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POLYAMINES AND LEAF SENESCENCE IN PYRROLIZIDINE ALKALOID-BEARING *HELIOTROPIUM* PLANTS

HELENA BIRECKA, TAMARA E. DINOLFO, WILLIAM B. MARTIN* and MICHAEL W. FROHLICH

Department of Biological Sciences and *Department of Chemistry, Union College, Schenectady, NY 12308, U.S.A.

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Key Word Index—*Heliotropium*; Boraginaceae; *Avena*; Gramineae; polyamines; putrescine; spermidine; homospermidine; spermine; 1,3-diaminopropane; pyrrolizidine alkaloids; senescence.

Abstract—Putrescine, spermidine and spermine were found in leaves and inflorescences of *H. angiospermum* and *H. indicum* plants; the levels of these amines declined with leaf age. In addition, homospermidine was identified in the inflorescence axes and youngest leaves of *H. indicum*. The youngest tissues exhibited the highest levels of both putrescine and pyrrolizidine alkaloids. The detection of homospermidine in the plants supports the theory that the pyrrolizidine moiety is derived from two molecules of putrescine with homospermidine as an intermediate. In the youngest organs, the pyrrolizidines represented over 5% of the total nitrogen content. Their level was 50–100 fold higher than that of the polyamines, including putrescine. When detached and kept in the dark for 100–120 hr, mature older *Heliotropium* leaves, with a very low polyamine content, exhibited only a weak senescence syndrome. By contrast, in detached, darkened leaves of *Avena sativa* and *Nicotiana alata* having high polyamine levels, the chlorophyll and protein degradation and increases in free amino acids were very pronounced.

INTRODUCTION

The classical hypothesis, according to which the amino alcohol (necine) moiety of pyrrolizidine alkaloids (PAs) derives from two putrescine (Put) molecules via a symmetric intermediate C_4–N–C_4 [1], has found strong support in recent studies on *Senecio isatideus*, and evidence for *sym*-homospermidine (homoSpd) as an intermediate in the biosynthetic pathway was obtained [2–6]. Labelled homoSpd was isolated from plants fed with D,L-[5-^{14}C] ornithine [4]; an *in vitro* transformation of intact homoSpd into trachelanthamidine was demonstrated using a pea diamine oxidase and liver alcohol dehydrogenase system [5].

Free homoSpd is a rather uncommon polyamine; it has previously been found only in *Santalum album* leaves and in some algae, bacteria and amphibia [7–11]. Contrary to the previously suggested biosynthetic route involving diamine oxidase [12], it has been recently shown that homoSpd can be formed from Put without any participation of the oxidase; an NAD-dependent enzyme catalysing homoSpd synthesis from Put with concomitant liberation of ammonia has been isolated from *Lathyrus sativus* seedlings, in which homoSpd could not be detected, and from sandal leaves in which this polyamine reaches 1.5% dry weight [13].

In young, non-flowering plants of *Heliotropium spathulatum*, exposed to pulse-labelling with $^{14}CO_2$, leaves were the main organs in which necine biosynthesis took place, this process being independent of light [14]. The youngest organs, leaves or inflorescences, exhibited the highest PA levels; an alkaloid gradient sharply decreasing with age was found in leaves of *H. spathulatum* and *H. indicum*, and, surprisingly, detached mature leaves of these species showed little or no loss of chlorophyll even after a 5–6 day exposure to darkness [15].

Detachment and darkness are known to accelerate senescence in leaves, in particular in oats [16, 17]. Senescing tissues often increase production of ethylene [18–20], a hormone also associated with a tissue response to wounding or infection [21–23]; in a few cases added C_2H_4 was shown to promote chlorophyll losses in leaves exposed to darkness [16, 19, 24]. In detached oat leaves, C_2H_4 had no or a slight effect in darkness, but greatly increased chlorophyll losses in light [16, 25]. Exogenous polyamines, including diamines, have been reported to retard chlorophyll losses, protein degradation and/or the increase in RNase activity in detached oat and radish leaves in dark [26–29] and also to reduce ethylene production during dark- or wound-induced tissue senescence [30–33]. In oat leaf segments, exogenous Put and spermidine (Spd) partially prevented chlorophyll losses in the dark, but greatly increased them in light [34].

