Label-free, direct localization and relative quantitation of the RNA nucleobase methylations m^6A, m^5C, m^3U, and m^5U by top-down mass spectrometry

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Calculation of average charge values and RNA $(M\text{-}nH)^{n-}$ ion yields

Average charge values, z_{av}, were calculated from $(M\text{-}nH)^{n-}$ ion abundances, A(n), and charge values, n, as

$$z_{av} = \sum_n \mathrm{n} \cdot \mathrm{A(n)} \,/\, \sum_n \mathrm{A(n)}$$

$(M\text{-}nH)^{n-}$ ion yields for each form i were calculated from $(M\text{-}nH)^{n-}$ ion abundances A(n,i) relative to all ion abundances as

$$yield = 100 \cdot \sum_n \mathrm{A(n,i)} \,/\, \sum_{n,i} \mathrm{A(n,i)}$$

Table S1. Average charge (z_{av}) and yield of $(M\text{-}nH)^{n-}$ ions from ESI of 1:1 mixtures (0.5 or 1 μM each in 1:1 H_2O/CH_3OH) of 27 nt RNAs **7** and **11** with 0 and 2 m^5C modifications, respectively, with ammonium acetate (20 mM), piperidine and imidazole (30 mM each), or piperidine (30 mM) as additives.

CH_3COONH_4	z_{av}	4-	5-	6-	7-	8-	9-	10-	11-	12-	13-	14-	15-	Σ	corrected yield [%]
RNA **7**	**5.51**	0.3	29.5	22.1	2.5	0.4	0.1							54.9	53.9
RNA **11**	**5.58**	0.2	21.5	20.6	2.4	0.3	0.1							45.1	46.1
piperidine/ imidazole															
RNA **7**	**6.21**	0.2	9.5	29.6	11.0	3.8	0.8							54.9	53.9
RNA **11**	**6.23**	0.1	7.6	23.4	9.9	3.3	0.6							45.1	46.1
piperidine															
RNA **7**	**11.44**					0.1	2.0	10.6	14.5	11.9	7.2	2.8	0.4	49.6	48.6
RNA **11**	**11.40**					0.0	1.1	12.0	15.2	12.9	6.4	2.5	0.3	50.4	51.4

n- corresponds to $(M - nH)^{n-}$, in %

Table S2. Fragments from CAD of $(M\text{-}6H)^{6-}$ ions at 96 eV laboratory frame energy, mass and m/z values refer to the monoisotopic peak.

m/z measured	z	relative abundance	m measured [Da]	assignment
679.08124	2	16,571,015	1360.17703	a_5-G
873.09819	3	40,747,040	2622.31641	a_9-G
1310.14839	2	42,201,822	2622.31134	a_9-G
988.11435	3	13,136,460	2967.36488	a_{10}-C
1089.79612	3	6,732,168	3272.41019	a_{11}-C
1297.82658	3	12,820,510	3896.50157	a_{13}_m-A
1293.15290	3	29,471,482	3882.48053	a_{13}-A

