 2012 and kept cool until stored at -20°C. These were then couriered frozen to the James Hutton Institute. Approximately 50 g of frozen fruit was homogenised (3 X 30 s pulses) in a solvent-proof Waring blender with 150 mL of ice-cold 0.2% FA in methanol then filtered through Whatman no. 1 paper. Aliquots (1 mL) were dried using Speed-Vac, flushed with N_2 and stored at -80ºC.

Extracts were re-suspended in 2 mL of 0.1 % FA in 75 % methanol/ UPW and analysed on a LC system consisting of a quaternary pump, autosampler and PDAD coupled to an Agilent 6224 Time-of-Flight mass spectrometer (TOF-MS, Agilent Technologies, Santa Clara, CA, USA). Triplicate samples (5 µL) were injected onto a 150 mm x 2 mm (4 µm) C18(2) column with a 4 × 2 mm C18 SecurityGuard™ cartridge (Phenomenex Ltd). The LC and MS conditions were detailed previously (Kallscheuer et al., 2019). ~~Sample and column temperature were maintained at 4 and 30ºC, respectively. The samples were eluted at 0.3 mL min^{-1} using a gradient consisting of two mobile phases (A= 0.1% FA in UPW; B = 0.1% FA in 50:50 ACN: UPW). The elution program started with 5 % B for 4 mins then a linear gradient was applied to 100% B at 32 mins, held at 100~~

~~% for 2 mins. returned to 5 % B at 36 mins and held for 4 mins at 5 % B. Mass detection was carried out using the TOF-MS using optimal ESI conditions. The mass range was set at 100-2000 and the instrument run in positive mode.~~

Peak areas of specific components at specific *m/z* $[M+H]^+$ values were obtained using Mass Hunter™ software (Agilent Technologies) after manual integration. The putative GSH-CGA derivatives were noted at retention time of ~ 14.3 mins with exact mass of 660.1708 giving a predicted formula of $C_{26}H_{34}O_{15}N_3S$ at < 2 ppm. Peak areas from triplicate samples were obtained and expressed as averages ± standard deviation.

2.6. Peroxidase-catalysed synthesis of glutathionyl-chlorogenic acid derivatives

The procedure followed a previous method (Panzella, Napolitano & d'Ischia, 2003). In short, 5 mL of 8 mM chlorogenic acid in 0.05 M phosphate buffer pH 7.4 was added to 5 mL of 25 mM glutathione in UPW followed by horseradish peroxidase (4 U/mL; Sigma type II) and then H_2O_2 was added in two portions at 5 min intervals to a final concentration of 4 mM. The reaction was terminated by addition of FA to 0.1 % (v/v) whilst on ice. The solution was passed through solid phase C18 extraction units (McDougall et al, 2017) to capture the phenolic components and dried in a Speed Vac prior to LC-MS analysis.

2.7. Potato extraction and LC-MS^n analysis

Potatoes (*Solanum tuberosum* vars. Pentland Dell & Maris Piper) were grown at the James Hutton Institute in summer 2014 and tubers stored at 9ºC for 4 weeks following harvest. The tubers were washed, and a proportion were peeled to obtain peel samples. Whole tuber and peel samples were frozen, freeze-dried then milled (Retsch ZM 200 Mill; 0.5 mm sieve) to a powder and stored at –20ºC. For each sample, 100 mg (± 1 mg) was weighed out in triplicate and 5 mL 50% ACN: UPW containing 0.2% FA was added, vortexed well and extracted for 30 min at 4ºC with rotation

at 90 rpm. The samples were centrifuged (2500 rpm, 10 min, 4ºC) and the supernatant removed. The pellets were re-extracted with 2.5 mL solvent and the supernatants combined. The total phenol content was measured, and 1 ml aliquots dried in the Speed-Vac.

The dried extracts were re-suspended in 500 μL of 10 % acetonitrile containing 0.1 % FA, vortex mixed and placed into 0.45 μM filter vials (Thomson Instrument Co.). The samples were analysed on a LC system consisting of a quaternary pump and a PDA detector coupled to a Fleet ESI ion trap mass spectrometer (Thermo Fisher Scientific). Duplicate 10 μL samples were injected on to a 150 mm x 2 mm (4 μm) Synergy Hydro-RP 80 with a C18 4 x 2 mm Security GuardTM cartridge (Phenomenex Ltd). Sample and column temperature were maintained at 6 ºC and 30 ºC respectively. Samples were analysed at a flow rate of 0.3 mL/min using a gradient of (A) 0.1% FA in UPW and (B) 0.1% FA in ACN (gradient: 0-2 min 2% B; 2-5 min 2-5% B; 5-25 min 5-45%B; 25-26 min 45-100% B; 26-29 min hold 100% B; 29-30 min 100-2% B; 30-35 min hold 2% B). Mass detection was carried out in negative and positive mode with full scan analysis (80-2000 m/z) followed by a data-dependent MS^2 scanning in wide-band activation mode. Standard solutions of chlorogenic acid were used in triplicate to produce a 16-point calibration curve ranging from 1 μM to 4 mM for quantification. Levels are expressed as averages of the six replicates (technical duplicate of triplicate extractions) ± standard deviation. Significant differences were assessed using two-tailed T-test at $p < 0.05$.

2.8. LC-MS^n analysis of Apple Juice

Apple juice concentrate was produced by Indulleida S.A. (Alguaire, Lleida, Spain) and diluted as required (Dobson, McDougall, Stewart, Cubero & Karjalainen, 2016). Pasteurization was carried out at Indulleida (92 °C for 5 min) and after cooling, unpasteurized and pasteurized juices were aliquoted into multiple 50 mL polypropylene tubes, frozen and couriered to the James Hutton Institute. Untreated and pasteurized apple juices were thawed at 4 °C and then stored at 20 °C. At weekly intervals, samples were flash frozen in liquid nitrogen and kept at -80 °C until analysed by

LC-MS. Frozen samples were thawed, filtered using 0.45 µm filter vials (Thomson Instrument Co.), and analysed on a Thermo Scientific LCQ-DECA ion trap mass spectrometer system with an ESI interface in both positive and negative modes as described previously (Dobson et al., 2016). ~~The full scan mass range was 80 to 2000 amu followed by data-dependent MS^2 scanning.~~