Polyamines, among which Put, Spd, and spermine (Spm) are the most common, occur at highest levels in young, dividing and/or differentiating tissues and decline with age in various organisms including higher plants; the bulk of these compounds has been localized in the vacuole, like alkaloids, or in the cytosol [35–40]. Their functions may be nonspecific, replaceable by other cations, or specific, nonreplaceable because of their specific linkages to specific macromolecules [41]. There is much evidence that polyamines fulfil essential roles in DNA, RNA, and protein synthesis, structure, and functions, and also in stabilizing plasma and organelle membranes [41–46]. It has been suggested that they may constitute a new class of plant hormones [47, 48].

There is no information available on naturally occurring polyamines in any PA-bearing plant species; all our knowledge is based on incorporation of introduced labelled Put, or its known precursors, ornithine and arginine, or its products, Spd and Spm, into PAs [2, 3, 49].

This study reports the qualitative and quantitative patterns of free polyamines in leaves and inflorescences of *H. indicum* and *H. angiospermum* plants. An attempt has also been made to find an explanation for the previously observed behavior of detached leaves in darkness; for comparison detached mature leaves of oats and of *Nicotiana alata* and the effects of exogenous C_2H_4 on leaf senescence were also studied.

RESULTS AND DISCUSSION

Both *H. indicum*, an annual, and *H. angiospermum*, a perennial, grow by a series of sympodial shoots which terminate in inflorescences. Renewal shoots form from axillary buds most commonly near the top of the older shoots, but occasionally near the base as well. Due to this growth pattern both old and young leaves occur at various heights on a plant.

In leaves and inflorescences of *H. indicum*—regardless of their age—over 94% of the PA necine moiety was represented by retronecine while the remainder was trachelanthamidine, supinidine and traces of necines with mass spectra of lindelofidine and pyrrolizidine-2,9-diol, [4, 50, 51]; nonesterified necines amounted to 10–15% of the total; about 70–80% of all PAs were found in the form of *N*-oxides. In leaves and inflorescences of *H. angiospermum*, 1β,2β-epoxy-l-hydroxymethyl-pyrrolizidine, previously identified in field collected and greenhouse grown plants of this species [15], amounted to over 97% of total PA, the remaining being represented by a pyrrolizidine-2,9-diol; the epoxypyrrolizidine was found only in the nonesterified forms; *N*-oxides amounted to 75–80% of total PAs.

Table 1 represents typical results obtained with leaves of various ages and very young inflorescences sampled three (*H. angiospermum*) and four (*H. indicum*) times from young and old plants between September 1982 and March 1983. The total N content per dry weight in *H. indicum* was somewhat higher than that in *H. angiospermum*; the share of the TCA insoluble portion amounted to about 70% of the total. Inflorescences and the youngest leaves, including apices, showed the highest total N; a decrease of about 20–25% with the leaf age was observed. In both species the necine contents were also the highest in the youngest organs, representing over 5% of the total N. As only one N atom from two Put molecules can be incorporated into a pyrrolizidine molecule, a very significant portion of the tissue N might have been involved in synthesis of Put; one deals here, apparently, with a huge 'factory' of Put. The biosynthetic studies mentioned above leave little room for the possibility that their results could have been affected by aberrant transformations.

