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Acyl Spermidines in Inflorescence Extracts of Elder (*Sambucus nigra* L., Adoxaceae) and Elderflower Drinks

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ABSTRACT: LC-UV-MS analyses of inflorescence extracts of *Sambucus nigra* L. (elder, Adoxaceae) revealed the presence of numerous acyl spermidines, with isomers of *N*,*N*-diferuloylspermidine and *N*-acetyl-*N*,*N*-diferuloylspermidine being most abundant. Pollen was the main source of the acyl spermidines in the inflorescence. Three of the major acyl spermidines were isolated and their structures determined by NMR spectroscopy as N^5,N^{10}-di-(*E*,*E*)-feruloylspermidine and the new compounds N^1-acetyl-N^5,N^{10}-di-(*Z*,*E*)-feruloylspermidine and N^1-acetyl-N^5,N^{10}-di-(*E*,*E*)-feruloylspermidine. An isomer of *N*,*N*,*N*-triferuloylspermidine was also obtained and identified as N^1,N^5,N^{10}-tri-(*E*,*E*,*E*)-feruloylspermidine. In addition to stereoisomers of the isolated acyl spermidines, other acyl spermidines detected by the positive ion LC-UV-MS were isomers of *N*-caffeoyl-*N*,*N*-diferuloylspermidine, *N*-coumaroyl-*N*,*N*-diferuloylspermidine, *N*-caffeoyl-*N*-feruloylspermidine, *N*-coumaroyl-*N*-feruloylspermidine, *N*-acetyl-*N*-caffeoyl-*N*-feruloylspermidine, and *N*-acetyl-*N*-coumaroyl-*N*-feruloylspermidine. Analysis of commercial elderflower drinks showed that acyl spermidines were persistent in these processed elderflower products. Examination of inflorescence extracts from *Sambucus canadensis* L. (American elder) revealed the presence of acyl spermidines that were different from those of *S. nigra*.

KEYWORDS: *elderflower, Sambucus nigra, feruloylspermidine, NMR, LC-MS*

INTRODUCTION

Sambucus nigra L. (Adoxaceae; elder, elderberry, European elder) has numerous traditional uses in Europe, both as a food and as a medicinal plant, with almost every part being employed for some purpose.[1] Among the traditional uses of the inflorescences of *S. nigra* (commonly referred to as "elderflower") is their incorporation into preparations to treat the symptoms of influenza, colds, and sinusitis due to their diaphoretic properties.[2] Today though, the most familiar use of elderflower is in the production of elderflower drinks that are now sold widely in Europe.[3] Less than 30 years ago the production of elderflower drinks was local and small-scale, but increasing popularity saw the market grow, and by 2001 it was estimated that in the United Kingdom alone over 100 tonnes of *S. nigra* inflorescences per year were being gathered.[4] This demand has encouraged the commercial cultivation of elder, with data from 2002 indicating that the then largest commercial drinks company in the United Kingdom obtained about half of its raw material from cultivated plants.[5]

The volatile compounds from elder inflorescences and extracts have been the subject of a number of comprehensive investigations undertaken to analyze the characteristic aroma.[6] Studies on the less volatile constituents have concentrated on the acyl quinic acids (chlorogenic acids and related compounds) and flavonoids proposed to be associated with the medicinal uses of elderflower.[7] This has led to flavonoid analysis by thin layer chromatography being recognized as the approved method in the European Pharmacopoeia for distinguishing between *S. nigra* and its nonmedicinal relative, *Sambucus ebulus* L.[8] Analysis of flavonoids by high-performance liquid chromatography (HPLC) has also been used to distinguish inflorescence material of *S. nigra* from *Sambucus australis* Cham. & Schltdl.[9]

When we examined the quality of commercial elderflower extracts using ultrahigh-performance liquid chromatography coupled to photodiode array detection and mass spectrometry (LC-UV-MS), it was noted that several of the constituents giving the greatest response to positive electrospray ionization (ESI) were neither flavonoids nor acyl quinic acids but acyl spermidines. Although compounds of this type have been reported widely in angiosperms,[10,11] they had not been reported previously in Adoxaceae. Thus, given the widespread consumption of elderflower products, the present study was undertaken to investigate the occurrence of acyl spermidines in *S. nigra* inflorescences in greater detail, through the isolation and structural determination of some of the compounds detected, and assess their relevance to the traditional uses of elderflower. Another objective was to determine whether these compounds were also present in commercial elderflower drinks. The acyl spermidines in inflorescences of three other species of *Sambucus*, including *Sambucus canadensis* (American elder), were also examined.

MATERIALS AND METHODS

Chemicals. MeOH and HCOOH (both of HPLC grade) were obtained from Fisher Scientific (Loughborough, UK). All other chemicals were of analytical grade. Deionized H_2O was obtained from an in-house Milli-Q Plus System (Millipore Inc., Billerica, MA, USA) at 18.2 mΩ.

