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A RECYCLING PATHWAY FOR CYANOGENIC GLYCOSIDES EVIDENCED BY THE COMPARATIVE METABOLIC PROFILING IN THREE CYANOGENIC PLANT SPECIES

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ABSTRACT

Cyanogenic glycosides are phytoanticipins involved in plant defence against herbivores by virtue of their ability to release toxic HCN upon tissue disruption. In addition, endogenous turnover of cyanogenic glycosides without the liberation of HCN may offer plants an important source of reduced nitrogen at specific developmental stages. To investigate the presence of putative turnover products of cyanogenic glycosides, comparative metabolic profiling using LC-MS/MS and HR-MS complemented by ion-mobility mass spectrometry was carried out in three cyanogenic plant species: cassava, almond and sorghum. In total, the endogenous formation of 36 different chemical structures related to the cyanogenic glucosides linamarin, lotaustralin, prunasin, amygdalin and dhurrin was discovered, including di- and triglycosides derived from these compounds. The relative abundance of the compounds was assessed in different tissues and developmental stages. Based on results common to the three phylogenetically unrelated species, a potential recycling endogenous turnover pathway for cyanogenic glycosides is described in which reduced nitrogen and carbon are recovered for primary metabolism without the liberation of free HCN. Glycosides of amides, carboxylic acids and "anitriles" derived from cyanogenic glycosides appear as common intermediates in this pathway and may also have individual functions in the plant. The recycling of cyanogenic glycosides and the biological significance of the presence of the turnover products in cyanogenic plants open entirely new insights into the multiplicity of biological roles cyanogenic glycosides may play in plants.

Abbreviations: HR-MS, high-resolution mass spectrometry; EIC, extracted ion chromatogram; IM-MS, ion-mobility mass spectrometry; ATD, arrival time distribution; CID, collision-induced dissociation

SUMMARY STATEMENT

A potential recycling pathway for cyanogenic glycosides is presented wherein reduced nitrogen and carbon are recovered for primary metabolism without HCN liberation. Common types of glycosylated pathway intermediates were found in three cyanogenic plant species: cassava, almond and sorghum.

INTRODUCTION

Cassava (*Manihot esculenta,* Euphorbiaceae), almond (*Prunus dulcis,* Prunaceae) and sorghum (*Sorghum bicolor,* Poaceae) are well-known representatives of more than 2600 species of higher plants known to possess an ingenious defence mechanism: cyanogenesis. The hidden power of cyanogenic plants resides in their ability to produce amino acid-derived cyanogenic glycosides, from which toxic hydrogen cyanide (HCN) may be released through the action of specific β-glycosidases and α-hydroxynitrilases whenever plant tissue is disrupted – for example by chewing herbivores or by humans preparing foods from cyanogenic crops [1, 2] The generated HCN is toxic for the plants themselves and has to be detoxified. This entails two steps: (1) β-cyanoalanine synthase-catalysed conversion of HCN and cysteine into β-cyanoalanine and H_2S; (2) NIT4 family nitrilase-catalysed conversion of the toxic β-cyanoalanine into ammonia, aspartic acid and asparagine, all of which can be further utilized in plant metabolism (Figure 1) [3-7].

Despite the established role of cyanogenic glycosides in plant chemical defence, it is apparent that they possess additional physiological functions which improve the plant's phenotypic plasticity during specific developmental stages and under environmental stress [1, 2, 7-12]. Studies on *Olinia* species suggest that cyanogenic glucosides may be involved in modulating oxidative stress by scavenging reactive oxygen species (e.g. H_2O_2 in chlorotic leaves) [13]. Cyanogenic diglucosides in *Hevea brasiliensis* (rubber tree) have been demonstrated to function as a transport and storage form of renewable nitrogen, carbon and glucose for both germination and latex production [14, 15]. In cassava, girdling experiments demonstrated that cyanogenic glucosides are synthesized in the shoot apex and transported to the root. RNAi cassava plants in which the cyanogenic glucoside content was reduced to below 25% of the average cyanide potential of wild-type plants exhibited a distinct morphological phenotype when grown *in vitro* [16]. The plants had long and slender stems with long internodes, the leaves at the nodes were small and withered quickly, and the majority of the plants did not produce roots *in vitro*. When the growth medium was supplemented with nitrogen, growth was partly restored, demonstrating the function of cyanogenic glucosides as a nitrogen source [16].

The scattered literature data on the endogenous turnover of cyanogenic glycosides prompted us to explore the possible existence of a recycling pathway in which reduced nitrogen is released in the form of ammonia, without the liberation of HCN (Figure 1). Our investigation embraced three phylogenetically distinct plant species: cassava (containing the cyanogenic glucosides linamarin **1** and lotaustralin **9)**, almond (containing prunasin **13** and amygdalin **19)** and sorghum (containing dhurrin **25)** (Figures 2A and 2B). All three species are amongst the world's most important crops. Cassava and sorghum are extremely drought-resistant crops, used as staple foods and as the main sources of carbohydrates for hundreds of millions of people in tropical and subtropical countries. In 2013, 277 Mt of cassava and 62 Mt of sorghum were produced in the world (http://faostat.fao.org/). Almond kernels have high nutritional and economic value and their global annual production in 2013 amounted to 3 Mt (http://faostat.fao.org/).

In this study we use comparative metabolic profiling and structural characterization of turnover products of cyanogenic monoglucosides and their di- and triglycosides to identify common features of an endogenous recycling pathway in cassava, almond and sorghum. The temporal as well as spatial occurrence of these turnover intermediates is investigated, and their function in plant physiological processes proposed. We also highlight the application of a new method involving the use of ion-mobility mass spectrometry (IM-MS) as an analytical tool capable of sequencing the monosaccharide building blocks within the glycosides studied [17].

EXPERIMENTAL

Plant material

Manihot esculenta Crantz var. TME12 (cassava): the maternal plants were provided by the International Institute of Tropical Agriculture, Ibadan, Nigeria. Stem segments (1-2 cm) were taken from 3-month-old and 9-month-old plants, and tubers from 9-month-old plants. The plants were cultivated in soil in a greenhouse under a cycle of 16 h light (28 °C) and 8 h darkness (25 °C).
Prunus dulcis (Mill.) D.A.Webb syn. *Prunus amygdalus* Batsch genotype S3067 (bitter almond): seeds were provided by the Centro de Edafología y Biología Aplicada del Segura-Consejo Superior de Investigaciones Científicas (CEBAS-CSIC). After imbibition (8 weeks, covered by moist vermiculite, 4 °C), the developing almond seedlings were grown in a greenhouse on vermiculite/perlite for 17 weeks under 16 h light (24 °C) and 8 h darkness (17 °C), and the collected samples stored at -80 °C.
Sorghum bicolor (L.) Moench var. BTX 623 (public sorghum line): seeds were kindly provided by Dr. Peter Stuart (University of Queensland, Australia). The plants were cultivated in soil in a greenhouse under a cycle of 16 h light (24 °C) and 8 h darkness (17 °C). Leaf sheaths (the basal part of the leaf) and leave blades were collected 13 and 30 days after sowing and stored at -80 °C.

Sample extraction

Frozen plant material was ground in liquid nitrogen with mortar and pestle and weighed frozen in a 2 ml screw-lid Eppendorf tube. The plant material was boiled in 85% aq. MeOH (300-500 µl, 5 min) and immediately cooled on ice. Subsequently, the samples were diluted 5-100 times in H_2O, an internal standard was added (50 µM prunasin in the case of cassava, 50 µM lotaustralin in the case of almond, 50 µM linamarin in the case of sorghum), and samples were filtered through a membrane filter (0.45 µm, Merck Millipore, MA, U.S.A.) by centrifugation (3000 rpm, 5 min).

LC-MS/MS analysis

Analytical LC-MS/MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Karlsruhe, Germany) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). A Zorbax SB-C18 column (Agilent; 1.8 µm, 2.1 x 50 mm) maintained at 35°C was used for separation. The mobile phases were: A, water with 0.1% (v/v) HCOOH and 50mM NaCl; B, acetonitrile with 0.1% (v/v) HCOOH. The gradient program was: 0 to 0.5 min, isocratic 2% B; 0.5 to 7.5 min, linear gradient 2 to 40% B; 7.5 to 8.5 min, linear gradient 40% to 90% B; 8.5 to 11.5 min isocratic 90% B; 11.6 to 17 min, isocratic 2% B. The flow rate was 0.2 mL min^{-1} but increased to 0.3 mL min^{-1} in the interval 11.2 to 13.5 min. ESI-MS^2 was run in positive mode. Extracted ion chromatograms for specific $[M+Na]^+$ adduct ions were used to locate compounds, and their MS, MS^2 and RT were used to identify peaks. Calibration curves covered the range 0.1 µM to 400 µM of **1**, **9**, **13**, **19** and **25**, all chemically synthesised (M. S. Motawia, unpublished work), and were used for absolute quantification. The relative quantities of their respective derivatives were calculated as the ratio of the peak area (normalized with an internal standard) to the sample weight, and expressed as a percentage of the absolute concentrations of the parent compounds. Authentic standards of compounds **4**, **14** (*S* and *R*), **15**, **16**, **18b**, **20** (crude), **21** (*S* and *R*; crude), **22** (crude), **26** (crude) and **28** were likewise synthesised (M. S. Motawia, unpublished work) and compared as above (MS, MS^2, RT) to the corresponding peaks from the plant samples. The ionization efficiency of cyanogenic glucosides and their derivatives might vary by a factor of approximately two (Supplementary Figure S1), suggesting that reported relative levels in Table 1 are correct in an absolute sense within this span. The LC-MS data were analysed using Bruker-DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany). Accurate mass measurements (HR-MS) were made under similar conditions using a micrOTOF-*Q* instrument (Bruker Daltonics, Bremen, Germany).