TLC of dansylated polyamines and GC/MS of trifluoroacetylated derivatives revealed the presence of Put, Spd and Spm in all tested organs of both species. No reliable data could be obtained for agmatine (Agm) due to (a) significant losses of authentic Agm during dansylation, (b) Agm R_f value similar to that of Put in both solvents used for TLC and (c) apparently unsuccessful trifluoroacetylation of Agm, followed by GC/MS. However, it is unlikely that Agm, if present, could have significantly affected the quantitative determination of Put; the differences between Put and Spd in the peak heights and total abundances observed during GC/MS resembled those observed after TLC. The search for homoSpd was successful with *H. indicum*; it was detected by GC/MS in traces in the youngest leaves and in amounts of about 15–20% of the Spd content in the inflorescence axis, i.e. in organs with the highest PA and polyamine contents. This is the first report of homoSpd naturally occurring in a PA-bearing plant species. The search for homoSpd in *H. angiospermum* by other methods is continuing; homoSpd apparently is of very transient existence in the tissues.

Of the three common polyamines found in the plants, Put was the dominant and Spd the minor one. The inflorescence, especially in *H. angiospermum*, exhibited relatively high Put levels; it seems that this plant part may be also an important site of pyrrolizidine biosynthesis. The polyamines, amounting only to 1–2% of the PA contents, declined with leaf age. However this decline, like that of N, was relatively small as compared with the sharp, age-related decrease in PAs. A previous study [15] indicated that the decrease may be due to PA export rather than to degradation *in situ*. This study supplies additional evidence strongly supporting the proposed biosynthetic pathway of pyrrolizidines and indicates a concurrence of a high rate of tissue growth and high Put and pyrrolizidine levels. However, nothing is known about the enzymes involved in synthesis of these compounds *in situ*.

The fact that labelled L-ornithine and L-arginine are good precursors of retronecine in *Senecio* indicates only that ornithine and arginine decarboxylases may both be operating in the plants. However, this cannot serve as a reliable index of the relative involvement of these enzymes in Put biosynthesis *in situ*; of course, nothing is known about the age-related activities of these decarboxylases in PA-bearing plants. The report that labelled Spd and Spm were as efficient precursors as was Put [2] raises some questions. When oxidized by polyamine oxidases, these two polyamines yield, in cereals, pyrroline and aminopropylpyrroline, respectively, but do not yield Put [37]. The formation of Put from Spd and Spm is known to occur in mammalian cells and requires *N*-acetylation of the polyamines prior to oxidation [52]; no acetylated polyamines have been reported to occur in plants. However, even if acetylation does occur, one would expect that the internal dilution factors during conversions would decrease the efficiency of the label incorporation into the pyrrolizidines.

If, in fact, very high Put production is a specific feature of nitrogen metabolism in *Heliotropium* or other PA-bearing genera, one might speculate that conversion of Put into pyrrolizidines may be an effective way of removing (controlling) and detoxifying this diamine and also of releasing the nitrogen for reutilization in the cells, in addition to any other possible role of the polyamines and/or PAs.

Representative results obtained with detached leaves in several experiments with each of the four species are included in Table 2; the experimental errors were relatively small, especially with *Heliotropium* and *N. alata*, due to the use of half-leaves as controls. Unfortunately, it was impossible to establish with full certainty the name of one of the two oat cultivars tested. The cv. Garry is genetically very close to cv. Victory which has been used in most investigations on oat leaf senescence; in a previous study [17] its first leaf responses to kinetin, dipyridyl or EDTA in darkness were similar to those reported for cv. Victory. This study shows chlorophyll degradation in both varieties, decrease in soluble protein, and increase in free amino acid levels closely resembling responses of oat

Table 1. Chlorophyll, nitrogen, soluble protein, alkaloid and polyamine contents (per fresh wt)