3. RESULTS AND DISCUSSION

Extracts of fruits of a range of blueberry cultivars and genotypes grown at the Hutton contained a major peak of 5-caffeoylquininc acid (Fig. 1a, panel A & C) which dominated a characteristic mixture of partly separated anthocyanins mainly hexosides and arabinoses of malvidin ~~(Mv)~~, peonidin ~~(Pn)~~, cyanidin ~~(Cy)~~, delphinidin ~~(Dp)~~ and petunidin ~~(Pt)~~ (Fig. 1a; panel B) as noted previously in highbush blueberry cultivars (Wu & Prior, 2005; Barnes, Nguyen, Shen & Schug, 2009) with no acylated anthocyanins apparent. In most samples, there was a clear peak at ~14.8 mins that preceded the major 5-caffeoylquininc acid peak and had a PDA maximum around 320 nm, which is characteristic of hydroxycinnamate derivatives (Macheix & Billot, 1990). This peak contained three main signals at *m/z* $[M+H]^+$ = 291, 465 and 660 (Fig. 1a; panel D & Fig. 1b, panel A). The *m/z* $[M+H]^+$ = 465.1016 gave a predicted formula of $C_{21}H_{21}O_{12,}$ the *m/z* $[M+H]^+$ = 291.0859 gave $C_{15}H_{15}O_6$ and the major CGA peak at RT 15.32 mins had a *m/z* $[M+H]^+$ = 355.1016, predicted formula $C_{16}H_{19}O_9$; all at< 1.5 ppm error. The *m/z* 465 peak was identified as delphinidin glucoside which arose as the front shoulder of the anthocyanin peak at RT 15.52 and the *m/z* 291 peak was identified as epicatechin which co-eluted with the *m/z* 660 feature. The *m/z* $[M+H]^+$ = 660.1694 gave the predicted formula $C_{26}H_{34}O_{15}N_3S$. This formula does not match any compound previously identified in blueberry fruit. In negative mode, the same peak gave *m/z* $[M+H]^+$ = 658.1559 gave $C_{26}H_{32}O_{15}N_3S$ at 1.02 ppm error. However, these formula match previously synthesized compounds which were identified as 2-*S*-glutathionyl chlorogenic acid (Panzella et al., 2003; Xie, Zhong & Chen, 2013; Diagram 1). Interestingly, three peaks with *m/z* 660 were apparent, the major one at 14.8 mins and two minor peaks at 10.1 and 12.3 mins with similar MS properties.

Considering their relative abundance in blueberry, the largest peak could represent the glutathionyl conjugate with 5-caffeoylquinic acid and the other smaller peaks being glutathionyl conjugates with 4- and 3- caffeoylquinic acids (Rothwell et al., 2013).

In positive mode, the MS^2 spectrum of *m/z* 660 showed fragments at 585 and 531 (Fig. 1b, panel B), neutral losses of 75 and 129 which could arise from glycine and pyroglutamic acid, characteristic of fragmentation of the peptide bonds of glutathione (GSH). Indeed, neutral loss scanning at 129 has been used to identify GSH derivatives (Xie et al., 2013). The fragment at *m/z* 468 arises through neutral loss of 192 which can be assigned with a loss of quinic acid. As observed previously in MS^2 data of GSH conjugates with CGA and other aromatic components (Xie et al., 2013), no evidence for fragmentation at the C-S bond between GSH and CGA was noted in positive mode.

However, in negative mode (Fig. 1b, panel C), fragmentation of *m/z* 658 gave product ions at *m/z* 385 and 272 which can be putatively assigned to typical fragmentation of the GSH-CGA conjugate at the cysteinyl C-S bond (Xie et al., 2013). The loss of 192 to *m/z* 466 suggests loss of quinic acid and the presence of the signal at *m/z* 306 can also be assigned to C-S bond fragmentation to yield GSH with a neutral loss of 352 as CGA.

The *m/z* $[M+H]^+$ signals at 660 consistent with the GSH-CGA conjugate were present in extracts of fruits from all blueberry cultivars and genotypes harvested at the Hutton in 2012. These peaks also gave MS^2 data essentially identical to those shown in Fig. 1b. The GSH-CGA component was present in all 16 blueberry extracts but at different levels (Fig. 1c). As the blueberry cultivars and genotypes were grown at the same location, under the same agronomic conditions and in the same season, these differences may reflect genetic control. The levels of the GSH-CGA conjugate (*m/z* $[M+H]^+$ 660) were compared to the levels of its putative precursor, 5-caffeoylquinic acid (*m/z* $[M+H]^+$ 355) in the varieties (Fig. 1c). There was no simple relationship between 5-caffeoylquinic acid levels and GSH-CGA levels. Based on MS responses relative to the internal standard, the

cultivars “Poppins” and “RH55” had the lowest and highest contents of 5-caffeoylquinic acid respectively. “Berkeley” had the lowest content of the GSH-CGA conjugate and “Nui” had the highest content. Considering that 5-caffeoylquinic acid is the major phenolic component in blueberries (Rothwell et al., 2013), the GSH-CGA conjugates are relatively abundant components and comparable in abundance to individual anthocyanin species, which have been implicated in a wide range of health beneficial bioactivities (Takikawa et al., 2010; Tsuda, 2008; Norberto et al., 2013; Prior et al., 2010) attributed to blueberries. Mining of previously obtained Hutton LC-MS data showed that this component was present in all highbush blueberry extracts studied including those prepared for other studies on specific bioactivities (Brown, Gill, Stewart & McDougall, 2016).