Preparative HPLC

The procedure is described in detail in the Supplementary Data. Briefly, pre-cleaned crude plant extracts were subjected to preparative HPLC using an analytical HPLC (LC-10AT pump with an SPD-M10AVP PDA detector; Shimadzu, Kyoto, Japan). The Luna phenylhexyl column and Luna C-18 column (both 5 μm; 250 mm x 4.6 mm i.d.) (Phenomenex, Torrance, CA, U.S.A) were operated at 1 mL/min with typically 100-200 μL injection volume and using the following gradient program: 2 min of isocratic H_2O, 48 min of a linear gradient from 0 to 60% MeOH, a brief wash in MeOH and equilibration for 7 min in H_2O. The purity of collected peaks was assessed by LC-MS/MS.

NMR

Purified compound **8** was analyzed on a Bruker AC-400 Avance spectrometer (Bruker Biospin, Fällanden, Switzerland) by ^{1}H NMR and 2D NMR (COSY, HSQC, HMBC AND JRES). The ^{13}C chemical shifts were retrieved from the HSQC spectra. They were in agreement with literature values as were the ^{1}H shifts and coupling constants for the two anomeric protons [18, 19]. ^{1}H and ^{13}C NMR spectra for purified compounds **18a** and **18b** were recorded on an 800 MHz Bruker Avance II spectrometer equipped with a TCI z-gradient cryoprobe (Bruker Biospin, Fällanden, Switzerland) and are described in detail in the Supplementary Data. 2D NMR (DQF-COSY, ROESY, TOCSY, HSQC) yielded signals for the aromatic CHs group indicative of a benzyl aglycone, while chemical shifts and scalar coupling patterns confirmed β-glucopyranosyl-residue glycosidically linked at O-6 to α-arabinopyranosyl residue in **18a,** and to β-xylopyranosyl residue in **18b**.

IM-MS/MS analysis

Samples were analysed according to the protocol reported by Both *et al.* [17]. Briefly, plant glycosides were diluted to approx. 5 μM in 50% methanol containing 50 μM lithium chloride (Sigma) prior to infusion into a Synapt G2 HDMS (Waters, Manchester, U.K.). The capillary voltage, cone voltage and source temperature were typically set to 0.8-1.2 kV, 40 V and 80 ^{o}C respectively. The IM travelling wave speed and height were set throughout the experiment to 700 m/s and 40 V, respectively. Collision-induced dissociation (CID) was induced by raising the trap collision energy from 4 kV to 30-40 kV. The mass measurements were calibrated to product ions generated by CID of Glu-fibrinopeptide (500 nM; 1:1 acetonitrile/water, 0.1% formic acid). The mobility measurements were calibrated to a series of small molecules whose rotationally averaged collision cross sections are known [20]. Mass spectra were processed using MassLynx V4.1 (Waters) and ATDs using OriginPro 9.1 (OriginLab Corporation, MA, U.S.A).

RESULTS

Identification and quantification of cyanogenic glucosides and their structural derivatives in cassava, almond and sorghum

In this section, we describe the results obtained from LC-MS/MS metabolic profiling in cassava, almond and sorghum. For simplicity, all the derivatives of cyanogenic glucosides have been named with their trivial names in relation to the parent cyanogenic glucosides from which they are putatively derived. In addition to amide and carboxylic acid derivatives of known cyanogenic glycosides, we repeatedly encountered structures that we suggest be named "anitriles": compounds resembling cyanogenic glycosides but with the nitrile (CN) functionality replaced by a hydrogen atom. We use this term in trivial names throughout the manuscript. The systematic chemical names are presented in the legend of Table 1; the corresponding structures are shown in Figure 2A; extracted ion chromatograms of all the compounds identified can be found in Figure 2B, and their respective MS and MS^2 spectra in Supplementary Figure S2. The identification of all structures is supported by HR-MS, with mass accuracy less than 4 ppm (Table 1A-C).

Metabolic profiling in cassava (*Manihot esculenta* Crantz)

Methanolic extracts of upper and lower stem segments collected from 3-month- and 9-month-old cassava plants, and from the cortex and starchy pulp of cassava tuberous roots of the 9-month-old plants, were subjected to LC-MS/MS analysis in order to investigate qualitatively and quantitatively the presence of the cyanogenic glucosides linamarin **1** and lotaustralin **9** and of their potential turnover products.

As summarized in Table 1A, linamarin is the major cyanogenic constituent in cassava, and its amount exceeds that of lotaustralin by 1-2 orders of magnitude in all tissues and at both developmental stages. Linamarin amide **2**, acid **3** and anitrile **4** were detected in low amounts in both young and old cassava plants, with the exception of **4**, which was not detected in young plants. The diglycosides linamarin glucoside (known as linustatin) **5** [21], linamarin anitrile glucoside **6**, linamarin apioside **7** (**a** and **b** isomers) and linamarin anitrile apioside **8** were also found in certain tissues. In contrast to **5,** which occurs at low levels and only in the lower part of old cassava stems, the apioside **8** reached levels exceeding those of its possible precursor linamarin, especially in the tuber cortex. Indeed, the levels of **8** enabled ^{1}H and ^{13}C NMR, thus confirming the identity, based on comparison with literature data [18, 19]. The occurrence of **7** in two isomers is probably due to different linkage positions between glucose and apiose moieties [c.f. 22]. In addition, lotaustralin acid **10** and cyanogenic diglycoside lotaustralin apioside **11** were detected at low levels in some of the plant parts investigated. Although lotaustralin anitrile (2-butanoyl-*O*-β-D-glucopyranoside) itself was not detected, lotaustralin anitrile apioside **12** (in parallel with **8**) was found to be the most abundant structural derivative of lotaustralin. In the lower stem and tuber cortex, it was detected in amounts comparable to those of lotaustralin.

For the structures described above, MS^2 fragmentation gave ion peaks corresponding to pseudomolecular ions $[M - H_2O + Na]^+$ and $[M + Na]^+$ of hexose (Hex), Hex + Hex, pentose (Pen), and Hex + Pen moieties, whose identities have been deduced from data in the literature [18, 19, 23, 24] and for **1**, **4**, **5**, **8**, **9**, **12** confirmed as: glucose (Glc) at *m/z* 185 $[C_6H_{12}O_6 - H_2O + Na]^+$ and/or at *m/z* 203 $[C_6H_{12}O_6 + Na]^+$, Glc + Glc at *m/z* 347 $[C_{12}H_{22}O_{11} - H_2O + Na]^+$ and at *m/z* 365 $[C_{12}H_{22}O_{11} + Na]^+$, and Glc + apiose (Api) at *m/z* 317 $[C_{11}H_{20}O_{10} - H_2O + Na]^+$ and at *m/z* 335 $[C_{11}H_{20}O_{10} + Na]^+$. Product ions associated with the aglycons of linamarin amide **2** and acid **3**, and of lotaustralin acid **10** were recorded as: 2-hydroxy-2-methylpropanamide at *m/z* 126 $[C_4H_9NO_2 + Na]^+$**,** 2-hydroxy-2-methylpropanoic acid at *m/z* 127 $[C_4H_8O_3 + Na]^+$**,** and 2-hydroxy-2-methylbutanoic acid at *m/z* 141 $[C_5H_{10}O_3 + Na]^+$, respectively. Compound **3** was also detected as $[M + 2Na - H]^+$ adduct (*m/z* 311), indicating the exchange of the carboxylic group proton with a sodium ion – a diagnostic feature for the identification of carboxylic acids [25]. Identification of **4** was confirmed by comparison with a synthetic standard.

For diglycosides, product ions corresponding to their respective monoglucosides appeared. Thus, fragmentation of **5** and **7** gave a fragment corresponding to **1** ($[C_{10}H_{17}NO_6 + Na]^+ = 270$). Similarly, fragmentation of **6** and **8** both gave rise to a fragment corresponding to **4** ($[C_9H_{18}O_6 + Na]^+ = 245$), while compounds **11** and **12** gave fragments corresponding to **9** ($[C_{11}H_{19}NO_6 + Na]^+ = 284$) and lotaustralin anitrile ($[C_{10}H_{20}O_6 + Na]^+ = 259$), respectively.

For all cyanogens, **1**, **5,** 7**, 9,** and **11**, the fragment ion $[M - HCN + Na]^+$ was detected, showing loss of HCN (*m/z* 27), which is diagnostic for cyanogenic compounds. Interestingly, fragmentation of cyanogenic diglycosides also resulted in a formation of fragments corresponding to the cyanide derivatives of glycans at *m/z* 374 $[Glc + Glc - H_2O + HCN + Na]^+$ for **5**, and at *m/z* 344 $[Glc + Api - H_2O + HCN + Na]^+$ for **7** and **11**. This rearrangement might be ascribed to the fact that CN^- is a known nucleophile, and thus during the loss of cyanide from cyanogens it could well attack a nearby glycan.

Metabolic profiling in bitter almond (*Prunus dulcis* (Mill.) D.A.Webb)

As it has previously been hypothesized that the cyanogenic glucosides in the seeds of *Prunus* species are metabolized during germination [8], extracts of different tissues of germinated bitter almonds were investigated by LC-MS/MS. Almond seeds 8 weeks after imbibition, denoted as stage A (with open cotyledons but as yet no roots), and germinated almonds 11-12 weeks after imbibition, denoted as stage B (with roots 3-10 cm in length and leafy shoots 4.5-10 cm in length), were investigated to show

the presence and amounts of cyanogenic glucosides prunasin and amygdalin and of their derivatives. The absolute quantities of prunasin **13** and amygdalin **19** displayed opposing trends (Table 1B): from 8 to 12 weeks of development, the level of amygdalin in the cotyledons decreased 6 times, and it was hardly detectable in other parts (root, stem and leaf), whilst the prunasin level increased 8 times in the cotyledons, 13 times in the stem and 29 times in the leaves (Table 1B).

