Food Chemistry 119 (2010) 1535–1543

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Structural identification of the main ellagitannins of a boysenberry (*Rubus loganbaccus* × *baileyanus* Britt.) extract by LC–ESI-MS/MS, MALDI-TOF-MS and NMR spectroscopy

Marijn M. Kool [a], Daniel J. Comeskey [b], Janine M. Cooney [c], Tony K. McGhie [b,*]

[a] Food Chemistry Laboratories, Wageningen University and Research Centre, The Netherlands
[b] The New Zealand Institute for Plant and Food Research Ltd., Private Bag 11600, Palmerston North 4442, New Zealand
[c] The New Zealand Institute for Plant and Food Research Ltd., Private Bag 3123, Hamilton 3240, New Zealand

ARTICLE INFO

*Article history:*
Received 4 May 2009
Received in revised form 3 August 2009
Accepted 9 September 2009

*Keywords:*
Ellagitannins
Ellagic acid
Boysenberry
NMR spectroscopy
LC–MS
MALDI-TOF-MS

ABSTRACT

Four ellagitannins from boysenberry, a cross between *Rubus loganbaccus* and *Rubus baileyanus* Britt., were isolated by preparative HPLC and the exact structures determined by a combination of LC–ESI-MS/MS, MALDI-TOF-MS and NMR spectroscopy. The two most abundant ellagitannins were identified as sanguiin H-6, which is known to be abundant in *Rubus* species, and the other was identified as an isomer of sanguiin H-10, which has not previously been reported in *Rubus*. The two less abundant ellagitannins were identified as sanguiin H-2 and [galloyl–bis-HHDP–glucose]$_2$-gallate. Sanguiin H-2 has been previously reported in *Rubus*, whereas both sanguiin H-2 and [galloyl–bis-HHDP–glucose]$_2$-gallate have been previously reported as hot-water degradation products of lambertianin C. Even though lambertianin C is reported to be a major ellagitannin in other *Rubus* species, it was not found in any of the fractions, suggesting that both sanguiin H-2 and [galloyl–bis-HHDP–glucose]$_2$-gallate are present naturally in boysenberry.



## 1. Introduction

Numerous studies have indicated that fruit and vegetables have health-promoting properties, which are often attributed to the presence of polyphenolic compounds (Joshipura et al., 2001; Knekt et al., 2002; Steinmetz & Potter, 1996). Berry fruits are particularly rich sources of polyphenols, with anthocyanins, ellagitannins, flavonol conjugates and hydroxycinnamic acids being most abundant (Hancock, McDougall, & Stewart, 2007; Kahkonen, Hopia, & Heinonen, 2001; Zadernowski, Naczk, & Nesterowicz, 2005).

Ellagitannins belong to a group of compounds known as hydrolysable tannins and are polymers of hexahydroxydiphenic acid ([1,1′-biphenyl]-2,2′-dicarboxylic acid, 4,4′,5,5′,6,6′-hexahydroxy-) and a polyol, which is usually either glucose or quinic acid (Cerda, Tomas-Barberan, & Espin, 2005; Clifford & Scalbert, 2000; Gupta, Al-Shafi, Layden, & Haslam, 1982; Hager, Howard, Liyanage, Lay, & Prior, 2008; Koponen, Happonen, Mattila, & Torronen, 2007; Vrhovsek et al., 2006). Ellagitannins occur in a variety of fruits, such as pomegranates, strawberries, raspberries, blackberries, muscadine grapes and persimmons. They are also present in nuts, such as walnuts and hazelnuts, in oak-aged wines and occur in plant-derived products, such as fruit juices, tea, and beer (Cerda et al., 2005; Clifford & Scalbert, 2000; Koponen et al., 2007; Wada & Ou, 2002a).

Ellagitannins are readily hydrolysed in an acidic or basic environment to release ellagic acid. This degradation reaction is often used to quantify the amount of ellagitannin in a product (Cerda et al., 2005; Clifford & Scalbert, 2000; Vrhovsek et al., 2006). *Rubus* berries are known to be rich sources of ellagic acid, with concentrations reported for raspberries and blackberries to be as high as 1.2–2.5 and 1.5–2 mg/g, respectively (Clifford & Scalbert, 2000), of which the majority is released during hydrolysis, indicating that most of the ellagic acid is present in complex polymers like ellagitannin (Vrhovsek et al., 2006). Both ellagic acid and ellagitannins are believed to have multiple health benefits. They function as antioxidants (Mullen et al., 2002) and antiviral agents (Cortout, Peiters, Claeys, Vanden Berghe, & Vleitinck, 1991), prevent different kinds of tumours, like colon (Rao et al., 1991), lung, oesophagus (Kresty et al., 2001) and prostate cancer (Seeram et al., 2007).

Several monomeric and oligomeric ellagitannin structures have been identified in *Rubus* berries, using LC–MS/MS and NMR. The basic ellagitannin structure found in *Rubus* fruit is a bis-hexahydroxydiphenoyl (HHDP)–glucopyranose, known as pedunculagin (Fig. 1A) (Hager et al., 2008), which is also often present in its galloylated form, galloyl–bis-HHDP–glucopyranose, known as casuarictin or potentillin (Fig. 1B), which are two isomeric forms (Hager et al., 2008). Several oligomeric structures of *Rubus*

* Corresponding author. Tel.: +64 6 953 7684; fax: +64 6 356 6731.
*E-mail address:* Tony.Mcghie@plantandfood.co.nz (T.K. McGhie).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.09.039