A bulk extract from fruit of the variety, Berkeley, was produced in 2016 and also contained the characteristic mixture of hexosides and arabinoses of malvidin, peonidin, cyanidin, delphinidin and petunidin as noted previously in highbush blueberry cultivars (Wu & Prior 2005; Barnes et al., 2009). After ion exchange solid phase extraction (Ion-Ex SPE), the bound sample retained the bulk of the red coloration and had approx. 3-fold higher total anthocyanin concentration than the original blueberry extract (results not shown). Indeed, LC-MS analysis of the original and bound fractions confirmed this 3-fold enrichment (see supplementary data, Fig. S1a) with only minor differences in anthocyanin composition (Fig. S1b) which confirms that this SPE procedure provides anthocyanin-rich fractions representative of the original extract (He & Giusti, 2011). Several non-anthocyanin components were relatively enriched in the SPE bound fraction (Fig. 2 & Table 1) including the amino acid, tryptophan and the nucleosides, adenosine and guanosine. Peak 5 had significant absorbance at 325 nm and gave a strong MS signal at *m/z* 660 in positive mode and at *m/z* 658 in negative mode (Table 1). The MS^2 data was essentially identical to those noted for synthesized glutathionyl (GSH) conjugates of chlorogenic acid (Panzella et al., 2003; Xie et al., 2013) in both positive and negative modes (Table 1). Exact mass data was consistent with the previous results; *m/z* $[M+H]^+$ 660.1702 which gave a predicted formula of $C_{26}H_{34}O_{15}N_3S$, and $C_{26}H_{32}O_{15}N_3S$ in negative mode, both at ~1 ppm. By repeating the *in vitro* peroxidase-catalyzed

conjugation of CGA with GSH as described by Panzella et al. (2003), a GSH-CGA conjugate with identical PDA, MS and MS^2 properties could be prepared (see Supplementary data; Fig. S3; Table S1).

Peaks with *m/z* [M+H] values of 674 were also present in the anthocyanin-rich bound SPE fraction and appear to be methylated versions of GSH-CGA. MS data strongly suggested that the methyl groups were attached to the quinic acid moiety (see Supplementary data Fig. S2). These arose from the use of methanol in the ion exchange SPE procedure, possibly as quinic acid groups are particularly prone to methylation (e.g. Jaiwal & Kuhnert, 2011), and avoidance of methanol prevented this artefact. However, although GSH-CGA could readily be detected in the original blueberry extract, the methylated *m/z* 674 components were absent suggesting that they were formed during the SPE fractionation procedure.

Interrogation of previously obtained time of flight-MS data from fruits from different *Vaccinium* species gathered from different locations in the BACHBERRY project (http://www.bachberry.eu/) confirmed that the GSH-CGA component was present in highbush blueberry (*V. corymbosum*) extracts with exact mass $[M+H]^+$ *m/z* data that matched previous data (Fig. 3). This component was also present in other *Vaccinium* species including wild lowbush blueberry (*V. angustifolium*) and Darrow's blueberry (*V. darrowii*), was less abundant in rabbit eye blueberry (*V. virgatum*) and lingonberry (*Vaccinium vitis-idaea*) but was not detected in Bog bilberry (*V. uliginosum*). Mining of other previous Hutton LC-MS data found that the conjugate was not detectable in extracts of non-Vaccinium berries, such as blackcurrant (Boath, Stewart & McDougall, 2012), strawberry (Josuttis, Verrall, Stewart, Kruger & McDougall, 2013) or raspberry (Coates, Popa, Gill, McCann et al., 2007).

Focusing on other plants known to be rich in 5-caffeoylquinic acid and mining previously obtained data, *m/z* $[M+H]^+$ signals consistent with the putative GSH-CGA conjugate were also identified in

extracts of potato tubers. These components gave PDA, MS and MS^2 data essentially identical to that shown in Table 1 (Supplementary data, Fig. S4). The GSH-CGA conjugate was relatively enriched in the peel of the two varieties, Maris Piper (MP) and Pentland Dell (PD), as was 5-caffeoylquinic acid (Fig. 4), whereas glutathione levels were much lower in the peel compared with the whole tuber extracts. However, glutathione is very prone to oxidation and these levels are only at best relative and indicative. The levels of 5-caffeoylquinic acid in MP tuber, MP peel, PD tuber and PD peel respectively were 0.647 ± 0.035, 1.818 ± 0.055, 1.110 ± 0.052 and 3.587 ± 0.157 mg /g dry weight (n = 6). The higher abundance of 5-caffeoylquinic acid in potato peels is well known (Weidel, Schantz & Richlingi, 2014) and the levels of the putative GSH-CGA conjugate mirrored those of 5-caffeoylquinic acid in the different varieties and tissues (Fig. 4). If quantified against 5-caffeoylquinic acid, the levels of GSH-CGA conjugate were in the same concentration range (i.e. from < 0.5 mg/g DW in the whole tuber to ~ 5 mg/g DW in the peel). However, this quantification is unwise as it may overestimate the content of the GSH-CGA conjugate since it appears to ionize more readily than CGA, particularly in positive mode. Further work with a greater supply of pure GSH-CGA is required to quantify this component. LC-MS data from previous metabolomic studies carried out at the Hutton suggested that the *m/z* 660 conjugate is ubiquitous in potato tubers and is largely stable after cooking (results not shown).

Examining data previously obtained from studies on the stability of commercial apple juices (Dobson et al., 2016), the putative GSH-CGA conjugate was also identified with essentially identical PDA, MS and MS^2 properties to the synthesized component (Supplementary data, Fig. S5). It was present in non-pasteurized apple juice (Fig. 5) but was slightly, but significantly, more abundant ($p < 0.05$; T-test) in juice after pasteurization. This increase may be due to increased extraction as other fruit juices showed increases in total phenol content after pasteurization (Dobson et al., 2016). The *m/z* 660 component was relatively stable to long term storage at 20 ºC with 94 % recovery of the original amount present after 8 weeks. The non-pasteurized juice also

had substantial amounts (82 % original) of the conjugate remaining after 8 weeks at 20 ºC but there was a notable drop in levels between one and two weeks.

Although glutathionyl hydroxycinnamate conjugates, including *S*-glutathionyl caftaric acid and *S*-glutathionyl caffeic acid (Cejudo-Bastante, Pérez-Coello, & Hermosín-Gutiérrez, 2010; Ferreira-Lima, Vallverdú-Queralt, Meudec, Mazauric et al., 2016), have been reported in wines, there have been few reports in commonly eaten plant foods. To date, *S*-linked glutathione conjugates of *p*-coumaric, ferulic and sinapic acids have only been described in pineapple fruits (Steingass, Glock, Schweiggert, & Carle, 2015) and there have been no reports of *S*-linked glutathionyl derivatives of chlorogenic acids.