As summarized in Table 1B, prunasin amides **14a** and **14b**, acid **15** and anitrile **16** were detected in low amounts in all parts and at both stages explored (with the exception of **14b**, which was not present in leaves); **15** was the most abundant derivative. Similarly, amygdalin amide **20**, acids **21a** and **21b** and anitrile **22** were found in all parts (with the exception of **21a**, which was present in the imbibed seeds and seedling cotyledons only); **22** was the most abundant derivative of amygdalin. Two isomers (with the same sum formula deduced from HR-MS but different retention times) were registered as the '**a**' and '**b**' isomers of both compounds **14** and **21**, due to the presence of *R* and *S* enantiomers of the aglycons (based on comparison with the chemically synthesised standards). Similarly to cassava diglycosides, prunasin "pentoside" **17**, prunasin anitrile arabinoside **18a**, and prunasin anitrile xyloside **18a**, were found in low amounts in most of the plant parts investigated. Interestingly, the triglucoside amygdalin glucoside **23** appeared in three isomeric forms (**a**, **b**, **c**), with **23a** being found in all parts, and **23b** and **23c** in seeds or seedling cotyledons only. Amygdalin anitrile glucoside **24** was found in two isomers (**a** and **b**), with **24a** present in all parts except for leaves and **24b** in seeds and seedling cotyledons only. According to the ion mobility analyses presented below, the existence of three isomers of **23** is attributed to the presence of different linkage positions between the Glc moieties. The same may be the case for **24**; note that the presence of the two isomers of **24** cannot be ascribed to *S* and *R* isomers as the benzyl aglycone is achiral.

The identification of compounds **13**, **14, 15, 16, 18b, 19**, **20, 21, 22** is based on comparison with their chemically synthesised authentic standards, and on ^{1}H and ^{13}C NMR in the case of **18a** and **18b**. However, only partially identified "compound **17**" is apparently a mixture of almost co-eluting vicianin (prunasin-6′-arabinoside) and lucumin (prunasin-6′-xyloside), based on the LC-MS/MS comparison with leaf extracts from *Phlebodium aureum* and *Clerodendrum grayi*, which contain high amounts of vicianin and lucumin, respectively [26, 27] (Supplementary Figure S3). Fragments at *m/z* corresponding to mono- and disaccharides Glc, Glc + Glc, and Glc + pentose (as described above) were observed for the respective compounds listed in Table 1B. Additionally, a Glc + Glc + Glc trisaccharide fragment ion at *m/z* 509 $[C_{18}H_{32}O_{16} - H_2O + Na]^+$ was generated from dissociation of **23** and **24**. Peaks at *m/z* 174 corresponding to mandelamide $[C_8H_9NO_2 + Na]^+$, the aglycone of the structures **14** and **20**, and at *m/z* 175 corresponding to mandelic acid $[C_8H_8O_3 + Na]^+$, the aglycone of **15,** were also observed.

Various other informative fragmentations were detected. The carboxylic acids **15** and **21a, b** showed characteristic $[M + 2Na - H]^+$ disodium adducts (at *m/z* 359 and 521, respectively) as did the carboxylic acid from cassava. Dissociation of **18a** and **18b** both gave a peak at *m/z* 293 of **16,** consistent with their being glycosides of **16**. Similarly, fragmentation of amygdalin derivatives **20**, **21**, and **22** resulted in fragments at *m/z* 336, 337, and 293, respectively, corresponding to $[M+Na]^+$ of the corresponding monoglucosides **14**, **15**, **16**; fragmentation of **23a**, **23b**, and **23c** each gave a peak at *m/z* 480, corresponding to $[M+Na]^+$ of **19**; and finally, fragmentation of **24** provided peaks at *m/z* 293 and 455, corresponding to $[M+Na]^+$ of **16** and **22**, respectively. Further, the loss of *m/z* 27 (HCN), typical for cyanogenic compounds, was seen in the fragmentation patterns of **13**, **17**, **19** and **23**, as well as the addition of *m/z* 27 to the disaccharide and trisaccharide fragments in **17**, **19** and **23**. Representative tandem mass spectra of compounds **13**, **19** and **23** are shown in Figure 3.

Metabolic profiling in sorghum (*Sorghum bicolor* (L.) Moench)

The monocotyledonous cyanogenic plant sorghum is known to metabolize its cyanogenic glucoside during development from seedling to adult plant [28, 29], and it was therefore analysed in the same manner as cassava and almond. Leaf sheaths and leaf blades of sorghum plants were subjected to LC-MS/MS analysis, 13 days (2-leaf-stage) and 30 days (6-leaf-stage) after sowing. As shown in Table 1C, the content of the cyanogenic glucoside dhurrin **25** decreased more than 3 times in leaf blades, and twice in leaf sheaths, during the third and fourth weeks of sorghum development. Dhurrin derivatives

appeared as compounds of very low abundance, with *p*-glucosyloxyphenylacetic acid **28** reaching the highest levels of all dhurrin derivatives.

Fragments at *m/z* corresponding to Glc and Glc + Glc were observed, as described above. The identification of dhurrin amide **26** and dhurrin acid **27** was based on their fragments at *m/z* 190 and 173, corresponding to 4-hydroxymandelamide $[C_8H_9NO_3 + Na]^+$ and 4-hydroxymandelic acid $[C_8H_8O_4 - H_2O + Na]^+$, respectively. Compound **28** was found to be *p*-glucosyloxyphenylacetic acid, with a fragment *at m/z* 175, corresponding to 4-hydroxyphenylacetic acid $[C_8H_8O_3 + Na]^+$. Both dhurrin acid and *p*-glucosyloxyphenylacetic acid were also identified as $[M + 2Na - H]^+$ adducts (at *m/z* 375 and 359, respectively). In addition, the identities of **26** and **28** were confirmed by comparison with their chemically synthesised standards. Glucosides **29a, b** and **c** of dhurrin were also observed, analogously to linustatin and amygdalin within cassava and almond, respectively (above). These isomeric diglucosides eluted as three clustered peaks with *m/z* = 496, as confirmed by HR-MS (Table 1C). Fragmentation of all three provided fragments at *m/z* 469 (corresponding to the loss of *m/z* = 27, HCN). The presence of the three peaks could be explained by different linkage positions between the two hexose moieties. Two additional isomers with a similar fragmentation pattern but eluting considerably later, were identified as caffeic acid ester regioisomers of dhurrin **30** (**a** and **b** isomer) according to their accurate mass. Currently the position at which the caffeoyl group is attached to Glc remains unspecified. Caffeic acid ester isomers of dhurrin acid **31a** and **b** were also detected. Compounds **30** and **31** gave a fragment at *m/z* 347 corresponding to the sodium adduct of caffeoyl anhydroglucose, proving the position of the acyl group to be on the glucoside rather than on the aromatic ring. Finally, as also seen in the other two plants, all carboxylic acids (**27**, **28** and **31**) showed the expected characteristic disodium adducts $[M + 2Na - H]^+$ (*m/z* 375, 359 and 537, respectively).

Remarkably, anitriles were not detected in sorghum, despite our careful search for such structures.

Ion-mobility MS characterization of cyanogenic glycosides and their derivatives

LC-MS/MS data alone does not suffice to ascertain the stereochemical nature of the glycans in the glycosides identified. Previous reports on the presence of diglycosides suggested that the glycosides identified are probably glucose-based and predominantly exist as the β-(1→6) linkage [19, 21, 30-32]. In order to confirm this, we analyzed selected glycosides by ion-mobility MS (IM-MS), a rapid and sensitive technique that separates gas phase ions based on their mobility (inversely proportional to a molecule's collision cross-section area) as they traverse through an inert buffer gas [33, for review see 34]. IM-MS has been previously reported as being able to discern the identity of hexose residues based on the arrival time distribution (ATD) of monosaccharide product ions, generated by collision-induced dissociation (CID) of either glycopeptides or free reducing-saccharides [17]. Recently it has also been shown that IM-MS has the potential to discern the stereo-/regio-chemistry of the glycosidic bond of glucose oligosaccharides, based on the ATDs of these monosaccharide product ions compared to synthetic standards (C.J. Gray, B. Schindler, M. Pičmanová, L. Migas, A.R. Allouche, A.P. Green, S. Mandal, S. Motawia, N. Bjarnholt, R. Sánchez-Pérez, B.L. Møller, I. Compagnon, P. Barran, S.L. Flitsch and C.E. Eyers, unpublished work). This technique is therefore eminently suited for confirming the glycan structures identified from the LC-MS/MS data. Plant glycoside samples were pre-fractionated and lithiated prior to analysis, as lithiated saccharides, consisting primarily of hexose residues, have a greater fragment ion yield compared to their sodiated equivalents [35]. Glycosides **1**, **5**, **8** and **12** from cassava, and glycosides **13**, **16**, **19, 20**, **22** and **23** from almond samples were identified within the fractions. The mass spectra and ATD of all hexose monosaccharide product ions recorded suggests that the cyanogenic glycosides consist of β-linked glucose units (Supplementary Figure S4 and S5).

The tandem mass spectra of dihexosides **5**, **19**, **20**, **22** suggest that these glycosides are likely predominantly to correspond to either the 1→4 or 1→6 linkage, based on the presence of the $^{0,2}A_2$ cross-ring fragment. To establish which linkage predominates, the ATD of the cross-ring fragment at *m/z* 289 was measured. The ATD matches with that of the β-(1→6) standard (gentiobiose) and not with that of the β-(1→4) standard (cellobiose), thus confirming that these saccharides consist of the Glc-β-(1→6)-Glc linkage type (confirmed by the synthesised standards in the case of **19**, **20**, and **22**).

However, certain extracted ion chromatograms described above suggested the presence of multiple isomeric structures (e.g. glycoside **23**). IM-MS analysis of fractions containing **23a**, **23b** and **23c** revealed the presence of Glc-β-(1→6)-Glc, Glc-β-(1→4)-Glc and Glc-β-(1→2)-Glc linkages, respectively, based on the ATD of selected cross-ring fragments (C.J. Gray, B. Schindler,M. Pičmanová, L. Migas, A.R. Allouche, A.P. Green, S. Mandal, S. Motawia, N. Bjarnholt, R. Sánchez-Pérez, B.L. Møller, I. Compagnon, P. Barran, S.L. Flitsch and C.E. Eyers, unpublished work).