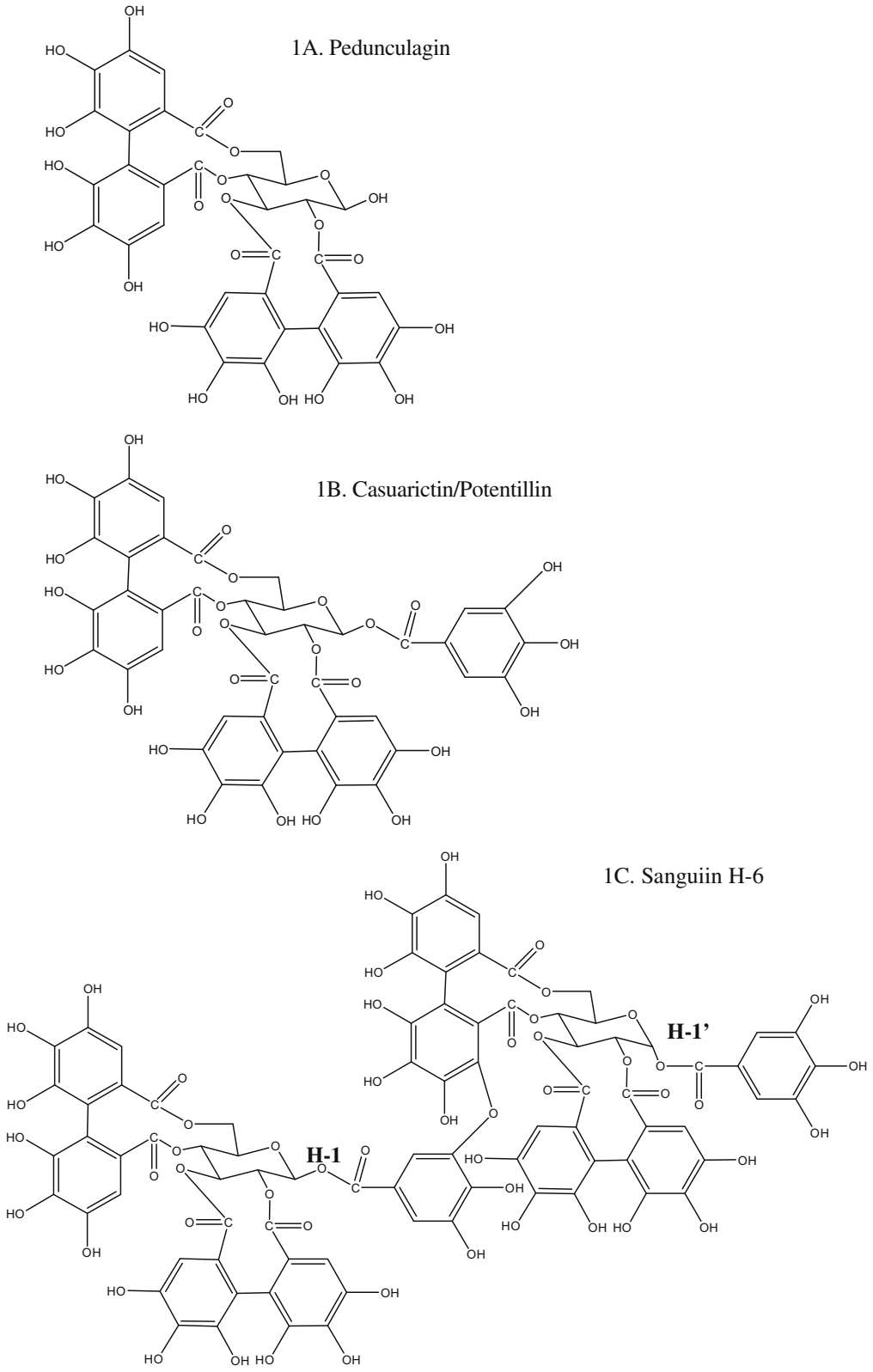


**Fig. 1.** Structures of ellagitannin compounds reported to be present in *Rubus* plants.

galloylated ellagitannin are known, of which the dimeric sanguiin H-6 (Fig. 1C) and lambertianin A (Fig. 1D) isomers are present at the highest concentrations in both raspberries and blackberries (Clifford & Scalbert, 2000; Mullen, Yokota, Lean, & Crozier, 2003). Other ellagitannin structures identified in *Rubus* species are the dimeric sanguiin H-10 (Fig. 1E), and lambertianin B, the trimeric lambertianin C (Fig. 1F), and the tetrameric lambertianin D (MW = 3740) (Clifford & Scalbert, 2000; Gupta et al., 1982; Koponen et al., 2007; Mullen et al., 2003). Ellagitannins identified in other plants include punicalagin (pomegranate), and juglanin

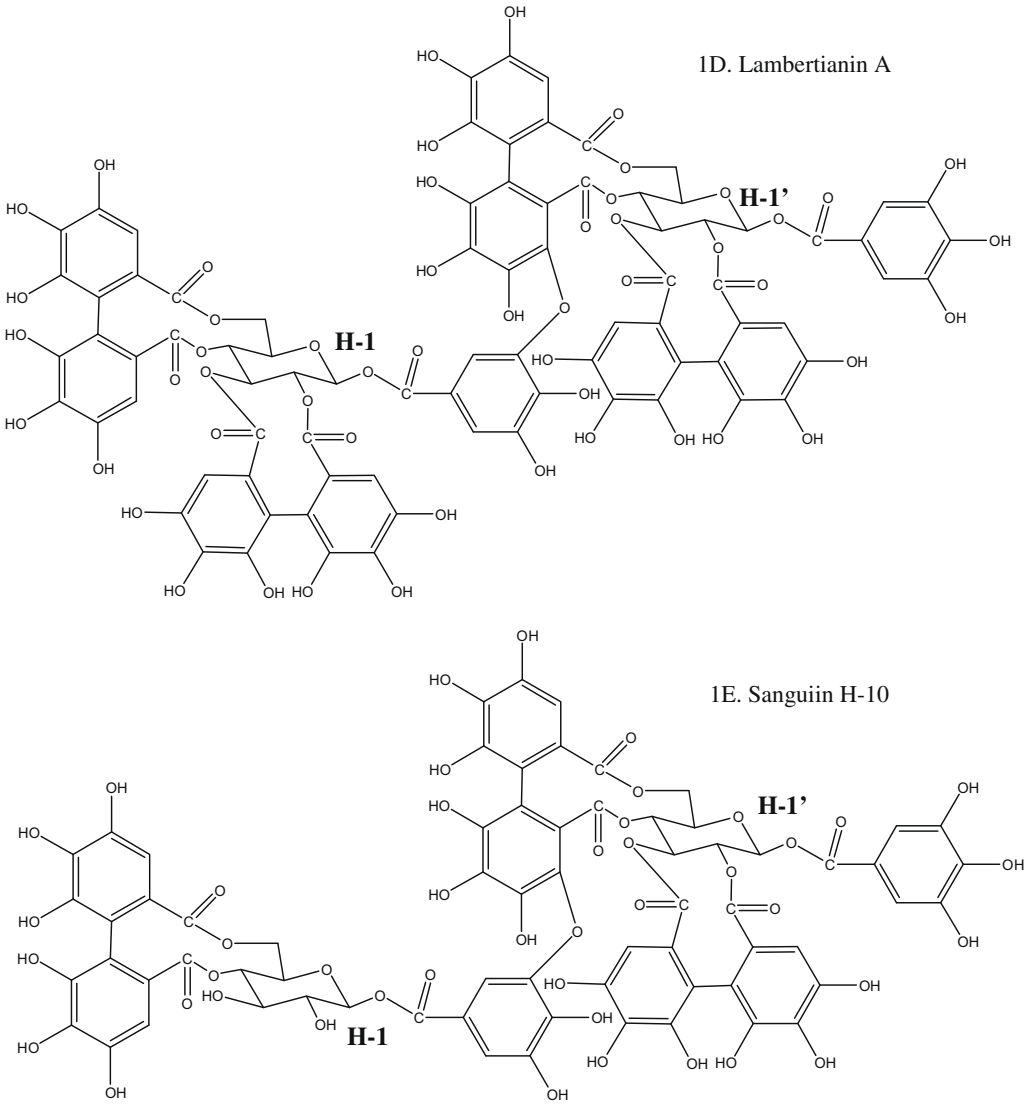


**Fig. 1** (*continued*)

(walnuts) (Adams et al., 2006; Clifford & Scalbert, 2000; Gupta et al., 1982).

Boysenberry is a hybrid *Rubus* berry, believed to be derived from a cross between loganberry (*Rubus loganbaccus*) and blackberry (*Rubus baileyanus* Britt.) and has distinctive flavour and pigment profiles, compared with blackberry (Hall & Langford, 2008; McGhie, Hall, Ainge, & Mowat, 2002; Stanley, McGhie, Rowan, Langford, & Hall, 2002). Commercial production of boysenberry occurs in New Zealand, and the USA (Oregon, California), with about 5000 tonne produced annually. The major market for the fruit is in processed foods, although fresh fruit are becoming available as new cultivars are introduced (Hall & Langford, 2008). The ellagitannins present in boysenberries have not been reported but several studies show that acidic hydrolysates contain substantial amounts of ellagic acid, suggesting boysenberries contain ellagitannins (Bushman et al., 2004; Hurst & McGhie, 2007; McGhie T. K., 2007; Wada & Ou, 2002b).

