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ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2011

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Deciphering the Complexity of Sainfoin (*Onobrychis viciifolia*) Proanthocyanidins by MALDI-TOF Mass Spectrometry with a Judicious Choice of Isotope Patterns and Matrixes

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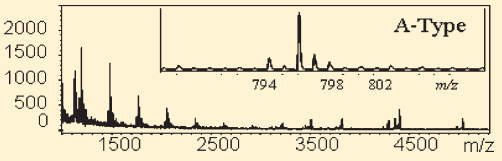
Supporting Information

ABSTRACT: Use of superdihydroxybenzoic acid as the matrix enabled the analysis of highly complex mixtures of proanthocyanidins from sainfoin (*Onobrychis viciifolia*) by MALDI-TOF mass spectrometry. Proanthocyanidins contained predominantly B-type homopolymers and heteropolymers up to 12-mers (3400 Da). Use of another matrix, 2,6-dihydroxyacetophenone, revealed the presence of A-type glycosylated dimers. In addition, we report here how a comparison of the isotopic adduct patterns, which resulted from Li and Na salts as MALDI matrix additives, could be used to confirm the presence of A-type linkages in complex proanthocyanidin mixtures. Preliminary evidence suggested the presence of A-type dimers in glycosylated prodelphinidins and in tetrameric procyanidins and prodelphinidins.



Tannins are the fourth largest group of natural plant products after cellulose, hemicellulose, and lignin.[1] Different plant species produce distinct mixtures of gallotannins, ellagitannins, and condensed tannins or proanthocyanidins (PAs; Figure 1).[2,3] As a result the analysis of these mixtures is by no means straightforward.[4]

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) has been used extensively to elucidate the complexity of these tannin mixtures and has provided the first evidence of homo- and heteropolymeric PA mixtures in foods and animal feeds.[5−8] Since PAs have a wide range of important biological properties, such as pro- and antinutritional,[3,9] nematicidal,[10] antimicrobial,[11,12] and anticancer[13] effects, a sound understanding of their structure−activity relationships is needed to derive optimum benefits from them.

Livestock farming presents two opportunities for practical PA applications which are of considerable interest. First, PAs have the potential for optimizing nitrogen use and for reducing N_2O and CH_4 greenhouse gas emissions from ruminant production systems.[14,15] Second, they can reduce parasitic worm burdens, which are a worldwide problem threatening livestock farming in some areas.[10,16,17] Forage legumes are a valuable source of home-grown protein, but most do not contain PAs, and as a result ruminants use this protein inefficiently. Up to 35% of the dietary nitrogen is lost as ammonia from the rumen, and most of this is excreted in urine and into the environment.[18] However, a small number of forage legumes, such as *Lotus corniculatus*, *Lotus pedunculatus*, and sainfoin (*Onobrychis viciifolia*), do contain PAs.[3,19] While MALDI-TOF MS has been used previously to detect PAs up to heptamers in *Trifolium* and decamers in *Lotus* species,[5,20] it has not yet been applied to sainfoin tannins. Some reports suggest that sainfoin PAs have a particularly low astringency and are difficult to extract.[21,22] Up to now, only low molecular weight PAs have been identified in sainfoin; PA dimers and trimers were detected by LC−MS,[23] and up to dodecamers were measured by phloroglucinol degradation.[24] However, sainfoin appears to also have highly polymeric PAs.[25]

To harness the economic potential of sainfoin and its PAs, it is important to develop analytical tools that are capable of analyzing these tannin mixtures. Such techniques will help to underpin the molecular breeding of new sainfoin and other forage chemotypes with desirable PA compositions. This study describes the application of MALDI-TOF MS for detecting several new compounds within the complexity of sainfoin tannin mixtures.

EXPERIMENTAL SECTION

Reagents and Plant Samples. The compounds 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), 2,6-dihydroxyacetophenone (DHAP), 2,4,6-trihydroxyacetophenone (THAP), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Gillingham, U.K.). Protein calibration standards and peptide calibration standards (ClinProt) were supplied by