Plant Materials. Fresh inflorescences of species of *Sambucus* were obtained from the sources listed in Table 1. Herbarium vouchers were made from each species studied and lodged at RBG Kew (sheet

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Table 1. Sample Details of *Sambucus* Inflorescences and Elderflower Products Studied

species/product name	source/company	collection reference; flowering voucher	sample identifier
Sambucus adnata Wall. ex DC.	*ex hort.* RBG Kew	1988-4540; *G.C.Kite/BI-20884* (K)	BI-19727
Sambucus canadensis L.	*ex hort.* RBG Kew	1986-1685; *G.C.Kite/BI-20881* (K)	BI-19730
Sambucus canadensis L.	*ex hort.* RBG Kew	2004-453	BI-19735
Sambucus ebulus L.	*ex hort.* RBG Kew	1979-4507	BI-19804
Sambucus ebulus L.	*ex hort.* RBG Kew	1990-3553; *G.C.Kite/BI-20883* (K)	BI-19805
Sambucus nigra L.	*ex hort.* RBG Kew	1998-605; *G.C.Kite/BI-20882* (K)	BI-19723
Sambucus nigra 'Aureomarginata'	*ex hort.* RBG Kew	1950-25507	BI-19733
Sambucus nigra 'Laciniata'	*ex hort.* RBG Kew	1973-18515	BI-19734
Sambucus nigra L.	wild, RBG Kew	–	BI-19719
Sambucus nigra L.	wild, RBG Kew	–	BI-19707
Sambucus nigra L.	wild, Pinkneys Green, Berkshire, UK	–	BI-19717
Sambucus nigra L.	wild, Cowlease wood, Oxon, UK	–	BI-18735
Elderflower Cordial	Belvoir Fruit Farms	–	BI-19487
Twinings Cranberry, Raspberry and Elderflower tea	R. Twining and Co. Ltd.	–	BI-19546
Elderflower Bubbly fizzy drink	Lovely Drinks Ltd.	–	BI-19668
Elderflower & Rose fizzy drink	Lovely Drinks Ltd.	–	BI-19669

references are given in the table). A bulk sample of dry inflorescences of *S. nigra* from a commercial grower was provided by Mactavish Consulting, Ltd., and commercial elderflower drinks were purchased from local shops (Table 1).

Extraction and Isolation. For LC-UV-MS analysis, either 100 mg of dried powdered inflorescences was extracted in 80% MeOH or 200 mg of the fresh flowers removed from the inflorescences were extracted in 1 mL of MeOH, each for 3 h. After centrifugation, the supernatant was diluted 10-fold in 80% MeOH before analysis. For one sample of *S. nigra* the anthers were dissected from the flowers, and any pollen was washed from the emasculated flowers with water; the anther and emasculated floral tissue were then extracted separately in MeOH. A sample of pollen was collected from the plastic bag in which inflorescences had been dried and also extracted in MeOH.

For compound isolation, dry inflorescences (200 g) were milled to a powder and extracted twice with 2 L of 80% MeOH. Extracts were combined, filtered, and evaporated to dryness under vacuum at 40 °C using a rotary evaporator. The dried sample was suspended in 600 mL of H_2O and partitioned against an equal volume of BuOH. The BuOH phase was rotary evaporated to dryness under vacuum at 40 °C, yielding 48 g of residue, of which 16 g was loaded onto a Sephadex LH20 column (GE Healthcare, Pittsburgh, PA, USA) for column chromatography after dissolution with 50% MeOH. A stepwise elution of 50, 60, 70, 80, 90, and 100% MeOH (500 mL of each) was used, and 25 mL fractions were collected. Guided by LC-UV-MS analyses, these fractions were pooled into a total of 13 combined fractions (A–I). Fraction A (700 mg) was dissolved in 5 mL of 80% MeOH and subjected to HPLC fractionation (Waters 600 controller, 717plus autosampler, and 2996 photodiode array detector; Waters, Milford MA, USA) fitted with a Lichrospher 250 × 4.6 mm i.d., 5 μm, column (Phenomenex, Torrance, CA, USA) using a 1 mL/min linear mobile phase gradient of 42–65% MeOH over 20 min and then to 100% MeOH over 1 min. Elution was monitored by UV absorption at 254 and 313 nm, and peaks collected between 16.9 and 17.5 min were evaporated to dryness and further purified by HPLC under isocratic conditions (41% MeOH, 1 mL/min) to yield **2b** and **2c** (<1 mg) and **2d** (2–3 mg).

Another 16 g of vacuum-dried BuOH partition phase was suspended in 400 mL of H_2O and partitioned with an equal volume of EtOAc. The EtOAc layer was dried under rotary evaporation at 40 °C, reconstituted in 40% MeOH, and loaded onto a Sephadex LH20 column. A stepwise elution of 40, 50, 60, and 80% MeOH (500 mL of each) followed by 1200 mL of 100% MeOH was used, and 30 fractions were collected. These were combined into five fractions (A_{II}–E_{II}). Fraction E_{II} (114.9 mg) was dried under rotary evaporation, dissolved in 20% MeOH, and loaded onto a Sephadex LH20 column eluting with 20% (350 mL) and 30, 40, and 100% (700 mL each) MeOH to yield 25 fractions. These were then combined to give four fractions (E_{IIa}–E_{IId}) according to LC-UV-MS analysis. Compound **1d** (8 mg) was obtained from fraction E_{IIb} and compound **3d** (1 mg) was obtained from fraction E_{IId} using analytical scale HPLC (isocratic conditions at 55% MeOH, 1 mL/min).

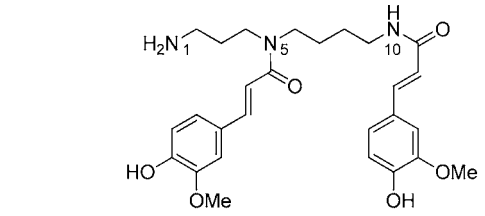
The commercial samples were prepared, when necessary, as for consumption and then freeze-dried and redissolved in 80% aqueous MeOH before LC-UV-MS analysis.