To identify the ellagitannin compounds in boysenberry, we have isolated four ellagitannins from a boysenberry extract by preparative HPLC and determined their structures by liquid chromatography–mass spectrometry (LC–MS/MS), matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) and nuclear magnetic resonance (NMR) spectroscopy.

## 2. Materials and methods

### 2.1. Ellagitannin extraction and purification

Ellagitannins were extracted from a dry boysenberry powder provided by New Zealand Extracts Ltd. (Blenheim, New Zealand). The powder was prepared by treating boysenberry waste from juice production with hot water, followed by drying. Since the boysenberry waste contains a high proportion of seeds this powder was a rich source of ellagitannins. A diagram of the procedure used to isolate and purify boysenberry ellagitannins is given in Fig. 2. Five grams of a dry boysenberry powder were dissolved in 200 ml EtOH:$H_2O$:formic acid (80:20:1). To remove the anthocyanins, 50 ml of the extract were applied to a 100 mm × 42 mm i.d. ion exchange column (Dowex 50W-X8, 20–50 U.S. mesh H; BDP product No. 55038), which had been conditioned with 200 ml of 2 M NaOH followed by 300 ml EtOH:$H_2O$:formic acid (80:20:1). The ellagitannins were eluted from the column with 150 ml of EtOH:$H_2O$:formic acid (80:20:1), followed by 125 ml EtOH:$H_2O$:HCl (50:50:10) to elute the anthocyanins and finally with 100 ml of 2 M NaOH to recondition the column. The ellagitannin-rich fractions of four isolations were combined, dried under vacuum, and dissolved in 100 ml of aqueous 5% formic acid (FA).

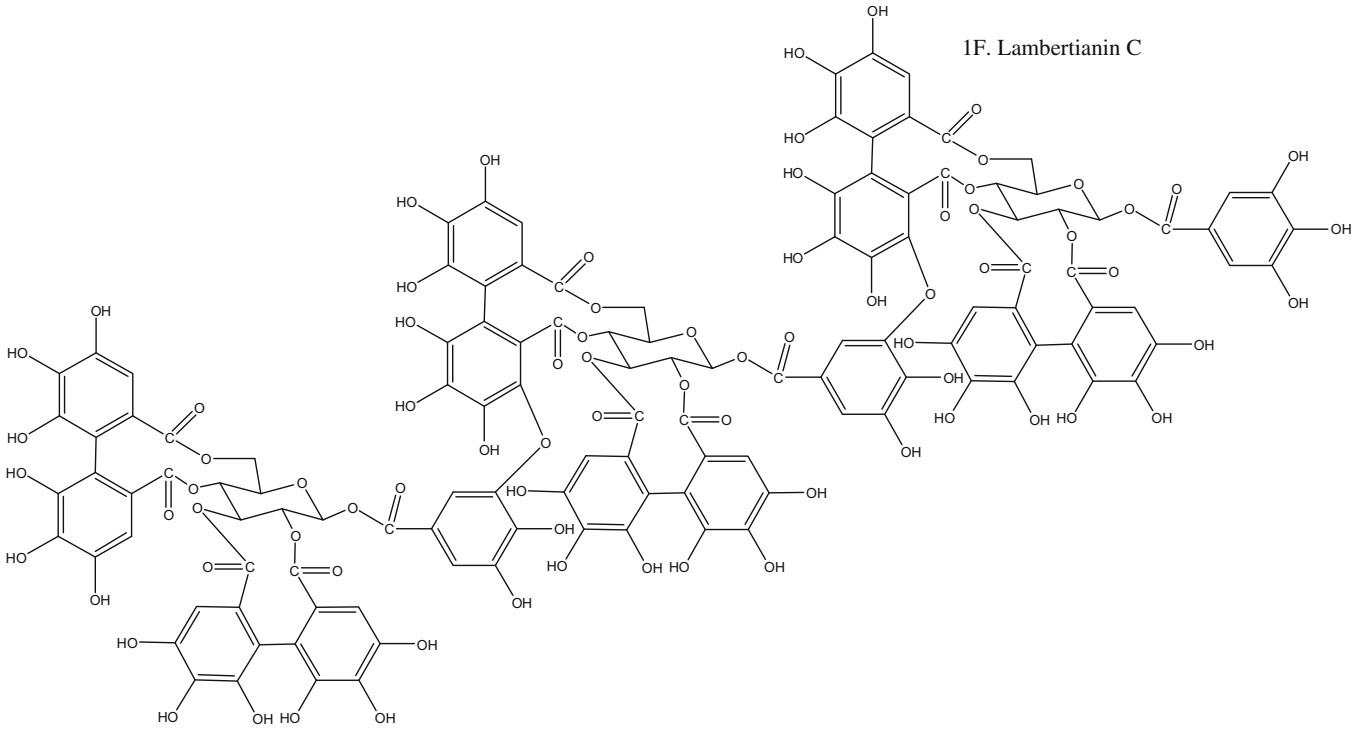


**Fig. 1** (*continued*)

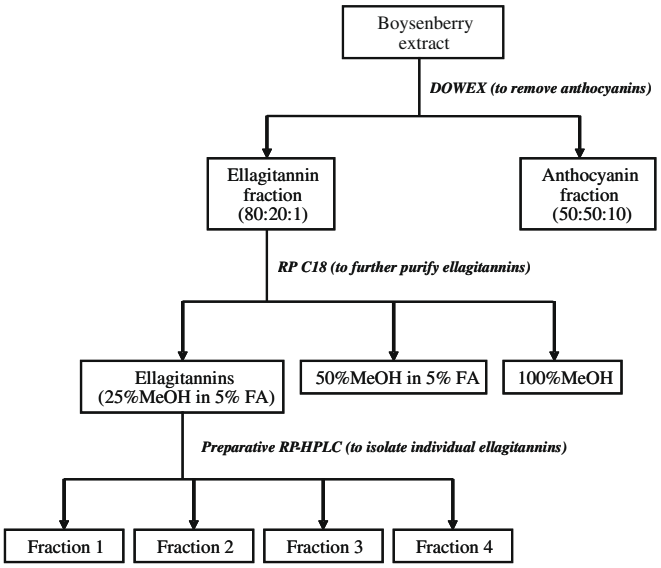


**Fig. 2.** The isolation and purification scheme used to purify the ellagitannin components of boysenberry.

To remove further non-ellagitannin compounds, 30 ml of this ellagitannin-rich extract was loaded onto a 160 mm × 30 mm i.d., 50 μm reversed phase C18-EC 70 Å column (Phenomenex, Torrance, CA), which had been conditioned with 150 ml of 100% MeOH and then 300 ml of aqueous 5% FA. The column was eluted sequentially with 25 ml of 5% FA, 200 ml of MeOH:5% aqueous FA (25:75), 100 ml of MeOH:5% aqueous FA (50:50), and finally with 100% MeOH. This was repeated twice. The fractions eluting with 25% MeOH in 5% FA solution contained the ellagitannins and these were combined, concentrated by rotary evaporation (30 °C) to ~20 ml and stored at −20 °C.