Received: February 14, 2011
Accepted: April 13, 2011
Published: April 13, 2011

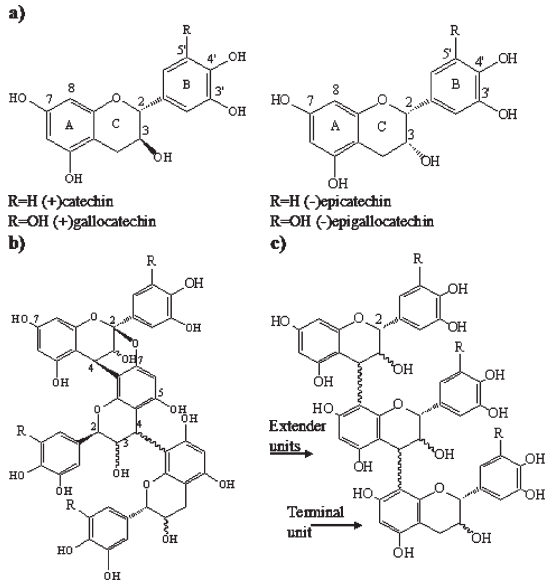


Figure 1. (a) (Epi)catechin (C) and (epi)gallocatechin (G) monomers. (b) A-type and (c) B-type linkages in proanthocyanidins: R = H (procyanidins), R = OH (prodelphinidins).

Bruker Daltonics (Bremen, Germany), and acetonitrile, methanol (HPLC grade), and lithium bromide were from Fisher Scientific (Loughborough, U.K.). Sodium chloride (ultra grade) and 2-hydroxy-5-methoxybenzoic acid (HMB) were from Fluka (Gillingham, U.K.), and water was purified using a Milli-Q system (Millipore, Watford, U.K.).

Sainfoin samples were harvested and freeze-dried as previously reported.[26] The Cotswold Common-1 variety was used for most of the experiments. The Supporting Information provides information on four additional sainfoin varieties: NIAB 1123 (CPI 63763), 1127 (CPI 63767), 1165 (Rees "A"), and 1262 (Cotswold Common-2).

PA Isolation. Samples were extracted from finely ground (<1 mm), freeze-dried sainfoin (*O. viciifolia*; 25 g) with 200 mL of acetone/water (7:3, v/v) using a magnetic stirrer for 40 min at room temperature. The solution was filtered under vacuum, concentrated on a rotary evaporator (<35 °C), and phase-separated twice in a separator funnel with dichloromethane (200 mL). The aqueous phase was concentrated and freeze-dried to yield the crude PA mixture (5.7 g).

Toyopearl HW-50F Fractionation. The fractionation column (23 cm × 3 cm) was packed with Toyopearl HW-50F (Hichrom Ltd., Theale, U.K.). The crude PA mixture (2 g) was dissolved in Milli-Q water (20 mL) and eluted first with water (300 mL), then with methanol/water (1:1, v/v; 300 mL) followed by acetone/water (7:3, v/v; 300 mL), and finally with acetone (100 mL). The fractions were concentrated at <35 °C, and the aqueous residues were freeze-dried. The aqueous methanol and aqueous acetone fractions were used for subsequent MALDI-TOF MS analysis.

MALDI-TOF MS Analysis. MALDI MS spectra were recorded on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with delayed extraction and a nitrogen laser (337 nm). In the positive reflectron mode an accelerating voltage of 25.0 kV and a reflectron voltage of 26.3 kV were used. For all spectra the data of 200 shots were accumulated. The instrument was calibrated with ClinProt standards (protein standards plus peptide standards) (Bruker Daltonics). The decision to use ClinProt standards was made because little was known about the molecular weight of sainfoin PAs. Therefore, with these standards it was possible to cover a wider MW range (600−17500 Da).

Sample Preparation. The DHB matrix solution was prepared by dissolving 5 mg in 1 mL of 50% aqueous acetonitrile containing 0.1% TFA. The s-DHB (DHB/HMB, 9:1, w/w) matrix solution was prepared by dissolving either 5 mg in 1 mL of acetonitrile/water (3:7, v/v) containing 0.1% TFA or 20 mg in 1 mL of acetonitrile/water (7:3, v/v) containing 0.1% TFA. The CHCA, DHAP, and THAP matrix solutions were prepared by dissolving 20 mg in 1 mL of acetonitrile/water (7:3, v/v) containing 0.1% TFA. The resulting pH of these matrix solutions was 2, 3, and 3, respectively.

The dried PA samples were reconstituted in acetonitrile/methanol (1:2, v/v) to give a final sample concentration of 4 mg mL^{-1}. Aqueous solutions (0.2 M) of sodium chloride and lithium bromide were prepared with Milli-Q water. Solutions containing matrix/sample/cationization agent were combined in a 1:1:0.5 volume ratio. The solutions were deposited on an AnchorChip steel target and left to dry at room temperature.

Peak Assignments. PA masses were calculated according to the following formula:

$$m/z\,(\mathrm{PA}) = (m/z\ 288.08)n + (m/z)(2 \times 1.0078) + (m/z\ 16)a + (m/z\ 162)b + (m/z)s$$

where m/z 288.08 corresponds to the molecular weight of one extender catechin or epicatechin unit (MW = 288.08 amu), n denotes the degree of polymerization (DP), 2 × 1.0078 accounts for one H in each of the top and bottom units (Figure 1c), a represents the number of gallocatechin or epigallocatechin units (Δm = 16 amu), b represents the number of glycosyl residues (Δm = 162 amu), and s is the molecular weight of the cation used for the ionization (Na or Li).