LC-UV-MS Analysis. The LC-UV-MS system comprised an Accela system 1250 pump, autosampler, and diode array detector, interfaced via an Ion Max electrospray source to an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Scientific, Waltham MA, USA). Chromatography was performed using a 100 mm × 2.1 mm (i.d.), 1.9 μm, Hypersil GOLD C18 column (Thermo Scientific) using a 400 μL/min mobile phase gradient of 90:0:10 (0–5 min), 60:30:10 (25 min), 0:90:10 (50–55 min), 90:0:10 (57–60 min) H_2O/MeOH/MeOH + 5% HCOOH. Analyses were undertaken in positive ion mode, and ions generated by the source were measured at 30000 resolution and 2 ppm mass accuracy by the orbitrap analyzer. MS^n spectra were obtained by performing collision-induced dissociation of isolated precursor ions in the ion trap (isolation window, ±2 m/z; collision energy, 35%) and recording either low-resolution spectra with the ion trap analyzer or 7500 resolution spectra with the orbitrap analyzer.

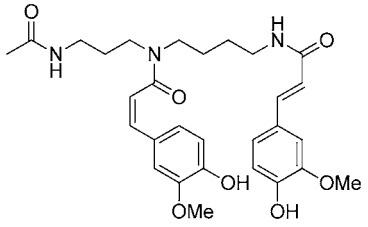
Spectroscopic and Spectrometric Measurements. NMR spectra were acquired in CD_3OD at 30 °C using a Bruker Avance 400 MHz instrument. Standard pulse sequences and parameters were used to obtain 1D ^1H, 1D ^{13}C, gradient-enhanced correlation spectroscopy (gCOSY), total correlation spectroscopy (TOCSY), gradient-enhanced heteronuclear single-quantum coherence (gHSQC), and gradient-enhanced heteronuclear multiple-bond correlation (gHMBC) spectra. Chemical shift referencing was carried out with respect to internal TMS at 0.00 ppm.

HRESIMS, MS^2, and UV data were obtained from LC-UV-MS analyses as described above.

*N^5,N^{10}-di-(E,E)-Feruloylspermidine (**1d** see Figure 1):* off-white solid; UV (mobile phase) λ_{max} 295sh, 319 nm; ^1H and ^{13}C NMR (CD_3OD, 400 MHz) see Table 2; HRESIMS m/z 498.2600 $[M + H]^+$ (calcd for $C_{27}H_{36}N_3O_6^+$ = 498.2599); ion trap MS^2 (m/z 498) 481 (25), 348 (5), 322 (64), 305 (14), 248 (9), 234 (100), 177 (21), 145 (3).



1d



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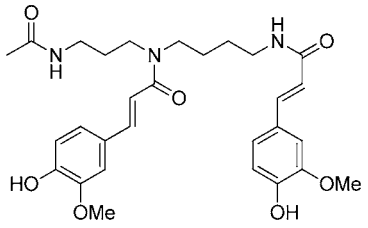
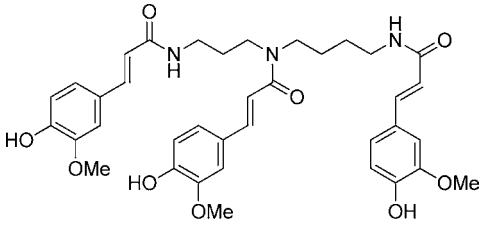


2d



3d

Figure 1. Structures of isolated acyl spermidines.

*N^1-Acetyl-N^5,N^{10}-di-(Z,E)-feruloylspermidine (**2c**):* off-white solid; UV (mobile phase) λ_{max} 232, 293, 312sh nm; ^{1}H and ^{13}C NMR (CD_3OD, 400 MHz) see Table 2; HRESIMS m/z 540.2703 $[M + H]^+$ (calcd for $C_{29}H_{38}N_3O_7^+$ = 540.2704). MS^n data were nearly identical to those of the (*E*,*E*) isomer **2d**.

*N^1-Acetyl-N^5,N^{10}-di-(E,E)-feruloylspermidine (**2d**):* off-white solid; UV (MeOH) λ_{max} 236, 297sh, 319 nm; ^{1}H and ^{13}C NMR (CD_3OD, 400 MHz) see Table 2; HRESIMS m/z 540.2704 $[M + H]^+$ (calcd for $C_{29}H_{38}N_3O_7^+$ = 540.2704); ion trap MS^2 (m/z 540), m/z (int) 480 (5), 364 (76), 346 (100), 171 (14); ion trap MS^3 (m/z 540 → 364), m/z (int) 347 (67), 265 (39), 248 (100), 214 (22), 188 (73), 177 (54), 171 (27), 145 (8), 117 (6).

*N^1,N^5,N^{10}-Tri-(E,E,E)-Feruloylspermidine (**3d**):* off-white solid; UV λ_{max} 295sh, 319 nm; ^{1}H NMR of feruloyl residues (CD_3OD, 400 MHz) N^5-feruloyl, δ 6.85 and 6.90 (both d, J = 15.3 Hz, H-α) 7.53 and 7.51 (both d, J = 15.3 Hz, H-β), 7.21 and 7.15 (both br m, H-2), 6.78 and 6.73 (both br m, H-5), 7.10 and 7.08 (both br m, H-6), 3.88 and 3.85 (both br s, 3-OCH_3); N^1 and N^{10}-feruloyl, δ 6.44 and 6.43 (both d, J = 15.6 Hz, H-α) 7.45 (d, J = 15.6 Hz, H-β), 7.11 and 7.04 (both br m, H-2), 6.79 and 6.78 (both br m, H-5), 7.02 and 6.98 (both br m, H-6), 3.87 (br s, 3-OCH_3); ^{13}C NMR of feruloyl residues (CD_3OD, 100 MHz) N^5-feruloyl, δ 115.1 (C-α), 144.5 (C-β), 112.0 (C-2), 116.3 (C-5), 123.2 (C-6), 56.4 (3-OCH_3); N^1 and N^{10}-feruloyl, δ 118.6 (C-α), 141.9 (C-β), 111.4 (C-2), 116.3 (C-5), 123.0 (C-6), 56.2 (3-OCH_3); HRESIMS m/z 674.3069 $[M + H]^+$ (calcd for $C_{37}H_{44}N_3O_9^+$ = 674.3072); ion trap MS^2 (m/z 674) m/z (int) 498 (42), 480 (100), 305 (2); ion trap MS^3 (m/z 674 → 498) m/z (int) 481 (26), 427 (2), 348 (6), 322 (66), 305 (12), 248 (9), 234 (100), 177 (20), 145 (3).