1606.52867	3	24,108,883	4822.60784	a_{16}-G
1463.42452	4	20,217,943	5857.72720	a_{19}-G
1705.71079	4	30,946,969	6826.87227	a_{22}_**m**-G
1632.40336	5	13,432,977	8167.05316	a_{26}_**m**-A
634.07070	1	74,185,032	635.07798	c_2
939.11238	1	276,980,586	940.11965	c_3
622.06483	2	61,779,766	1246.14421	c_4
1245.13512	1	173,761,710	1246.14239	c_4
794.58894	2	132,737,067	1591.19244	c_5
947.10992	2	264,593,876	1896.23439	c_6
891.58847	2	10,296,201	1785.19149	c_6-C
1100.12048	2	216,198,429	2202.25552	c_7
1032.59458	2	14,153,533	2067.20371	c_7-A
835.08794	3	73,327,348	2508.28565	c_8
1253.13595	2	223,010,735	2508.28644	c_8
950.10310	3	108,950,292	2853.33112	c_9
899.75517	3	10,279,405	2702.28734	c_9-G
1051.78414	3	122,006,718	3158.37426	c_{10}
1056.45430	3	55,709,726	3172.38474	c_{10}_**m**
1153.46464	3	127,958,183	3463.41574	c_{11}
1158.13554	3	63,264,810	3477.42844	c_{11}_**m**
1255.14566	3	164,849,480	3768.45880	c_{12}
1259.81391	3	94,313,724	3782.46355	c_{12}_**m**
1204.79402	3	9,699,088	3617.40389	c_{12}-G
1364.82930	3	77,642,600	4097.50974	c_{13}
1369.50216	3	47,047,117	4111.52831	c_{13}_**m**
1466.83382	3	75,269,704	4403.52330	c_{14}
1471.50869	3	37,680,640	4417.54790	c_{14}_**m**
1568.51275	3	186,623,931	4708.56009	c_{15}
1573.18430	3	88,992,294	4722.57473	c_{15}_**m**
1518.17038	3	10,203,683	4557.53296	c_{15}-G
1262.39589	4	26,977,716	5053.61265	c_{16}
1683.53367	3	130,257,206	5053.62284	c_{16}
1265.90378	4	12,256,063	5067.64421	c_{16}_**m**
1688.20717	3	71,363,624	5067.64333	c_{16}_**m**
1348.66206	4	49,047,854	5398.67736	c_{17}
1798.53992	3	64,286,066	5398.64160	c_{17}
1352.16420	4	23,640,986	5412.68590	c_{17}_**m**
1803.21135	3	31,861,861	5412.65589	c_{17}_**m**
1434.92266	4	67,319,517	5743.71975	c_{18}
1913.55950	3	34,802,879	5743.70033	c_{18}
1438.43087	4	overlaps with $(M-6H)^{6-}$	5757.75259	c_{18}_**m**
1918.22888	3	17,215,131	5757.70847	c_{18}_**m**
1521.18272	4	39,154,302	6088.75997	c_{19}
1524.69048	4	18,480,989	6102.79104	c_{19}_**m**
1597.44046	4	30,456,848	6393.79093	c_{20}
1600.94188	4	67,767,806	6407.79664	c_{20}_**m**
1563.18787	4	11,614,931	6256.78059	c_{20}_**m**-G
1677.20249	4	280,840,253	6712.83906	c_{21}_**m**
1643.43828	4	22,339,513	6577.78224	c_{21}_**m**-A
1639.43905	4	49,585,368	6561.78532	c_{21}_**m**-G
1682.69945	4	53,181,165	6734.82691	c_{21}_**m**+Na
1763.46644	4	90,314,569	7057.89487	c_{22}_**m**
1729.70644	4	11,836,733	6922.85488	c_{22}_**m**-A
1725.70790	4	26,182,423	6906.86072	c_{22}_**m**-G
1471.58385	5	104,412,022	7362.95565	c_{23}_**m**
1839.72614	4	173,144,390	7362.93368	c_{23}_**m**
1805.95635	4	16,237,689	7227.85449	c_{23}_**m**-A