RESULTS AND DISCUSSION

MALDI Matrix Selection for High Molecular Weight Proanthocyanidins. Previous studies based on degradation with phloroglucinol have shown that most sainfoin varieties contain approximately 70−80% prodelphinidins and 30−20% procyanidins.[21,24,25] However, less agreement exists currently on the molecular size distribution of sainfoin PAs.[27−29] Most studies reported molecular weights ranging from ca. 1000 to 3800 Da, i.e., trimers to dodecamers, but highly polymerized PAs appear to exist as well.[21,25] Therefore, we explored MALDI-TOF MS for analyzing purified PA fractions with two conventional (DHB, CHCA),[20,30] and three less widely used (THAP,[31] DHAP, and s-DHB) MALDI matrixes for their suitability for analyzing PA mixtures from sainfoin.

Table 1 shows the predicted and observed values for Li adducts of homo- and heteropolymeric B-type PAs containing all possible combinations of catechin/epicatechin (C) and gallocatechin/epigallocatechin (G) units up to hexamers and with DPs ranging from 2 to 12. All the predicted m/z values were calculated as exact monoisotopic values. Some observed m/z values were not within 50 ppm to the expected ones. It is likely that s-DHB gave more of the hot-spot phenomena and more differences in the topology between desorption spots and therefore less mass accuracy at higher MW compared to CHCA, for instance.

Among the five tested MALDI matrixes, only s-DHB, at a concentration of 20 $\mu g\ \mu L^{-1}$ in 70% aqueous acetonitrile, enabled

Table 1. Predicted and Observed Monoisotopic m/z Values for the Na Adduct Ions of Proanthocyanidins[a]

		composition of oligomers						
chain length		nC	$(n-1)$C + 1G	$(n-2)$C + 2G	$(n-3)$C + 3G	$(n-4)$C + 4G	$(n-5)$C + 5G	$(n-6)$C + 6G
2-mer (n = 2)	pred	601.13	617.12	633.12				
	obsd	601.09	617.09	633.06				
3-mer (n = 3)[b]	pred	889.19	905.19	921.18	937.18			
	obsd	889.10	905.10	921.10	937.14			
4-mer (n = 4)	pred	1177.26	1193.25	1209.25	1225.24	1241.24		
	obsd	1177.18	1193.17	1209.17	1225.17	1241.17		
5-mer (n = 5)	pred	1465.31	1481.32	1497.31	1513.31	1529.30	1545.30	
	obsd	1465.25	1481.27	1497.25	1513.27	1529.26	1545.26	
6-mer (n = 6)	pred	1753.38	1769.38	1785.37	1801.37	1817.36	1833.36	1849.35
	obsd	1753.38	1769.39	1785.37	1801.36	1817.34	1833.40	1849.35
7-mer (n = 7)	pred	2041.44	2057.44	2073.44	2089.43	2105.43	2121.42	2137.42
	obsd	2041.38	2057.37	2073.45	2089.44	2105.39	2121.40	2137.39
8-mer (n = 8)	pred	2329.51	2345.51	2361.50	2377.50	2393.49	2409.49	2425.48
	obsd		2345.85	2361.68	2377.94	2393.59	2409.52	2425.63
9-mer (n = 9)	pred	2617.57	2633.57	2649.57	2665.56	2681.55	2697.55	2713.54
	obsd		2633.45	2649.64	2666.21	2681.26		
10-mer (n = 10)	pred	2905.64	2921.64	2937.63	2953.62	2969.62	2985.61	3001.61
	obsd		2922.20	2937.65	2954.14	2968.71		
11-mer (n = 11)	pred	3193.70	3209.70	3225.69	3241.69	3257.68	3273.68	3289.67
	obsd		3210.11	3226.40	3242.16	3257.27		
12-mer (n = 12)	pred	3481.77	3497.76	3513.75	3529.75	3545.74	3561.74	3577.73
	obsd		3496.74	3513.45	3526.88	3544.26		

[a] Note: for Li adduct ion values, subtract 15.97. [b] Trimers were detected only as Na adducts (see Figure S-8, Supporting Information).