In Vitro Assays. Antioxidant activity was measured in a commercial Trolox equivalence antioxidant capacity assay kit (Sigma, Gillingham, UK), in accordance with the manufacturer's application notes. Absorbance measurements were performed in a 96-well plate format on an Infinite M200 (Tecan, Reading, UK).

RESULTS AND DISCUSSION

Analysis of Inflorescence Extracts of *S. nigra* and Identification of Isolated Compounds. LC-UV-MS analysis of a MeOH extract made from inflorescences of *S. nigra* revealed that among the major chromatographic peaks in the base ion chromatogram were two groups each of four peaks giving $[M + H]^+$ at m/z 498.260 (**1a**, **1b**, **1c**, **1d**) and m/z 540.270 (**2a**, **2b**, **2c**, **2d**) (Figure 2). For isomers **1a**–**d** accurate mass measurement predicted a likely molecular formula of $C_{27}H_{35}N_3O_6$, which suggested acylated spermidine derivatives,[12] and the ion trap MS^2 spectra of the protonated molecules, all of which were similar, showed abundant product ions at m/z 322, 234, and 177, in agreement with the main product ions reported in the collision cell spectra of protonated *N*,*N*-diferuloylspermidines.[13] Accurate mass measurements of these product ions (m/z 322.212 = $C_{17}H_{28}N_3O_3^+$; m/z 234.112 = $C_{13}H_{16}NO_3^+$; m/z 177.055 = $C_{10}H_9O_3^+$) were also in agreement with the fragments postulated by Youhnovski and co-workers.[13] Notably, the presence of an acyl fragment ion at only one m/z value (m/z 177 = feruloyl) supported the assignment of **1a**–**d** as diferuloylspermidines and not isomeric compounds acylated with two different acids that have the same combined molecular mass (such as *p*-coumaric and sinapic acids).

The assignment of the ^{1}H NMR spectrum of **1d** (Table 1) achieved by a combination of 1D and 2D methods was in good agreement with data previously published by Meurer and co-workers for N^5,N^{10}-di-(*E*,*E*)-feruloylspermidine (Figure 1),[14] although the additional spectral complexity ascribed by the latter to rotational isomerization of the N^5-feruloyl moiety was not observed. A full assignment of the ^{13}C NMR spectrum of **1d** is given for the first time in Table 2. Confirmation of the location and specific assignment of the N^5- and N^{10}-(*E*)-feruloyl moieties was obtained using connectivities detected in a gHMBC experiment between CH_2-4 and CH_2-6, and CH_2-9, with the noncoincident carbonyl carbons at δ_C 170.3 (N^5-feruloyl) and 169.5 (N^{10}-feruloyl), respectively.

Elution from C18 stationary phases of spermidine conjugates substituted at the same two positions with either the *E* and/or *Z* stereoisomers of a given hydroxycinnamic acid derivative has been found to be *ZZ*, *EZ*/*ZE*, *EE*.[13,15] The UV absorption spectra of the *E* and *Z* isomers of hydroxycinnammic acids differ, thus **1d**, bearing two ferulic acid groups both in the *E* configuration, had a UV spectrum showing a maximum at 319 nm with a shoulder at 295 nm, similar to that of (*E*)-ferulic acid and that recorded by Youhnovski and co-workers for a presumed *EE* isomer of diferuloylspermidine.[13] The UV spectrum of **1a** had an absorption maximum of 271 nm with a shoulder at 294 nm, similar to that recorded for a presumed *ZZ* isomer of diferuloylspermidine.[13] The UV spectra of **1b** and **1c** showed approximately equal intensity maxima at 293 and 312 nm and were similar to the spectrum of a mixture of **2b** and **2c**, the structures of which both comprise a (*Z*)- and an (*E*)-feruloyl moiety (see below). Thus, **1a** is likely to be N^5,N^{10}-di-(*Z*,*Z*)-feruloylspermidine, with **1b** and **1c** being the alternative *EZ* and *ZE* isomers.