1801.95771	4	33,114,735	7211.85993	c_{23}**_m**-G
1845.21704	4	35,954,119	7384.89727	c_{23}**_m**+Na
1540.58774	5	194,601,490	7707.97509	c_{24}**_m**
1513.57694	5	15,375,051	7572.92109	c_{24}**_m**-A
1510.37957	5	54,691,886	7556.93422	c_{24}**_m**-G
1536.98557	5	8,847,989	7689.96423	c_{24}**_m**-H_2O
1544.98408	5	43,524,450	7729.95680	c_{24}**_m**+Na
1609.60051	5	87,553,971	8053.03893	c_{25}**_m**
1579.39097	5	28,372,840	7901.99124	c_{25}**_m**-G
1396.00446	6	54,640,662	8382.07041	c_{26}**_m**
1370.83297	6	15,517,003	8231.04147	c_{26}**_m**-G
994.12896	1	28,071,918	995.13624	**GG**cP internal fragment
652.08156	1	24,620,899	653.08884	w_2
997.12822	1	74,538,213	998.13549	w_3
670.58397	2	10,277,568	1343.18249	w_4
823.10496	2	80,521,204	1648.22448	w_5
995.62947	2	19,946,624	1993.27349	w_6
1148.14791	2	20,139,178	2298.31037	w_7
1155.15982	2	9,164,595	2312.33419	w_7**_m**
866.77729	3	3,977,196	2603.35370	w_8
1300.67061	2	20,596,467	2603.35578	w_8
871.45036	3	9,886,686	2617.37290	w_8**_m**
1307.67513	2	49,620,368	2617.36482	w_8**_m**
986.46556	3	16,807,248	2962.41850	w_9**_m**
1096.80958	3	9,473,177	3293.45057	w_{10}
1101.48028	3	23,460,610	3307.46267	w_{10}**_m**
1211.82417	3	12,773,059	3638.49434	w_{11}
1216.49643	3	28,525,614	3652.51112	w_{11}**_m**
1331.51488	3	6,859,993	3997.56647	w_{12}**_m**
1535.19937	3	21,184,212	4608.61995	w_{14}**_m**
1462.18987	4	27,368,341	5852.78857	w_{18}**_m**
1949.93158	3	19,802,012	5852.81656	w_{18}**_m**
1777.72989	4	16,808,435	7114.94867	w_{22}**_m**
1395.16752	6	19,338,884	8377.04878	w_{26}**_m**
572.11478	1	75,291,262	573.12205	y_2
917.16210	1	532,453,169	918.16938	y_3
630.60148	2	140,882,532	1263.21751	y_4
1262.20766	1	320,095,829	1263.21494	y_4
783.12169	2	145,644,597	1568.25794	y_5
955.64534	2	480,725,898	1913.30524	y_6
888.11798	2	10,652,173	1778.25051	y_6-A
880.12125	2	26,978,252	1762.25705	y_6-G
1108.16537	2	137,334,230	2218.34529	y_7
1115.17505	2	68,894,275	2232.36465	y_7**_m**
1260.68447	2	43,966,216	2523.38349	y_8
1267.69673	2	86,777,990	2537.40802	y_8**_m**
955.13704	3	25,462,325	2868.43295	y_9
1433.21600	2	36,552,890	2868.44656	y_9
959.80941	3	54,976,734	2882.45005	y_9**_m**