Their means of biosynthesis remain opaque but glutathione *S*-transferases from maize have been shown to accept hydroxycinnamic acids as substrates *in vitro* (Dean, Devarenne, Lee & Orlofsky, 1995). However, it is also possible that oxidation of hydroxycinnamates in the presence of glutathione e.g. by peroxidase (Panzella et al, 2003) or cytochrome P450 (Thompson, Constantin-Teodosiu, Egestad, Mickos & Moldeus, 1990) is involved. Along with other roles such as in detoxification of xenobiotics (e.g. herbicides), glutathione-*S*-transferase catalyzed reactions (Zhao, 2015) have been proposed to be involved in producing glutathionyl-phenolic derivatives as intermediates in biosynthesis or for the transport of phenolic components into vacuoles (Dixon, Skipsey & Edwards, 2010). Glutathionylation enhances water solubility and provides a ligand modification recognized by ATP-binding cassette (ABC) transporters for cross membrane transport (Rea, 2007) and that could function to direct phenolic accumulation into vacuoles, for example, during berry ripening. Indeed, a range of gene transcripts with significant homologies to glutathionyl transferases were expressed during late ripening in blackcurrant fruit (Jarret, Morris, Cullen, Gordon et al., 2018) and were postulated to be involved in vacuolar transport of phenolics. Operating in this role, one might expect that these glutathionyl conjugates would be relatively short-lived and be recycled after transport, but the survival and detection of these derivatives may

be a consequence of the relatively high abundance of 5-caffeoylquinic acid in blueberries, potato tubers and apples.

4. CONCLUSION

This study provides evidence for the presence of S-linked glutathionyl derivatives of chlorogenic acids in commonly eaten foods, i.e. blueberries, potato tubers and apple juice. The role of these compounds *in planta* and their possible bioactivities as widely ingested phytochemicals deserves further study.

5. SUPPORTING INFORMATION

This contains further information on the MS properties of the GSH-CGA conjugate from the berry extracts, potato tuber extracts, the apple juice samples and the *in vitro* synthesis of the conjugate.

6. ACKNOWLEDGEMENTS

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7. FIGURE LEGENDS

Fig. 1a. LC-MS-PDA traces from blueberry variety "Nui"

Panels A and B show the UV traces at 280 and 520 nm respectively. Panels C and D show the MS response at base peak *m/z* $[M+H]^+$ = 355 and at base peak *m/z* $[M+H]^+$ = 660. FSD = full scale deflection

Fig. 1b. MS and MS^2 spectra of peak at RT 14.81 min

Panel A shows the Full MS spectra at RT = 14.81; Panel B shows the MS^2 fragmentation pattern of the *m/z* 660 component in positive mode and panel C the MS^2 fragmentation pattern of the *m/z* 658 component in negative mode. Signals in bold are mentioned in the text. FSD = full scale deflection

Fig. 1c. Comparison of levels of GSH-CGA and 5CQ in blueberry fruit extracts

Response factors (averages of triplicate values ± SE, n = 3) were obtained by dividing the peak areas of selected peaks by those of the internal standard, morin. M+H 355 = 5CQ = 5-caffeoylquinic acid; M+H 660 = GSH-CGA conjugate. Varieties are ordered by highest content of the GSH-CGA conjugate as % of total 5CQ (i.e. figures above each set of bars).

Fig. 2. LC-MS-PDA traces of the anthocyanin-rich Ion-Ex SPE bound fraction

Trace A is the UV absorbance at 280 nm and trace B is at 520 nm. FSD = full scale deflection. The PDA and MS properties of the denoted peaks are discussed in the text and in Table 1.

Fig. 3. Levels of putative glutathionyl-CGA derivative (*m/z* 660) in fruit from different Vaccinium species

All values are averages of triplicate extractions ± SE. The glutathionyl chlorogenic acid derivative was noted at *m/z* 660.1708 at RT ~14 min.

Fig. 4. Relative abundance of glutathione, putative glutathionyl-CGA derivative and 5CQ in tubers and peel samples from Maris Piper and Pentland Dell tubers

The MS peak areas are averages ± SE obtained from duplicate injections of three separate extractions (n = 6) of the material. MP = Maris Piper & PD = Pentland Dell.

Fig. 5. Stability of *m/z* 660 component in stored pasteurized and non-pasteurized apple juice at 20 ºC

MS peak areas are averages ± SE obtained from triplicate samples and expressed as % of the zero-time non-pasteurized apple juice. Past = pasteurized and NP = non-pasteurized. Asterisks denote that the pasteurized values differ from the non-pasteurized values (T-test; $p < 0.05$).

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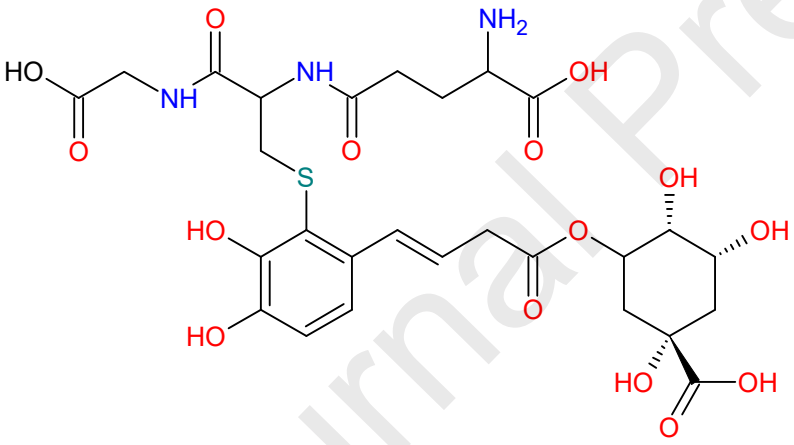
Declaration of interests

☒ 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:

Diagram 1. Structure of putative glutathionyl chlorogenic acid derivative



FIGURES

Fig. 1a LC-MS-PDA traces from blueberry variety "Nui"

