

## Multiplexed Quantification of Metabolites with MISSILE

Jones DR<sup>1\*</sup>, Wang X<sup>2</sup>, Shaw T<sup>2,3</sup>, Cho JH<sup>2</sup> and Peng J<sup>2-5\*</sup>

<sup>1</sup>Department of Biochemistry and Molecular Pharmacology, Langone Medical Center, New York University, NY, 10016, USA

<sup>2</sup>St. Jude Proteomics Facility, St. Jude Children's Research Hospital, Memphis, TN 38105, USA

<sup>3</sup>Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA

<sup>4</sup>Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA

<sup>5</sup>Department of Structural Biology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA

### Abstract

We tested a strategy for multiplexed (4-plex) quantification of metabolites using the MISSILE identification method with liquid chromatography coupled to tandem mass spectrometry. We applied this methodology to study the metabolic effect of the proteasome inhibitor and chemotherapeutic drug Bortezomib in yeast cells. Using JUMPm software version 1.1 we simultaneously identified and quantified 95 metabolites across four experimental conditions and found that Bortezomib increased the accumulation of dipeptides but decreased the levels of specific lipid molecules (e.g. phosphatidylethanolamines) in a dose-dependent manner. This method combines metabolite identification and quantification, making untargeted metabolomics experiments more informative.

**Keywords:** Metabolomics; Metabolome; Mass spectrometry; Stable isotope labeling; Liquid chromatography; Metabolite quantification; Multiplex; Bortezomib; Proteasome inhibition; MISSILE

**Abbreviations:** MISSILE: Metabolome Identification by Systematic Stable Isotope Labeling Experiments; nUPLC-HRMS: Nanoscale Ultra-Performance Liquid Chromatography–High Resolution Mass Spectrometry

### Introduction

We recently described a formula-centric strategy for the identification of metabolites in untargeted metabolomics experiments [1,2]. The method uses stable isotope labeling [3-5] to identify the formula and structure of metabolites in an automated fashion with support for false discovery rate estimation [6,7]. Here we have expanded on this strategy by adding a fourth isotope labeling condition to enable 4-plex relative quantification of metabolites in LC-MS experiments. Each label represents an independent experimental condition so that we can compare the relative levels of metabolites from pairwise duplicates, time course, or dose-response experimental designs. Multi-plexed analyses offer many advantages over traditional label-free methods [8], but current strategies rely on specific functional group tags to chemically label metabolites [9] which limit the scope of quantified metabolites and introduce complexity to the sample preparation workflow. As a test of our methodology, we performed a dose-response analysis on the effect of the proteasome inhibitor Bortezomib [10] in yeast cells. This drug is an effective therapy for multiple-myeloma, but drug-resistance invariably develops due to incompletely characterized mechanisms [11,12]. The effect of proteasome inhibition on protein [13] and gene regulation [14] has been studied in a variety of contexts, but much less is known about the role of metabolism in this process. We report our preliminary investigation and demonstrate the analytical tools for carrying out multiplexed metabolomics analyses.

### Methods

Materials—LC-MS grade acetonitrile (ACN), water, and formic acid (Sigma), glass beads (Next Advance), Difco™ yeast nitrogen base, DMEM (BD Biosciences), nanoLC column (Waters), <sup>13</sup>C-6 glucose, <sup>15</sup>N-2 ammonium sulfate, (Cambridge Isotope Laboratories).

### MISSILE protocol

For *Saccharomyces cerevisiae* (Fleischmann) labeling, cells were grown in four different minimal media conditions. A control media consisted of natural isotopic abundance components; Difco™ yeast nitrogen base without amino acids and ammonium sulfate (BD Biosciences), with 5 g/L ammonium sulfate (Sigma), and 20 g/L glucose (Sigma). For carbon-13 labeling, the media remained the same except that <sup>13</sup>C-6 glucose (Cambridge Isotope Laboratories) was used in place of standard glucose. Similarly for nitrogen-15 labeling, <sup>15</sup>N-2 ammonium sulfate was substituted into the media. Each culture was maintained for ~30 generations in the labeled media before drug treatment and metabolite extraction. For Bortezomib treatment, cultures were seeded to an OD<sub>600</sub> of 0.1 and allowed to grow to 1 (~6 generations).