### 2.2. Preparative reversed-phase HPLC

To isolate the individual ellagitannins, the extract prepared above was injected into a Shimadzu (Kyoto, Japan) preparative HPLC system comprising two LC-8A pumps, SIL-10AP autosampler, CTO-20A column oven, SPD-20A detector, FRC-10A fraction collector and a CBM-20A controller. The separation column was a 250 × 15 mm i.d., 4 μm, Synergi Hydro-RP (Phenomenex) with solvents 100% water (A) and 100% acetonitrile (B). The gradient was 10% B to 5% B in 5 min, 5% B to 40% B in 13 min, 40% B to 80% B in 3 min, held at 80% B for 5 min, 80% B to 5% B in 5 min and 2 min conditioning on 5% B. The flow rate was 10 ml/min with the column maintained at 30 °C. The injection volumes were 50–200 μl and ellagitannins were detected at 280 nm. Fractions were collected separately for multiple chromatographic runs and like fractions combined. To prevent degradation of the purified ellagitannin, the acetonitrile was removed by rotary evaporation (40 °C) and the purified ellagitannin stored at −80 °C.

### 2.3. Analytical reversed-phase HPLC

A Waters Alliance W2690 HPLC system with a PDA detector (Model 996) was used to determine the purity of the ellagitannins isolated by preparative HPLC. Compound separation was achieved using a Zorbax Rapid Resolution SB–C18, 1.8 μm, 4.6 × 150 mm column (Agilent, Santa Clara, CA) with a flow rate of 0.8 ml/min and 5 μl injection volume. Solvents were concentrated formic acid:$H_2O$ (5:95; A), and 100% acetonitrile (B). The ellagitannins were separated using the following gradient system. The solvent composition programme was as follows: initial 5% B, 5–20% B in 10 min, 20–80% B in 3 min and held at 80% B for 4 min; before returning to the initial conditions. Chromatograms were recorded at 280 nm.

### 2.4. LC–MS/MS

LC–MS experiments employed a Surveyor™ HPLC and PDA detector coupled to a LCQ Deca ion trap mass spectrometer fitted

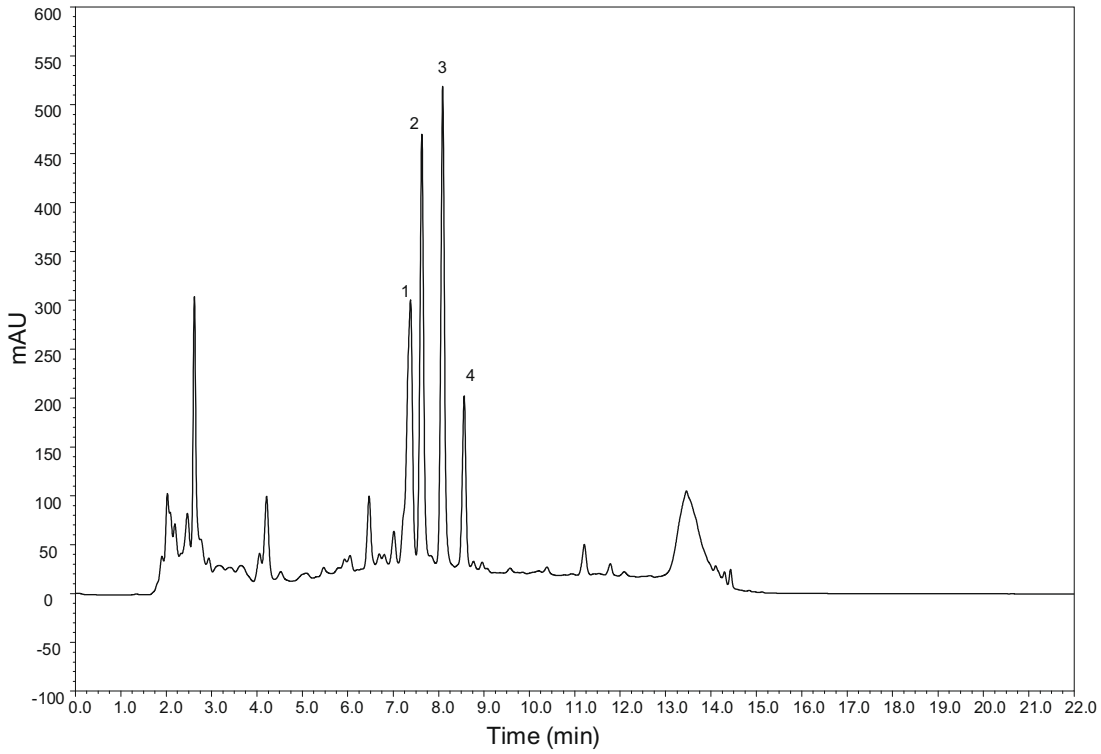


**Fig. 3.** A chromatogram trace of the crude polyphenolic extract of boysenberry obtained by ion exchange and reversed-phase column chromatography, showing four major ellagitannin components. Detection is at 280 nm.

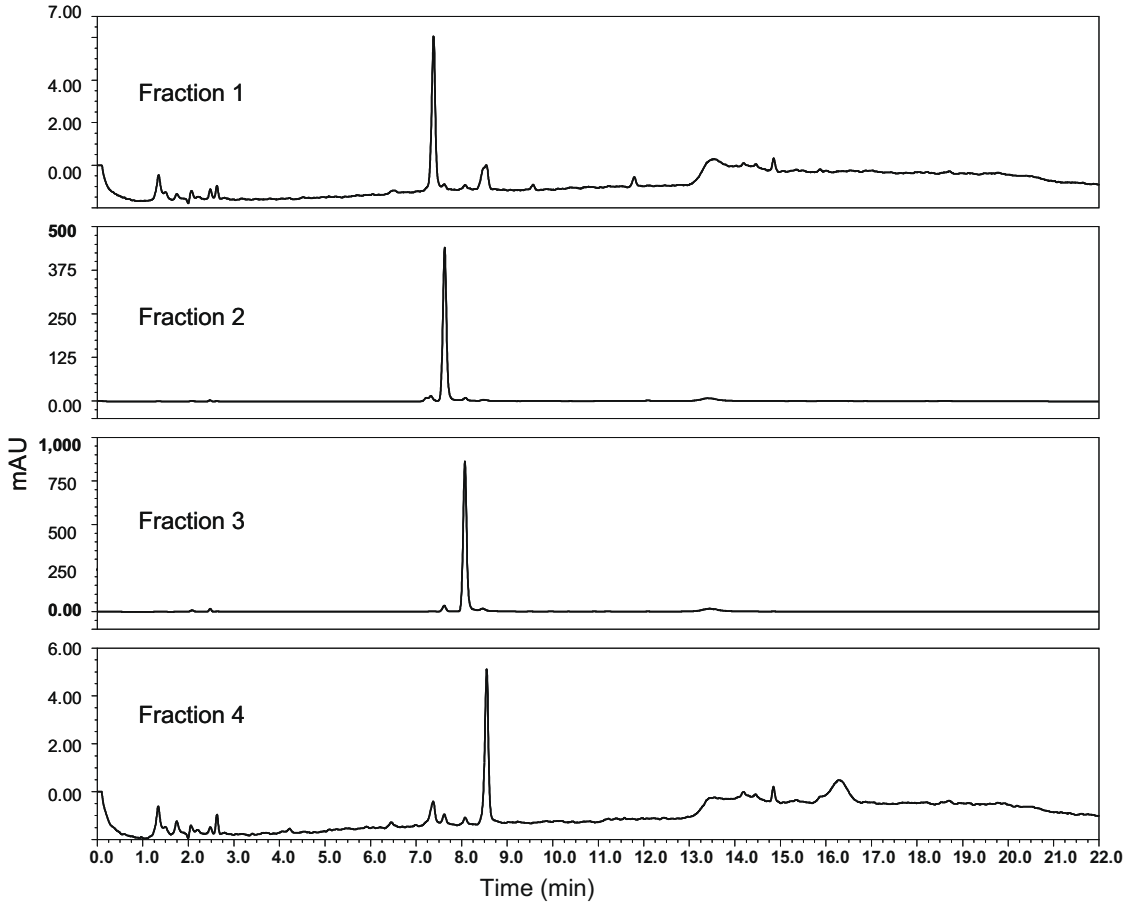


**Fig. 4.** Analytical RP-HPLC chromatogram traces of the ellagitannin fractions after purification by preparative HPLC. Detection is at 280 nm.

