MALDI-TOF mass spectrometric determination of 11 phenothiazines with heavy side chains in urine

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Abstract A rapid screening method was developed for the determination of phenothiazines by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In this method, α-cyano-4-hydroxy cinnamic acid (CHCA) was used as the matrix to assist the ionization of phenothiazines. The identification of 11 phenothiazines with heavy side chains was performed by their protonated molecular ions $[M + H]^+$ at m/z = 340 – 447, and the quantification was achieved using trifluprομazine at m/z = 353 as the internal standard (IS). The relative ionization efficiencies of 11 phenothiazines and IS on MALDI-TOF-MS were different from those on ESI-TSQ-MS, but the product ion spectra on MALDI-MS-MS were quite similar to those on ESI-MS-MS except in the case of flupentixol. The limit of detection was 0.3 ng/ml with its quantification range of 1 – 50 ng/ml urine in the best case, and limit of detection was 5 ng/ml with its quantification range of 10 – 100 ng/ml urine in the worst case for 10 phenothiazines except thiethylperazine. Present method provides a simple and high throughput method for the screening of these phenothiazines using only 20 μl of urine. To our knowledge, this study is the first trial to analyze phenothiazines by MALDI-TOF MS (-MS).

Keywords MALDI-TOF mass spectrometry・α-Cyano-4-hydroxy cinnamic acid (CHCA)・Phenothiazines・Urine

Introduction

Phenothiazine drugs are being prescribed for psychosis treatment, such as schizophrenia,

and they are also used as antiemetic and antihistamic medicines. Many poisoning cases taking these drugs were reported in Japan [1]. For the detection of phenothiazines, gas chromatography (GC) [2] and GC coupled to mass spectrometry (MS) [1,3] were currently used. Phenothiazines with heavy side chains, however, have to be measured by LC [4] and LC-MS (-MS) [5-7] owing to their low volatility [6,7].

Matrix assisted laser desorption ionization time of flight (MALDI-TOF) MS is a very popular and powerful tool that is used in the analysis of high-mass biomolecules [8], because the signals of matrices decrease with increasing *m/z* values, and hence they do not interfere with the signals of high-mass molecules. Recently, however, the analysis of low-mass (*m/z* <500) molecules using MALDI has been reported [9-11], but the work on phenothiazines by MALDI was not reported yet.

Phenothiazines can be excited with UV laser light, and the *m/z* values of phenothiazines with heavy chains, 340 – 477, belong to the *m/z* regions that are not seriously interfered with by the signals of matrices. Therefore, we tried the detection of these phenothiazines by MALDI-TOF-MS (-MS) for the first time.

The purification of phenothiazines with liquid-liquid extraction (LLE) [2,4,5] or solid phase extraction (SPE) [3,6,7] was performed to eliminate large amounts of interfering substances contained in biological samples. Comparing the result of LLE used for serum in [4] with that of SPE used for plasma in [3], they were equivalent in terms of recovery, linearity and accuracy, and LLE was selected in the present work because of its simplicity, rapidity and low cost. Phenothiazines usually exhibit low concentration levels in blood but high- or medium- concentration levels in urine of the medicine taker [1], and hence urine was selected as the sample material. Multi-analyte

procedures for screening as well as quantification of drugs with a single sample extract contribute to the saving of times and resources in forensic and clinical toxicology. Therefore, our study focused on the simultaneous detection of heavy side chain-substituted phenothiazines.

Materials and methods

Materials

Perazine, propericiazine, prochlorperazine, perphenazine, trifluoperazine, fluphenazine, and clospirazine were obtained from Yoshitomi Pharmaceutical Ind., Osaka, Japan; thioridazine and thiethylperazine from Sandoz AG, Basel, Switzerland; thioproperazine from Shionogi, Osaka, Japan; flupentixol from Takeda Chem. Ind., Osaka, Japan; and triflupromazine, α-cyanono-4-hydroxy cinnamic acid (CHCA), trifluoroacetic acid and angiotensin I were obtained from Sigma-Aldrich, St. Louis, MO, USA. Acetonitrile (ACN) and methanol, suitable for LC-MS, cetrimonium bromide (CTB) and other chemicals of analytical grade were obtained from Wako Pure Chemicals, Osaka, Japan. Pure water with a specific resistance of 18 $M\Omega \cdot cm$ was used (Millipore, Bedford, MA, USA). Urine samples from normal subjects under permission were used as blank samples and those spiked with several amounts of phenothiazines were used as positive samples.

Standard solutions

Individual stock solutions of 11 phenothiazines and trifluprom azine as the internal standard (IS) were prepared separately by dissolving appropriate amounts of each drug in methanol at a 500 μg/ml and stored at – 20 ℃, respectively. To compare MS and MS-MS by MALDI with those by ESI, a standard solution containing phenothiazines with heavy chains at 10 μg/ml each and IS at 50 μg/ml in methanol was prepared and stored at – 20 ℃. At the detection, it was diluted to 10-fold with 80 % ACN solution containing CHCA at 1.6 mg/ml for MALDI and with 30 % methanol solution for ESI, respectively. Working calibration solutions and quality control solutions were prepared daily by dilutions of the stock solutions with blank urine at 0.3 – 100 ng/ml.

Urine preparation for the assay

To 20 μl of urine in a tube (Eppendorf AG, Hamburg, Germany), 1 μl of IS and 1 μl of 25 % NH_4OH were added. Add 40 μl of 1-chlorobutane (CHB) to the solution and vortex-mix for 1 min, and centrifuge it at 10,000 ｇ for 1 min. Then collect the CHB layer to a new tube, and the layer was evaporated to dryness using a centrifugal dryer, Savant Speed Vac Plus SC110A (Savant, Holbrook, NY, USA). The residue was reconstituted with 20 μl of CHCA-solution prepared (1.6 mg CHCA and 6 μg CTB/ml in 80 % ACN solution). One μl of the solution was loaded on a stainless steel MALDI sample target plate (V700666, AB SCIEX, Framingham, MA, USA) and allowed to dry.

Instrument

A reflector type of time-of-flight (TOF) mass spectrometer (MS) QSTAR Elite Hybrid (AB SCIEX) was used in the positive mode. A stainless steel MALDI target plate with samples on it was inserted into the machine. Mass spectra were obtained using 355-nm radiation from Nd:YAG laser system (AB SCIEX). The instrument was equipped with a video camera for displaying the sample image on a monitor and permitting the laser to be focused on a specific well within the area of the target. All spectra were obtained as 20 – 200 shots averages. Mass calibration was conducted using fragments produced from CHCA standard at *m/z* 172.0393 and angiotensin I standard at *m/z* 1296.6848, respectively.