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Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data for 1d, 2c, and 2d (CD_3OD, 30 °C)[a]

	1d		2c		2d	
atom	δ ^{1}H (J in Hz)	δ ^{13}C	δ ^{1}H (J in Hz)	δ ^{13}C	δ ^{1}H (J in Hz)	δ ^{13}C
spermidine						
2	2.93 m	38.2	3.19, 3.06 m	38.4, 38.1	3.22, 3.19 m	38.1, 38.2
3	1.97 m	27.1	1.81, 1.64 m	28.6, 29.7	1.87, 1.80 m	30.6, 28.8
4	3.59 m	44.1	3.45, 3.35 m	44.2, 48.0	3.55, 3.49 m	47.1, 45.7
6	3.60 m	48.8	3.45, 3.37 m	46.2, 49.9	3.58, 3.48 m	49.0, 47.7
7	1.74 m	27.8	1.69, 1.50 m	26.1, 27.1	1.73, 1.68 m	28.0, 26.4
8	1.65 m	28.0	1.57, 1.44 m	28.1, 27.8	1.63, 1.59 m	28.0, 28.0
9	3.37 m	39.8	3.34, 3.21 m	40.2, 39.9	3.36, 3.34 m	39.9, 40.2
N^1-acetyl						
CO				173.5, 173.4		173.6, 173.4
CH_3			1.94, 1.89 s	22.8, 22.7	1.94, 1.91 s	22.7
N^5-feruloyl						
CO		170.3		172.1		169.4
α	6.91 d (15.3)	114.6	5.99, 5.96 d (12.6)	121.5	6.89, 6.85 d (15.5)	115.5
β	7.58 d (15.3)	145.5	6.58 br d (12.6)	135.2	7.51 d (15.3)	144.7
1		128.4		128.8		128.7
2	7.23 d (2.0)	112.4	7.02 d (2.1)	113.1	7.21, 7.19 d (2.0)	112.4
3		149.5		149.0		149.5
4		150.0		148.8		150.2
5	6.80 d (8.2)	116.6	6.74, 6.73 d (8.2)	116.4	6.79 d (8.2)	116.6
6	7.11 dd (8.2, 2.0)	123.7	6.85, 6.84 dd (8.2, 1.9)	123.8	7.10 dd (8.2, 2.0)	123.5
3-OCH_3	3.89 s	56.6	3.81, 3.80 s	56.7	3.90, 3.89 s	56.7
N^{10}-feruloyl						
CO		169.5		169.3		169.4
α	6.39 d (15.6)	118.7	6.43, 6.37 d (15.7)	118.9	6.43, 6.38 d (15.7)	118.9
β	7.42 d (15.6)	142.2	7.45, 7.42 d (15.6)	142.2	7.44, 7.42 d (15.7)	142.2
1		128.3		128.4		128.5
2	7.05 d (2.0)	111.7	7.13, 7.12 d (2.1)	111.8	7.12, 7.06 d (2.0)	111.7
3		149.4		149.4		149.5
4		150.0		150.3		150.1
5	6.79 d (8.2)	116.6	6.80 d (8.2)	116.7	6.79 d (8.2)	116.6
6	6.98 dd (8.5, 2.0)	123.2	7.03 m	123.3	7.03 m, 6.99 dd (8.2, 2.0)	123.2
3-OCH_3	3.87 s	56.5	3.89, 3.88 s	56.6	3.88, 3.87 s	56.5

[a]Extensive resonance doubling was observed in the NMR spectra of **2c** and **2d** (multiplicity labels for duplicated resonances refer to both entries).

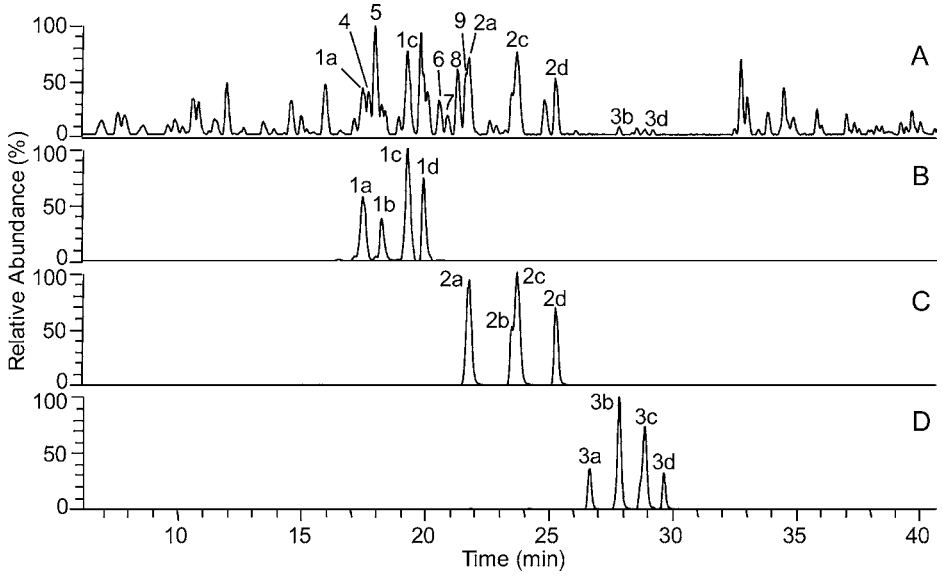

Figure 2. Positive ion LC-UV-ESI/MS analysis of an 80% aqueous MeOH extract of dried inflorescences of *Sambucus nigra*: (A) base ion chromatogram; (B) single ion chromatogram m/z 498; (C) single ion chromatogram m/z 540; (D) single ion chromatogram m/z 674. N^5,N^{10}-Diferuloylspermidine isomers (**1a–d**), N^1-acetyl-N^5,N^{10}-diferuloylspermidine isomers (**2a–d**), N^1,N^5,N^{10}-triferuloylspermidine isomers (**3a–d**), quercetin 3-*O*-glucoside (**4**), quercetin 3-*O*-rutinoside (**5**), kaempferol 3-*O*-glucoside (**6**), kaempferol 3-*O*-rutinoside (**7**), isorhamnetin 3-*O*-glucoside (**8**), isorhamnetin 3-*O*-rutinoside (**9**).