**Table 1**
Summary of the properties of the ellagitannin compounds present in the four fractions isolated and purified from boysenberry.

| Fraction # | $t_r$ (min) | Compound identification | $[M-H]^-$ ($m/z$) | Fragment ions ($m/z$) |
|---|---|---|---|---|
| 1 | 7.38 | Galloyl–sanguiin H-6 (possible artefact of lambertianin C) | 2037 | 1567 ($[M-H]^-$–HHDP–galloyl), 1103 ($[M-H]^-$–HHDP–HHDP–galloyl–glu), 933 ($[M-H]^-$–galloyl–galloyl–HHDP–HHDP–glu) |
| 2 | 7.63 | Sanguiin H-10 (an isomer) | 1567 | 1265 ($[M-H]^-$–HHDP), 1103 ($[M-H]^-$–HHDP–glu), 933 ($[M-H]^-$–HHDP–glu–galloyl), 631 ($[M-H]^-$–HHDP–glu–galloyl–HHDP) |
| 3 | 8.06 | Sanguiin H-6 | 1869 | 1567 ($[M-H]^-$–HHDP), 1265 ($[M-H]^-$–HHDP–HHDP), 933 ($[M-H]^-$–HHDP–HHDP–glu–galloyl), 631 ($[M-H]^-$–HHDP–HHDP–glu–galloyl–HHDP) |
| 4 | 8.53 | Sanguiin H-2 | 1103 | 1059 ($[M-H]^-$–$CO_2$), 935 ($[M-H]^-$–$CO_2$–tri-hydroxy-benzene), 633 ($[M-H]^-$–$CO_2$–galloyl–HHDP) |

glu, glucose; HHDP, hexahydroxydiphenoyl.

with an ESI interface (ThermoQuest, Finnigan, San Jose, CA). Compound separation was achieved using a Prodigy 5 μm ODS(3) 100 Å (Phenomenex) 150 × 2 mm analytical column maintained at 35 °C. A 0.2 μm in-line filter (Alltech, Deerfield, IL) was installed before the column. Solvents were 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) and the flow rate was 200 μl/min. The solvent composition programme was as follows: initial 5% A held for 5 min, 5–10% A in 5 min, 10–17% A in 15 min, 17–23% A in 5 min, 23–30% A in 10 min, 30–97% A in 8 min, and held for 5 min before returning to the initial conditions. Sample injection volume was 10 μl and UV–vis detection was measured by absorbance at 200–600 nm.

MS data were acquired in negative ion mode using a data-dependent LC–$MS^3$ method with dynamic exclusion enabled and a repeat count of 2. This method isolates and fragments the most intense parent ion twice to give $MS^2$ data, then isolates and fragments the most intense daughter ion twice ($MS^3$ data). Each ion examined is then added to an exclusion list for a period of 1 min to enable mass spectral fragmentation data to be obtained from less intense ions. The ESI voltage, capillary temperature, sheath gas pressure and auxiliary gas were set at −47 V, 300 °C, 50 psig, and 10 psig, respectively.

### 2.5. Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS)

LC–MS/MS was not able to adequately analyse ellagitannins with a higher molecular mass (>2000 Da), because it is beyond the regular working range of the instrument. To confirm the presence of higher molecular mass compounds, MALDI-TOF-MS was performed on the four purified ellagitannins.

The purified ellagitannin samples were mixed in a 1:1 ratio with a 0.1 M solution of 2,5-dihydroxybenzoic acid (DHB) in 90% methanol, and 2 μl of the mixture were spotted onto a ground stainless steel MALDI target, for MALDI analysis using the dry droplet method. A Micromass M@LDI-LR ($N_2$ laser: 337 nm, source voltage: 15 kV, reflectron voltage: 2 kV) was used in the MALDI analysis, and all the data were obtained in positive ion reflection TOF mode.

### 2.6. Nuclear magnetic resonance (NMR) spectroscopy

Samples (3–5 mg) were dissolved in acetone-$d_6$:$D_2O$ (Fraction 2, 3:1; Fraction 3, 1:1). $^1H$ nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance 500 NMR spectrometer. Chemical shifts ($\delta$) are in parts per million relative to acetone-$d_6$ at 2.15 ppm.

## 3. Results and discussion

Analytical HPLC analysis of the ellagitannin-rich boysenberry extract, after ion exchange and C18 column chromatography, revealed four ellagitannins (Fig. 3). All four ellagitannin components (Fractions 1–4) were isolated using preparative HPLC. Isolated fractions were examined by analytical HPLC, which demonstrated that all fractions were relatively pure with respect to ellagitannins (280 nm) (Fig. 4). The structural characteristics of Fractions 1–4, based on LC–ESI-MS/MS, MALDI-TOF-MS and NMR data are outlined below and summarised in Table 1.

### 3.1. Identification of ellagitannins

Fraction 1 gave an ion at $m/z$ 1018 by ESI-MS that was shown to be doubly charged ion by zoom scan analysis, suggesting a true molecular weight of 2038. Sample concentration was too low for ESI-MS–MS analysis but full-scan ESI-MS showed singly charged fragments at $m/z$ 1567, 1103, and 933 (Table 1). These fragments were interpreted as follows: $m/z$ 1567 (2037 − 302 − 168, loss of HHDP and galloyl), $m/z$ 1103 (2037 − 604 − 168 − 162, loss of 2 HHDP, galloyl, and glucose), $m/z$ 933 (2037 − 604 − 338 − 162, loss of 2 HHDP, 2 galloyl, and glucose). The MALDI-TOF-MS spectrum of Fraction 1 showed ions at $m/z$ 2062.9 and 2078.9 (Fig. 5), which are most likely the sodium and potassium adducts of the ellagitannin compound identified by ESI-MS with a molecular weight of 2038, confirming that the molecular ion at $m/z$ 1018, observed in the ESI-MS spectra, was doubly charged. It has been previously reported that treatment of lambertianin C (Fig. 1F) with hot water, results in three hydrolysis products, one of which has a molecular mass of 2038 (Tanaka et al., 1993). These data suggest that the compound in Fraction 1 is galloyl–sanguiin H-6 with a structure as shown in Fig. 6A. It is possible that galloyl–sanguiin H-6 itself is present in boysenberry fruit, or that it was produced by degradation of lambertianin C during the hot water treatment used in the manufacture of the boysenberry extract. However, no evidence of lambertianin C was observed in any of the analyses performed in this study. Another possibility is that galloyl–sanguiin H-6 is derived by hot-water degradation of another high molecular weight ellagitannin polymer.