For comparison, ESI-TSQ-MS (-MS) was performed using a TSQ 7000 LC-triple stage quadrupole mass spectrometer equipped with flow-injector (ThermoQuest, Japan) in the positive ion mode. Methanol was flowed as the mobile phase at 300 ml/min and the capillary temperature was set at 230 ℃. The electrospray voltage was set at 4.5 kV, multiplier voltage at 1.3 kV, and collision voltages at 27 and 32 V. Nitrogen was used as the sheath gas (469 kPa) and also as an auxiliary gas (8 units) and argon was used as the collision gas (134 kPa). One μL of the sample was directly flow-injected using an auto-sampler into ESI-MS-MS apparatus.

Results and discussion

Optimizations in MALDI detection of phenothiazines

The concentrations of CHCA were varied at 0, 0.5, 1, 1.4, 1.6, 1.7, 1.8, 2, 2.5, 3, 4 and 5 mg/ml in 80 % ACN solution, and 1.6 – 1.8 mg/ml gave the highest signal. Since blank signals increased with the concentrations, 1.6 mg CHCA/ml in 80 % ACN solution (named CHCA solution here after) was used in the present work. When CHCA was not contained, the signals of phenothiazines decreased to <0.5 % of the highest ones, and

hence it could be said that CHCA contributed greatly to the ionization of phenothiazines. Although trifluoroacetic acid often enhanced signals in MALDI detections, it did not work in the present case, and was not used. 2,5-Dihydroxy benzoic acid is also a matrix commonly used in MALDI but was not used in the present assay because the signals of phenothiazines with the matrix were < 6 % of those with CHCA.

The number of shots was varied from 20 to 200. Twenty shots at 40 % power level was enough at most concentrations, but 200 shots was used at the concentrations of limit of detection (LOD) and the limit of quantification (LOQ).

MS and MS-MS spectra using MALDI and ESI

One μl of CHCA solution containing phenothiazines with heavy side chains at 1 ng each and IS at 5 ng were placed on a target well and detected with 20 shots by MALDI-MS as shown in Figs. 1 a and b. These spectra were shown in two figures, a and b, because *m/z* 446 of clospirazine numbered 10 differed by only one *m/z* unit from *m/z* 447 of thioproperazine numbered 11. All phenothiazines showed the protonated ion as the main ion, respectively, and were listed together with respective isotope ions in Table 1. The heights of main peak of respective phenothiazine differed from 5,000 to 48,500 counts due to the differences in their ionization efficiency.

For comparison between MALDI and ESI, phenothizines at 1 ng each and IS at 5 ng in 1 μl of 30 % methanol solution was directly flow-injected and detected by ESI-TSQ-MS as shown in Figs. 2 a and b. Comparing Fig. 1 with Fig. 2, relative ionization efficiencies of 11 phenothiazines and IS in MALDI were different from those in ESI. The largest difference was observed in IS that is a phenothiazine having a light side chain. That is, the peak height of IS (5 ng per well) in MALDI was nearly the middle one among peak heights of 11 phenothiazines (1 ng each per well), whereas the peak height of IS in ESI was more than 5 times that of 11 phenothiazines.

The results of MS-MS in MALDI were listed in Table 1 where the protonated ion was selected as the precursor ion, respectively. The spectra of collision voltage of 30 and 40 V in MALDI-MS-MS were the nearly the same spectra of collision voltage of 27 and 32 V in ESI-MS-MS, respectively, in cases of 10 phenothiazines as shown in Figs. 3 a and 4 a, whereas different spectra were observed in case of flupentixol as shown in Figs. 3 b and 4 b.

Quantification of phenothiazines

Most of the main peaks of phenothiazines were separated each other, and their simultaneous quantifications could be performed using IS, respectively. As only one exception, the main peak of thioproperazine at *m/z* 447 numbered 11 was interfered with by the isotopic ion of clospirazine numbered 12. Therefore, the quantification of thioproperazine should be done after subtracting the contribution from the isotopic signal of clospirazine when two phenothiazines are contained together.

When the concentrations of phenothiazines were low, the interference from the signals of matrix CHCA were observed, especially in the detection of thiethylperazine at *m/z* 400 numbered 5, although the interference from blank urine extract was not observed. Therefore, a surfactant CTB was used to suppress the signals of CHCA [9]. The concentrations of CTB were varied from 2 – 10 μg/ml in CHCA solution. Figure 5 indicated the MS spectrum of blank urine extract using CTB at 6 μg/ml in CHCA solution where the *m/z* values of 11 phenothiazines and IS were indicated by arrows. By addition of CTB at 6 μg/ml, signals of CHCA decreased to 18 % whereas the signals of all phenothiazines decreased to 40 %, respectively. Although the signals of CHCA at *m/z* 400 numbered 5 and *m/z* 435 numbered 8 showed 350 counts and 70 counts, respectively, the other signals at the *m/z* corresponding to the other 9 phenothiazines and IS were less than 15 counts. The signal of CHCA at *m/z* 400 having 350 counts shown

in Fig. 5 was equivalent to 50 pg of thiethylperazine, and seriously interfered with the detection of thiethylperazine. As a consequence, the limit of detection of thiethylperazine became 100 pg corresponding to 100 ng/ml urine, and hence the quantification of thiethylperazine in urine was abandoned in the present determination. On the other hand, the signal of CHCA at *m/z* 435 having 70 counts was equivalent to only 2 pg of flupentixol, and did not seriously interfere with the detection of flupentixol.

Here, CTB showed only a signal at *m/z* = 284.53 [9] that did not interfere with the detection of phenothiazines and IS but it suppressed the signals of CHCA to 18 % and those of phenothiazines to 40 %, respectively. Therefore, quantification of phenothiazines was performed using the 1.6 mg CHCA and 6 μg CTB/ ml in 80 % ACN solution as described in the section of assay.

Extraction and detection of phenothiazines in urine

The extraction efficiencies were compared by changing the amount of an alkalizing agent, 25 % NH_4OH, from 0 to 4 μl and the amount of extractor, CHB, from 20 to 60 μl for 20 μl of urine sample, respectively. The condition, 1 μl of 25 % NH_4OH and 40 μl of CHB, was found to be the best one. Although CHB and butyl methyl ether exhibited nearly the same extraction efficiencies, the former showed a sharper interface between solvent layer and aqueous layer, and hence was selected.

When the CHB extract of phenothiazines was mixed with the CHCA solution, they could not make uniform deposits on the MALDI target due to hydrophobicity of CHB. Therefore, the CHB extract was evaporated to dryness to eliminate CHB. Twelve extracts, 40 μl each, could be evaporated to dryness in a few minutes using a centrifugal dryer, Savant.