Pentose monosaccharide product ions were also observed in some of the samples studied. Our current understanding of the behaviour of pentose product ions is poor, thus precluding the unequivocal identification of the pentose residues. The ATDs of a pentose moiety of **12,** however, appeared to be identical and displayed the same ATD as linamarin anitrile apioside **8**, a structure we have characterised by NMR (Figure 4).

DISCUSSION

Our investigation of three phylogenetically unrelated plant species – cassava, almond, and sorghum – demonstrates that cyanogenic glucosides co-occur with a series of structural derivatives: amides, acids and anitriles. Moreover, we provide confirmation that cyanogenic glucosides and their corresponding derivatives are further glycosylated to form di- and triglycosides, whilst in sorghum they may also be acylated. Based on LC-MS/MS fragmentation patterns, accurate mass determination as well as (in particular cases) comparisons with authentic standards and NMR, in total 36 structural derivatives of the cyanogenic glucosides linamarin, lotaustralin, prunasin, amygdalin and dhurrin were identified in this study. Five of the derivatives described had been previously found in the same plant species: linamarin-6′-apioside, lotaustralin-6′-apioside, linamarin anitrile-6′-apioside and the (*S*) stereoisomer of lotaustralin anitrile-6′-apioside (the substitution of the CN- in (*R*)-lotaustralin with hydrogen results in a formal change of the absolute configuration from *R* to *S*, according to CIP conventions) in cassava [18, 19, 24], and dhurrin-6′-glucoside in sorghum [32]. Linustatin, the diglucoside of linamarin identified in flaxseed (*Linum usitatissimum*) [21] and in the rubber tree (*Hevea brasiliensis*) [36, 37], had not hitherto been unequivocally identified in cassava – although it had been suggested as a transport form of linamarin for its translocation from the site of its biosynthesis in the shoot apex and young leaves downwards to the sink in tuberous roots [16, 23, 38]. Here we report on the presence of linustatin in the lower stem of tuberous cassava plants, which would appear to be consistent with a transport function.

In addition to the above findings, other cyanogenic plant species have been reported to contain non-cyanogenic compounds that, upon close examination, turn out strikingly to resemble the structures of the cyanogenic glucosides. Glycosylated amides and acids – putatively derived from cyanogenic glycosides including prunasin, sambunigrin, amygdalin, and lucumin – have been isolated from different species of the families Rosaceae, Oliniaceae, Dennstaedtiaceae, Fabaceae, Adoxaceae and Sapotaceae (Supplementary Table S1 and references therein). The glycosylated anitrile products (namely the glycosides of 2-propanol, 2-butanol, benzyl alcohol and 4-hydroxybenzyl alcohol) have been found in various plant species [39], including cassava [18, 19], *Prunus persica* [40], *Sambucus nigra* [41] and *Passiflora morifolia* [42]. In all the above examples, glycosylated alcohols that can be described as anitriles of cyanogenic glycosides co-occur with their cyanogenic counterparts.

In previous reports, the co-occurrence of amides and acids with their cyanogenic counterparts has been considered to be a consequence of chemical hydration of the nitrile group to the amide during sample processing (mostly during the drying of plant material), and the structures have been reported as artifacts [43-45], although the authors have acknowledged the necessity for enzymatic catalysis of such a reaction [44]. However, Nahrstedt and Rockenbach, who identified prunasin together with prunasin amide in the air-dried leaves of *Olinia ventosa*, argued that the detected amide was not an artificial product, in view of its high amount and the conservation of its stereochemistry [13]. Similarly, in the present study, artificial hydrolysis of nitriles to amides and acids during processing can be ruled out, as it requires strong acidic or basic conditions that could not have been provided during extraction in 85% methanol. Moreover, all enzymatic activities are inhibited by snap freezing of the plant material in liquid nitrogen and by its subsequent boiling in methanol.

The higher amounts of linamarin and lotaustralin in the upper stem of young and old cassava plants and in the tuber cortex of old plants correspond to the sites of their biosynthesis [16, 46], and no significant change in their distribution in the stem can be seen during development. In contrast, the levels of amygdalin and prunasin are altered dramatically during almond early ontogeny. Amygdalin was found to be stored in high amounts in non-germinated seeds and its level dropped down significantly upon initiation of germination, whilst the prunasin level rapidly rose. This is primarily due to the hydrolysis of amygdalin into prunasin in the beginning of germination. A similar transformation has previously been described in black cherry [8]. During almond ripening, the rapid decline of the level of prunasin in fruit tegument and a concomitant increase in amygdalin level in the cotyledons, suggest that prunasin is a direct precursor to amygdalin biosynthesis [10]. Conversely, the almond seedling may utilize amygdalin stored in cotyledons for prunasin formation. The higher levels of prunasin in the stem and leaves of the almond seedling may be attributed to transport from the cotyledons after amygdalin hydrolysis, as well as to *de novo* biosynthesis from L-phenylalanine.

With few exceptions, the derivatives of cyanogenic glucosides are present in very low amounts, making it very difficult to isolate them and confirm their structures by NMR. Their levels often represent thousandths of the content of their respective parent compounds, and no specific trends with respect to their formation during plant ontogeny have been identified. The exceptions are linamarin anitrile apioside and lotaustralin anitrile apioside, which occur in amounts respectively corresponding to those of linamarin and lotaustralin, in the lower stem and tuber cortex of tuberous cassava plants. Similarly, amygdalin anitrile is present in amounts similar to those of amygdalin during the early stages of germination. The qualitative and in part also quantitative variation observed in different tissues at different developmental stages in the three plant species suggests that the metabolism of cyanogenic glucosides is very dynamic, and that cyanogenic glucosides are decidedly not merely present as stagnant defence compounds.

As summarized in Table 2, putative turnover products of cyanogenic glucosides – amides and acids – have been detected in all three species investigated. It is noteworthy that these compounds only occur as monoglucosides. In cassava and almond, both cyanogenic glucosides and their corresponding anitriles co-occur as di- and triglycosides, which strongly suggests a metabolic relationship – as also speculated by Jaroszewski and co-workers in connection with the co-occurrence of linamarin and isopropyl primaveroside (linamarin anitrile-6′-xyloside) in *Passiflora morifolia* [42]. Sorghum, as a monocotyledonous plant phylogenetically distant from cassava and almond, represents a slightly different system. The *p*-hydroxy group attached to the mandelonitrile unit in dhurrin provides yet another site of possible glucosylation, resulting in the apparent formation of two different acids: 4-hydroxymandelic acid glucoside (**27**) and 4-glucosyloxyphenylacetic acid (**28**), of which the latter is the more abundant. In contrast to the detected anitriles in almond and cassava, no dhurrin anitrile was found in sorghum. It also appears that dhurrin and its acid are present in sorghum plants as caffeoyl esters, whereas these were not found in cassava and almond. Interestingly, caffeoyl-dhurrin has the same *m/z* as dhurrin-6′-glucoside – previously reported to be present in sorghum [32] – but its accurate mass and molecular formula correspond to the cyanogenic compound nandinin, previously identified in the plant *Nandina domestica* [47]. The structure of nandinin was confirmed as *p*-glucosyloxymandelonitrile esterified with caffeic acid at the C-6 position of the Glc moiety. Notably, nandinin from leaf extract of *N. domestica* does not co-elute with the compound assigned here as caffeoyl-dhurrin, and, moreover, the fragmentation patterns of these two compounds differ significantly (Supplementary Figure S6).

Although the first indications of the existence of endogenous metabolism of cyanogenic glycosides appeared in the literature more than twenty years ago [13, 48], the evidence has been lacking. For sorghum, it has been suggested that this metabolism could take place through the conversion of dhurrin to a nitrile *via* an unknown pathway. This nitrile can be metabolized to the corresponding acid (the aglucone of **28**) by specific nitrilases (EC 3.5.5.1) in a process involving the release of ammonia [7]. This proposed turnover pathway for the recovery of reduced nitrogen from cyanogenic glucoside avoids the release of toxic HCN.

Here we suggest – on the basis of the comparative metabolic profiling presented for three different plant species, and supported by data from the literature – an alternative recycling pathway for the turnover of cyanogenic glycosides in cassava, almond and sorghum characterized by several common features constituting a “unifying principle” in the metabolism of cyanogenic glycosides

across unrelated plant species. This proposed recycling pathway likewise avoids the release of HCN, and recovers the reduced nitrogen and carbon from the nitrile group as ammonia and carbon dioxide. The reduced nitrogen and carbon released would then be available for use in plant primary metabolism – for instance during specific phases of plant ontogeny. Glutamine synthetase catalyses the assimilation of ammonia into glutamine with glutamate as co-substrate. Alternatively, ammonia may be incorporated into glutamate in a reaction catalysed by glutamate dehydrogenase with 2-oxoglutarate as co-substrate [49]. Both amino acids may be incorporated into proteins or be used as reduced nitrogen donors in transamination reactions. Glutamate is also a key precursor in the biosynthesis of tetrapyrroles (chlorophyll, heme etc.) [50]. Similarly, the carbon dioxide released in the proposed recycling pathway for cyanogenic glucosides may be incorporated into organic compounds in the dark reactions of photosynthesis. In our current study, cyanogenic glycosides and some of their derivatives were discovered to be present in relatively high amounts in a spatially as well as temporally defined manner (cf. levels of compounds **1**, **8**, **13**, **15**, **19**, **22**). In the tips of etiolated sorghum seedlings, dhurrin constitutes 30% of the dry matter [51] and in the epidermal cell layer of barley leaves, glucose bound in hydroxynitrileglucosides constitute 90% of the total soluble carbohydrate content [52]. In newly formed shoots of *Eucalyptus cladocalyx*, as much as 25% of leaf nitrogen can be tied up in prunasin [53]. In specific plant tissues, the nitrogen and sugar bound within cyanogenic glycosides may therefore represent significant percentages of the total organic matter present [12]. Recycling of cyanogenic glycosides may thus serve to balance resource demands in primary metabolism. As an example, the prunasin content in leaves of almond seedlings is 23 mg/g (fresh tissue). Theoretically, this may provide the growing plant with 1.4 mg NH_3/g of fresh plant material, which may afford 12 mg glutamine or glutamate/g (fresh tissue), i.e. approximately 12 times as many nitrogen equivalents than present in the total free amino acid pool in dried almond seeds [54].