### Bortezomib treatment

Bortezomib was solubilized in 10 mM, 1 mM, and 0.1 mM stock solutions (water) so that equal volumes could be spiked in for each treatment. For each 25 mL yeast culture, the OD<sub>600</sub> was monitored and recorded every hour. Bortezomib was added to each culture at OD<sub>600</sub>=0.5 absorbance units.

### Sample preparation

Control and drug treatment yeast cultures were harvested and their metabolites were extracted. The liquid cultures were transferred to 15 mL conical vials and centrifuged at 1,000 g for 3 min to obtain

\*Corresponding author: Jones DR, Department of Biochemistry and Molecular Pharmacology, Langone Medical Center, New York University, 550 1st Avenue, NY, 10016, USA, Tel: 646-501-2097; E-mail: [Drew.Jones@nyumc.org](mailto:Drew.Jones@nyumc.org)

Peng J, Department of Structural Biology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA, Tel: (901) 595-7499; Fax: (901) 595-3032; E-mail: [Junmin.Peng@stjude.org](mailto:Junmin.Peng@stjude.org)

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a cell pellet. The supernatant was discarded and 1 mL of freezing 80% acetonitrile was added. Each vial was subjected to 3 min of vortexing at 3,000 rpm in a 1 on/1 off pattern to maintain sample temperature [2]. The lysate was transferred to a fresh vial to exclude the glass beads, and then centrifuged at 21,000 g for 5 min to clarify the liquid phase.

### Spectrophotometric determination of multiplex mixing ratio

The absorption (300 nm) of each supernatant was measured in a 1 mL cuvette and used to calculate the mixing ratio of the 4 individual cultures. For each culture we multiplied the absorbance reading by the volume of supernatant recovered (900  $\mu$ L) as an estimate of the total amount of metabolites present. We then calculated the amount of each sample needed to equal 90% of the lowest measured label. Using these values we mixed the supernatants of the 4 labeled samples into a single tube (~3 mL), vortexed, and aliquoted the mixture into 4 separate tubes.

### Sample reconstitution

The mixed or individual aliquots were dried under centrifugal vacuum and resolubilized to 75  $\mu$ L in buffer A and transferred to inserts for LC-MS analysis.

### LC-MS analysis and parameters

Bortezomib treated yeast samples were analyzed on an Orbitrap Elite (Thermo Scientific) coupled to an Easy nLC™ system as previously described [2]. In summary, we used a nano Acquity UPLC column (75  $\mu$ m  $\times$  100 mm) packed with 1.7  $\mu$ m BEH C18 beads with 0.2% formic acid in water (Mobile Phase A) or acetonitrile (Mobile Phase B). Sample injection volume was 2  $\mu$ L. LC-MS analysis was performed in positive ion (3 kV) mode with a 15  $\mu$ m, 5 cm PicoTip emitter (New Objective). A top 5 data-dependent method was used to target ions for fragmentation (MS/MS) for later structural identification.

## Results and Discussion

### Proteasome inhibition of yeast cells and LC-MS analysis

Yeast cells in a respiring liquid culture were subjected to proteasome inhibition by addition of Bortezomib. We treated cells with three concentrations of drug; at the reported IC<sub>50</sub> [15], 10-fold below, and 10-fold above, with a no-drug control (Figure 1a). To distinguish these four experimental conditions, carbon (i.e., glucose) and nitrogen (ammonium sulfate) sources in the culture were exchanged for various heavy stable isotope labeled compounds (i.e., <sup>13</sup>C-6-glucose and <sup>15</sup>N-ammonium sulfate). There was a decrease in the yeast growth rate with increasing concentrations of Bortezomib (Figure 1b). We subjected each condition to LC-MS analysis alone (Figure 1c) or multiplexed (mixed together) and observed changes in the base-peak chromatogram due to drug-treatment.