The recovery was calculated by comparing the peak height obtained from the extract of spiked urine with the peak obtained from the phenothiazines dissolved in 80 % ACN

solution. The recoveries of phenothizines at 1, 3, 6, 10, 50 and 100 ng/ml urine were in the range of 90 – 100 %. The signals from impurities in urine did not overlap with the signals of phenothiazines and IS, as shown in Fig. 5.

Reliability of the method

Reliability of the present method was examined by spiking phenothizines at 1, 3, 6, 10, 50 and 100 ng/ml to blank urine (n = 3) for calibration curves and spiking at 0.3, 0.5, 1, 2, 4, and 5 ng/ml for LODs (S/N = 3), respectively, and they were listed in Table 2. The MALDI-MS spectrum in case of the worst LOD and that in case of best LOD were shown in Figs. 6 b and d, respectively. Precision and accuracy were assessed by analyzing urine spiked with phenothiazines three times a day as well as on three different days as listed in Table 3. The concentrations in Table 3 indicated that the coefficient of variation was <24 % and the accuracy was 80–125 % for intra-day and inter-day variations, and could be considered as the quantification ranges.

Conclusion

The demand for high analysis speed using a minute amount of specimen is especially important in poisoning cases [1]. MALDI-MS analysis can be performed within 30 s per sample. Furthermore, MALDI is moderately tolerant to salts, and hence the sample preparation required in the present work was only liquid-liquid extraction and solvent evaporation for the detection of phenothiazines in 20 μl of urine.

Acknowledgement This work was supported by Grant-in Aid for Scientific Research (number 22590631) from the ministry of Education, Science, Sports and Culture of Japan.

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Figure captions

Fig. 1 MALDI-TOF-MS spectrum of 6 phenothiazines at 1 ng each and IS at 5 ng per well (a) and the other 5 phenothiazines at 1 ng each and IS at 5 ng per well (b)

Fig. 2 ESI-TSQ-MS spectrum of 6 phenothiazines at 1 ng each and IS at 5 ng in 1 μl of 30 % methanol solution injected (a) and that of the other 5 phenothiazines at 1 ng each and IS at 5 ng in 1 μl of 30 % methanol solution injected

Fig. 3 MALDI-MS-MS product ion spectrum of perazine from the precursor ion at *m/z* 340 (a) and that of flupentixol from the precursor ion at *m/z* 435 (b) at collision voltage of 40 V

Fig. 4 ESI-MS-MS product ion spectrum of perazine from the precursor ion at *m/z* 340 (a) and that of flupentixol from the precursor ion at *m/z* 435 (b) at collision voltage of 32 V, respectively. The collision voltage of 32 V was selected in ESI-MS-MS because it gave the most resembled spectrum to that at collision voltage of 40 V in MALDI-MS-MS of 10 phenothiazines, respectively, except ESI-MS-MS of flupentixol shown in (b)

Fig. 5 MALDI-MS spectrum of the extract from blank urine. The arrows indicated the *m/z* of 11 phenothiazines and IS

Fig. 6 MALDI-MS spectrum of the extract from blank urine (a) and that from urine spiked with perazine at 0.5 ng/ml (b) detected at *m/z* 332-348, respectively, and that from blank urine (c) and that from urine spiked with thioproperazine at 0.03 ng/ml (d) detected at *m/z* 439-455

Table 1 *m/z* values and their relative intensities (%) of main ions in MS, and *m/z* values and their relative intensities (%) of main product ions in MS-MS

		MS	MS-MS (at 30V)	MS-MS (at 40V)
1. perazine	*m/z*	340, 341, 342	141, 113, 70, 212, 239	113, 141, 70, 212, 180
	%	100 21 7	100 55 8 7 3	100 66 39 14 12
2. propericiazine	*m/z*	366, 367, 368	142, 114, 98, 237, 265	114, 142, 98, 237, 205
	%	100 22 6	100 52 14 6 3	100 75 14 12 6
3. thioridazine	*m/z*	371, 372, 373	126, 98, 258, 324, 58	126, 98, 258, 58, 70
	%	100 24 11	100 49 4 1 1	100 10 6 6 4
4. prochlorperazine	*m/z*	374, 376, 375	141, 113, 70, 246, 239	113, 141, 70, 246, 98
	%	100 36 21	100 51 6 6 3	100 62 31 12 8
5. thiethylperazine	*m/z*	400, 401, 402	141, 113, 202, 199, 239	113, 141, 70, 202, 272
	%	100 24 10	100 32 5 4 4	100 99 26 13 9
6. perphenazine	*m/z*	404, 406, 405	171, 143, 100, 246, 98	143, 171, 100, 98, 246
	%	100 34 21	100 43 5 5 4	100 85 26 20 13
7. trifluoperazine	*m/z*	408, 409, 410	141, 113, 280, 111, 70	113, 141, 70, 280, 98
	%	100 22 6	100 59 6 6 6	100 70 26 12 9
8. flupentixol	*m/z*	435, 436, 437	128, 100, 390, 305, 265	100, 128, 305, 265, 97
	%	100 20 6	100 72 66 52 31	100 84 83 80 68
9. fluphenazine	*m/z*	438, 439, 440	171, 143, 258, 398, 280	143, 171, 100, 98, 280
	%	100 22 6	100 43 6 5 5	100 85 22 17 13
10. clospirazine	*m/z*	446, 448, 447	213, 185, 260, 246, 274	213, 185, 246, 260, 156
	%	100 42 26	100 27 5 4 2	100 59 8 6 4