1440.19492	2	overlaps with $(M-6H)^{6-}$	2882.40438	y_9**_m**
1070.15459	3	55,138,627	3213.48560	y_{10}
1074.82469	3	101,710,787	3227.49589	y_{10}**_m**
1612.74295	2	46,150,431	3227.50046	y_{10}**_m**
1024.47590	3	11,945,591	3076.44953	y_{10}**_m**-G
1019.80185	3	6,695,167	3062.42738	y_{10}-G
1185.16853	3	64,343,063	3558.52742	y_{11}
1189.84288	3	141,475,842	3572.55047	y_{11}**_m**
1785.26660	2	22,847,560	3572.54776	y_{11}**_m**

1139.49009	3	18,644,302	3421.49211	y_{11}_**m**-G
1134.82119	3	13,980,151	3407.48539	y_{11}-G
1300.18662	3	86,851,297	3903.58168	y_{12}
1304.85509	3	167,744,310	3917.58710	y_{12}_**m**
1254.50981	3	40,797,590	3766.55125	y_{12}_**m**-G
1249.83442	3	22,873,249	3752.52508	y_{12}-G
1401.86455	3	31,892,563	4208.61547	y_{13}
1406.54036	3	71,825,994	4222.64292	y_{13}_**m**
1503.87380	3	19,979,011	4514.64322	y_{14}
1508.54377	3	46,262,608	4528.65313	y_{14}_**m**
1458.19718	3	12,306,888	4377.61337	y_{14}_**m**-G
1613.55969	3	47,667,362	4843.70090	y_{15}
1618.23329	3	92,767,678	4857.72170	y_{15}_**m**
1715.23159	3	34,450,180	5148.71659	y_{16}
1719.90879	3	75,963,400	5162.74820	y_{16}_**m**
1669.55548	3	15,089,481	5011.68826	y_{16}_**m**-G
1816.92064	3	37,378,183	5453.78376	y_{17}
1821.59567	3	63,618,959	5467.80883	y_{17}_**m**
1771.24472	3	22,758,405	5316.75599	y_{17}_**m**-G
1923.26909	3	65,017,677	5772.82909	y_{18}_**m**
1528.46718	4	98,938,786	6117.89784	y_{19}_**m**
2038.27684	3	33,126,958	6117.85234	y_{19}_**m**
1490.69730	4	17,106,026	5966.81829	y_{19}_**m**-G
1604.96475	4	85,078,280	6423.88811	y_{20}_**m**
1567.20383	4	28,353,736	6272.84442	y_{20}_**m**-G
1681.47538	4	117,505,554	6729.93064	y_{21}_**m**
1643.71456	4	27,803,786	6578.88735	y_{21}_**m**-G
1686.97369	4	22,136,948	6751.92387	y_{21}_**m**+Na
1757.73037	4	69,791,727	7034.95060	y_{22}_**m**
1719.97176	4	18,307,038	6883.91616	y_{22}_**m**-G
1475.00021	5	30,968,984	7380.03744	y_{23}_**m**
1843.99325	4	58,345,103	7380.00211	y_{23}_**m**
1536.20088	5	84,083,082	7686.04080	y_{24}_**m**
1505.99587	5	13,357,995	7535.01574	y_{24}_**m**-G
1597.20696	5	51,730,122	7991.07119	y_{25}_**m**
1658.41849	5	87,382,577	8297.12882	y_{26}_**m**
1628.20373	5	23,410,650	8146.05502	y_{26}_**m**-G