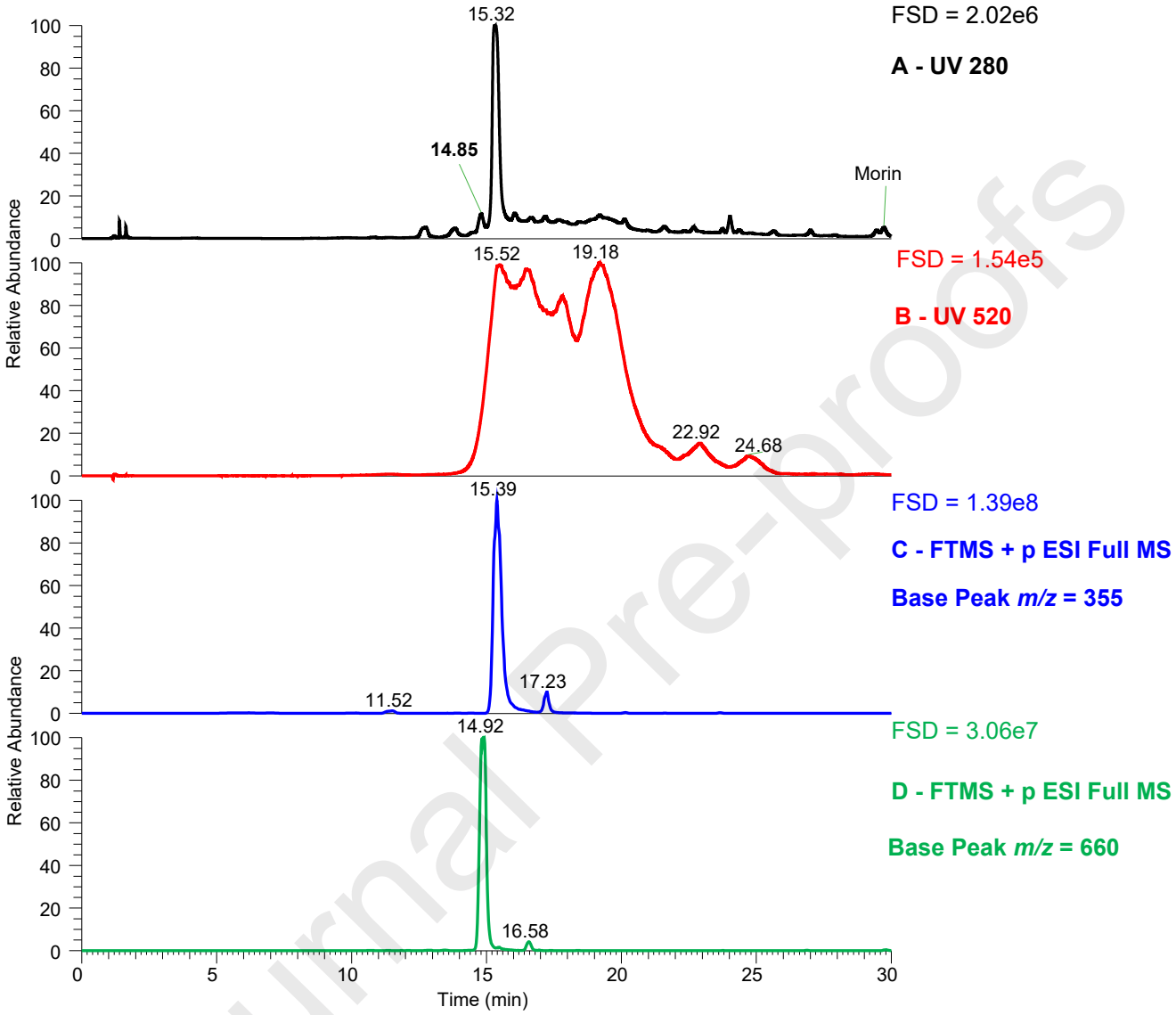


Fig. 1b MS and MS^2 spectra of peak at RT 14.81 min

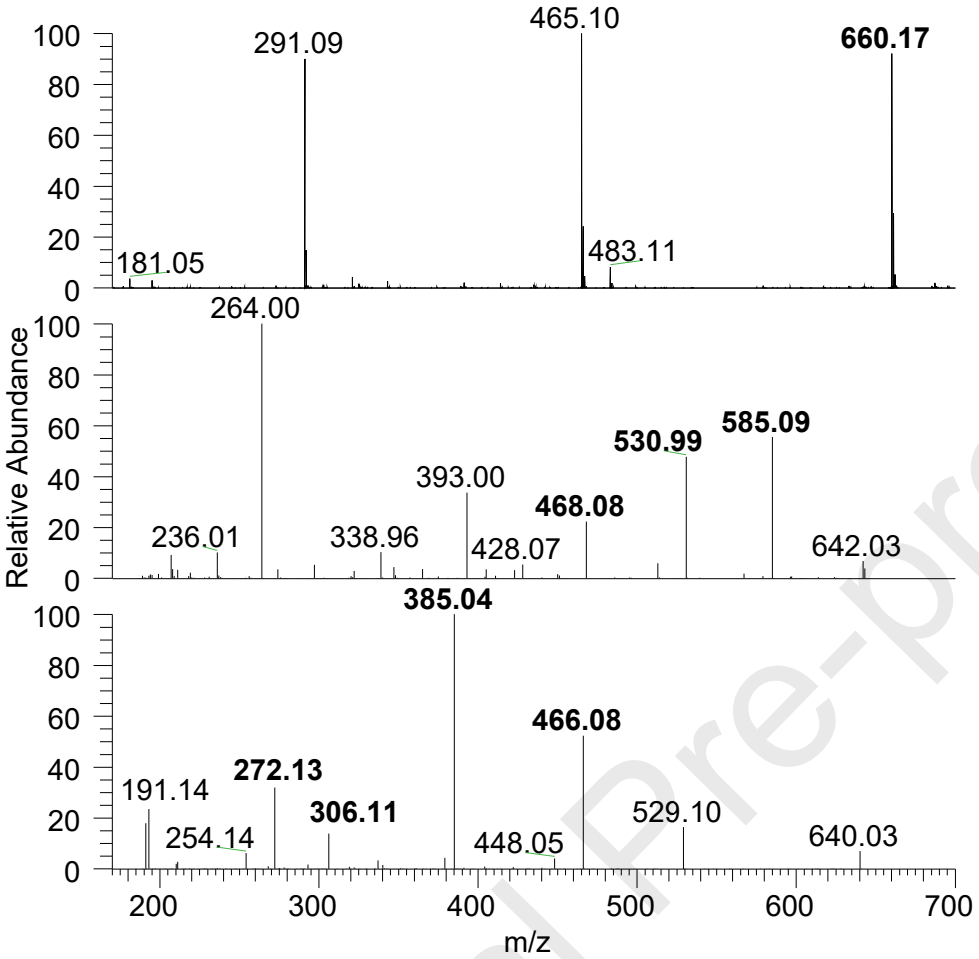


Fig. 1c Comparison of levels of GSH-CGA and 5CQ in blueberry fruit extracts