the detection of larger PAs up to DP ≤ 12 (Figure 2a). Signals could also be seen at higher m/z values, but resolution above the 12-mers was insufficient to estimate the degree of polymerization accurately. The tested MALDI matrixes differed considerably in their ability to assist ionization of PAs: CHCA yielded acceptable spectra for PA dimers (Figure 2c) but was much less satisfactory for the tetramers than s-DHB (Figure 2d) and did not detect the pentamers at all (Figure S-1, Supporting Information). However, not all the concentrations of s-DHB were suitable for the detection of Na adduct ions. When s-DHB at a concentration of 5 μg μL^{-1} in 30% aqueous acetonitrile was used as the matrix, it enabled the detection of trimeric proanthocyanidins but not of higher MW oligomers. However, the detection of trimeric proanthocyanidins was not possible when the same matrix was used at a higher concentration of 20 μg μL^{-1} in 70% aqueous acetonitrile (Figure S-2, Supporting Information). Oligomers up to trimers were only detected in the 50% aqueous methanol fraction and higher molecular weight PAs were only detected in the aqueous acetone fraction (Figure S-3, Supporting Information).

The s-DHB matrix concentration and solvent composition also had a marked effect on the signal-to-noise (S/N) ratio. The best s-DHB matrix concentration for detecting larger PAs was 20 μg μL^{-1} in 70% aqueous acetonitrile. However, in the low mass region s-DHB at 5 μg μL^{-1} in 30% aqueous acetonitrile produced much better resolved peaks. In addition to the oligomeric PA series with DP from 2 to 12 (Figure 2a), a second series of peaks differing by approximately 16 Th could be observed within each of the oligomers. For instance, Figure 3a depicts the tetrameric series of Li adduct ions. Li was used to confirm the molecular weights depicted in Figure 2e and then chosen because of a better S/N ratio.

These peaks can be explained in terms of successive substitution of catechin/epicatechin units (C) with gallocatechin/epigallocatechin units (G) (Figure 1, Table 1). This suggests that sainfoin PAs contain not only homopolymers consisting of either procyanidins or prodelphinidins but also all possible combinations of heteropolymers up to the 12-mers. Although past MALDI-TOF MS analysis of PAs used MALDI matrixes such as sinapinic acid, 5-chlorosalicylic acid, CHCA, and 9-nitroanthracene, only *trans*-3-indoleacrylic acid (t-IAA) and 2,5-dihydroxybenzoic acid (DHB) have provided suitable spectra for the analysis of catechin oligomers.[7]

Interestingly, Table 1 reveals that homopolymers of prodelphinidins (up to six G units) and procyanidins (up to eight C units) could be detected. This observation is supported by several previous reports where not all possible combinations of C and G units in proanthocyanidins were detected.[7,32,33]

MALDI Matrix Selection for Low Molecular Weight Proanthocyanidins. The poor resolution given by s-DHB for the lower masses (Figure 4d) necessitated the use of other matrixes. The tested matrixes yielded substantially different ionization results (Figure 4). While DHAP and THAP generated similar spectra, the THAP peaks were less intense despite being better resolved. DHB gave strong peaks at m/z 779.1 and m/z 763.1, but the peak at m/z 795.1 had disappeared, and a new peak at m/z 793.1 could be seen instead. DHB and s-DHB yielded similar spectra. These additional ions were not a background from the matrix as they did not appear in the blanks and they were not observed in the acetone fraction under the same conditions (Figure S-8, Supporting Information). Furthermore, since 2,6-DHAP is known to be a very soft/cold matrix, it is very unlikely to have caused any fragmentation. The peaks in Figure 4 could be assigned to a series