### Formula and structure identification of Bortezomib regulated metabolites

We used JUMPm software to globally analyze the metabolites from yeast cells treated with Bortezomib. As an example, we manually examined one of the down-regulated metabolites (a phosphatidylethanolamine). The MS1 scan shows four ions of varying mass, reflecting total incorporation of heavy stable isotopes into the chemical structure (Figure 1d). The mass shift of the four ions can also be used to determine the chemical formula of the metabolite (Figure 1e) as previously described. Each of the four parent ions for phosphatidylethanolamine was manually extracted to confirm their co-elution and identity as isotope labels (Figure 1f). The intensity of these

peaks was detected by JUMPm and serves as the basis for the relative quantification. The structure identity was determined by JUMPm in a search of its associated MS/MS spectra (Figure 1g). These tandem mass spectra were collected in a Top 5 data-dependent fashion. For phosphatidylethanolamine, MS2 spectra were acquired from two of the four labeled parent ions, the <sup>12</sup>C parent (unlabeled control) and the double labeled <sup>13</sup>C<sup>15</sup>N parent ion. Two structural fragments were observed for each parent with nearly identical relative intensities. Overall, we identified 181 metabolite formulas and quantified 95 metabolite structures among the four treatment conditions with a false discovery rate of less than 1% (Table 1).

### Multiplexed quantification of Bortezomib regulated metabolites

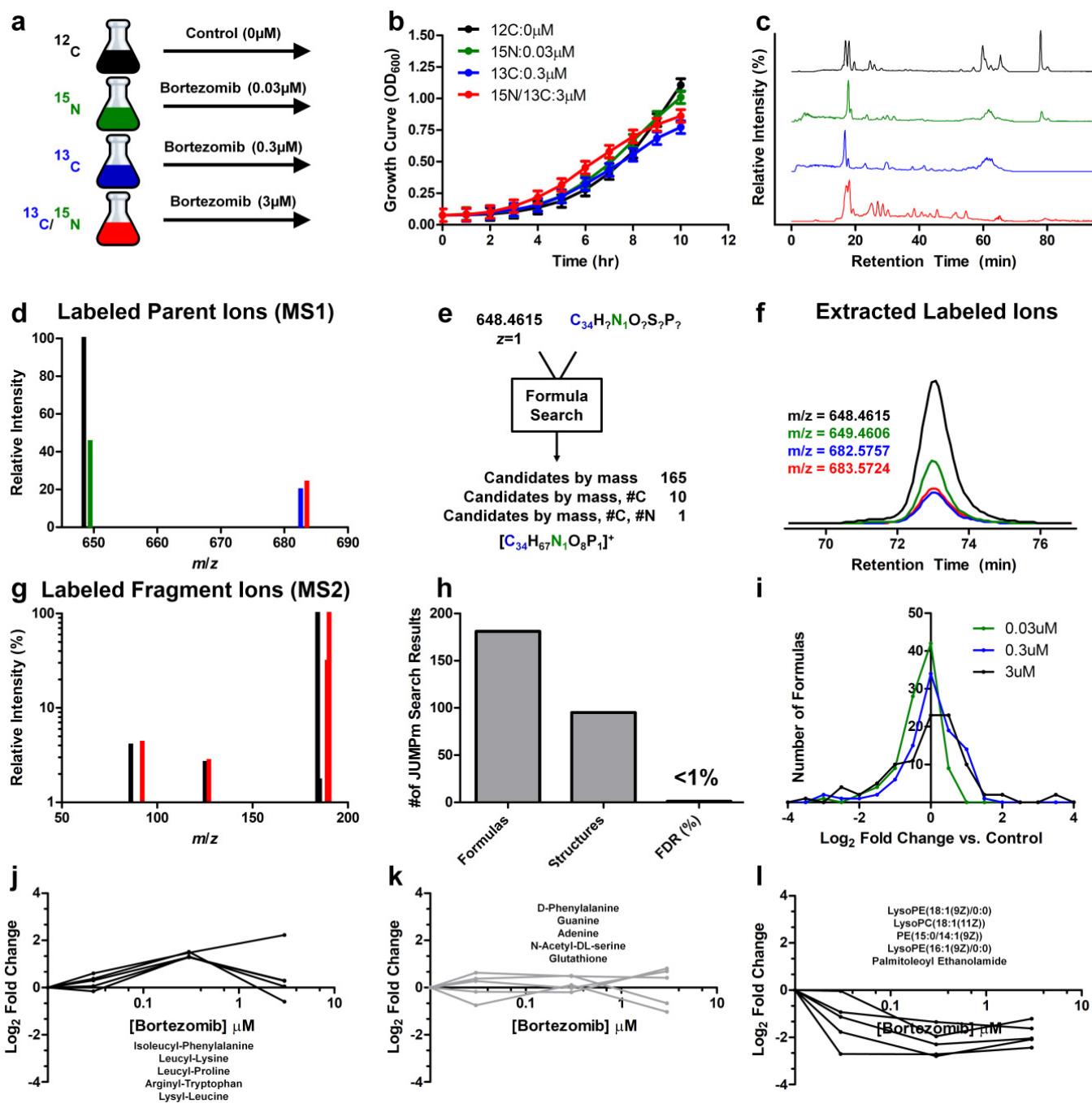
We observed that higher Bortezomib concentrations had broader impacts on the yeast metabolic profile, with a greater proportion of metabolites showing a larger fold change in peak size relative to control (Figure 1i). The lowest concentration of drug had a relatively minor effect on metabolite levels, whereas the higher concentrations increasingly segregated metabolites into up- or down-regulated populations. This trend was also true for individual metabolites (Figure 1d). As expected we observed that Bortezomib treatment increased the levels of incomplete protein catabolites e.g., di-peptides (Figure 1j). Metabolites that were unaffected by drug-treatment included nucleobases (e.g. adenine) and other “housekeeping” metabolites such as glutathione. We also observed that membrane components and signaling lipids were strongly down-regulated with higher Bortezomib concentrations (Figure 1l and 1k; Table 1).