Fraction 2 gave an $[M-H]^-$ at $m/z$ 1567 by ESI-MS data which produced MS–MS fragments at $m/z$ 1265 (1567 − 302, loss of HHDP), $m/z$ 1103 (1567 − 302 − 162, loss of HHDP and glucosyl), $m/z$ 933 (1567 − 302 − 162 − 170, loss of HHDP, glucosyl and galloyl), $m/z$ 631 (1567 − 604 − 162 − 170, loss of two HHDP, glucosyl and galloyl). Previous results for raspberry reported by Mullen et al. (2003) gave the same ESI-MS–MS fragments for the ellagitannin sanguiin H-10. Based on the ESI-MS fragmentation pattern, the compound in Fraction 2 was identified as sanguiin H-10 (Fig. 1E). Fraction 2 gave ions at $m/z$ 1591.5 and $m/z$ 1607.5 in the MALDI-TOF-MS spectrum (Fig. 5), which are consistent with the sodium and potassium adducts of sanguiin H-10, respectively, confirming the LC–ESI-MS/MS results.

To determine the absolute configuration of the two glucose units present the $^1H$ NMR spectrum was measured and showed

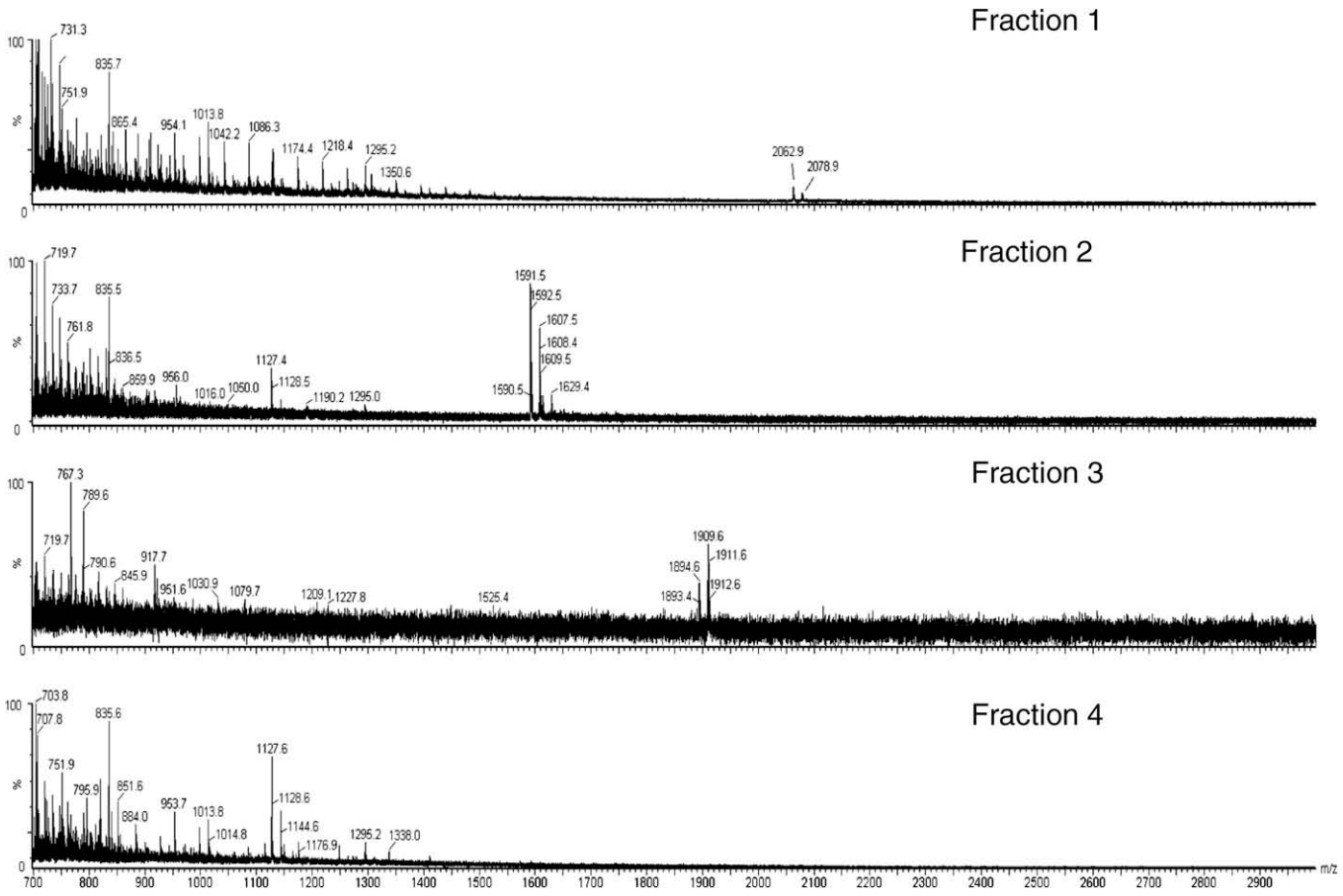


**Fig. 5.** MALDI-TOF-MS spectra of the four purified ellagitannin fractions showing putative $Na^+$ and $K^+$ adducts of the ellagitannin component present in each fractions.

two anomeric protons at $\delta$ 6.5 ($J$ = 3.5 Hz; H-1) and $\delta$ 6.0 ($J$ = 8.5 Hz; H-1′). The small (3.5 Hz) and larger (8.5 Hz) coupling constants indicate α- and β-glycoside configurations, respectively (Fossen & Andersen, 2006). This shows that glucose one is in the β-configuration and glucose two in the α-configuration, which differs from the previously reported structures of sanguiin H-10 (Fig. 1E) which had two β-glucose units (Hager et al., 2008; Mullen et al., 2003). We therefore report the compound in Fraction 2 as an isomer (Fig. 6B) of sanguiin H-10.

Fraction 3 gave an $[M-H]^-$ at $m/z$ 1869 by ESI-MS, which produced MS–MS fragments at $m/z$ 1567 (1869 – 302, loss of HHDP), $m/z$ 1265 (1869 – 604, loss of two HHDP), $m$/z 1103 (1869 – 604 – 162, loss of two HHDP and glucosyl), $m$/z 933 (1869 – 604 – 162 – 170, loss of two HHDP, glucosyl, and galloyl), m/z 631 (1869 – 906 – 162 – 170, loss of three HHDP, glucosyl, and galloyl). This MS–MS spectrum was similar to that previously reported for sanguiin H-6/lambertianin A (Hager et al., 2008; Mullen et al., 2002, 2003). This compound is therefore identified as one of the isomers of sanguiin H-6/lambertianin A. The MALDI-TOF-MS spectrum of Fraction 3 (Fig. 5) gave ions at $m/z$ 1894.6 and 1909.6, which is consistent with the sodium and potassium adducts of sanguiin H-6/lambertianin A, respectively, confirming the LC–ESI-MS/MS results. $^1$H NMR data of Fraction 3 showed two anomeric protons at $\delta$ 6.4 ($J$ = 4.5 Hz; H-1′) and $\delta$ 6.0 ($J$ = 8.3 Hz; H-1), indicating that glucose one is in the β-configuration and glucose two in the α-configuration, which is consistent with the previously reported structure of sanguiin H-6.