Table S3. Stoichiometry of 23 nt RNA isomers **18**-**20** (m^5C) electrosprayed from a 3 μM (1 μM each) solution in 3:1 H_2O/CH_3OH with 25 mM ammonium acetate derived from CAD of $(M-5H)^{5-}$ ions (average from triplicate measurements at 85, 87.5, and 90 eV laboratory frame energy).

RNA	% from CAD
18	35.24 ± 0.75
19	35.84 ± 2.23
20	28.92 ± 1.48

Table S4. Stoichiometry of a mixture of 23 nt RNA isomers **4**, **16**, and **17** (m^6A) electrosprayed from a 3.1 μM (RNA **4**: 1 μM, RNA **16**: 1 μM, RNA **17**: 1.1 μM) solution in 1:1 H_2O/CH_3OH with 20 mM ammonium acetate derived from CAD of $(M-5H)^{5-}$ ions (average from triplicate measurements at 75, 77.5, and 80 eV laboratory frame energy).

RNA	% in solution	% from CAD
16	32.14	29.74 ± 0.47
17	32.14	31.36 ± 1.32
4	35.72	38.90 ± 0.85

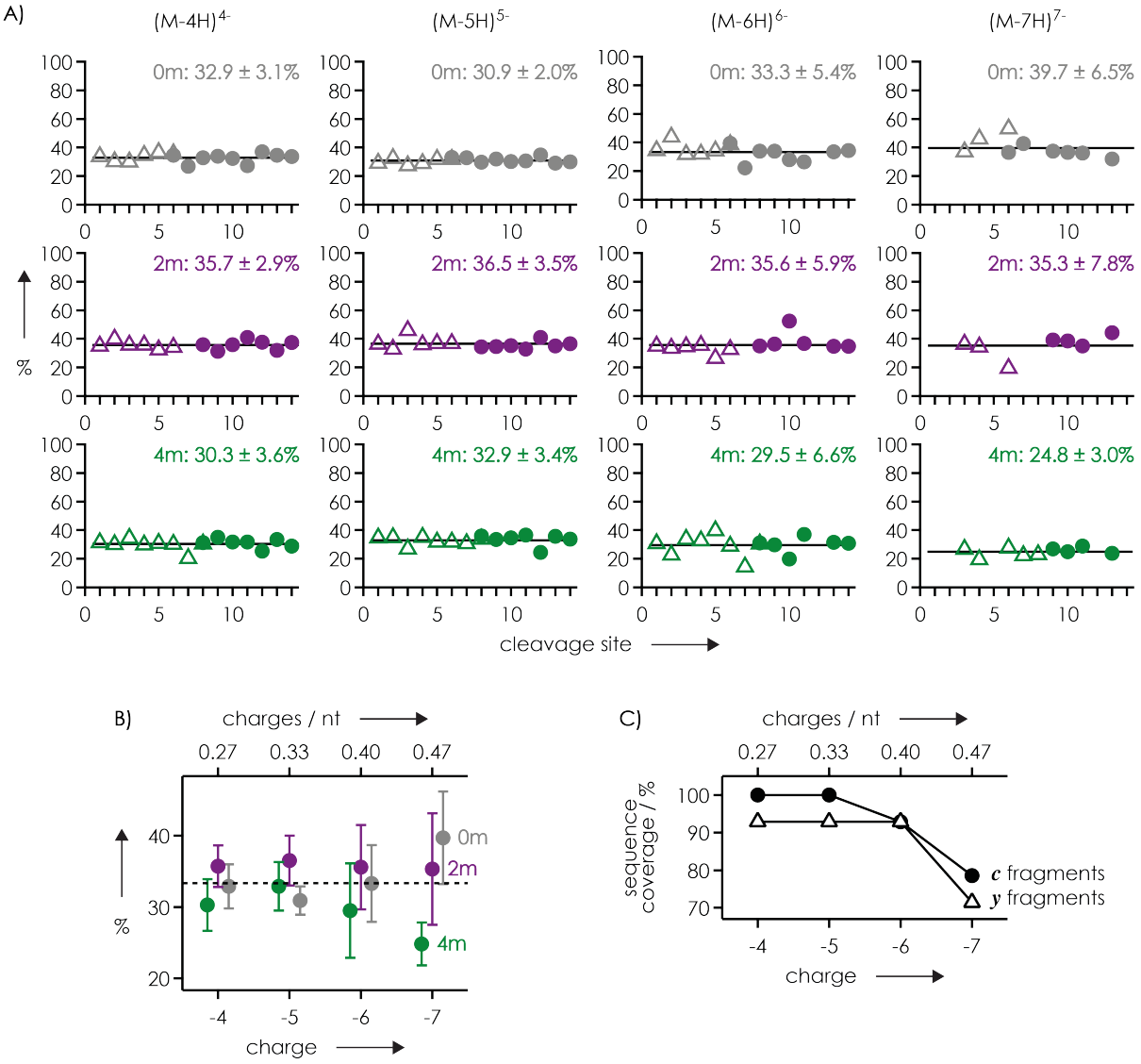

Figure S1. A) Fraction of c (circles) and y (triangles) fragments with 0 to 4 methylations from CAD of $(M\text{-}nH)^{n-}$ ions electrosprayed from 1:1:1 mixtures of 15 nt RNAs **1**, **2**, and **3** in 1:1 H_2O/CH_3OH with 20 mM piperidine and 20 mM imidazole as additive versus cleavage site; B) average fractions with standard deviations shown as error bars and C) sequence coverage from c (circles) and y (triangles) fragments versus $(M\text{-}nH)^{n-}$ ion charge. Color coding refers to the number of m^5U residues: gray for 0 (RNA **1**), violet for 2 (RNA **2**), and green for 4 (RNA **3**).