dx.doi.org/10.1021/ac2003856 |*Anal. Chem.* 2011, 83, 4147–4153

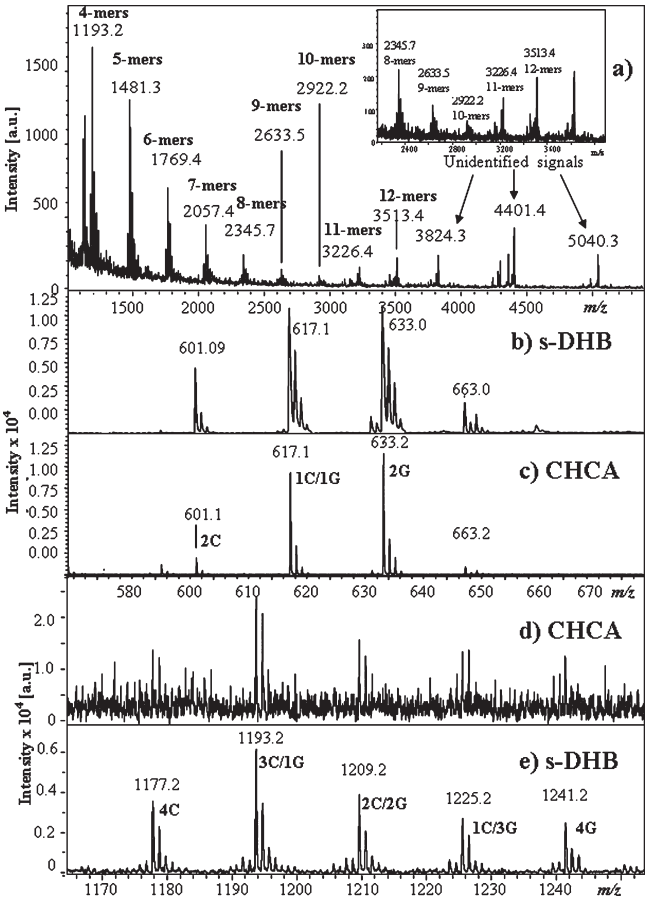


Figure 2. MALDI-TOF MS spectra (reflectron mode) of (a) Na adducts of an oligomeric proanthocyanidin series in the aqueous acetone fraction obtained using s-DHB (20 $\mu g\,\mu L^{-1}$, acetonitrile/water (7:3, v/v)) as the matrix, Na adduct dimers of proanthocyanidins in the aqueous acetone fraction obtained using either (b) s-DHB or (c) CHCA (20 $\mu g\,\mu L^{-1}$, acetonitrile/water (7:3, v/v)) as the matrix, and Na adduct tetramers of proanthocyanidins in the aqueous acetone fraction obtained using either (d) CHCA or (e) s-DHB (20 $\mu g\,\mu L^{-1}$, acetonitrile/water (7:3, v/v)) as the matrix.

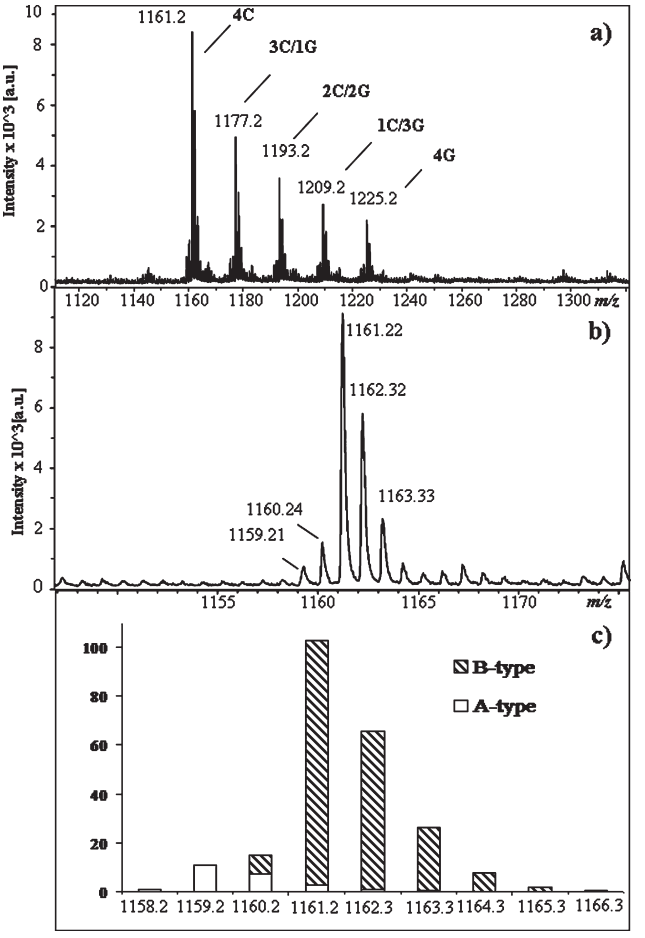


Figure 3. (a) MALDI-TOF MS spectrum (reflectron mode) of a series of Li adduct proanthocyanidin tetramers in the aqueous acetone fraction obtained using s-DHB (20 $\mu g\,\mu L^{-1}$, acetonitrile/water (7:3, v/v)) as the matrix. (b) Enlarged MALDI-TOF MS spectrum (reflectron mode) of the isotopic Li adduct cluster of proanthocyanidin tetramers. (c) Calculated isotopologue distribution for the A/B-type proanthocyanidin mixture in (b).

of dimeric PA glycosides (Table 2). The best MALDI matrix for detecting these glycosylated dimers (Table 2) proved to be DHAP (20 $\mu g\,\mu L^{-1}$, acetonitrile/water (7:3, v/v)) using either Na or Li as the cationizing agent. The best condition for detecting all other oligomers was s-DHB (20 $\mu g\,\mu L^{-1}$, acetonitrile/water (7:3, v/v)) as the matrix and Na as the cationizing agent (Figure 2).