Organ	Dry matter (%)	Chlorophyll Total (mg/g)	Chlorophyll Chlor. *a* (% total)	Nitrogen-% Total	Nitrogen-% TCA insol.*	Soluble proteins† (%)	Alkaloid‡ (μmol/g)	Polyamines§ (nmol/g) Put	Polyamines§ (nmol/g) Spd	Polyamines§ (nmol/g) Spm
				Heliotropium angiospermum—sampled October 7, 1982						
Inflorescence	27.5	0.86	70	0.60	0.43	0.71	32.5	585	163	87
Leaves										
Youngest and apices	24.9	2.64	66	0.61	0.44	0.62	34.7	205	64	43
Half-expanded	22.5	4.18	69	0.56	0.39	0.37	13.6	140	49	31
Mature	22.0	3.77	69	0.49	0.35	0.28	7.5	101	22	30
Yellowing	21.1	1.51	67	0.37	0.28	0.26	2.2	28	20	15
				Heliotropium indicum—sampled October 9, 1982						
Inflorescence	21.4	0.83	67	0.50			34.7	215	83	32
Leaves										
Youngest and apices	19.6	2.94	74	0.48			17.6	101	60	31
Half-expanded	17.7	4.52	78	0.41			8.3	66	53	27
Mature	16.4	2.89	75	0.36			1.2	40	35	22
Yellowing	15.1	1.52	77	0.34			0.8	23	36	15
				Heliotropium indicum—sampled January 6, 1983						
Inflorescence										
Flowers	19.7	0.29	63	0.56	0.38	0.51	27.1	187	90	38
Axes.	18.1	0.76	74	0.59	0.40	0.41	26.5	347	126	40
Leaves										
Youngest and apices	17.8	2.86	75	0.56	0.36	0.40	18.4	78	46	26
Fully expanded	17.0	3.11	76	0.48	0.33	0.38	3.1	59	29	18
Old green	16.7	2.31	77	0.44	0.31	0.33	1.5	28	27	14

*Nitrogenous compounds insoluble in 5% trichloroacetic acid.

†Determined using BSA as the standard protein.

‡Calculated as epoxyhydroxymethylpyrrolizidine and retronecine for *H. angiospermum* and *H. indicum* respectively.

§Putrescine, Put; spermidine, Spd; spermine, Spm. Homospermidine was detected in *H. indicum*; in the youngest leaves as traces and in the inflorescence axes at about 15–20% of the Spd content.

Table 2. Chlorophyll, nitrogen, soluble protein, free amino acid, alkaloid and polyamine contents of detached leaves exposed to darkness* (per fresh wt)

Exposure	Hours†	Chlorophyll Total (mg/g)	Chlorophyll Chlor. *a* (% total)	Nitrogen-% Total	Nitrogen-% TCA insol.	Soluble proteins (%)	Free amino acids‡ (%)	Alkaloid (μmol/g)	Polyamines (nmol/g) Dap§	Put	Spd	Spm
					Heliotropium angiospermum							
Air	0	3.22	67	0.47	0.34	0.28	0.025	2.32		35	23	14
	100	2.85	73	0.49	0.33	0.24	0.037	2.20		31	21	13
C_2H_4	0	3.27	65	0.48	0.35	0.29	0.021	2.45		32	22	12
	100	2.80	75	0.48	0.32	0.26	0.036	2.51		34	20	13
					Heliotropium indicum—October							
Air	0	2.96	72	0.37	0.28	0.32	0.027			26	30	11
	102	2.50	74	0.36	0.28	0.30	0.047			28	24	13
C_2H_4	0	2.89	76	0.35	0.27	0.33	0.026			27	28	12
	102	2.48	75	0.35	0.26	0.29	0.049			28	31	11
					Heliotropium indicum‖—January							
Air	0	2.20	77	0.44	0.31	0.36	0.028	1.50		24	28	11
	96	2.23	75	0.44	0.32	0.32	0.067	1.53		22	29	15
					Nicotiana alata‖							
Air	0	1.68	57	0.25		0.22	0.011			174	161	40
	102	0.76	68	0.24		0.15	0.025			159	166	46
					Avena sativa cv. Astro (or Orbit)‖							
Air	0	2.14	65	0.47	0.33	0.38	0.049		170	1186	98	64
	72	0.80	63	0.44	0.19	0.29	0.158		202	1005	103	79
	106	0.26	66	0.44	0.16	0.22	0.158		187	1202	87	85
					Avena sativa cv. Garry							
Air	0	1.88	72						82	465	87	28
	72	0.69	75						91	424	95	30

*In experiments with *H. angiospermum*, *H. indicum*, and *N. elata*, old leaves were halved; one half was sampled immediately (time 0), the other half exposed to darkness. See also footnotes to Table 1.