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The molecular formula determined for each of the four isomers **2a**−**d** giving protonated molecules at m/z 540.270 was $C_{29}H_{37}N_3O_7$, giving a difference from the formula of **1a**−**d** of C_2H_2O (corresponding to an acetyl group). The MS^2 spectra were all similar and showed major product ions as m/z 364.223 ($C_{19}H_{30}N_3O_4^+$), 346.212 ($C_{19}H_{28}N_3O_3^+$), and 171.149 ($C_9H_{19}N_2O^+$). The formula of m/z 171 is in accordance with an ammonia-loss ion of protonated *N*-acetylspermidine and, this, together with the observed loss of a feruloyl moiety ($C_{10}H_8O_3$) from $[M + H]^+$ to give the ion at m/z 364, suggested that **2a**−**d** could be *N*,*N*,*N*-acetyldiferuloylspermidines.

The ^1H NMR spectra of **2c** and **2d** exhibited extensive resonance doubling (Table 2). Use of gCOSY, gHSQC, and gHMBC data enabled the duplicated sets of methylene resonances of the spermidine moieties to be assigned to the 3- and 4-carbon sequences from CH_2-2 to CH_2-4 and CH_2-6 to CH_2-9, respectively. As expected, the methylene resonances of CH_2-2 were downfield shifted compared to **1d**, indicating that the N^1 nitrogen atom was acylated in addition to N^5 and N^{10} (Table 2). Analysis of the ^1H and ^{13}C NMR spectra confirmed that the *N*-acyl groups comprised one acetyl and two feruloyl residues, as predicted by MS^2. The magnitudes of the coupling constants for the α-H and β-H resonances of the feruloyl residues confirmed that **2c** contained an (*E*)- and a (*Z*)-feruloyl group and **2d**, two (*E*)-feruloyl groups (Table 2). Thus, for the (*Z*)-feruloyl group of **2c**, $J_{\alpha,\beta}$ = 12.6 Hz, and for the (*E*)-feruloyl groups of **2c** and **2d**, $J_{\alpha,\beta}$ = 15.3−15.7 Hz. The specific location of each acyl group was determined using connectivities detected in gHMBC spectra. For **2c**, the key correlations observed were from CH_2-2 to the carbonyl carbon of the acetyl group (δ_C 173.5, 173.4), CH_2-4 to the carbonyl carbon of the (*Z*)-feruloyl group (δ_C 172.1), and CH_2-9 to the carbonyl carbon of the (*E*)-feruloyl group (δ_C 169.3). Similarly for **2d**, CH_2-2 correlated to the carbonyl carbon of the acetyl group (δ_C 173.6, 173.4) and both CH_2-4 and CH_2-9 to the carbonyl carbons of the two (*E*)-feruloyl groups (δ_C 169.4). Thus, **2c** and **2d** were N^1-acetyl-N^5,N^{10}-di-(*Z*,*E*)-feruloylspermidine and N^1-acetyl-N^5,N^{10}-di-(*E*,*E*)-feruloylspermidine, respectively. The downfield shift of H-α of the N^5-(*E*)-feruloyl group (6.85−6.91 ppm) compared to that of the N^{10}-(*E*)-feruloyl group (6.38−6.43 ppm) in ^1H NMR spectra acquired in CD_3OD for these diferuloylspermidines is consistent with previous correlations of chemical shift values[14] and provides a useful diagnostic feature for assigning sites of *N*-acylation (Table 2). In the sample containing **2c**, a second *N*,*N*,*N*-acetyldiferuloylspermidine that also comprised an (*E*)- and a (*Z*)-feruloyl group was present as a minor component (**2b**). The H-α and H-β resonances of the latter at δ_H 6.87 (overlapped; δ_C 115.6) and δ_H 7.51 (br d, J = 15.3 Hz; δ_C 144.8), respectively, were thus those of a N^5-(*E*)-feruloyl group. For the (*Z*)-feruloyl group, the H-α and H-β resonances were duplicated at δ_H 5.85 and 5.80 (both d, J = 12.6 Hz; δ_C 122.1) and δ_H 6.63 and 6.61 (d, J = 12.6 Hz and obscured; δ_C 138.2), respectively. Thus, **2b** was tentatively assigned the structure of N^1-acetyl-N^5,N^{10}-di-(*E*,*Z*)-feruloylspermidine. The UV spectra of **2a**, **2b**/**c**, and **2d** were similar to those of **1a**, **1b**/**c**, and **1d**, respectively. Thus, **2a** is likely to be N^1-acetyl-N^5,N^{10}-di-(*Z*,*Z*)-feruloylspermidine.

Among the minor chromatographic peaks observed in the extract of *Sambucus* inflorescences were a group of at least four peaks (**3a**−**d**) giving protonated molecules at m/z 674.307 ($C_{37}H_{44}N_3O_9^+$) (Figure 2), which, upon MS^2 analysis, showed the loss of a feruloyl moiety ($C_{10}H_8O_3$) to yield an ion at m/z 498.260 ($C_{27}H_{36}N_3O_6^+$) that had a product ion spectrum similar to that of the diferuloylspermidines **1a**−**d** following MS^3 (m/z 674 → 498) analysis. The last-eluting of these triferuloylspermidines (**3d**) had a UV spectrum consistent with the *EEE* isomer, the configurational assignment of which was confirmed by NMR analysis of the isolated compound. Although the ^1H NMR spectrum of **3d** in CD_3OD exhibited extensive resonance doubling and broadening, the magnitudes of $J_{\alpha,\beta}$ for the α-H and β-H resonances of the constituent feruloyl residues were all within the range of 15.3−15.6 Hz, as expected for the *E* configuration. Thus, **3d** was N^1,N^5,N^{10}-tri-(*E*,*E*,*E*)-feruloylspermidine, a known derivative reported previously from the pollen of *Hippeastrum* × *hortorum* and later as keayanidine C from the roots of *Microdesmis keayana*.[16,17] Adequate UV spectroscopic data on **3a**−**c** were difficult to obtain from the extract analysis, but these suggested that **3a** was the *ZZZ* isomer, whereas **3b** and **3c** were two or more of the *EZZ* and *EEZ* isomers.