### Advantages and limitations

The described multiplexed design has several advantages over classical unlabeled strategies for metabolite quantification. Peak alignment between analyses is a major confounding factor for unlabeled strategies, and much effort has been focused on resolving this issue. For multiplexed samples, peak alignment is unnecessary because the treatment conditions are analyzed simultaneously. Carbon and nitrogen stable isotope labeling does not affect the retention time of small molecules, so each labeled form co-elutes with the other treatment conditions. This co-elution is a major advantage because it controls for time and sample-dependent variations in retention time and ionization efficiency, a major source of technical error during metabolite quantification.

Using the current strategy we can expect to identify and quantify metabolites from four independent labeled conditions. The labels are comprised of light or heavy carbon and nitrogen atoms. Therefore we observe all four possible mass labels for a given metabolite if the chemical formula contains at least one nitrogen atom. We previously observed that ~50% of known metabolite formulas contain nitrogen while the rest do not. For metabolites without nitrogen, our strategy can only provide two independent labels. In that case, the two peaks represent the average of two cultures (i.e., cultures 1&2 or 3&4). Therefore the duplicate experimental design may be more suitable for studies focused on such metabolites.

We introduce a new capability to perform 4-plex relative quantification of metabolites in untargeted metabolomics experiments using the MISSILE strategy in combination with JUMPm software. Using this method, we can now analyze metabolites from two experimental conditions with duplicates, or with up to 4 independent experimental conditions (e.g. time series or dose response data). The