The proposed recycling pathway for cyanogenic glycosides is shown in Figure 5. In contrast to the route proposed by Jenrich *et al.* [7] for sorghum, which entails the formation of a non-glucosylated nitrile, the pathway proposed here proceeds *via* the transformation of glycosides. This turnover pathway is envisioned to operate in parallel with the classical cyanogenesis pathway releasing HCN from cyanogenic glucosides by the action of a β-glycosidase. In this binary system, compartmentalization is essential to prevent autotoxicity and to ensure that the "bomb" is detonated as a targeted response, e.g. to herbivore attack [55]. HCN released by this route may be further reassimilated via β-cyanoalanine into aspartic acid, asparagine and ammonia [3, 4] – all of which can be utilized in primary metabolism [8, 10, 14].

The conversion of nitriles into amides in plants can be explained either by: (1) the *in vivo* occurrence of a Radziszweski process, driven by the presence of hydrogen peroxide evolved upon oxidative stress such as air-drying, light exposure, senescence, or as a result of the oxidative burst caused by a fungal infection [1, 56]; or by (2) the endogenous turnover of cyanogenic glucosides catalysed by specific turnover enzymes – probably bifunctional nitrilase (EC 3.5.5.1) / nitrile hydratase (EC 4.2.1.84) enzymes that can form amides as major products from nitriles [5, 57]. The formation of amides might be followed by the enzymatic hydrolysis of the amide function into a carboxylic acid function, and thereby release ammonia. Such reactions are catalysed by amidase (EC 3.5.1.4). However, amidase activity has been reported to be negligible in another amide-forming plant [57], so alternative fates should be borne in mind. In principle, cyanogenic glucosides may also be converted directly to their corresponding acids – i.e. without the previous formation of amides, under catalysis by specific nitrilases (EC 3.5.5.1). However, we are not aware of any instance where any of the aforementioned enzymes exert their activity on glycosides. An analogous example of the possible formation of glycosylated amide and acid from their structurally-related glucosinolate sinigrin in *Alliaria petiolata* has been recently published [58]. Further on in the pathway, enzymatic decarboxylation of the acid might follow, accompanied by the release of carbon dioxide. Finally, the possible involvement of alternative enzyme systems such as a multifunctional enzyme – "decyanase" – which removes the nitrile group from cyanogenic glucosides to form anitriles directly cannot be excluded [59].

The co-occurrence of mono-, di- and triglucosides, as well as of apiosides, arabinosides and xylosides of cyanogenic glucosides, indicates the presence of: (1) specific glycosyltransferases, and (2) specific β-glycosidases which sequentially or simultaneously hydrolyze the glycosidic bonds [37]. Glucosyl transferases producing linamarin and lotaustralin (UGT85K4, UGT85K5) [60], prunasin

(UGT85A19) [61] and dhurrin (UGT85B1) [62] have been described in cassava, almond and sorghum, respectively. Nothing is known about the glucosyltransferases and apiosyl-, xylosyl- and arabinosyltransferases which produce diglycosides in these plants. Similarly, the enzymes catalysing the hydrolysis of cyanogenic di- and triglycosides have not been described, with the exception of amygdalin hydrolase from *Prunus serotina* [30, 63], vicianin hydrolase from *Vicia angustifolia* [64, 65] and *Davallia trichomanoides* [30, 66], and linustatinase from *Hevea brasiliensis* and *Linum usitatissimum* [14, 67].

The formation of cyanogenic diglycosides may also be considered to exemplify a general principle in the metabolism of cyanogenic glucosides. Several types of glycosylation of cyanogenic glucosides have been described in the literature – in particular glucosylation [14, 32], apiosylation [18, 19], arabinosylation [64, 68], xylosylation [27, 31] and allosylation [69]. It has been proposed that the cyanogenic diglucosides linustatin, neolinustatin, amygdalin and dhurrin-6´-glucoside may function as a protected transport form of their corresponding monoglucosides linamarin, lotaustralin, prunasin and dhurrin, respectively [14, 32, 36], for the long-distance translocation from the site of their biosynthesis to the sink. This hypothesis is supported by the fact that cyanogenic diglucosides cannot be hydrolyzed by the β-glucosidases that cleave off the Glc moiety in the corresponding cyanogenic monoglucosides; it has been demonstrated that prunasin hydrolase does not act on amygdalin [70], and that linamarase does not act on either linustatin or neolinustatin [36]. Since cyanogenic β-glucosidases are predominantly localised in the apoplast [71, 72], cyanogenic diglucosides passing through the apoplastic space remain resistant to the hydrolysis [14]. However, if glycosidases capable of cleaving off oligosaccharide units from the glycosides do exist, then such units may serve to signal a further defence response [12].

The role of the introduction of pentose moieties into the chemical structures of cyanogenic glucosides and their derivatives remains a mystery. Linamarin anitrile apioside is known to be more bitter than linamarin itself, and as such contributes to the overall deterrent bitter effect of cassava tubers [18]. The co-occurrence of linamarin apioside and lotaustralin apioside with their corresponding apiosylated anitriles might indicate that apiose serves as a label for cyanogenic glucosides to initiate their turnover into anitriles, with a concomitant production of ammonia and carbon dioxide – exploitable in certain developmental stages, such as for tuber formation. Pentoses present in plant glycosides may also be involved in signalling pathogen attack, or may act as anti-pathogen agents. Likewise, the role of the acylation of dhurrin and its derivatives remains unexplained, but transport and signalling are again plausible purposes served by this type of decoration.

To test the suggested metabolic relationship between cyanogenic glycosides and their putative turnover products, extensive genomic and enzymological studies need to be carried out. Our data, supplemented by scattered reports in the literature, strongly suggests that the co-occurrence of cyanogenic glycosides with compounds glaringly resembling their structure is no mere coincidence. With the thorough investigation of the occurrence and the identity of turnover products formed from cyanogenic glycosides presented in our current study, the scene is now set for embarking upon the identification of the enzyme systems catalyzing this pathway.

Understanding the enzymes and mechanisms involved in the recycling pathway for cyanogenic glycosides and the importance of this pathway in plant physiological processes and plant plasticity will open up the option to regulate the content of cyanogenic glycosides in crops on the basis of a new rationale. Studies indicate that the presence of cyanogenic glycosides plays an important role at some stages of development [8, 14, 16]. Therefore, in some plants the complete knockout of the biosynthesis of cyanogenic glycosides may have undesirable side effects, whereas modulation of their recycling pathway may provide a more feasible way of reducing the content of potentially toxic cyanogenic glycosides in food crops.

AUTHOR CONTRIBUTION

Martina Pičmanová, Birger Lindberg Møller, Nanna Bjarnholt, Raquel Sánchez-Pérez, Elizabeth H. Neilson and Kirsten Jørgensen designed and directed the project. Nanna Bjarnholt, Raquel Sánchez-Pérez, Elizabeth H. Neilson and Martina Pičmanová grew and collected plant material. Martina Pičmanová performed the metabolic profiling and wrote the first draft of the manuscript, which was

then commented upon by all co-authors. Carl Erik Olsen acquired all LC-MS data and NMR data of compound **8**. Sebastian Meier acquired NMR data of compounds **18a** and **18b**. Mohammed S. Motawia chemically synthesized reference compounds. Niels Agerbirk, Martina Pičmanová and Daniele Silvestro fractionated plant extracts. Sabine Flitsch and Christopher J. Gray designed and performed IM-MS/MS analysis.

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TABLES

Table 1 Identification and quantification of cyanogenic glucosides and their derivatives putatively formed as part of a recycling pathway in cassava (A), almond (B) and sorghum (C). Compounds marked with an asterisk were compared to their authentic standards. The parent cyanogenic glucosides **1**, **9**, **13**, **19**, and **25** were quantified absolutely using standard curves based on pure reference compounds; their structural derivatives were quantified relatively, and their levels are expressed as a percentage of the parent cyanogenic glucoside content (in %). The average values ± SD are shown; n.d. = not detected. (A) n = 4; Upper stem = 1 cm stem segment under the first unfolded leaf; Lower stem = 1 cm stem segment approx. 5 cm above soil. (B) n = 5; Stage A = 8 weeks after imbibition; Stage B = 11-12 weeks after imbibition. (C) n = 6. IUPAC nomenclature for the compounds listed: **1**, 2-β-D-Glucopyranosyloxy-2-methylpropanenitrile; **2**, 2-β-D-Glucopyranosyloxy-2-methylpropanamide; **3**, 2-β-D-Glucopyranosyloxy-2-methylpropanoic acid; **4**, Isopropyl β-D-glucopyranoside; **5**, 2-[β-D-Glucopyranosyl-(1→6)-β-D-glucopyranoyloxy]-2-methylpropanenitrile; **6**, Isopropyl β-D-glucopyranosyl-β-D-glucopyranoside; **7**#, 2-(β-D-apiofuranosyl-β-D-glucopyranosyloxy)-2-methylpropanenitrile; **8**, Isopropyl β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside; **9**, 2-β-D-glucopyranosyloxy-2-methylbutanenitrile; **10**#, 2-β-D-Glucopyranosyloxy-2-methylbutanoic acid; **11**#, 2-(β-D-Apiofuranosyl-β-D-glucopyranoside)-2-methylbutanoic acid; **12**#, 2-Butyl β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside; **13**, 2(*R*)-(β-D-Glucopyranosyloxy)phenylacetonitrile; **14**, 2-(β-D-Glucopyranosyloxy)phenylacetamide; **15**, 2(*R*)-(β-D-Glucopyranosyloxy)phenylacetic acid; **16**, Benzyl β-D-glucopyranoside; **17**#, 2-[β-D-Pentosyl-(1→6)-β-D-glucopyranosyloxy)]phenylacetonitrile; **18a**, Benzyl α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside; **18b**, Benzyl β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside; **19**, 2(*R*)-[β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyloxy]phenylacetonitrile; **20**, 2-[β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyloxy]phenylacetamide; **21**, 2-[β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyloxy]phenylacetic acid; **22**, Benzyl β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside; **23a**, 2(*R*)-[β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyloxy]phenylacetonitrile; **23b**, 2(*R*)-[β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyloxy]phenylacetonitrile; **23c**, 2(*R*)-[β-D-Glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyloxy]phenylacetonitrile; **24**#, Benzyl β-D-glucopyranosyl-β-D-glucopyranosyl-β-D-glucopyranoside; **25**, 2(*S*)-β-D-Glucopyranosyloxy-(4-hydroxyphenyl)acetonitrile; **26**, 2(*S*)-β-D-Glucopyranosyloxy-(4-hydroxyphenyl)acetamide; **27**#, 2-β-D-Glucopyranosyloxy-(4-hydroxyphenyl)acetic acid; **28**, 4-Glucosyloxyphenylacetic acid; **29**#, 2-(β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy-(4-hydroxyphenyl)acetonitrile; **30**#, 2-[(3,4-Dihydroxybenzoyl)-β-D-glucopyranosyl)]oxy-(4-hydroxyphenyl)acetonitrile; **31**#, 2-[(3,4-Dihydroxybenzoyl)-β-D-glucopyranosyl)]oxy-(4-hydroxyphenyl)acetic acid. # stands for an unknown absolute configuration and/or unknown linkage position between sugar moieties or between acyl and sugar.