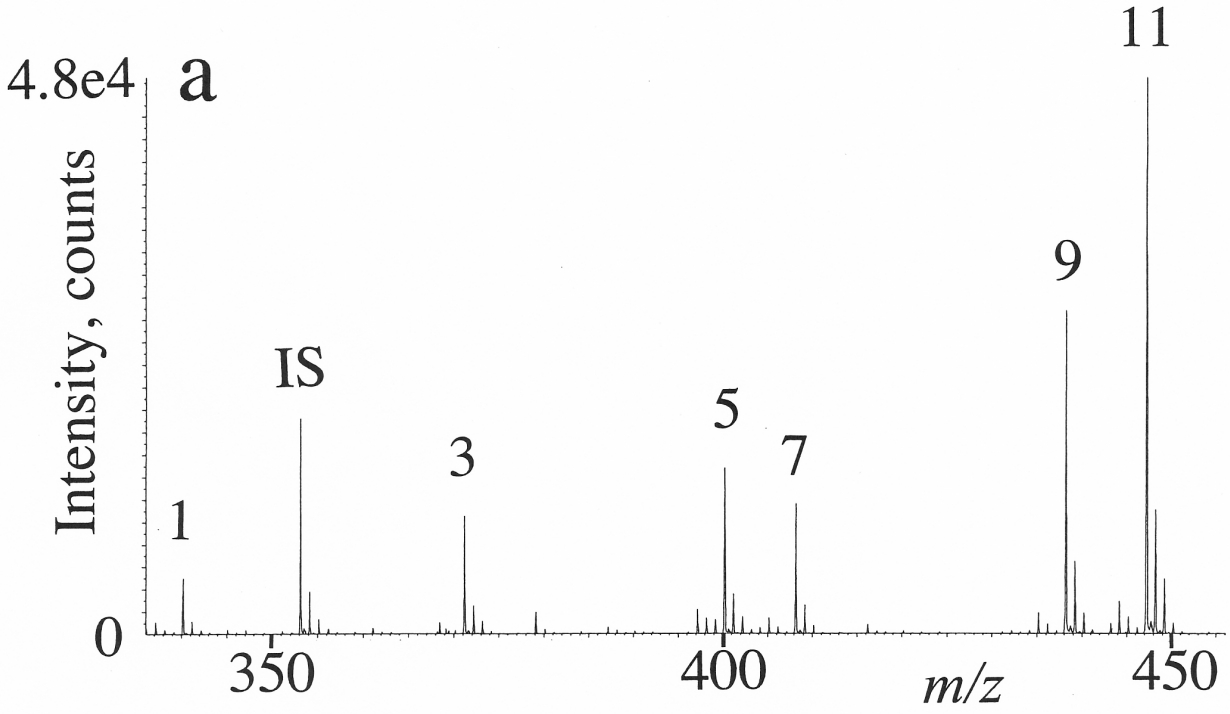
11. thioproperazine	*m/z*	447,	448,	449		141,	113,	238,	319,	70		113,	141,	70,	319,	238
	%	100	22	10		100	39	5	4	3		100	99	21	10	8

Table 2 Data on regression equation, quantification range and detection limit of 11 phenothiazines extracted from urine

	Equation	Correlation coefficient	Quantification range (ng/ml)	Detection limit (ng/ml)
1. perazine	$y = 8.139x + 16.9$	0.998	10 — 100	5
2. propericiazine	$y = 37.259x + 9.7$	0.998	3 — 100	1
3. thioridazine	$y = 10.874x + 2.9$	0.998	3 — 100	1
4. prochlorperazine	$y = 3.950x + 4.5$	0.995	6 — 100	2
5. thiethylperazine				100
6. perphenazine	$y = 21.273x + 6.9$	0.995	3 — 100	1
7. trifluoperazine	$y = 11.209x + 2.4$	0.999	3 — 100	1
8. flupentixol	$y = 35.310x + 79.2$	0.999	6 — 100	4
9. fluphenazine	$y = 76.743x + 8.8$	0.999	1 — 50	0.5
10. clospirazine	$y = 36.994x + 6.2$	0.999	3 — 100	1
11. thioproperazine	$y = 96.968x + 3.9$	0.999	1 — 50	0.3

Table 3 Intra-day (3 times) and inter-day (3 days) accuracy/precision of phenothiazines spiked into urine