Fraction 4 gave an $[M-H]^-$ at $m/z$ 1103 by ESI-MS, which produced MS–MS fragments at $m/z$ 1059 (1103 – 44, loss of $CO_2$), $m$/z 935 (1103 – 44 – 124, loss of $CO_2$ and trihydroxybenzene), and $m/z$ 633 (1103 – 44 – 124 – 302, loss of $CO_2$, trihydroxybenzene and HHDP). The MALDI-TOF-MS spectra of Fraction 4 (Fig. 5) showed ions at $m/z$ 1127.6 and 1144.6, which are the sodium and potassium adducts of sanguiin H-2. Based on these LC–ESI-MS/MS and MALDI-TOF-MS data, the compound in Fraction 4 is identified as sanguiin H-2 (Fig. 6C), which is previously reported by Tanaka et al. (1993) to be present in *Rubus* leaves and as a hot-water degradation product of lambertianin C.

A previous report by Hager et al. (2008) detected 11 ellagitannins in various parts of blackberry fruit, a probable progenitor of boysenberry. However, in this study we isolated and characterised only four different ellagitannins from boysenberry waste material. The results reported here suggest that the composition of ellagitannin compounds present in boysenberry is different to that of other *Rubus* berries. In this study the two major ellagitannins isolated from boysenberry waste were identified as sanguiin H-6 (Fig. 1D) and an isomer of sanguiin H-10 (Fig. 6B). Previous studies on *Rubus* fruit found that sanguiin H-6/lambertianin A are the most abundant ellagitannin compounds in blackberries and raspberries (Clifford & Scalbert, 2000; Hager et al., 2008; Mullen et al., 2002, 2003). However, the second most abundant ellagitannin in boysenberry, sanguiin H-10, is only reported as a minor ellagitannin in other *Rubus* species. Moreover, the isomeric form of sanguiin H-10 found in this study and identified by NMR spectroscopy has not been reported before. To the best of our knowledge, NMR spectroscopy on sanguiin H-10 has not been performed before. The confirmation of the presence of the sanguiin H-10 isomer found in this study suggests that including NMR spectroscopy would be a very useful tool to more fully identify the exact structures of ellagitannins in other *Rubus* species.

The ellagitannin compounds present in Fractions 1 and 4 were apparently lower in concentration than Fractions 2 and 3. Sanguiin

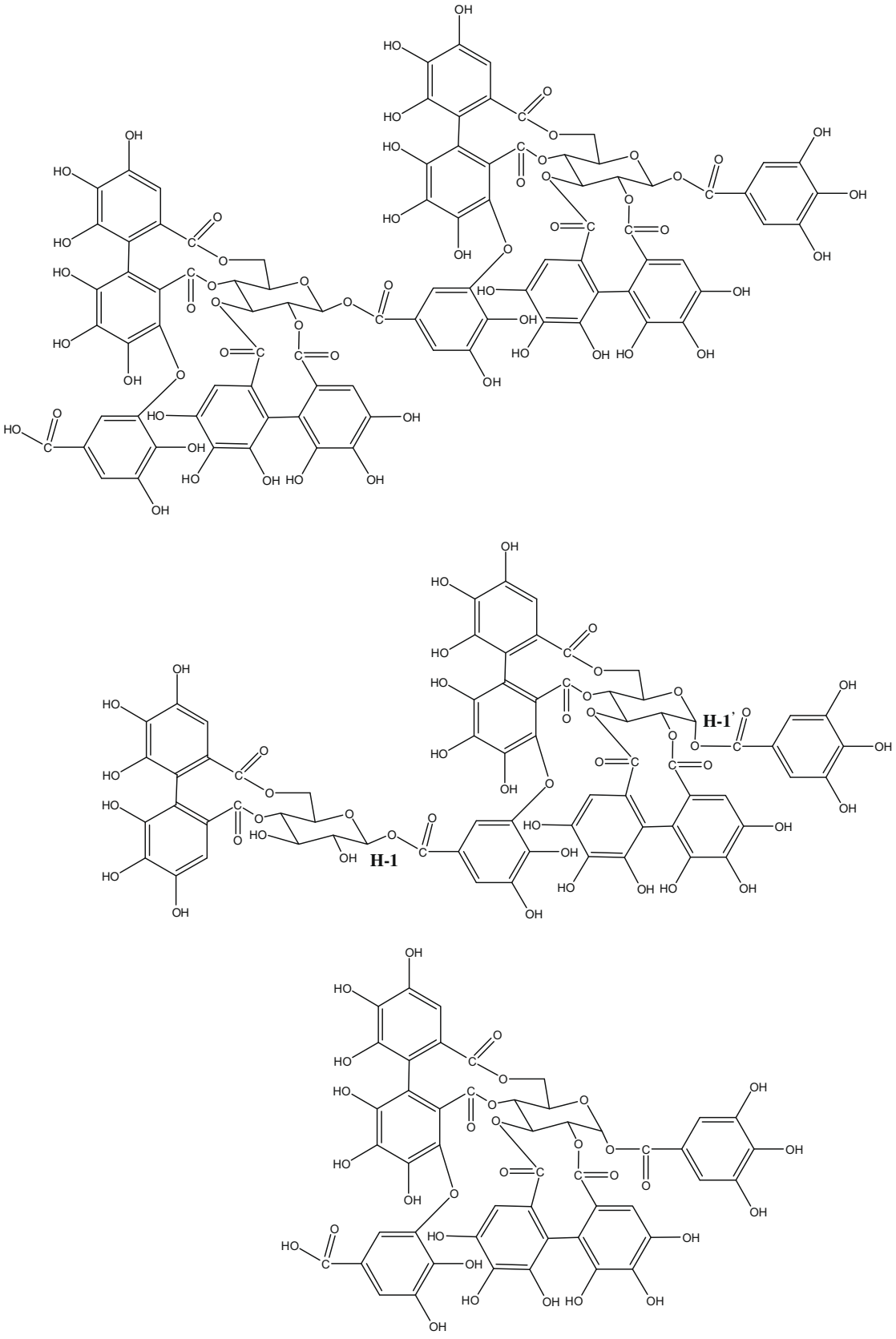


**Fig. 6.** Possible chemical structures for the ellagitannin compounds in Fractions 1, 2 and 4.

H-2 (Fraction 4) has been reported in *Rubus* leaves and as a minor hot-water degradation product of lambertianin C (Tanaka et al., 1993). The compound in Fraction 1, galloyl–sanguiin H-6 (Fig. 6A), has only been reported as a hot-water degradation product of lambertianin C (Tanaka et al., 1993). Lambertianin C is relatively abundant in *Rubus* fruit but in the present study it was not

detected in any of the fractions, suggesting that lambertianin C is not present in boysenberry. It is possible that lambertianin C is initially present in boysenberry and that it was degraded by the hot-water extraction used, resulting in the production of sanguiin H-2 and [galloyl–bis-HHDP–glucose]$_2$-gallate. So while the presence of, sanguiin H-2 and [galloyl–bis-HHDP–glucose]$_2$-gallate may be an artefact of the extraction procedure, severe conditions were required to only partially hydrolyse lambertianin C to these products (Tanaka et al., 1993). Therefore the inability to detect any lambertianin C suggests that sanguiin H-2 and [galloyl–bis-HHDP–glucose]$_2$-gallate may occur naturally in boysenberry, but this needs to be verified by further research.