Comparison of PAs from Sainfoin and Other Plants. Sainfoin PAs consisted of homo- and heteropolymers (Table 1). The s-DHB MALDI matrix enabled detection of homopolymeric procyanidins (up to the 7-mers) and homopolymeric prodelphinidins (up to the 6-mers). It was also possible to detect heteropolymers (up to 12-mers, about m/z 3500) which included between one and six catechin/epicatechin and one and eleven gallocatechin/epigallocatechin units.

The s-DHB matrix resulted in much better mass resolution for homo- and heteropolymers than previously reported for mixed procyanidins/prodelphinidins from *L. corniculatus* and hop (*Humulus lupulus* L.), which had been detected using *trans*-3-indoleacrylic acid as the MALDI matrix.[5,32] The use of s-DHB enabled the detection of homopolymeric PDs, which were not detected with *trans*-3-indoleacrylic acid.[5,32]

However, thiolytic degradation has shown that PAs in the sainfoin Cotswold Common-1 variety had a mean degree of polymerization of 10.3 (about 3000 Da).[26] In comparison, PAs with masses of up to 8394 Da have been detected by MALDI-TOF MS previously: the largest PAs from brown soybean seed coat had a DP = 30,[34] those from pear juice had a DP = 24,[35] and those from apples had a DP = 15.[36] It is worth noting, however, that all of these particular PAs consisted of procyanidin homopolymers. Most other MALDI TOF MS studies detected PAs only up to ca. 3000 Da.[5,37−39] Purification of the PA mixtures into different size fractions tended to improve the detection of larger polymers.[8,32] For instance, after purification hop PAs with DP up to 20 and cranberry PAs with DP up to 23 could be detected. These contained homopolymeric procyanidins plus some heteropolymers, which included one to four units of prodelphinidins. A high heterogeneity was also observed in sorghum PAs, which arose from repeating flavan-3-ol (procyanidins) and flavanone units (fast atom bombardment (FAB) MS studies)[40] plus prodelphinidins and procyanidins up to nonamers,[37] which contained A-type and B-type interflavanol linkages and glycosides. In comparison, the s-DHB MALDI matrix allowed resolution of the sainfoin PA mixtures,

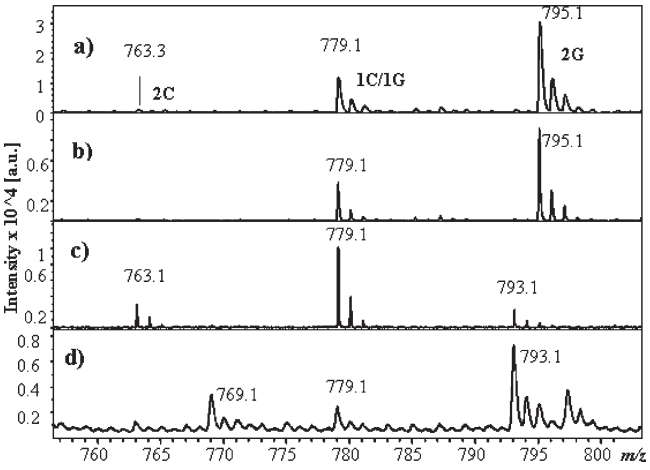


Figure 4. MALDI-TOF MS spectra (reflectron mode) of Na adduct ions of the glycosylated dimers in the aqueous methanol fraction obtained using (a) DHAP, 20 μg μL^{-1}, acetonitrile/water (7:3, v/v), (b) THAP, 20 μg μL^{-1}, acetonitrile/water (7:3, v/v), (c) DHB, 20 μg μL^{-1}, acetonitrile/water (7:3, v/v), or (d) s-DHB, 20 μg μL^{-1}, acetonitrile/water (7:3, v/v), as matrixes.

Table 2. Predicted and Observed *m/z* Values for Na Adduct Ions of the Glycosylated A-Type and B-Type Dimers

	m/z values			
	B-type		A-type	
composition of dimers	pred	obsd	pred	obsd
2C	763.08	763.33	761.08	
1C + 1G	779.08	779.14	777.08	
2G	795.08	795.10	793.08	793.10

which included pentamers to dodecamers that contained between one and six prodelphinidin units. Arguably, the complexity of these sainfoin PAs prevented optimal cocrystallization with the matrix and thus the detection of the higher molecular weight PAs by MALDI-TOF MS.