A. CASSAVA	Molecular formula	*m/z* $[M+Na]^+$	Measured mass	Calculated mass	r.t. [min]	3-month-old plants		9-month-old plants			
						Upper stem	Lower stem	Upper stem	Lower stem	Tuber cortex	Tuber pulp
1 Linamarin* (in mg/g of fresh tissue)	**$C_{10}H_{17}NO_6$**	**270**	**247.1055**	**247.1056**	**2.3**	**2.9±0.75**	**1.2±0.27**	**2.4±0.65**	**1.3±0.33**	**5.3±4.25**	**1.1±0.10**
Linamarin derivatives (in % of Linamarin)											
2 Linamarin amide	$C_{10}H_{19}NO_7$	288	265.1158	265.1162	1.3	0.3±0.02	2.0±0.23	0.4±0.12	3.3±0.60	3.3±0.99	7.7±0.87
3 Linamarin acid	$C_{10}H_{18}O_8$	289	266.1007	266.1002	1.9	n.d.	0.8±0.15	0.03±0.01	0.4±0.06	0.4±0.18	0.7±0.21
4 Linamarin anitrile*	$C_9H_{18}O_6$	245	222.1107	222.1103	2.4	n.d.	n.d.	0.5±0.33	0.8±0.51	0.7±0.51	0.9±0.73
5 Linamarin-6´-glucoside (Linustatin)	$C_{16}H_{27}NO_{11}$	432	409.1590	409.1584	3.0	n.d.	n.d.	n.d.	1.0±0.81	n.d.	n.d.
6 Linamarin anitrile glucoside	$C_{15}H_{28}O_{11}$	407	384.1644	384.1632	3.3	n.d.	n.d.	n.d.	0.7±0.38	0.3±0.05	0.06±0.03
7a Linamarin apioside	$C_{15}H_{23}NO_{10}$	402	379.1491	379.1478	4.0	0.1±0.02	0.9±0.21	0.1±0.02	0.6±0.72	1.7±1.64	0.2±0.14
7b Linamarin apioside	$C_{15}H_{23}NO_{10}$	402	379.1492	379.1478	5.0	0.2±0.02	0.6±0.08	0.2±0.07	0.4±0.05	1.7±0.23	0.6±0.24
8 Linamarin anitrile-6´-apioside	$C_{14}H_{26}O_{10}$	377	354.1532	354.1526	4.6	3.7±0.42	37.5±8.53	14.0±2.65	116.6±26.74	134.6±38.16	18.6±6.09
9 Lotaustralin* (in mg/g of fresh tissue)	**$C_{11}H_{19}NO_6$**	**284**	**261.1217**	**261.1212**	**5.5**	**1.2±0.45**	**0.05±0.02**	**0.4±0.18**	**0.1±0.05**	**0.3±0.11**	**0.1±0.02**
Lotaustralin derivatives (in % of Lotaustralin)											
10 Lotaustralin acid	$C_{11}H_{20}O_8$	303	280.1163	280.1158	5.0	0.7±0.09	n.d.	n.d.	0.8±0.40	2.3 ± 0.82	5.6±0.89
11 Lotaustralin apioside	$C_{16}H_{27}NO_{10}$	416	393.1644	393.1635	5.6	n.d.	3.9±2.19	n.d.	3.3±1.91	7.1 ± 2.41	n.d.
12 Lotaustralin anitrile-6´-apioside	$C_{15}H_{28}O_{10}$	391	368.1689	368.1682	5.8	2.4±0.09	36.6±20.03	10.1±2.72	72.8±39.83	105.7 ± 63.41	9.0±3.95

B. ALMOND	Molecular formula	*m/z* $[M+Na]^+$	Measured mass	Calculated mass	r.t. [min]	Stage A	Stage B			
						Imbibed seed	Cotyledons	Root	Stem	Leaf
13 Prunasin* (in mg/g of fresh tissue)	**$C_{14}H_{17}NO_6$**	**318**	**295.1066**	**295.1056**	**7.0**	**0.8±0.33**	**6.5±0.51**	**5.8±1.33**	**10.5±3.49**	**22.8±4.76**
Prunasin derivatives (in % of Prunasin)										
14a Prunasin amide*	$C_{14}H_{19}NO_7$	336	313.1158	313.1162	4.4	1.2±0.44	0.6±0.29	0.5±0.39	0.1±0.07	1.3±1.12
14b Prunasin amide*	$C_{14}H_{19}NO_7$	336	313.1160	313.1162	5.0	0.2±0.09	0.3±0.37	0.01±0.01	0.01±0.003	n.d.
15 Prunasin acid*	$C_{14}H_{18}O_8$	337	314.1012	314.1002	5.7	1.5±0.41	18.4±8.90	0.5±0.26	0.4±0.48	4.7±3.48
16 Prunasin anitrile*	$C_{13}H_{18}O_6$	293	270.1116	270.1103	6.5	0.1±0.11	0.9±0.57	3.2±0.77	0.2±0.04	0.8±0.29
17 Prunasin pentoside	$C_{19}H_{25}NO_{10}$	450	427.1477	427.1478	6.9	n.d.	0.1±0.14	0.02±0.01	0.03±0.02	0.03±0.02
18a Prunasin anitrile-6´-arabinoside	$C_{18}H_{26}O_{10}$	425	402.1537	402.1526	6.6	n.d.	0.1±0.04	0.4±0.15	0.2±0.13	0.4±0.30
18b Prunasin anitrile-6´-xyloside*	$C_{18}H_{26}O_{10}$	425	402.1528	402.1526	6.8	0.8±0.74	0.2±0.10	1.5±1.11	0.2±0.15	0.5±0.35
19 Amygdalin* (in mg/g of fresh tissue)	**$C_{20}H_{27}NO_{11}$**	**480**	**457.1601**	**457.1584**	**6.6**	**24.6±5.07**	**4.3±2.11**	**0.004±0.006**	**0.01±0.01**	**0.002±0.002**
Amygdalin derivatives (in % of Amygdalin)										
20 Amygdalin amide*	$C_{20}H_{29}NO_{12}$	498	475.1688	475.1690	5.2	5.7±1.69	2.4±2.29	16.3±4.83	3.3±2.17	13.1±12.23
21a Amygdalin acid*	$C_{20}H_{28}O_{13}$	499	476.1533	476.1530	5.4	0.4±0.12	1.0±0.58	n.d.	n.d.	n.d.
21b Amygdalin acid*	$C_{20}H_{28}O_{13}$	499	476.1529	476.1530	5.8	4.4±1.34	14.7±6.90	31.1±14.10	15.7±7.41	92.1±63.57
22 Amygdalin anitrile*	$C_{19}H_{28}O_{11}$	455	432.1649	432.1632	6.2	19.7±6.03	42.2±11.49	69.8±9.52	56.0±24.41	223.9±343.80
23a Amygdalin-6´-glucoside	$C_{26}H_{37}NO_{16}$	642	619.2112	619.2112	6.2	0.3±0.11	2.9±0.51	5.7±3.67	4.8±2.50	7.0±1.36
23b Amygdalin-4´-glucoside	$C_{26}H_{37}NO_{16}$	642	619.2102	619.2112	6.4	n.d.	0.6±0.62	n.d.	n.d.	n.d.
23c Amygdalin-2´-glucoside	$C_{26}H_{37}NO_{16}$	642	619.2102	619.2112	6.6	0.4±0.03	1.4±0.55	n.d.	n.d.	n.d.
24a Amygdalin anitrile glucoside	$C_{25}H_{38}O_{16}$	617	594.2165	594.2160	5.9	0.2±0.07	1.1±0.20	2.2±1.23	2.1±0.82	n.d.
24b Amygdalin anitrile glucoside	$C_{25}H_{38}O_{16}$	617	594.2163	594.2160	6.4	0.1±0.04	0.5±0.12	n.d.	n.d.	n.d.