†At time 0, the dry matter contents of *H. angiospermum*, *H. indicum* (October), *H. indicum* (January), *N. elata* and *A. sativa* leaves were 22.1; 15.8; 16.9; 8.3; and 14.0%, respectively.

‡Leucine was used as the standard.

§1,3-Diaminopropane, Dap.

‖Similar results were obtained with leaves exposed to C_2H_4.

leaves to darkness reported by others. In C_2H_4-exposed oat leaf segments, the chlorophyll content was sometimes lower by 10–15% than in the air-exposed ones; however these differences were not consistent.

In both varieties Put, Spd, Spm and 1,3-diaminopropane (Dap) were identified. The presence of Dap has been previously reported in oats [39, 53], barley [36], *Helianthus tuberosus* [54] and as a trace in *Amaranthus hypochondriacus* [53]. Its occurrence in relatively large amounts may indicate a significant oxidation of free Spd and/or Spm although the half-lives of these polyamines are reported to range between 10 and 42 days, at least in mammals [52]. Dap contents in oat leaves were similar to combined amounts of Spd and Spm, but they were only about 15–20% of the Put levels. The latter were extremely high, even above those found in the inflorescences of *Heliotropium* and resembled Put levels reported for barley seedlings [36] rather than for oat seedlings [39, 53, 55].

In contrast to the reported decreases in polyamine levels in detached and darkened oat leaves as well as in similarly treated radish leaves which contained over 7 μmol of polyamines per g fresh weight [39, 56], no significant declines could be observed in either oat variety. Neither were decreases found in *N. alata* leaves which likewise exhibited the typical dark-induced senescence syndrome and revealed polyamine levels much above those in *Heliotropium* leaves. One should add that in this study the cutting injury inflicted on leaves was minimal; they were placed in a water vapor saturated atmosphere; they were not floated on water as in most studies of leaf senescence.

Of the four experiments with each of the *Heliotropium* species, in only two was even a slight chlorophyll decline observed after a 4–5 day dark exposure. However, even when no chlorophyll degradation was found, a small decrease in soluble proteins and a more pronounced increase in free amino acids was noticed. No significant changes in either the PA or polyamine levels could be detected. Thus, leaves with a huge content of polyamines revealed a strong senescence syndrome which—as reported—could be prevented by exogenous polyamines and *Heliotropium* leaves with extremely low polyamine levels exhibited only a weak senescence syndrome.

It has been suggested that promotion and prevention of senescence in the dark can be related to closure and opening of stomates, respectively [16, 57]. When tested with a stomatal porometer for 36 hr at 3–9 hr intervals, the stomates of *Heliotropium* leaves proved to be closed during dark exposure. The possible relation between chlorophyll degradation and accumulation of H_2O_2 which might be prevented by peroxidase [16, 17] was also tested. The soluble peroxidase activity (per fresh weight) in oat leaves was about 80 and 140% higher than that in leaves of *H. indicum* and *H. angiospermum*, respectively. In all three species only small increases in the enzyme activities occurred during dark exposure.