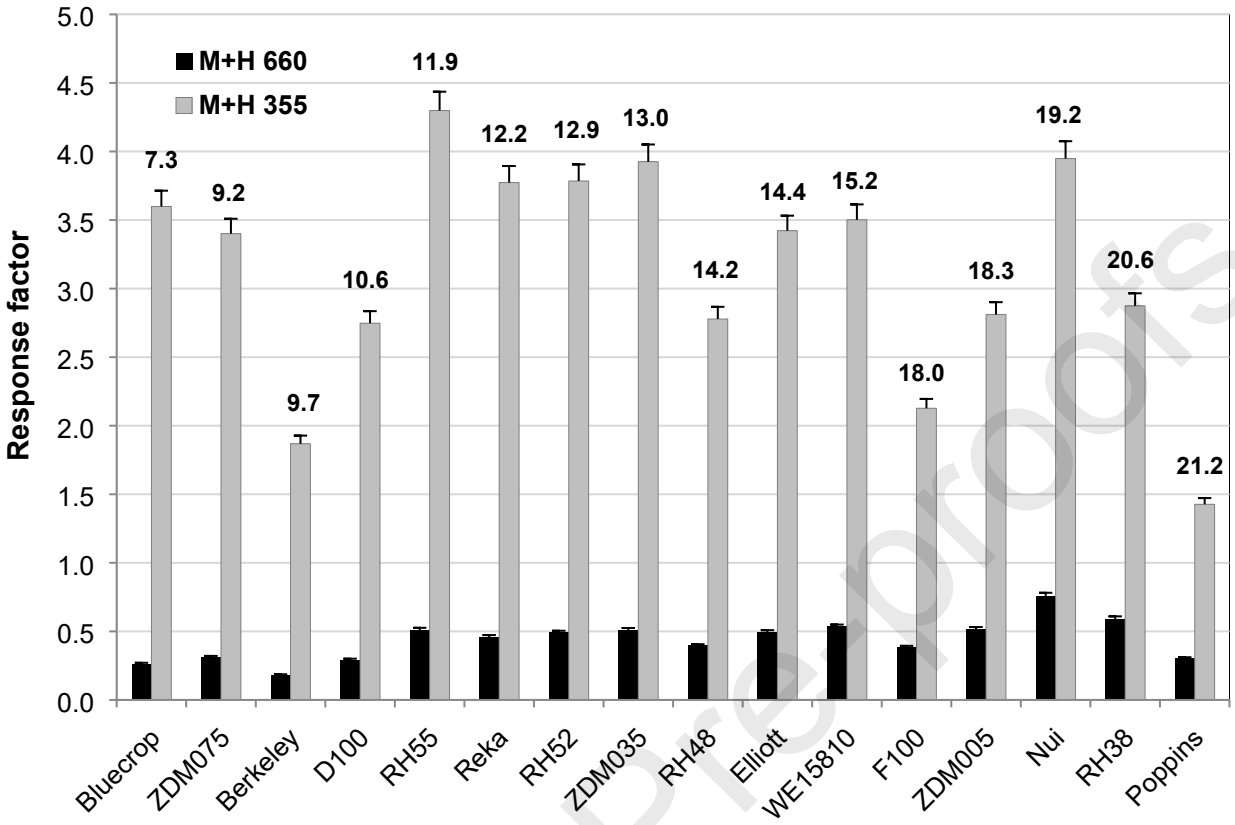


Fig. 2 UV profiles of the anthocyanin-rich Ion-Ex SPE bound fraction

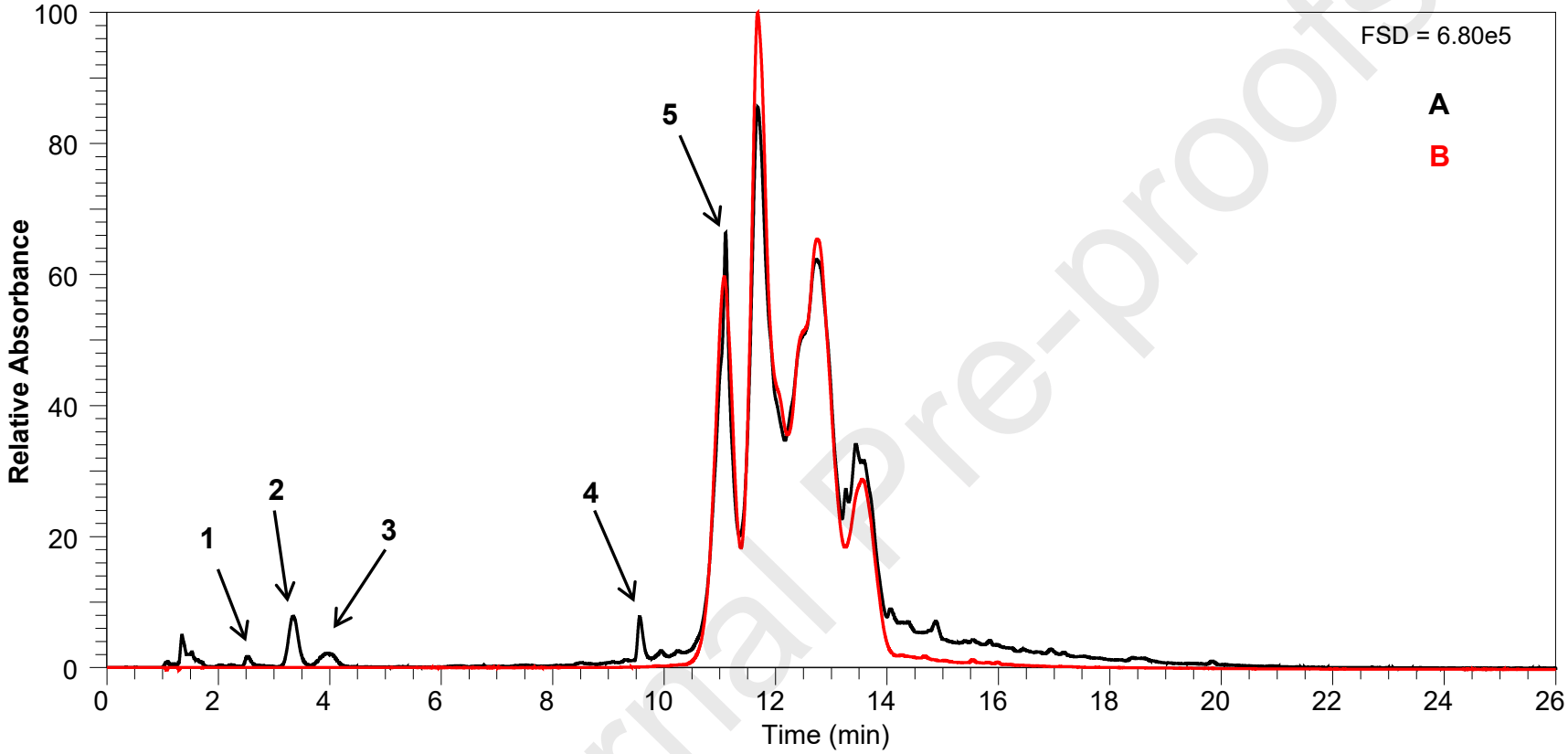


Fig. 3 Levels of putative glutathionyl CGA derivative (*m/z* 660) in fruit from different *Vaccinium* species