Several series of other minor acylated spermidines were detected in LC-UV-MS analyses of *S. nigra* inflorescence extracts, including *N*-caffeoyl-*N*,*N*-diferuloylspermidines ($[M + H]^+ = m/z$ 660.292, $C_{36}H_{42}N_3O_9^+$), *N*-coumaroyl-*N*,*N*-diferuloylspermidines ($[M + H]^+ = m/z$ 644.298, $C_{36}H_{42}N_3O_8^+$), *N*-caffeoyl-*N*-feruloylspermidines ($[M + H]^+ = m/z$ 484.245, $C_{26}H_{34}N_3O_6^+$), *N*-coumaroyl-*N*-feruloylspermidines ($[M + H]^+ = m/z$ 468.249, $C_{26}H_{34}N_3O_5^+$), *N*-acetyl-*N*-caffeoyl-*N*-feruloylspermidines ($[M + H]^+ = m/z$ 526.255, $C_{28}H_{36}N_3O_7^+$), and *N*-acetyl-*N*-coumaroyl-*N*-feruloylspermidines ($[M + H]^+ = m/z$ 510.260, $C_{28}H_{36}N_3O_6^+$).

Location of Acyl Spermidines in *Sambucus* Inflorescences. LC-UV-MS analyses of extracts from individual flowers dissected into anther tissue and the remaining emasculated floral tissue revealed acyl spermidines as the major compounds in the anther tissue but only trace components in the floral tissue, possibly from contamination with anther tissue or pollen (Figure 3). Pollen collected from *S. nigra* also contained acyl spermidines as the major components detected by LC-UV-MS analysis, with the only other major component being naringenin. Thus, acyl spermidines appear to be pollen specific metabolites in *S. nigra*, which is in accordance with reports on this class of compounds from pollen from several other sources.[13,14,16,18−21] LC-UV-MS analysis of the emasculated flower extract revealed numerous components among which were assigned the 3-*O*-glucosides and 3-*O*-rutinosides of quercetin (**4** and **5**, respectively), kaempferol (**6** and **7**), and isorhamnetin (**8** and **9**), and caffeoylquinic acid isomers, in accordance with previous studies.[9,22,23]

Occurrence of Acyl Spermidines in Commercial Elderflower Products. The major acyl spermidines detected in extracts of *S. nigra* inflorescences were readily revealed in LC-UV-MS analyses of three examples of commercially sold cold drinks containing elderflower by displaying relevant single ion chromatograms (Figure 3). In a hot water infusion made from the fruit/herbal "tea" bag, the acyl spermidines were among the largest peaks in the base ion chromatogram. Among other compounds noted in these drink products were many of the phenolic compounds detected in the *S. nigra* inflorescence analyses, with the flavonoids rutin (**5**) and isorhamnetin 3-*O*-rutinoside (**9**) producing prominent chromatographic peaks.

Occurrence of Acyl Spermidines in Other *Sambucus* Species. Acyl spermidines were detected in extracts from inflorescences of the three other *Sambucus* species examined. In two herbaceous species, *Sambucus ebulus* and *Sambucus adnata*, the chromatographic profiles of the acyl spermidines **1**−**3** were similar to that of *S. nigra*, and the profile in *S. nigra* was consistent among all examples of this species analyzed.

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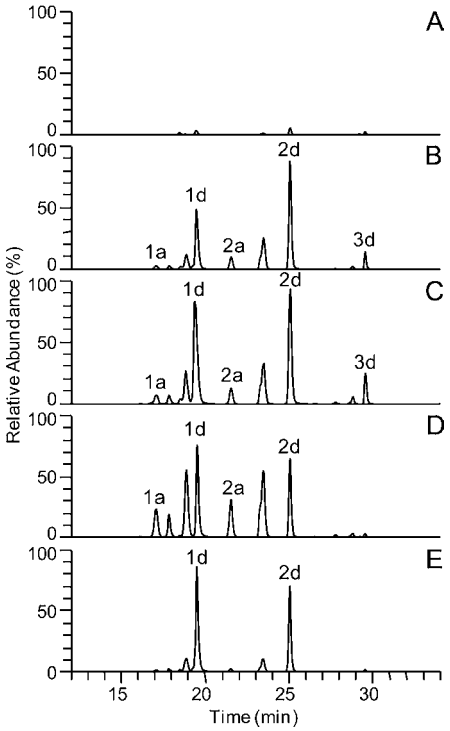

Figure 3. Combined single ion chromatograms at *m/z* 498 (showing **1a–d**), 540 (**2a–d**), and 674 (**3a–d**) from positive ion LC-UV-ESI/MS analyses of (A–C) 100% MeOH extracts of fresh *Sambucus nigra* tissues (A, emasculated flowers; B, anthers; C, pollen) and (D, E) commercial elderflower drinks (D, Elderflower & Rose, BI-19669; E, Cranberry, Raspberry and Elderflower fruit/herbal infusion, BI-19546). All chromatograms are scaled to the 100% relative abundance of **2d** in chromatogram C.