Assignments of Additional PA Peaks Based on Isotopic Masses. The observed masses listed in Table 1 are close to the predicted monoisotopic masses. However, because of the high resolution of MALDI-TOF in the reflectron mode, relatively complicated isotopic patterns of molecular adduct ions can be seen. For example, the observed and predicted monoisotopic mass of the Li adduct ions of the tetrameric procyanidin homopolymer was *m/z* 1161.22 (Figure 3b).

As lithium occurs naturally as a mixture of ^{6}Li (7.52%) and ^{7}Li (92.48%) isotopes, it can be expected that a minor peak 1 Th below the monoisotopic mass can be detected. Indeed, the enlarged MALDI-TOF MS spectrum (Figure 3b) also shows the contribution of a ^{6}Li isotope (*m/z* 1160.24) to the isotopic cluster. However, this still leaves one peak unaccounted for (*m/z* 1159.21). To elucidate the origin of this peak, different cationizing metals and matrixes were used.

We observed several MALDI MS ion signals that can be attributed to three types of PAs: B-type (Table 1), A-type (see below), and glycosylated PAs (Table 2 and below).

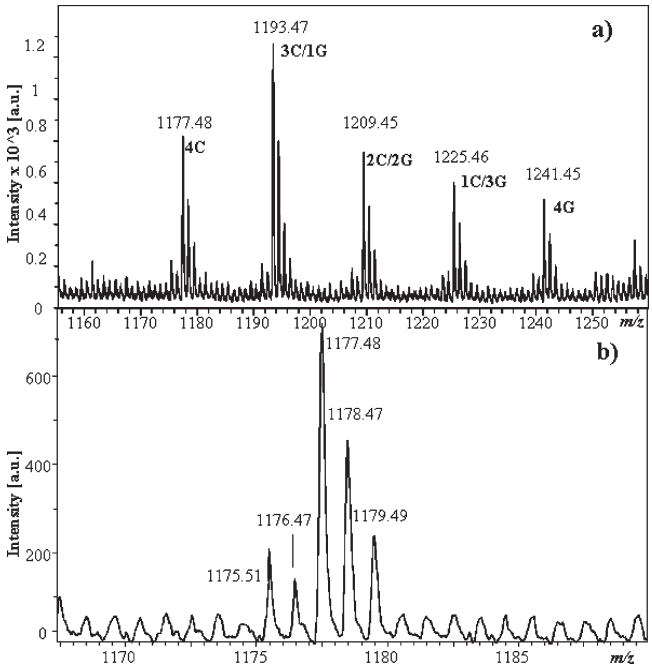


Figure 5. (a) MALDI-TOF MS spectrum (reflectron mode) of a series of Na adduct ions of tetrameric proanthocyanidins in the aqueous acetone fraction obtained using s-DHB (20 μg μL^{-1}, acetonitrile/water (7:3, v/v)) as the matrix. (b) Enlarged MALDI-TOF MS spectrum (reflectron mode) showing the isotopic cluster of Na adduct proanthocyanidin tetramers containing four (epi)/catechin units (4C) obtained using s-DHB (20 μg μL^{-1}, acetonitrile/water (7:3, v/v)) as the matrix.

The MALDI-TOF MS spectrum of Na adduct ions of tetrameric PAs is shown in Figure 5a, where the enlarged spectrum (Figure 5b) focuses only on the tetrameric procyanidin homopolymers. It reveals an isotopic pattern that significantly differs from the Li adduct ion pattern (Figure 3b): the peak lacking two mass units (*m/z* 1175.51) was more intense than the peak lacking just one mass unit (*m/z* 1176.47). This can be explained by the contribution of the isotope ^{6}Li to the overall isotopologue pattern (Figure 3c), thus being distinctly different from the Na adduct ion pattern, as Na has no other naturally occurring isotope than ^{23}Na.

These different isotopic patterns are even more obvious in the enlarged spectrum of the Na adducts of the glycosylated dimers. The peak at *m/z* 795.1 can be assigned to glycosylated dimers containing two (epi)/gallocatechin (G) units (Table 2, Figure 6; see also Figures S-4 and S-5 in the Supporting Information). Furthermore, sainfoin varieties appear to differ in their relative proportions of A-type and B-type PA dimers (Figure S-4, Supporting Information) and in the relative proportions of (epi)/catechin (C) and (epi)/gallocatechin (G) units (Figure S-6, Supporting Information).

Further examination of the isotopic patterns also reveals the presence of glycosylated A-type dimers when using THAP and especially DHAP as matrixes (Figure 4, Table 2). Preliminary evidence for A-type tetramers is presented in Figures S-6 and S-7 (Supporting Information).