**Figure 1:** Metabolomics analysis of Bortezomib treated yeast cells (a) Experimental design of the 4-plex study. Labels indicate the stable isotope culture condition. Condition 4 (red) used both stable isotope labels simultaneously. (b) Growth curve for the four independent yeast cultures, monitored by  $OD_{600}$ . (c) Base-peak chromatograms for the four cultures analyzed independently prior to multiplex analysis. (d) An example MS1 spectrum for a metabolite peak after mixing the four yeast samples together. Colored peaks reflect the increased mass of the metabolite from stable isotope labelling and also the relative intensity changes due to Bortezomib treatment. This metabolite was identified as PE (15:0/14:1(9Z)). (e) Automated chemical formula-determination workflow for the example metabolite peaks shown in (d). Candidates represent chemical formulas within 4 ppm mass tolerance of the unlabelled peak. With increasingly strict search criteria the number of possible formulas dramatically decreases. The determined formula represents the charged parent ion. All formulas in Table 1 have been discharged. (f) Extracted ion chromatogram for each of the four labeled versions of the yeast metabolite shown in (d). Retention time and peak shape are not affected by isotope labelling, while the peak intensity reflects the changes due to Bortezomib treatment. (g) Tandem mass spectrum (MS/MS) of the labeled precursors from the example metabolite. (d-f) Each structure fragment shows two peaks, representing the two isotopes labeled parent ions which were fragmented in the Top 5 data-dependent method. (h) Fold change distribution of quantified metabolites compared to the no-drug control. Higher Bortezomib concentrations were associated with increased variation in metabolite levels. (i) Summary of JUMPm global metabolite search results. The formula false discovery rate (FDR) was less than 1% as determined by JUMPm based on the relative frequency of target and decoy formulas detected. (j-l) Up-regulated, unaffected, and down-regulated metabolites respectively as a function of Bortezomib concentration. Labels indicate identified metabolites using JUMPm software.