	Intra-day		Inter-day	
Spiked (ng/ml)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
1. perazine				
10	111.1	22.6	124.8	15.3
30	105.0	5.1	92.7	8.4
100	110.6	2.9	95.2	2.0
2. propericiazine				
3	92.0	4.9	91.1	4.2
10	98.0	11.0	99.8	12.2
100	114.0	5.0	104.0	2.3
3. thioridazine				
3	114.4	17.3	89.8	8.7
10	104.6	3.7	92.3	16.9
100	114.3	1.9	101.8	9.6
4. prochlorperazine				
6	122.3	23.8	113.9	20.7
10	86.5	16.7	98.4	9.7
100	114.5	6.3	106.6	5.1
6. perphenazine				
3	90.5	8.2	80.6	4.5
10	106.2	2.9	114.3	11.5
100	124.4	0.9	109.4	8.0
7. trifluoperazine				
3	88.6	18.3	106.4	15.2
10	110.3	14.3	104.0	13.8
100	107.4	3.7	108.6	9.6
8. fluphenazine				
6	108.2	18.6	115.5	21.2
10	107.8	3.3	105.5	6.8
100	100.4	2.1	103.3	4.4
9. flupentixol				
1	89.9	9.3	89.2	7.8
10	112.6	8.0	117.4	7.0
50	96.1	5.6	105.4	3.8
10. clospirazine				
3	107.6	3.1	110.1	9.0
10	96.4	2.1	98.0	5.5
100	102.4	1.6	101.7	5.9
11. thioproperazine				
1	98.4	11.5	122.8	5.9
10	99.3	15.0	95.6	21.2
50	111.3	9.2	105.1	9.4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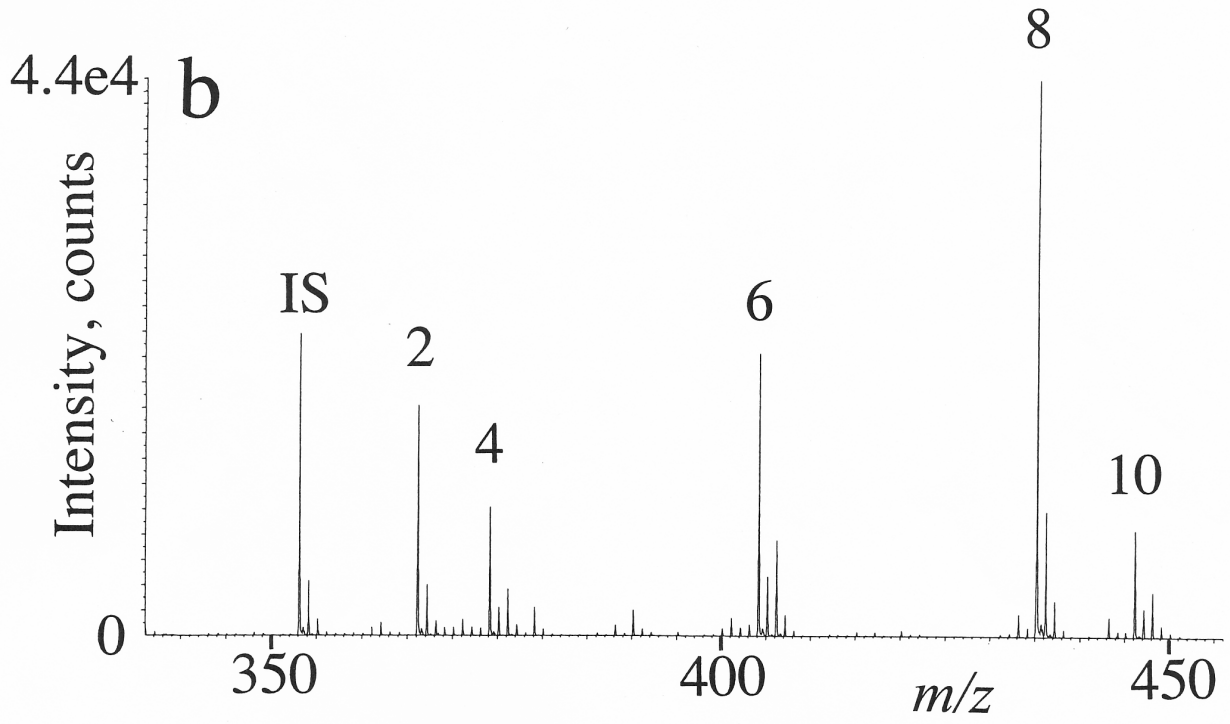