In senescing leaves of a non-yellowing *Festuca pratensis* mutant, the thylakoid membranes and chlorophyll were found to be preserved while the stroma matrix in the chloroplasts was destroyed [58]. Chlorophyll degradation is apparently associated sequentially with chloroplast degradation by vacuolar proteases, increased chlorophyllase activity and an increased access to chlorophyll for this enzyme due to thylakoid membrane destruction [24, 58, 59]. In dark exposed barley leaf discs, exogenous polyamines, apparently due to their cationic nature, prevented chlorophyll losses by preserving the thylakoid membrane structure, although protein losses and the decline in photosynthetic activities were greater in the treated discs than in the untreated ones [60, 61]. Thus, it is quite possible that also in other cases the exogenous polyamines could have a nonspecific action on thylakoids and/or tonoplast, an action apparently not exercised by endogenous polyamines in spite of their high accumulation. On the other hand, one could speculate that in *Heliotropium* endogenous polyamines might fulfil a specific function in relation to membranes. Obviously, there may be many other factors not related to polyamines, which could be responsible for the behavior of *Heliotropium* leaves in darkness.

EXPERIMENTAL

Plant material. Plants were grown in the greenhouse under a 16 hr photoperiod; *H. indicum* from seeds collected in 1980 from greenhouse grown plants [15], *H. angiospermum* from cuttings of plants grown from seeds collected in Queretaro, Mexico [62]. The youngest parts of inflorescences and leaves of various ages were collected from young and older plants and immediately frozen. *Nicotiana alata* and *Avena sativa* plants were grown from seed. One of the two oat varieties could have been Astro or Orbit; the other was cv. Garry previously used in studies on peroxidase [17]. All plants were grown in a rich general purpose soil mixture and fertilized periodically with N, P, and K.

In experiments with detached older leaves of *H. angiospermum*, *H. indicum* and *N. alata*, the leaves were washed with H_2O, blotted and divided along the mid-vein. Both half-leaves were weighed, one of them immediately frozen, the other one exposed to continuous darkness in the presence or absence of ethylene added at 500 μl/l. In experiments with oats, 5 cm long apical segments of the first leaves were excised from 9–11 day old plants, weighed and treated as above. For comparison, in some cases the bottom 4 cm segments or apical segments of the second leaves were used. They yielded results similar to those obtained with the apical segments of the first leaves. The leaves were placed in air-tight plexiglass boxes in a water vapor-saturated atmosphere as previously [22]. The dark exposure at room temp. ranged between 72 and 120 hr.

Analyses. The plant material was extracted with cold 80% Me_2CO for chlorophyll determination [63]. For total N, trichloroacetic acid (TCA)-insoluble N, and soluble proteins, the tissue was homogenized in 0.1 M KPi buffer, pH 6.8. A portion of the homogenate was directly digested, another one was precipitated with 5% TCA (final conc) and the ppt digested with conc. H_2SO_4 in Kjeldahl flasks. The remaining homogenate was centrifuged and the supernatant used for soluble protein and peroxidase determinations. Total and TCA insoluble N was determined by Nesslerization. Soluble protein was assayed using Coomassie Blue [64] with BSA as the standard. The results were compared with those obtained from the Lowry method (BSA as the standard), which required protein pptn with TCA, followed by resolubilization with 0.3 M NaOH. Significant quantitative differences, over 30%, were found between these two methods, but the observed trends were the same in both.

Peroxidase was determined as described earlier [21]. Free amino acids were extracted with 80% EtOH and assayed spectrophotometrically [65]. Alkaloids were exhaustively extracted with EtOH and assayed as previously [51]. Polyamines were extracted by homogenization with 5–6% $HClO_4$ using 200–400 mg fresh plant material per ml $HClO_4$. After centrifugation they were dansylated [66] using dansyl chloride at 15 mg/ml in Me_2CO and separated on washed (MeOH) and

activated (1 hr, 105°) LK6D silica gel plates (Whatman) using in most cases $CHCl_3$–triethylamine (25:2 v/v) and/or cyclohexane–EtOAc (5:4 v/v). Authentic polyamines were treated similarly and applied in 2–3 different amounts on the plates in order to permit a quantitative assessment. After chromatography, the plates were sprayed with Et_3N–2-propanol (1:4). Visualized by UV, bands of Dns derivatives were scraped, eluted in 2–3 ml EtOAc by vortexing and quantified using a Turner 430 spectrofluorimeter, with excitation at 350 nm and emission at 495 nm.