7255	175.1195	179.076	181.1366	185.1277	176.719	194.865	32.15860	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	7208	0.43	D-Arginine	NC@H[C@@H](C(=O)N)C(=O)O	ODS/FDXX/FXON-SCSABSYSAN	0.0	C12	1052
407	303.1462	307.1344	318.1987	322.1844	28925	30996	43694	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	480	0.44	Histidinyl-Phenylalanine	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	XIA/HMAA/TDOP-UHFFFAOYSAN	20.1	C12	14
7169	308.0919	311.0830	318.254	321.1165	40845	49574	679460	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>	7157	0.45	Glutathione	NC@H[C@@H](C(=O)N)C(=O)N(CCC(=O)N)C(=O)O	RWSXRVCMGZ/ZWBV-WSKDSNSAN	19.2	C12	66
378	308.0924	311.0835	318.259	321.1170	8695	11535	11839	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>	344	0.45	Glutathione	NC@H[C@@H](C(=O)N)C(=O)N(CCC(=O)N)C(=O)O	RWSXRVCMGZ/ZWBV-WSKDSNSAN	0.0	C13	88
799	175.1195	179.077	181.1366	185.1277	233049	2339478	3976705	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	732	0.45	D-Arginine	NC@H[C@@H](C(=O)N)C(=O)O	ODS/FYDXX/FXON-SCSABSYSAN	0.0	C13	1033
7255	147.1332	148.073	153.1333	155.1274	47461	18895	46568	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	7220	0.46	D-Lysine	NC(CCC(=O)N)C(=O)O	KDXKERNBSR/KRMOYKEDSAN	0.0	C12	13
243	308.0924	311.0835	318.263	321.1174	20323	27942	28097	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>	213	0.47	Glutathione	NC@H[C@@H](C(=O)N)C(=O)N(CCC(=O)N)C(=O)O	RWSXRVCMGZ/ZWBV-WSKDSNSAN	11.8 <th>C12</th> <th>193</th>	C12	193
378	303.1464	307.1346	318.1989	322.1850	1898	1715	2637	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	344	0.47	Histidinyl-Phenylalanine	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	XIA/HMAA/TDOP-UHFFFAOYSAN	26.0 <th>C13</th> <th>13</th>	C13	13
1593	253.1301	257.1183	264.1668	268.1551	366289	473371	505950	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	1650	0.48	Histidinyl-Proline	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	LNCF/UHPTMYLUB-UHFFFAOYSAN	28.6 <th>C13</th> <th>9</th>	C13	9
180	308.0924	311.0835	318.260	321.1173	4625	7479	6442	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>	213	0.48	Glutathione	NC@H[C@@H](C(=O)N)C(=O)N(CCC(=O)N)C(=O)O	RWSXRVCMGZ/ZWBV-WSKDSNSAN	11.8 <th>C12</th> <th>43</th>	C12	43
196	272.1731	277.1593	283.2100	288.1953	2973	4599	4193	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	222	0.50	Arginyl-Proline	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	LQJALCCPOTJG-UHFFFAOYSAN	62.8 <th>N15</th> <th>28</th>	N15	28
180	152.0574	157.0425	167.0742	162.0695	2590	3359	3670	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	143	0.50	Guanine	NC1=NC=O=C2N1CN=CN=C2	UYTURDROBUNYGS-UHFFFAOYSAN	0.0	C12	11
273	272.1731	277.1592	283.2099	288.1950	9722	13926	13854	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	224	0.51	Prolyl-Arginine	NC(N)C(=O)N(CCC(=O)N)C(=O)O	HMSRSLZAHRSIK-UHFFFAOYSAN	1.5 <th>C12</th> <th>43</th>	C12	43
180	175.1197	179.079	181.1399	185.1281	12831	17475	16553	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	141	0.53	L-Arginine	NC@H[C@@H](C(=O)N)C(=O)O	ODS/FYDXX/FXON-SCSABSYSAN	6.9 <th>C12</th> <th>131</th>	C12	131
1575	272.1724	277.1575	283.2092	288.1945	5033493	6237272	7491910	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	1626	0.56	Arginyl-Proline	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	LQJALCCPOTJG-UHFFFAOYSAN	26.7 <th>N15</th> <th>157</th>	N15	157
3529	352.1666	355.1576	372.2338	375.2249	160772	145919	238006	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	3625	0.57	Phenylalanyl-Tryptophan	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	JMCOUWKLXJERBU-UHFFFAOYSAN	0.0 <th>C13</th> <th>23</th>	C13	23
299	308.0924	311.0836	318.260	321.1174	1730	2790	2586	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>	213	0.58	Glutathione	NC@H[C@@H](C(=O)N)C(=O)N(CCC(=O)N)C(=O)O	RWSXRVCMGZ/ZWBV-WSKDSNSAN	11.8 <th>C12</th> <th>193</th>	C12	193
497	142.0890	145.0891	148.1181	151.0933	43398	67416	65584	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	517	0.60	L-Histidinol	NC@H[C@@H](C(=O)N)C(=O)O	ZSRSDGJUNB/VMA-YRFRYSAN	0.0 <th>C12</th> <th>37</th>	C12	37
8784	137.0641	141.0643	142.0630	146.0512	23356	24881	37970	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	6761	0.69	Algaumul	O=C(N)C=C(N)C=C12	OFCNMPARWPKY-UHFFFAOYSAN	0.0 <th>C13</th> <th>63</th>	C13	63
2290	302.1507	305.1419	318.2043	321.1946	468520	462842	767671	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	2319	0.69	Prolyl-Tryptophan	OC(=O)C(=O)N(CCC(=O)N)C(=O)O	UEYKROQHOZ-UHFFFAOYSAN	0.0 <th>C12</th> <th>59</th>	C12	59
273	289.1621	273.1602	281.2023	285.1904	7461	7597	12484	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	224	0.74	Leucyl-Histidine	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	XVGBNRDUDEY-UHFFFAOYSAN	0.9 <th>N15</th> <th>9</th>	N15	9
759	229.1553	231.1494	240.1923	242.1864	332933	247587	543330	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	804	0.75	Leucyl-Proline	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	VTJUNRYAHF-UHFFFAOYSAN	0.0 <th>C12</th> <th>88</th>	C12	88
250	288.2044	293.1895	300.2447	305.2299	6799	4910	10001	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	221	0.79	Arginyl-Leucine	CC(C)C(N)C(=O)N(CCC(=O)N)N(C)O	WYBBIJNWOJG-UHFFFAOYSAN	2.2 <th>C12</th> <th>34</th>	C12	34