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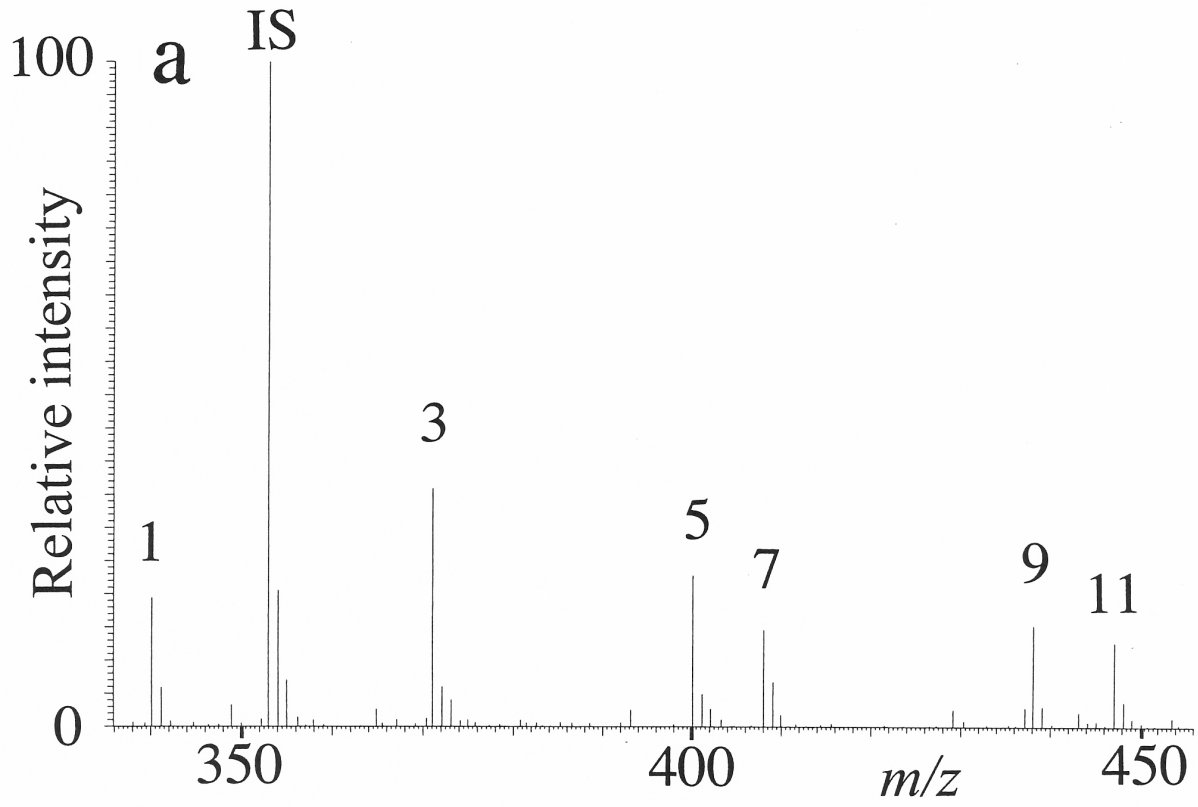
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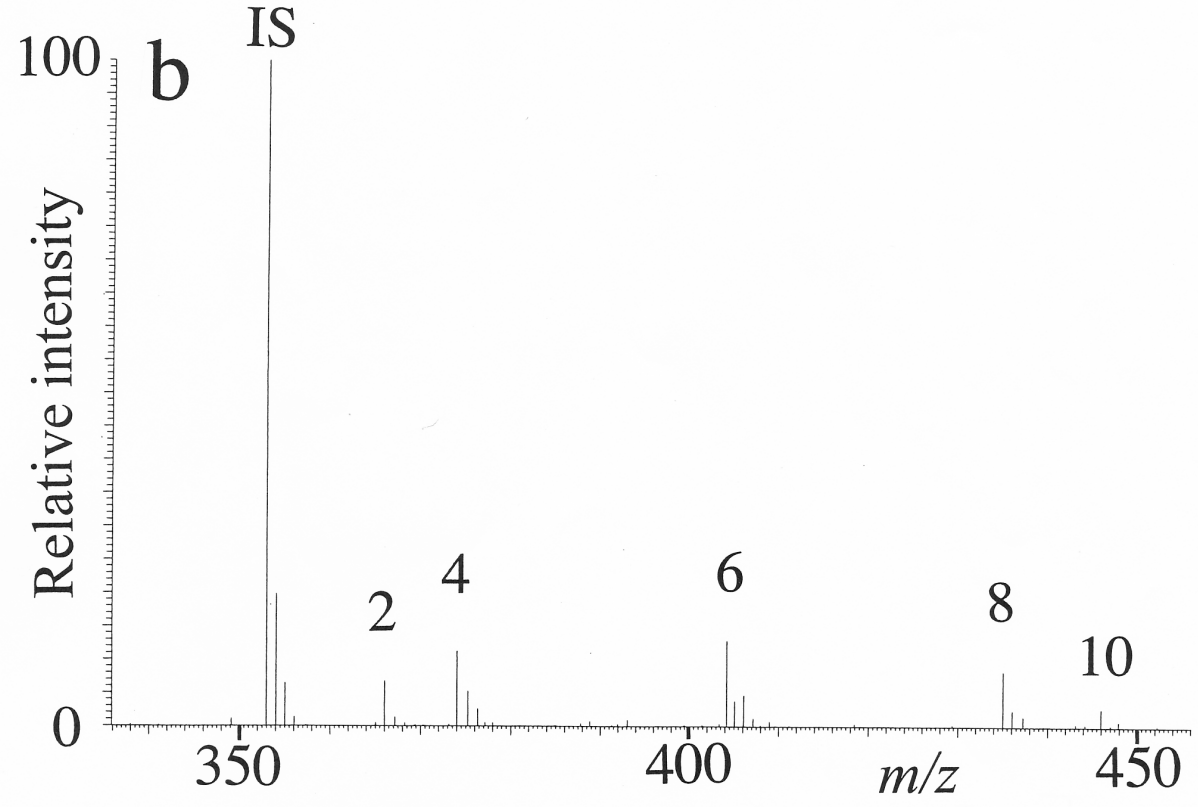
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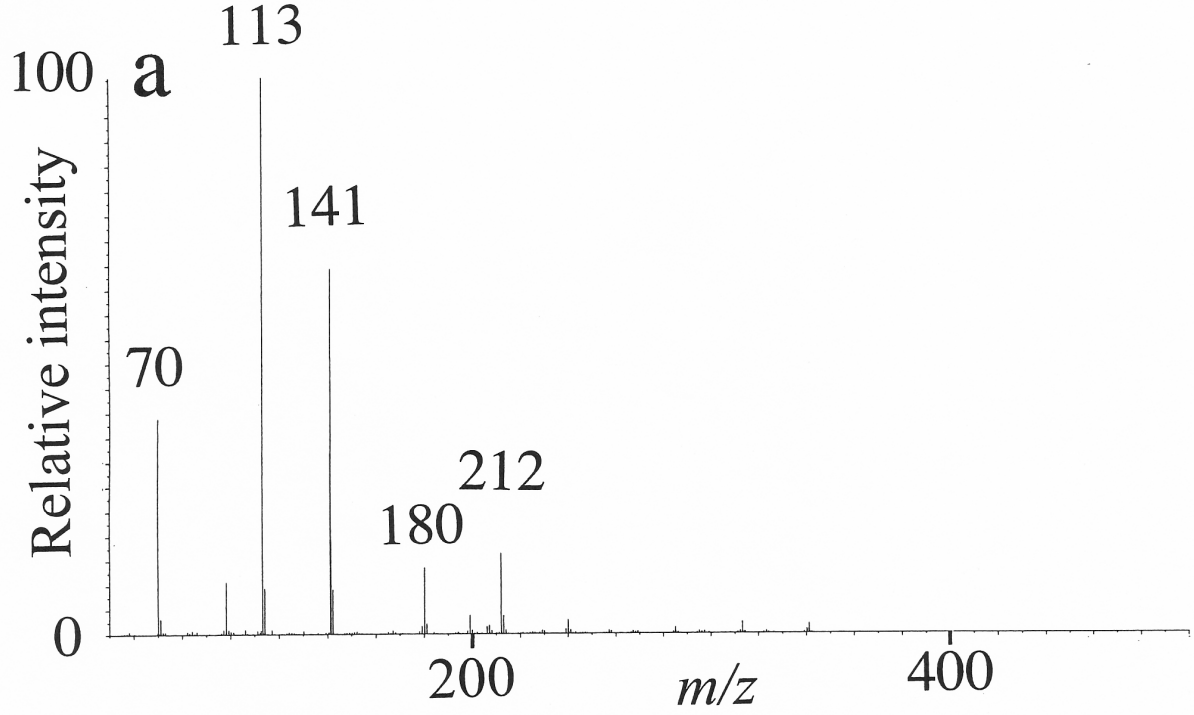
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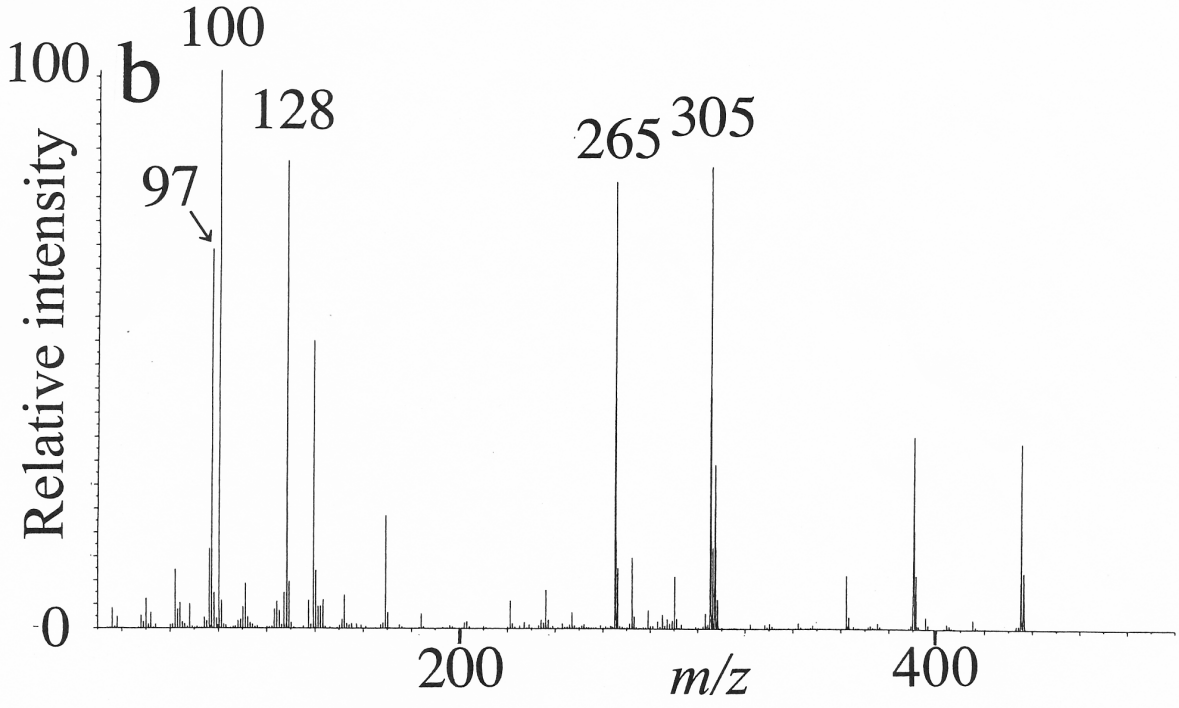
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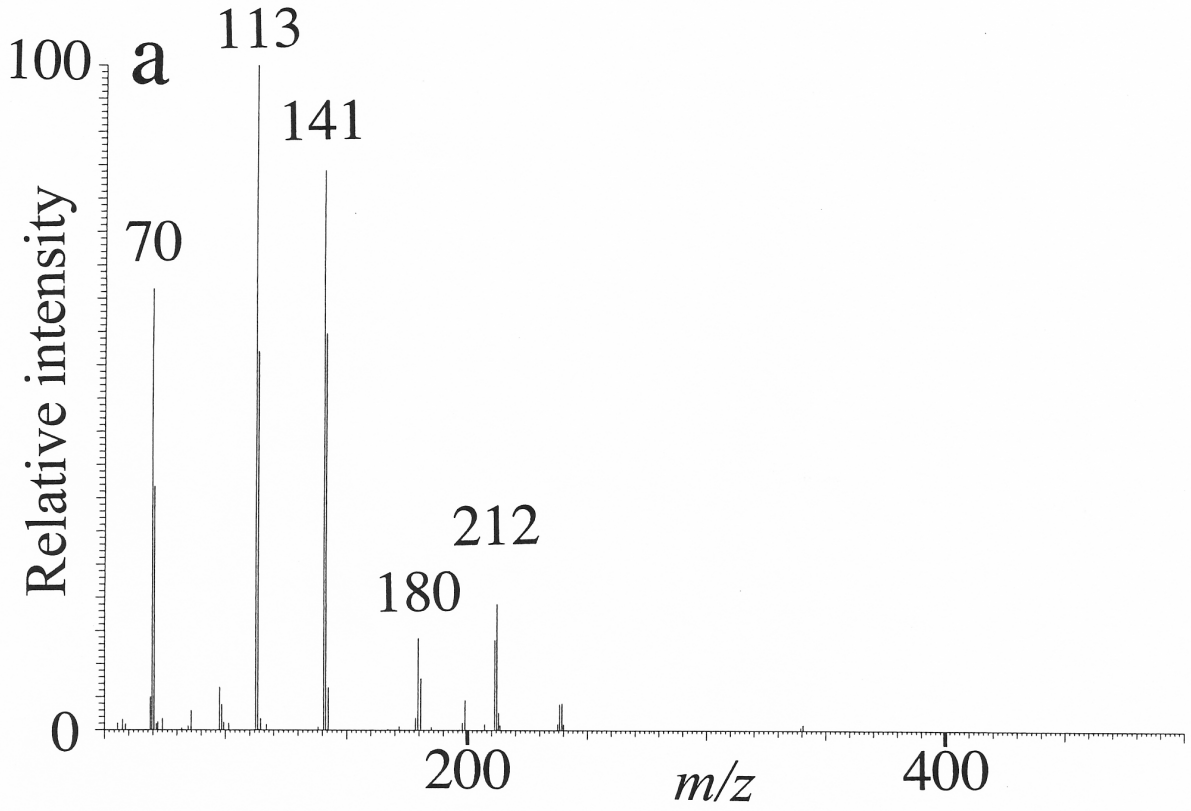