Substantial concentrations of ellagitannins are present in *Rubus* fruit and are possibly able to provide considerable health benefits as has been suggested for the ellagitannin content of pomegranate. Until this study, the ellagitannin content of boysenberry had only been identified as ellagic acid equivalents following acid hydrolysis. The information presented here, showing that an extract of boysenberry contains four different ellagitannins, provides a better understanding of the potential health benefits associated with boysenberry consumption. Future research will focus on identifying and measuring these compounds in boysenberry fruit and determining how field production and processing techniques influence the composition of ellagitannins in boysenberry-derived foods.

## Acknowledgements

The authors thank Dr. Adam Matich and Barry Bunn for their critical review of the manuscript. Dr. Pat Edwards and David Lun (Massey University) assisted with the NMR and MALDI-TOF analysis, respectively. Funding was provided by the NZ Foundation for Research Science and Technology (C06X0407) and is gratefully acknowledged.

## References

Adams, L. S., Seeram, N. P., Aggarwal, B. B., Takada, Y., Sand, D., & Heber, D. (2006). Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells. *Journal of Agricultural and Food Chemistry, 54*, 980–985.

Bushman, B. S., Phillips, B., Isbell, T., Ou, B., Crane, J. M., & Knapp, S. J. (2004). Chemical Composition of Caneberry (*Rubus* spp.) seeds and oils and their antioxidant potential. *Journal of Agricultural and Food Chemistry, 52*, 7982–7987.

Cerda, B., Tomas-Barberan, F. A., & Espin, J. C. (2005). Metabolism of antioxidant and chemopreventative ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: Identification of biomarkers and individual variability. *Journal of Agricultural and Food Chemistry, 53*, 227–235.

Clifford, M. N., & Scalbert, A. (2000). Ellagitannins – Nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture, 80*, 1118–1125.

Cortout, J., Peiters, L. A., Claeys, M., Vanden Berghe, D. A., & Vleitinck, A. J. (1991). Antiviral ellagitannins from *Spondia mombin*. *Phytochemistry, 30*, 1129–1130.

Fossen, T., & Andersen, O. M. (2006). Spectroscopic techniques applied to flavonoids. In O. M. Andersen & K. R. Markham (Eds.), *Flavonoids chemistry, biochemistry and applications* (pp. 37–142). Taylor and Francis, Boca Raton: CRC Press.

Gupta, R. K., Al-Shafi, S. M. K., Layden, K., & Haslam, E. (1982). The metabolism of gallic acid and hexahydroxydiphenic acid in plants. Part 2. Esters of (*S*)-hexahydroxydiphenic acid and D-glucopyranose ($^4C_1$). *Journal of the Chemical Society. Perkin Transactions, 1*, 2525–2534.

Hager, T. J., Howard, L. R., Liyanage, R., Lay, J. O., & Prior, R. L. (2008). Ellagitannin composition of blackberry as determined by HPLC–ESI-MS and MALDI-TOF-MS. *Journal of Agricultural and Food Chemistry, 56*, 661–669.

Hall, H. K., & Langford, G. (2008). The 'Boysenberry': Development of the cultivar and industries in California, Oregon and New Zealand. *Acta Horticulturae, 777*, 103–108.

Hancock, R. D., McDougall, G. J., & Stewart, D. (2007). Berry fruit as 'superfood': Hope or hype. *Biologist, 54*, 73–79.

Hurst, R. D., & McGhie, T. K. (2007). *The unassuming Boysenberry – A health-promoting superfruit too*. Auckland: HortResearch.

Joshipura, K. J., Hu, F. B., Manson, J. E., Stampfer, M. J., Rimm, E. B., Speizer, F. E., et al. (2001). The effect of fruit and vegetable intake on risk for coronary heart disease. *Annals of Internal Medicine, 134*, 1106–1114.

Kahkonen, M. P., Hopia, A. I., & Heinonen, M. (2001). Berry phenolics and their antioxidant activity. *Journal of Agricultural and Food Chemistry, 49*, 4076–4082.

Knekt, P., Kumpulainen, J., Jarvinen, R., Rissanen, H., Heliovaara, M., Reunanen, A., et al. (2002). Flavonoid intake and risk of chronic diseases. *American Journal of Clinical Nutrition, 76*, 560–568.

Koponen, J. M., Happonen, A. M., Mattila, P. H., & Torronen, A. R. (2007). Contents of anthocyanins and ellagitannins in selected foods consumed in Finland. *Journal of Agricultural and Food Chemistry, 55*, 1612–1619.

Kresty, L. A., Morse, M. A., Morgan, C., Carlton, P. S., Lu, J., Gupta, A., et al. (2001). Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries. *Cancer Research, 61*, 6112–6119.

McGhie, T. K. (2007). Unpublished results.

McGhie, T. K., Hall, H. K., Ainge, G. D., & Mowat, A. D. (2002). Breeding *Rubus* cultivars for high anthocyanin content and high antioxidant capacity. *Acta Horticulturae, 585*, 494–500.

Mullen, W., McGinn, J., Lean, M. E. J., MacLean, M. R., Gardner, P. T., Duthie, G. G., et al. (2002). Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. *Journal of Agricultural and Food Chemistry, 50*, 5191–5916.

Mullen, W., Yokota, T., Lean, M. E. J., & Crozier, A. (2003). Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruit by LC–MS$^n$. *Phytochemistry, 64*, 617–624.

Rao, C. V., Tokumo, K., Rigoty, J., Zang, E., Kellof, G., & Reddy, B. S. (1991). Chemoprevention of colon carcinogenesis by dietary administration of piroxicam, difluoromethlornithine, 16-beta-fluor-5-androsten-17-one and ellagic acid individually and in combination. *Cancer Research, 51*, 4528–4534.

Seeram, N. P., Aronson, W. J., Zhang, Y., Henning, S. M., Moro, A., Lee, R.-P., et al. (2007). Pomegranate ellagitannin-derived metabolites inhibit prostate cancer growth and localize to the mouse prostrate gland. *Journal of Agricultural and Food Chemistry, 55*, 7732–7737.

Stanley, C. J., McGhie, T. K., Rowan, D. R., Langford, G., & Hall, H. K. (2002). Boysenberry – A character description for commerce. *Acta Horticulturae, 585*, 247–252.

Steinmetz, K. A., & Potter, J. D. (1996). Vegetables, fruit and cancer prevention: A review. *Journal of the American Dietetic Association, 96*, 1027–1039.

Tanaka, T., Tachibana, H., Nonaka, G., Nishioka, I., Hsu, F.-L., Kohda, H., et al. (1993). Tannins and related compounds. CXXII. New dimeric, trimeric and tetrameric ellagitannins, lambertianins A–D, from *Rubus lambertianus* SERINGE. *Chemical and Pharmaceutical Bulletin, 47*, 1214–1220.

Vrhovsek, U., Palchetti, A., Reniero, F., Guillou, C., Masuero, D., & Mattivi, F. (2006). Concentration and mean degree of polymerization of *Rubus* ellagitannins evaluated by optimized acid methanolysis. *Journal of Agricultural and Food Chemistry, 54*, 4469–4475.

Wada, L., & Ou, B. (2002a). Antioxidant activity and phenolic content of Oregon caneberries. *Journal of Agricultural and Food Chemistry, 50*, 3495–3500.

Wada, L., & Ou, B. (2002b). Antioxidant activity and phenolic content of Oregon caneberries. *Journal of Agricultural and Food Chemistry, 50*, 3495–3500.

Zadernowski, R., Naczk, M., & Nesterowicz, J. (2005). Phenolic acid profiles in some small berries. *Journal of Agricultural and Food Chemistry, 53*, 2118–2124.