7163	258.1107	259.1078	266.1376	267.1346	57791	94433	133143	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	7121	0.79	Glycerocephalosoline	CN1[C@H](C(=O)O)C[C@H](O)C[C@H]1O	SYBQZJWYXORJMMKGPBSAN	0.0 <th>C12</th> <th>9</th>	C12	9
180	269.1620	273.1602	281.2023	285.1906	1380	1911	2500	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	224	0.86	Leucyl-Histidine	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	XVGBNRDUDEY-UHFFFAOYSAN	0.9 <th>N15</th> <th>9</th>	N15	9
273	219.1950	221.1290	228.1652	230.1591	1090	676	1977	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O	214	0.86	L-Lysine	CC(N)C(C)C(=O)O	ZZYZZAZDMRPS-UHFFFAOYSAN	6.0 <th>C13</th> <th>13</th>	C13	13
273	229.1556	231.1497	240.1926	242.1865	10214	7017	18710	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	227	0.87	Leucyl-Proline	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	VTJUNRYAHF-UHFFFAOYSAN	11.6 <th>C13</th> <th>41</th>	C13	41
497	319.1411	323.1293	334.1915	338.1796	119051	119051	199921	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	469	0.91	Tryptophyl-Histidine	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	ZOOYCYZBENIMC-UHFFFAOYSAN	37.3 <th>C13</th> <th>74</th>	C13	74
3439	361.1893	367.1814	378.2563	384.2396	972895	921215	1880096	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	3394	0.95	Arginyl-Tryptophan	NC(CCC(=O)N)C(=O)N(CCC(=O)N)N(C)O	GADERTWYXV-UHFFFAOYSAN	0.0 <th>N15</th> <th>126</th>	N15	126
200	229.1558	231.1498	240.1928	242.1868	6892	6588	17757	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	227	0.98	Leucyl-Proline	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	VTJUNRYAHF-UHFFFAOYSAN	11.6 <th>C13</th> <th>72</th>	C13	72
200	260.1617	263.1600	271.1984	274.1898	1607	1416	3278	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	224	1.03	Hydroxy-Lysine	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	VTJUNRYAHF-UHFFFAOYSAN	2.4 <th>C13</th> <th>12</th>	C13	12
2603	253.1190	255.1131	265.1593	267.1533	299885	423382	632884	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	2941	1.08	Tryptophyl-Alanine	CC(N)C(=O)N(CCC(=O)N)C(=O)O	BAZOKHDAYJAP-UHFFFAOYSAN	0.0 <th>C13</th> <th>51</th>	C13	51
273	269.1616	263.1629	271.1966	274.1895	1338	1509	2876	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	224	1.08	Hydroxyprolyl-Lysine	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	BAZOKHDAYJAP-UHFFFAOYSAN	2.4 <th>C13</th> <th>3</th>	C13	3
2111	361.1963	367.1815	378.2565	384.2396	210915	396508	453475	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	2201	1.10	Tryptophyl-Arginine	NC(CCC(=O)N)C(=O)N(CCC(=O)N)N(C)O	LQJALCCPOTJG-UHFFFAOYSAN	0.0 <th>C13</th> <th>30</th>	C13	30
1975	288.2035	293.1899	300.2440	305.2292	1884540	2072277	4053500	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	1954	1.10	Arginyl-Leucine	CC(C)C(N)C(=O)N(CCC(=O)N)N(C)O	WYBBIJNWOJG-UHFFFAOYSAN	0.0 <th>C12</th> <th>73</th>	C12	73
497	229.1555	231.1495	240.1924	242.1865	115922	119944	254268	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	467	1.14	Leucyl-Proline	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	VTJUNRYAHF-UHFFFAOYSAN	10.0 <th>N15</th> <th>56</th>	N15	56
180	288.2042	293.1895	300.2446	305.2299	1300	1485	2904	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	221	1.16	Arginyl-Leucine	CC(C)C(N)C(=O)N(CCC(=O)N)N(C)O	WYBBIJNWOJG-UHFFFAOYSAN	2.2 <th>C12</th> <th>15</th>	C12	15
2308	195.1134	197.1074	205.1469	207.1410	520378	994623	1877375	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	2335	1.19	Leucarn	CC1=CC=CC=C1C(=O)O	DAMBADJWLFTM-UHFFFAOYSAN	0.0 <th>C12</th> <th>49</th>	C12	49
273	269.1691	263.1682	272.2384	275.2293	4328	5171	9932	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	224	1.20	Lysyl-Lysine	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	ATPTGOTUXBK-UHFFFAOYSAN	2.2 <th>C13</th> <th>17</th>	C13	17
230	322.1890	327.1740	337.2392	342.2244	3944	4831	9093	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	278	1.21	Arginyl-Phenylalanine	NC(CCC(=O)N)C(=O)N(CCC(=O)N)C(=O)O	POBHSQZSLUR-UHFFFAOYSAN	28.4 <th>N15</th> <th>40</th>	N15	40
2693	279.1710	283.1697	294.2214	296.2155	288834	301991	70352	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	2534	1.28	Isoleucyl-Phenylalanine	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	WMDARISIMZOO-UHFFFAOYSAN	0.0 <th>N15</th> <th>31</th>	N15	31
568	269.1676	263.1687	272.2378	275.2290	145455	179186	357164	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	587	1.30	Leucyl-Lysine	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	VTJUNRYAHF-UHFFFAOYSAN	11.6 <th>C13</th> <th>45</th>	C13	45
180	229.1557	231.1497	240.1927	242.1868	2654	2390	6625	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	227	1.32	Leucyl-Proline	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	VTJUNRYAHF-UHFFFAOYSAN	0.0 <th>C13</th> <th>18</th>	C13	18
3421	361.1893	367.1815	378.2563	384.2396	251842	381961	701954	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	3394	1.48	Arginyl-Tryptophan	NC(CCC(=O)N)C(=O)N(CCC(=O)N)N(C)O	GADERTWYXV-UHFFFAOYSAN	0.0 <th>N15</th> <th>126</th>	N15	126
180	269.1690	263.1693	272.2384	275.2296	988	1293	2824	C <sub>16</sub> H <sub>18</sub> N <sub>2</sub> O	224	1.52	Lysyl-Lysine	CC(C)C(N)C(=O)N(CCC(=O)N)C(=O)O	ATPTGOTUXBK-UHFFFAOYSAN	2.2 <th>C13</th> <th>4</th>	C13	4