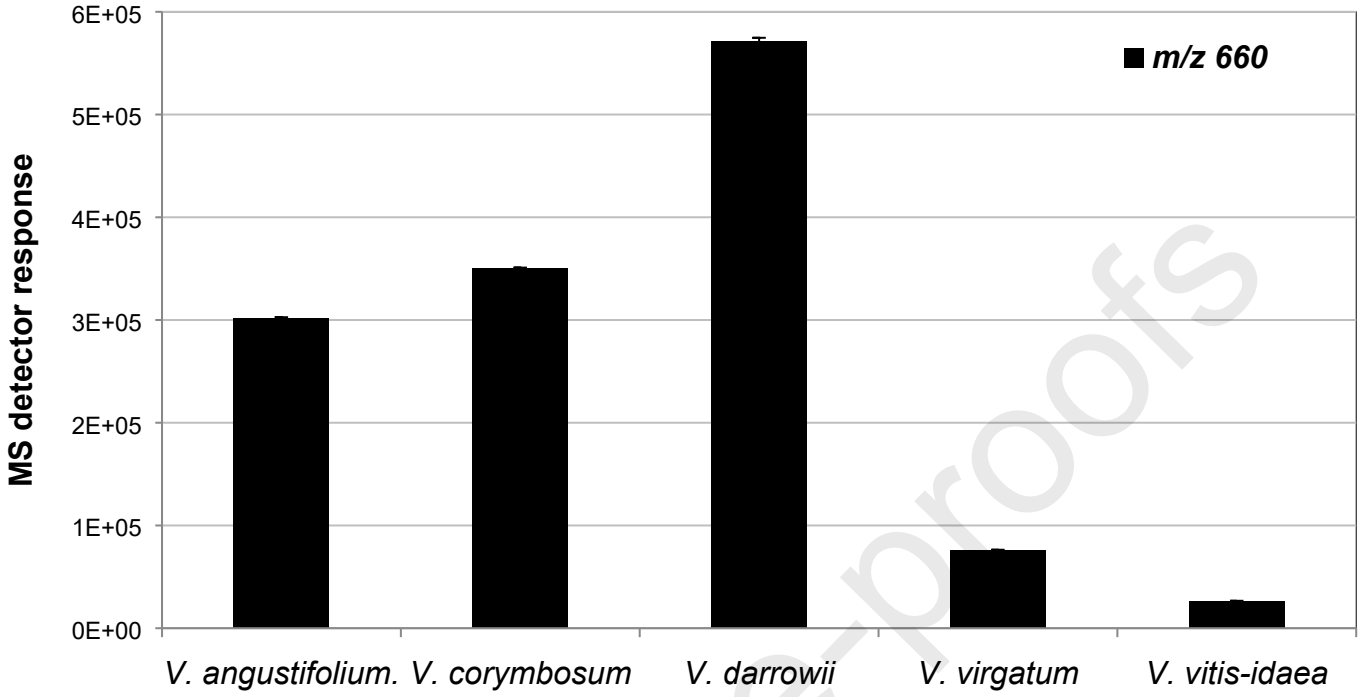


Fig. 4. Relative abundance of glutathione, putative glutathionyl-CGA derivative and 5-caffeoylquinic acid in tubers and peel samples from Maris Piper and Pentland Dell tubers