C. SORGHUM	Molecular formula	*m/z* $[M+Na]^+$	Measured mass	Calculated mass	r.t. [min]	13 days after sowing		30 days after sowing	
						Leaf	Sheath	Leaf	Sheath
25 Dhurrin* (in mg/g of fresh tissue)	**$C_{14}H_{17}NO_7$**	**334**	**311.1005**	**311.1005**	**5.7**	**3.7±0.74**	**2.0±0.45**	**1.1±0.18**	**1.0±0.20**
Dhurrin derivatives (in % of Dhurrin)									
26 Dhurrin amide*	$C_{14}H_{19}NO_8$	352	329.1099	329.1111	1.2	0.2±0.10	0.5±0.07	0.5±0.15	0.7±0.34
27 Dhurrin acid	$C_{14}H_{18}O_9$	353	330.0938	330.0951	1.4	0.6±0.17	1.9±0.78	2.4±0.94	1.8±0.19
28 p-Glucosyloxyphenylacetic acid*	$C_{14}H_{18}O_8$	337	314.1001	314.1002	5.3	5.7±1.00	2.6±0.57	5.5±1.14	1.8±0.20
29a Dhurrin glucoside	$C_{20}H_{27}NO_{12}$	496	473.1538	473.1533	5.6	0.4±0.10	0.6±0.25	0.6±0.13	0.2±0.03
29b Dhurrin glucoside	$C_{20}H_{27}NO_{12}$	496	473.1545	473.1533	5.8	0.1±0.02	0.1±0.03	0.1±0.02	0.1±0.06
29c Dhurrin glucoside	$C_{20}H_{27}NO_{12}$	496	473.1537	473.1533	6.1	0.6±0.20	1.3±0.60	0.3±0.14	0.8±0.32
30a Caffeoyl-dhurrin	$C_{23}H_{23}NO_{10}$	496	473.1314	473.1322	8.5	3.4±0.54	0.8±0.14	0.9±0.07	0.2±0.07
30b Caffeoyl-dhurrin	$C_{23}H_{23}NO_{10}$	496	473.1316	473.1322	8.8	0.4±0.11	0.1±0.03	0.1±0.03	n.d.
31 Caffeoyl-dhurrin acid	$C_{23}H_{24}O_{12}$	515	492.1272	492.1268	7.4	0.4±0.08	0.2±0.03	0.6±0.08	0.5±0.08

Table 2 Summary of the presence of the derivatives of cyanogenic glucosides in cassava, almond and sorghum.

Plant species	Cyanogenic Di-/Tri-glycosides	Derivatives of cyanogenic glucosides					
		Amide		Acid		Anitrile	
		Mono-glucoside	Di-/Tri-glycoside	Mono-glucoside	Di-/Tri-glycoside	Mono-glucoside	Di-/Tri-glycoside
Cassava	+	+	–	+	–	+	+
Almond	+	+	–	+	–	+	+
Sorghum	+	+	–	+	–	–	–

FIGURE LEGENDS

Figure 1 Simplified scheme depicting the metabolism of cyanogenic glycosides with representation of two different recycling processes.

Figure 2 Comparative metabolic profiling in cassava, almond and sorghum. (A) Structures of cyanogenic glycosides and their identified derivatives. Only one, namely β-(1→6), of the four possible linkage positions between sugar moieties in di- and triglycosides (**5-8, 11, 12, 17-24, 29**), and between caffeic acid and the monoglucoside (**30**, **31**), is shown. Hexoses in **2**, **3**, **6**, **7**, **10**, **11**, **17**, **24, 27** and **31** are deduced to be glucoses, based on the literature and IM-MS data. (B) Representative extracted ion chromatograms (EIC) at *m/z* corresponding to $[M+Na]^+$ of putative derivatives of linamarin and lotaustralin in cassava; of prunasin and amygdalin in almond; and of dhurrin in sorghum. The parent compounds are highlighted. The intensities result from different dilutions of the samples. The arrows indicate the peaks corresponding to the structures **1-31** (Table 1).

Figure 3 Tandem mass spectra corresponding to prunasin **13**, amygdalin **19** and amygdalin glucosides **23a, b** and **c** illustrating typical fragmentation pattern of cyanogenic mono-, di- and triglycosides.

Figure 4 Scheme depicting the collision-induced dissociation (CID) generated product ions for glycoside **8** and the nomenclature for the product ions [73]. Also shown are the tandem mass spectra associated with glycoside **8**, and the proposed structures of selected product ions. Ion mobility spectrometry (IMS) analysis of these selected product ions highlights the presence of apiose (magenta) linked β-(1→6) (green) within this characterized glycoside, and also glycoside **12**. The hexose and dehydrohexose ATD for all shown glycosides is identical to that of β-glucose (red and blue respectively) from the gentiobiose standard. Finally for glycoside **5**, the ATD for the $^{0,2}A_2$ cross-ring fragment (black) also appears to agree with gentiobiose, suggesting it is β-(1→6) linked.

Figure 5 Proposed recycling pathway for cyanogenic glycosides proceeding without release of toxic hydrogen cyanide. Reduced nitrogen and carbon from the CN group are recovered as ammonia and carbon dioxide for primary metabolism (violet dotted arrows). This hypothetical recycling route includes three possible ways of turnover (indicated in the scheme by magenta, green and blue arrows): (i) Cyanogenic glycosides are hydrolyzed into cyanogenic monoglucosides, which are further converted into glucosylated amides and/or acids and anitriles (magenta route); or, alternatively, (ii) into glucosylated anitriles directly (green route); the anitriles so formed can be further glycosylated; or, on the contrary, (iii) cyanogenic glucosides are glycosylated and converted into the corresponding glycosylated anitriles directly; they can be further hydrolysed into monoglucosides (blue route).