For GC/MS the polyamines were extracted with $CHCl_3$ from alkalized $HClO_4$ extracts [8]; purification on silica gel columns as applied to blood serum polyamines [67] was not successful. Trifluoroacetylation was performed using sonication [68]. After excess trifluoroacetic anhydride was removed, the derivatives were dissolved in a known vol. of CH_2Cl_2 prior to analysis. Standard polyamines were treated similarly and each of them was analysed in amounts between 2–10 nmol in order to permit a semiquantitative assessment of polyamines in the plant samples. The mass spectra were obtained using the same GC/MS system equipped with the same column as previously described [62]. The injection port temp. was 200° and the column temp. was programmed at 100° for 5 min, then to 250° at 10°/min; carrier gas He at 24 ml/min. The instrument had a low sensitivity in the region of fragments above 300 *m/z* as indicated by the autotune.

The stomatal diffusion resistance for water vapor was measured by a custom made stomatal diffusion porometer following the design of ref. [69]. The stomatal resistance of detached leaves in the dark was compared (in dim green light) with that of leaves attached to plants under the usual 16 hr photoperiod.

Chemicals. Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories); BSA, 1,3-diaminopropane. 2HCl, spermidine. 3 HCl, spermine. 4HCl, agmatine sulfate, L-proline, dansyl chloride, and trifluoroacetate anhydride were obtained from Sigma.

TLC. R_f values in $CHCl_3$–Et_3N (25:2) were: 0.27; 0.30; 0.31; 0.43; 0.58; 0.61; and 0.75 for dansylated Dap, Agm, Put, Cad, Spd, homoSpd, and Spm.

Mass spectra of TFA amines. Dap (R_t 9.8 min) 41 (80), 56 (35), 58 (31), 69 (100), 72 (30), 78 (28), 84 (12), 97 (6), 114 (6), 126 (91), 127 (30), 135 (15), 140 (22), 141 (8), 154 (16), 197 (4). *Put* (R_t 11.2 min) 41 (32), 55 (25), 69 (100), 78 (28), 86 (12), 97 (9), 114 (10), 124 (7), 126 (97), 127 (39), 140 (5), 149 (6), 154 (7), 166 (8), 167 (18), 211 (12). *Cad* (R_t 11.9 min) 41 (63), 55 (54), 68 (83), 69 (100), 78 (26), 100 (32), 114 (11), 126 (92), 127 (20), 140 (6), 168 (7), 180 (5), 225 (5). *Spd* (R_t 16.7 min) 41 (32), 55 (32), 56 (32), 69 (42), 78 (12), 84 (6), 114 (10), 126 (100), 140 (30), 152 (12), 154 (57), 166 (34), 168 (16), 209 (5), 223 (13), 279 (5), 293 (4), 307 (4), 320 (3), 334 (4), 336 (5), 364 (8). *homoSpd* (R_t 17.3 min) 41 (28), 55 (100), 56 (20), 69 (31), 78 (12), 84 (8), 114 (10), 126 (82), 140 (6), 165 (8), 166 (30), 168 (15), 180 (4), 223 (5), 275 (4), 293 (9), 321 (3), 334 (3), 350 (3), 378 (8). *Spm* (R_t 21.2 min) 41 (56), 43 (40), 55 (37), 57 (32), 69 (40), 71 (18), 73 (100), 126 (97), 140 (34), 147 (70), 152 (20), 154 (95), 166 (12), 168 (12), 180 (7), 194 (5), 207 (12), 209 (10), 221 (22), 267 (6), 279 (12), 281 (16), 295 (6), 320 (6), 341 (10), 355 (15), 356 (7), 376 (10), 429 (14), 490 (10), 518 (22).

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