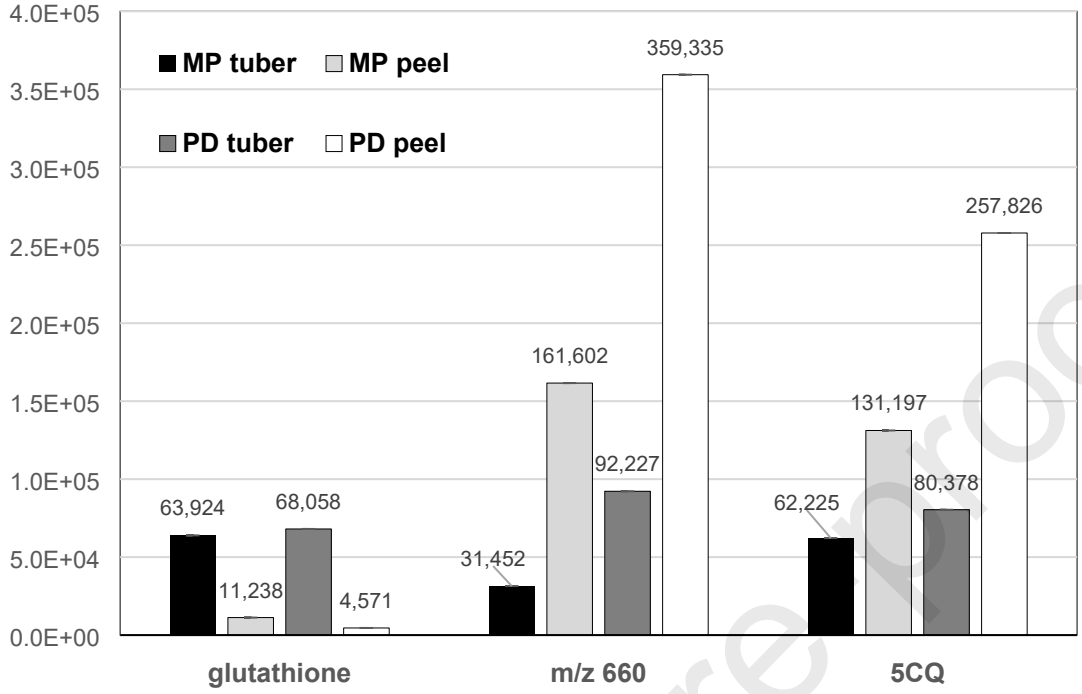
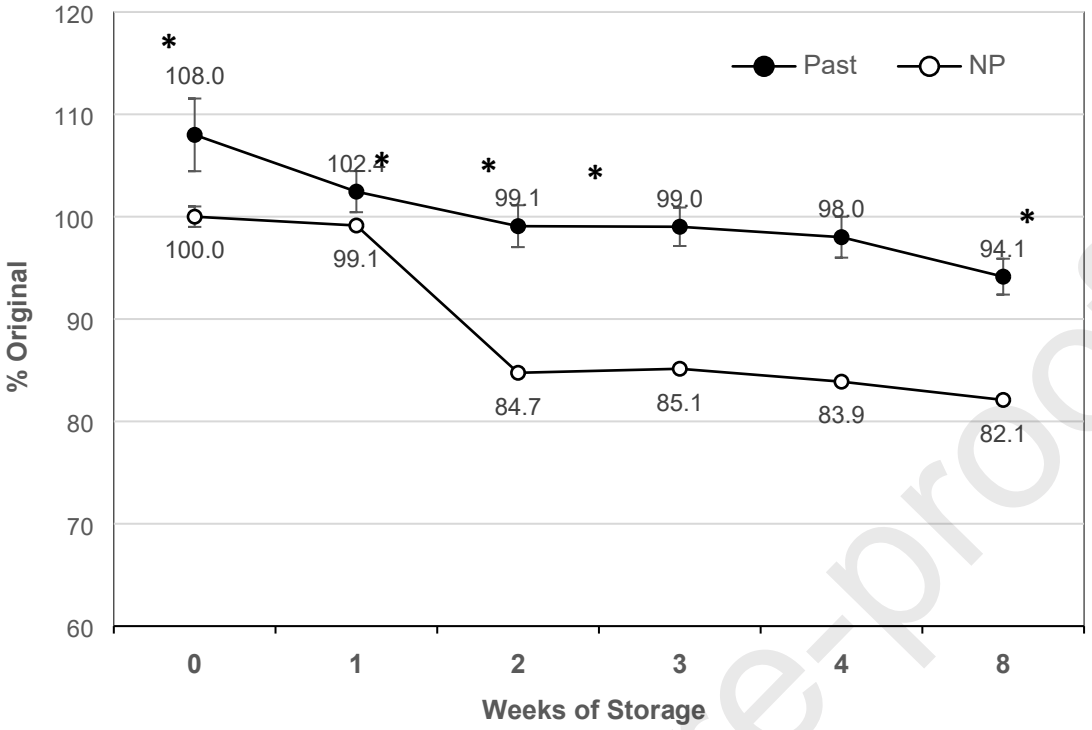


Fig. 5 Stability of *m/z* 660 component in stored pasteurized and non-pasteurized apple juice at 20 °C



Glutathionyl-*S*-chlorogenic acid is present in fruit of *Vaccinium* species, potato tubers and apple juice

- A novel glutathionyl-chlorogenic acid conjugate is reported in blueberry fruit
- The conjugate appears identical to an in vitro synthesised conjugate
- The conjugate is also present at appreciable levels in potato tubers and apple juice
- The biological function/ bioactivity of this ubiquitous component is discussed

TABLE 1. LC-MS^n characteristics of peaks in the Ion-Ex SPE bound fraction

Peak	RT	PDA max	*m/z* [M+H]$^+$	MS^2	Putative Identity

	(min)	(nm)			
				Journal Pre-proofs	
1	2.53	255	182.1	165.1, 136.1 (46) [b]	Ribose (as formate adduct)
2	3.38	255	268.1 [136.1] [a]	136.1 (132)	Adenosine
3	3.93	255	283.9 [152.1]	152.1 (132)	Guanosine
4	9.63	278	205.0 [188.1]	188.1 (17)	Tryptophan
5	11.10	325, 285	660.0	585.1 (75), 531.0 (129) 467.9 (192), 392.9 (265) 338.9 (321), 264.1 (396) 236.0 (424), 207.1 (453)	Glutathionyl chlorogenic acid derivative[c]
5	11.10	325, 285	658.2 [**M-H**]$^-$	529.1 (129), 466.1 (192) 448.0 (210), 385.0 (273), 306.2 (352), 272.1 (386), 254.0 (404), 193.1 (465), 191.1 (467)	Glutathionyl chlorogenic acid derivative[c]

a- Figures in square brackets represent in-source fragments.

b- Underlining denotes major fragments and figures in round brackets are neutral losses.

c- Xie et al. (2013).