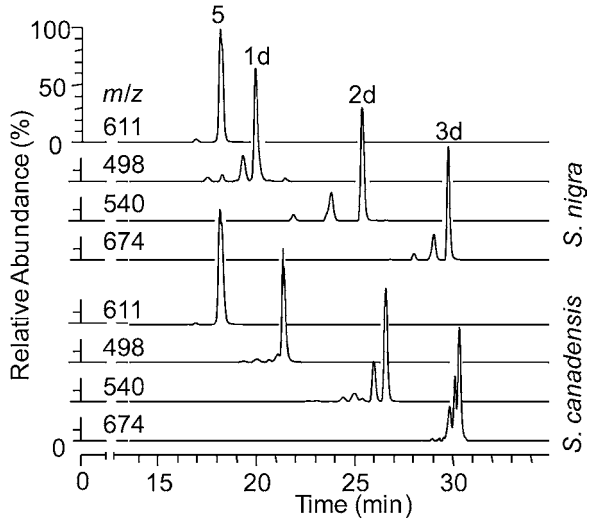

Figure 4. Single ion chromatograms from positive ion LC-UV-ESI/MS analyses of 100% MeOH extracts of fresh inflorescences of *Sambucus nigra* and *Sambucus canadensis* showing the elution profiles of acyl spermidines relative to rutin, **5** (showing the same t_R in both analyses).

However, the LC-UV-MS profile of acyl spermidines from the two specimens studied of *S. canadensis* (American elder), although similar to each other, differed from the profile obtained for *S. nigra*.

In the *S. canadensis* analyses, groups of acyl spermidine isomers having the same molecular formulas and similar MS^2 spectra as **1–3** were present, but each isomeric group had a longer retention time compared to the corresponding isomers in the *S. nigra* analyses (Figure 4). The MS^2 spectra of these compounds differed only from **1–3** in the relative abundance of some ions, and the difference in retention time was not due to chromatographic drift as common components in the extracts of the two species, such as rutin (**5**), had identical t_R values, and the compounds did not coelute in an analysis of a mixed extract of *S. nigra* and *S. canadensis*. Bigler and co-workers showed that the three positional isomers of *N,N*-diacylspermidines undergo transamidation under electrospray conditions and so give similar MS^2 spectra.[24] Thus, it is possible different positional isomers of *N,N*-diferuloylspermidine and *N*-acetyl-*N,N*-diferuloylspermidine are produced by *S. canadensis* compared with *S. nigra*; however, this does not explain the longer elution time of the triferuloylspermidines as the *N,N,N*-substituted form cannot have positional isomers. Eight stereoisomers of *N,N,N*-triferuloylspermidines are possible, and only four were resolved in the analyses of *S. nigra*, and so the remaining stereoisomers may account for these peaks in *S. canadensis*; alternatively, the compounds may be triferuloylspermidine isomers with one disubstituted and one unsubstituted amine function. Regardless of the identity of these compounds, the differing chromatographic profiles of acyl spermidines extracted from *S. canadensis* and *S. nigra* inflorescences, if found to be consistent with wider sampling, could provide a means of differentiating commercial elderflower extracts from these two sources.

Biological Significance of Acyl Spermidines in Elderflower. The first published survey of amides of spermidine and phenylpropanoic acids in plants reported these derivatives from 11 angiosperm families and implied that these compounds were widespread.[10] Further papers have confirmed this.[14,15,17–21,25–29] Recently, polyamine amides were reviewed by Bassard and co-workers,[11] who concluded that di- and trisubstituted hydroxycinnamoyl conjugates, particularly of spermidine and putrescine, are major metabolites of pollen. Their importance in flower development and reproduction has been substantiated by metabolomic and genomic studies.[30,31] Acylated spermidines are suggested to have an ecological role as defense compounds against viruses, bacteria, and fungi.[10,11] The spermidine amides show structural similarity to certain spider and wasp toxins and could deter herbivores from eating plants.[32,33] However, although the compounds show in vitro activity on neuroreceptors, their toxicity is low when ingested by insects.[34]

Given the widespread occurrence of acyl spermidines in pollen, it is surprising that they have not been reported previously in analyses of elderflower extracts. It is also of interest that a new series of N^1-acetyl-N^5,N^{10}-diferuloylspermidines should be found in elderflower and the widely consumed drinks made from this material. In previous HPLC analyses of elder inflorescence extracts, the major UV-absorbing compounds were associated only with flavonoids and phenolic acid derivatives comprising caffeoyl-, *p*-coumaroyl-, and di-*O*-caffeoylquinic acids.[7,9]

The occurrence of acyl spermidines in elder inflorescences may be of relevance to its traditional medicinal use. In activity-guided isolation studies of species of Asteraceae, tricoumaroyl spermidine was found to inhibit HIV-1 protease and a serotonin transporter.[35,36] In the present study, acyl spermidines tested for their antioxidant capacity in the TEAC assay showed negligible activity. However, the activities given for acyl spermidines in the literature indicate that the feruloyl spermidines reported in the

present work may contribute to the effects of elderflower preparations as traditionally used for alleviating common cold symptoms.

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ABBREVIATIONS USED

gCOSY, gradient-enhanced correlation spectroscopy; gHMBC, gradient-enhanced heteronuclear multiple-bond correlation; gHSQC, gradient-enhanced heteronuclear single-quantum coherence; TEAC, Trolox equivalence antioxidant capacity; TOCSY, total correlation spectroscopy

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