Table 1: Metabolite formulas and structures identified from LC-MS analysis of Bortezomib treated yeast using MISSILE and JUMPm.

**Columns:** <sup>1</sup>The MS1 scan number in the raw file in which the formula was detected. <sup>2-5</sup>The monoisotopic mass of the unlabelled (12C) and labeled (15N, 13C, 13C15N) peaks as detected. <sup>6-8</sup>The intensities of the detected peaks. <sup>10</sup>The chemical formula as determined by JUMPm. <sup>11</sup>The MS2 scan number in the raw file in which the structure was detected. <sup>12</sup>The Log2 ratio of the peak intensity for the 0.3µM Bortezomib treated sample divided by the control. <sup>13</sup>The best scoring structure candidate for the detected formula as determined by JUMPm. <sup>14</sup>The SMILES notation for the annotated structure. <sup>15</sup>The InChIKey for the annotated structure. <sup>16</sup>The Mascot score as determined by JUMPm, which scores the quality of the match between the observed MS2 fragment ions and the predicted MS2 fragment ions for the reported structure. <sup>17</sup>The type of label from which the MS2 scan was detected. <sup>18</sup>The signal to noise ratio for the detected peak in the MS1 scan.

isotope labels are used by JUMPm software to determine chemical formulas and perform relative quantification and false discovery control.

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