FIGURES

Figure 1

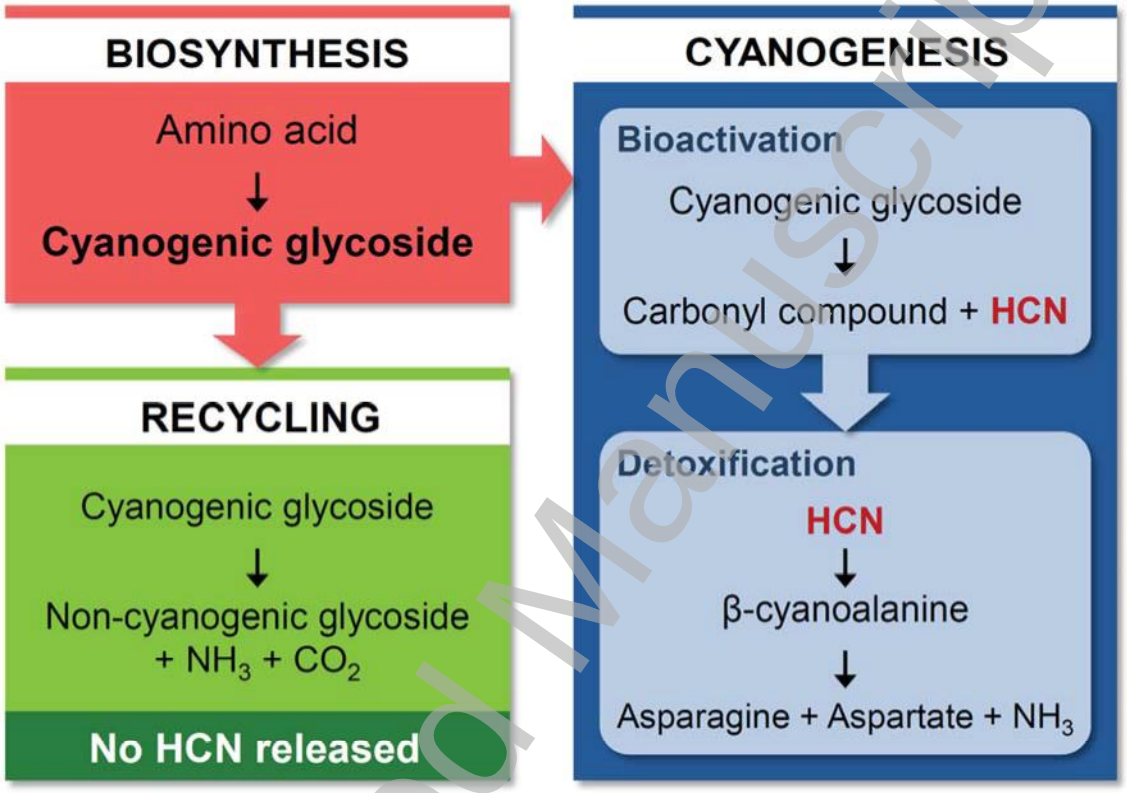

Figure 2

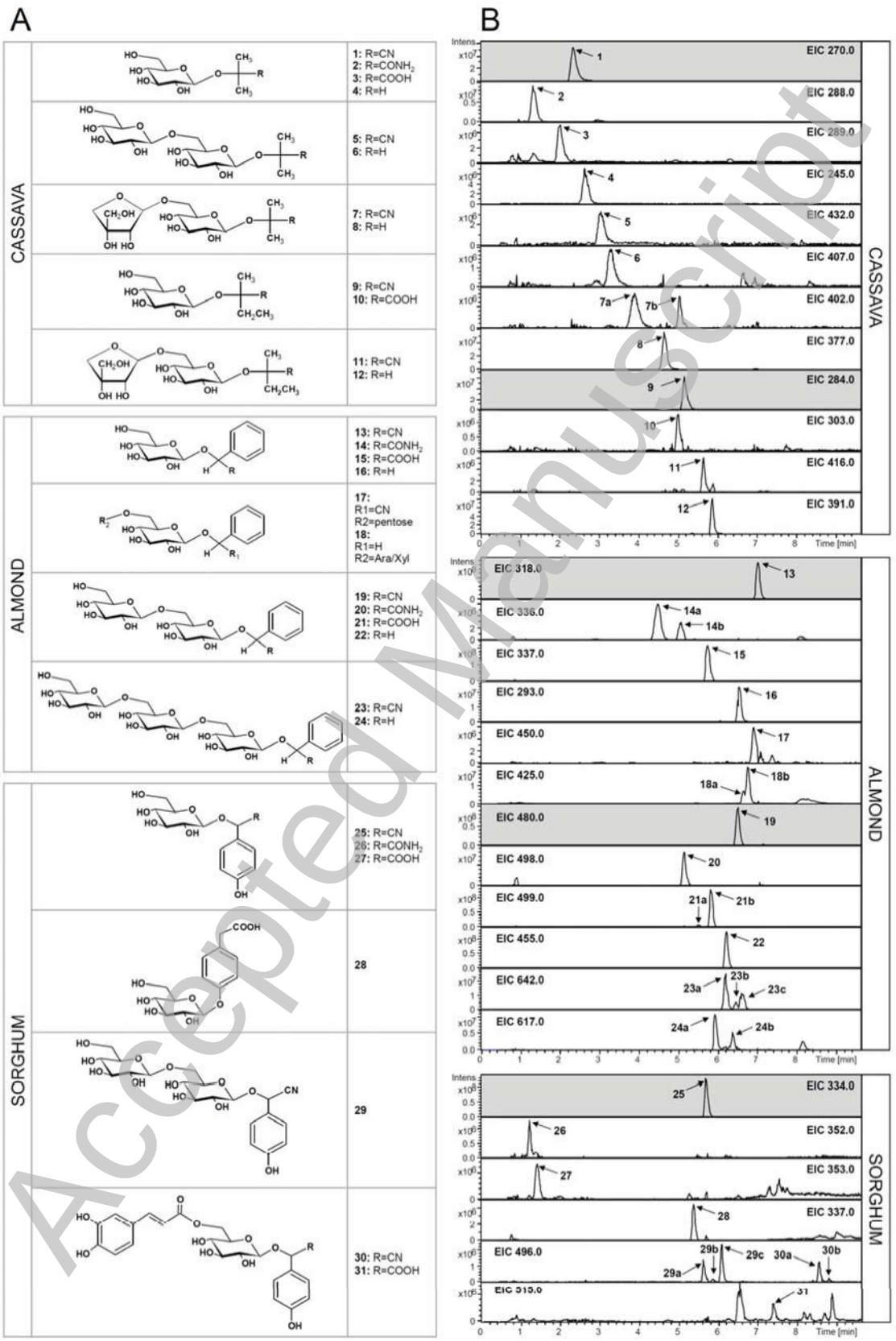

Figure 3

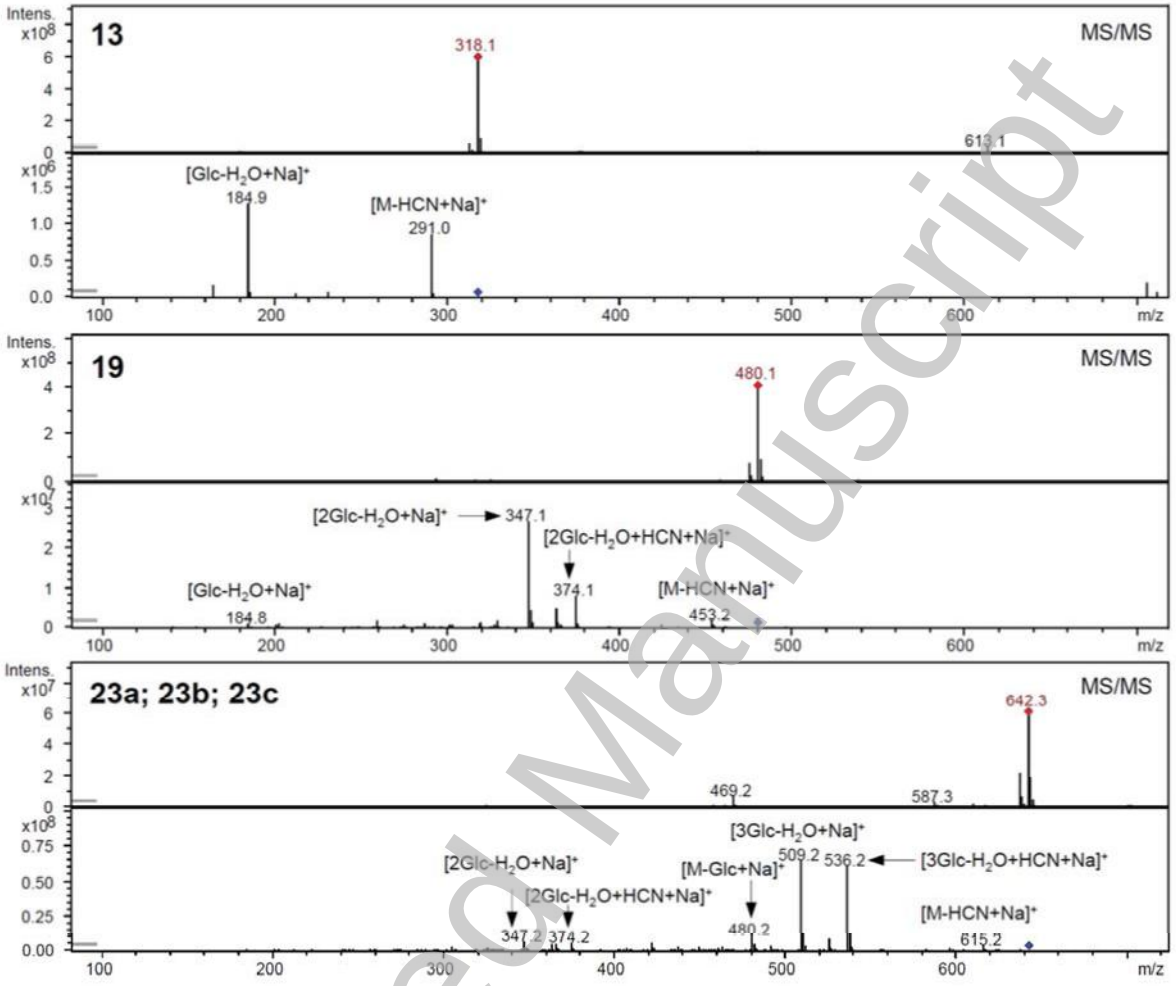

Figure 4

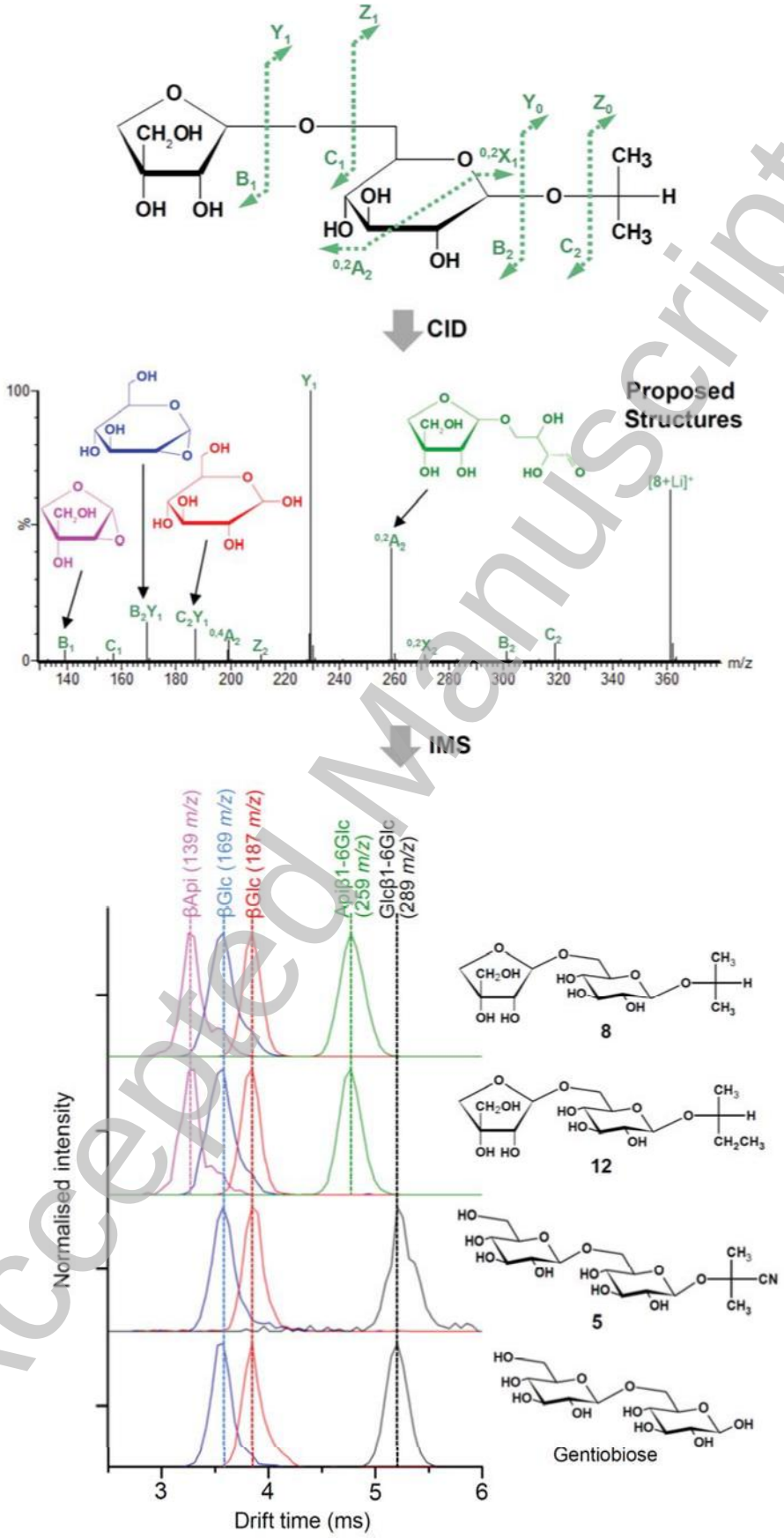

Figure 5