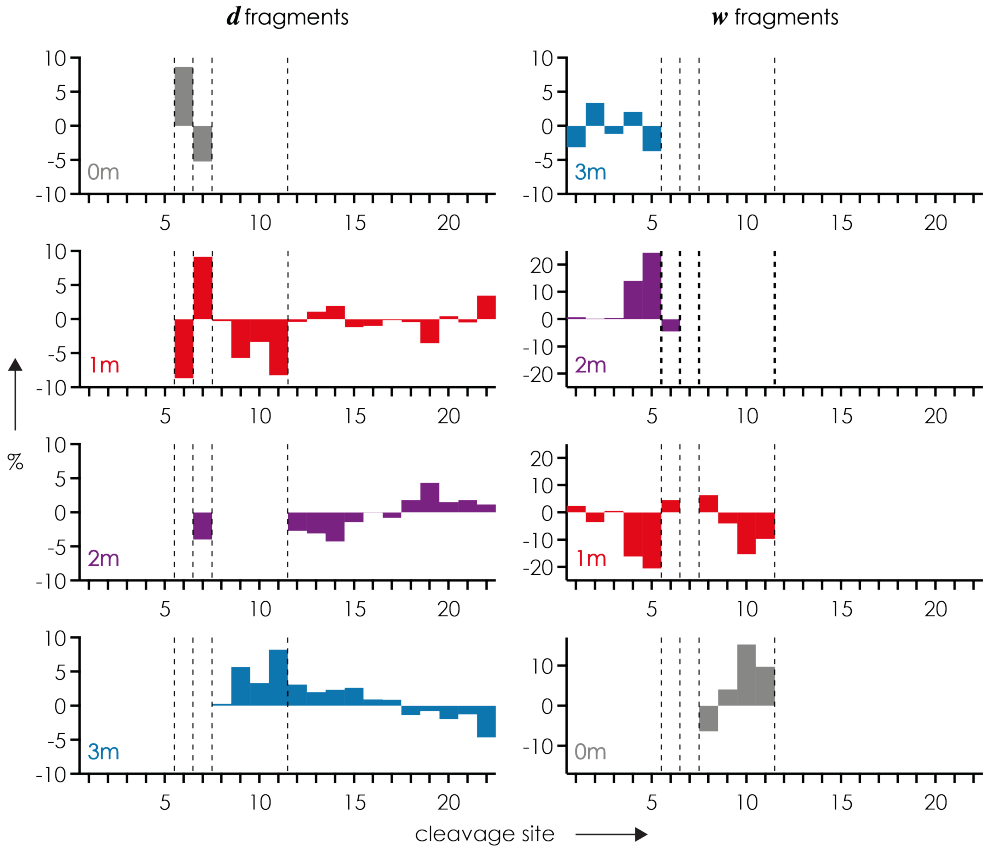

Figure S2. Deviations of *d* (left) and *w* (right) fragment yields from theoretical values (33.3 or 66.7%) from EDD of $(M\text{-}12H)^{12-}$ ions of a 1:1:1 mixture of the 23 nt RNA forms **4**, **5**, and **6** with 1, 2, and 3 m^6A residues, respectively, versus cleavage site; color coding indicates the number of methylations: gray=0, red=1, purple=2, blue=3; dashed lines mark cleavage sites next to m^6A residues at positions 6, 7, 8, and 12.

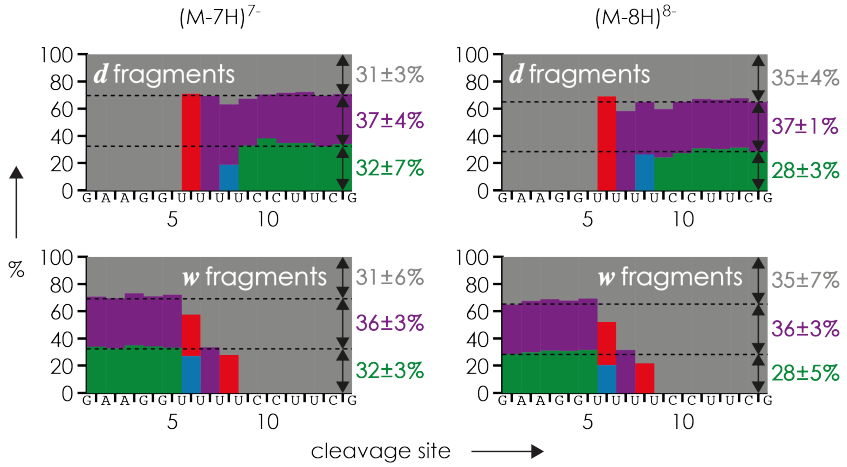

Figure S3. Fractions of ***d*** (top) and ***w*** (bottom) fragments from EDD of $(M\text{-}7H)^{7-}$ (left, average from three measurements at 24, 26, and 26 eV electron energy) and $(M\text{-}8H)^{8-}$ (right, average from three measurements at 22, 24, and 26 eV electron energy) ions from ESI of a 1:1:1 mixture of the 15 nt RNA forms **1**, **2**, and **3** with 0, 2, and 4 m^5U residues, respectively, 0.5 μM each, in 1:1 H_2O/CH_3OH with 20 mM piperidine versus cleavage site; color coding indicates the number of methylations of a fragment: gray=0, red=1, purple=2, blue=3, green=4.