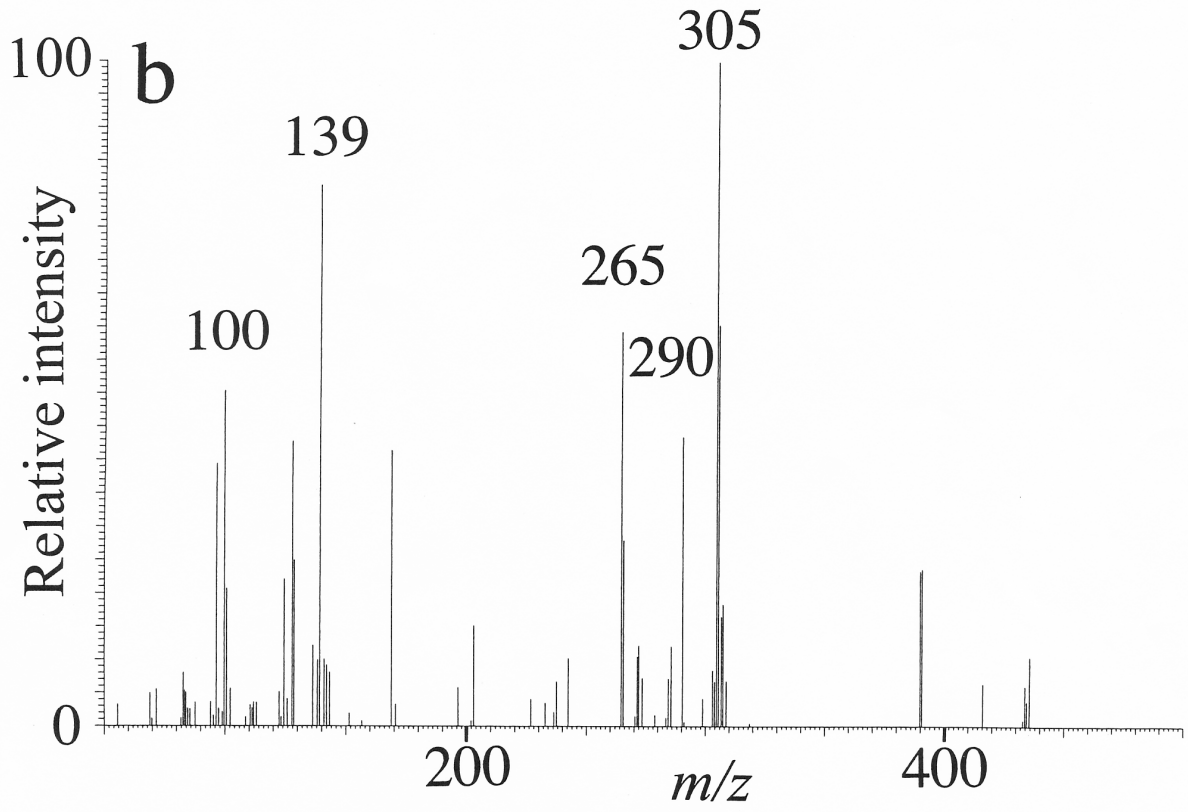
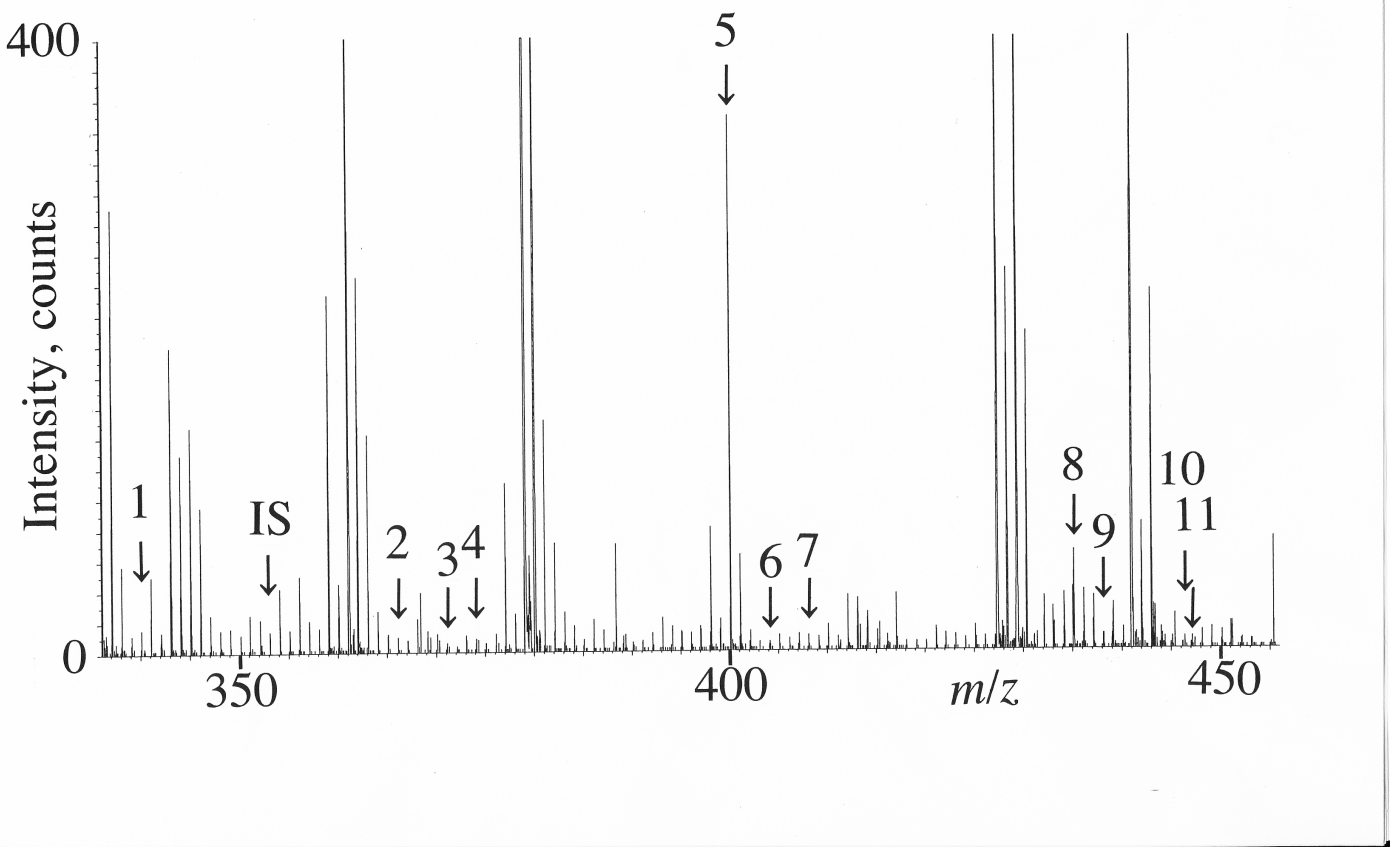
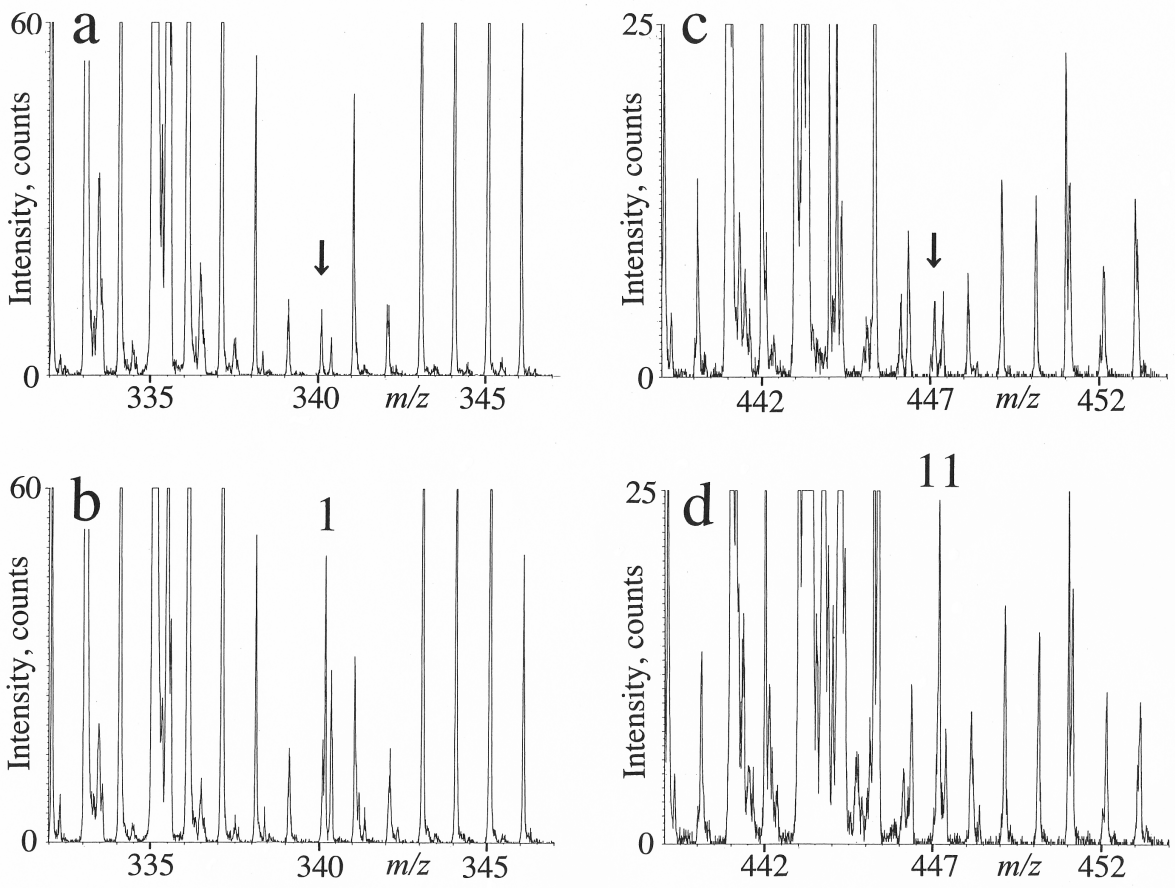
Relative intensity
100
128
97
265
305
200
m/z
400


a
100
0
Relative intensity
113
141
70
212
180
200
400
m/z


b
100
0
Relative intensity
305
139
265
100
290
200
400
m/z


400
0
Intensity, counts
350
400
450
m/z
1
IS
2
3
4
5
6
7
8
9
10
11


a
60
0
Intensity, counts
335
340
m/z
345
c
25
0
Intensity, counts
442
447
m/z
452
b
60
0
Intensity, counts
1
335
340
m/z
345
d
25
0
Intensity, counts
11
442
447
m/z
452