As A-type PAs have not been reported before in sainfoin, the question arises of whether the matrix system developed here enabled their detection or whether [M + Na − 2H] ions were generated under the experimental conditions. Indeed, a loss of two hydrogens is suggested in Figure 4 in the DHB and s-DHB, but not DHAP and

dx.doi.org/10.1021/ac2003856 |*Anal. Chem.* 2011, 83, 4147–4153

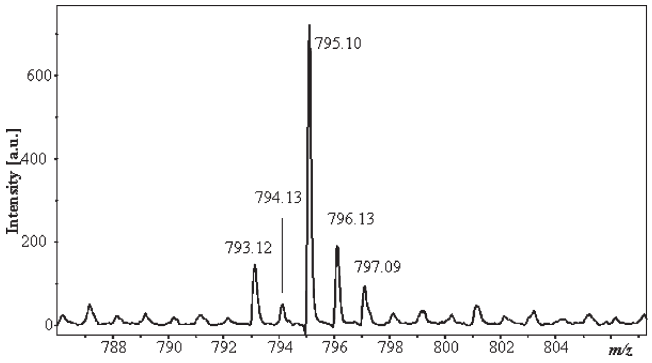


Figure 6. Enlarged MALDI-TOF MS spectrum (reflectron mode) showing the isotopic cluster of Na adduct ions of the glycosylated dimers containing (epi)gallocatechin units in the aqueous methanol fraction obtained using DHAP (20 $\mu g\ \mu L^{-1}$, acetonitrile:/water (7:3, v/v)) as the matrix. The peak at m/z 795.1 can be assigned to glycosylated dimers containing two (epi)gallocatechin units. The peak at m/z 793.1 can be assigned to the same molecule having an A-type instead of the B-type linkage.

THAP, matrixes. However, depending on the sainfoin variety, A-type PAs were also detected with DHAP (Figures S-4 and S-5, Supporting Information). A-type PAs have been detected previously by MALDI-TOF MS with DHB as the matrix in the presence of B-type PAs.[33] Evidence of A-type PAs has been reported in cranberries,[41,42] peanuts,[43] sorghum,[37] avocados,[44] and brown soybeans.[34]

Examination of the glycosylated dimer spectra might suggest that prodelphinidins but not procyanidins contained A-type linkages (Figures S-4 and S-5, Supporting Information). Several examples of a series of A- and B-type procyanidins and propelargonidins (B-ring with one OH group) or A- and B-type prodelphinidins can be found in the literature.[47,48] However, no propelargonidins have been reported for sainfoin in the literature.[23,25] In addition to this, prodelphinidins (B-ring with three OH groups) are known to be more reactive than procyanidins (B-ring with two OH groups) in terms of susceptibility to oxidation.[45] However, these and related NMR studies which compared B- to A-type conversions of prorobinetinidins (B-ring with three OH groups, A-ring with one OH group) and profisetinidins (B-ring with two OH groups, A-ring with one OH group)[46] were done at pH 10. Indeed, evidence for A-type linkages can also be seen in the tetrameric procyanidin homopolymers and procyanidin/prodelphinidin heteropolymers (Figures 3 and 5; see also Figure S-7, Supporting Information).

Taken together, these MALDI-TOF MS spectra revealed that A-type linkages were only detected in some sainfoin PA mixtures, i.e., tetrameric procyanidin and prodelphinidin homo- and heteropolymers, and in glycosylated prodelphinidin dimers.

CONCLUSIONS

This study demonstrated that sainfoin proanthocyanidins are complex mixtures of homo- and heteropolymers of B-type procyanidins and prodelphinidins. Careful selection of MALDI matrixes and analytical conditions made it possible to also detect ion signals that could be assigned to A-type plus glycosylated A-type proanthocyanidins. s-DHB is a particularly mild matrix that was introduced first to facilitate the detection of large proteins.[49] The experiments described here revealed that s-DHB enabled the detection of individual compounds in complex mixtures of sainfoin proanthocyanidins. This is the first report of using isotopic pattern analysis in MALDI-TOF MS for detecting A-type proanthocyanidins in the presence of B-type proanthocyanidins by using Li and Na salts as MALDI matrix additives.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

This investigation was supported by the European Commission (Marie Curie Research Training Network, "HealthyHay", Grant MRTN-CT-2006-035805). MALDI-TOF analyses were carried out at the BioCentre Facility (University of Reading).

The Cotswold Common-1 sainfoin variety was collected with kind permission from Hartley Farm, near Gloucester, U.K. CPI 63763, CPI 63767, Rees "A", and Cotswold Common-2 varieties were grown at the National Institute of Agricultural Botany, Cambridge, U.K., by Dr. Christine Hayot Carbonero (see the Supporting Information).

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