1 **Untargeted metabolomics suffers from incomplete data analysis**

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5 *Brief Communication*

6 **Abstract**

7 *Introduction:* Untargeted metabolomics is a powerful tool for biological discoveries. Significant
8 advances in computational approaches to analyzing the complex raw data have been made, yet it is
9 not clear how exhaustive and reliable are the data analysis results.

10 *Objectives:* Assessment of the quality of data analysis results in untargeted metabolomics.

11 *Methods:* Five published untargeted metabolomics studies acquired using instruments from
12 different manufacturers were reanalyzed.

13 *Results:* Omissions of at least 50 relevant compounds from original results as well as examples of
14 representative mistakes are reported for each study.

15 *Conclusion:* Incomplete data analysis shows unexplored potential of current and legacy data.

16 **Keywords**

17 metabolomics, mass spectrometry, data analysis, liquid chromatography

18 **Introduction**

19 Mass spectrometry-based metabolomics is a powerful tool for the discovery of novel compounds,
 20 metabolic capabilities, and biomarkers (Patti et al. 2012; Sévin et al. 2015). Successful discoveries
 21 are dependent on the ability to reliably detect relevant signals in raw data and to correctly interpret
 22 the underlying spectral features of compounds (Kind & Fiehn 2007; Dunn et al. 2013; Scheubert et
 23 al. 2013; Baran & Northen 2013; Kind et al. 2017). The challenging complexity of the data analysis
 24 process is well recognized and computational tools facilitating the data analysis process are
 25 available (Weber et al. 2017). However, it is not clear how exhaustive and reliable are the current
 26 data analysis results. The quality of the results is important not only in the context of exploratory
 27 research but even more more so in the context of a strengthening trend towards large scale
 28 integration of multi-omic datasets (Perez-Riverol et al. 2017). Public repositories of metabolomics
 29 data, such as the UCSD Metabolomics Workbench (Sud et al. 2016) or the MetaboLights (Haug et
 30 al. 2013) database, provide an opportunity to reanalyze published raw data to assess the coverage of
 31 relevant signals as well as the quality of mass spectra interpretation.

32 Five untargeted metabolomics datasets from public repositories acquired using instruments from
 33 different manufacturers were selected for reanalysis (Table 1, Supplementary Fig. 1-5). The
 34 selection was arbitrary with a focus leaning towards high complexity of the raw data (large numbers
 35 of detected compounds).

36 **Materials and Methods**

37 Raw datafiles along with accompanying data analysis results were downloaded from the
 38 respective data repositories (Table 1). Raw data files in original instrument manufacturers'
 39 proprietary data formats were converted to mzXML (Pedrioli et al. 2004) data format using
 40 ProteoWizard's msconvert tool (Chambers et al. 2012). Differences among datasets within a specific
 41 study (for ions not reported in original study results) were detected using direct comparisons
 42 between datasets binned along the m/z dimension as described previously (Baran et al. 2006). The

43 mass spectra and extracted ion chromatograms corresponding to candidate differences were then
44 inspected visually to assign related ions (e.g. $[M+H]^+$, adducts, multimers, in-source fragments,
45 isotopic peaks). To limit the extent of tedious manual curation, the aim of the reanalysis was to find
46 50 relevant omissions in each study.

47 To be considered an omission, none of the ions corresponding to the omitted compound could be
48 reported in the original results (even if the only reported ion corresponds to an isotopic peak of an
49 in-source fragment ion of a specific compound). Only raw data acquired in positive mode polarity
50 were used for re-analysis for each study. However, negative mode raw data and results were
51 examined as well. If none of the ions of a specific compound were reported in positive mode
52 results, but at least one ion related to the compound was reported in negative mode results, the
53 compound was not considered and not reported as an omission.

54 Multiple ions for omitted compounds along with their peak areas are listed in Supplementary
55 Data 1. These lists of ions are not exhaustive. Low intensity isotopic peaks or ions that could be
56 potentially related (but not showing clear similarities in chromatographic profiles, relative peak
57 areas across samples, or differences in m/z to other ions of typical chemical relationships) may have
58 been left out of these lists. However, records for even these possibly related ions were sought in
59 original results accompanying the study to make the best effort to report truly omitted compounds
60 in reanalysis results.

61 Peak areas were calculated using the trapezoidal integration method without any prior smoothing
62 of extracted ion chromatograms or baseline subtraction. Integration bounds were set manually. The
63 ion with the largest peak area from a group of related ions was selected as a "representative" ion for
64 a given compound and used for extracted ion chromatograms (Fig. 1, Supplementary Fig. 6-10).
65 Few representative mistakes found during the reanalysis process were mostly related to ion type
66 (mis)interpretation in the original results and are shown in Supplementary Figures 12-16. A rough
67 comparison of relevance of omitted compounds to the original results was based on peak areas of

68 "representative" ions and a measure of a statistical significance of a difference among the groups of
 69 replicate samples in a study, if applicable (Supplementary Fig. 11). Peak areas calculated by the
 70 trapezoidal method were normalized to peak areas in the original results (Supplementary Fig. 17-
 71 21) for this comparison.

72 **Results and Discussion**

73 The raw data were reanalyzed as described in the Materials and Methods section to look for
 74 omissions of relevant compounds as well as examples of common mistakes in the original data
 75 analysis results accompanying the study data. To limit the extent of tedious manual curation of the
 76 data, a goal of finding 50 relevant omissions in each study was set. For a compound to be
 77 considered omitted, none of its ions (e.g. $[M+H]^+$, adducts, multimers, in-source fragments, isotopic
 78 peaks) could be reported in the original results. Figure 1a-d shows a few examples of omissions
 79 from one of the reanalyzed studies, and Supplementary Figures 6-10 show examples of at least 50
 80 omissions from each study. These omissions are relevant in the context of reported results, since
 81 these compounds show either intense signals or differ significantly among the study groups
 82 (Supplementary Fig. 11). In addition to omissions, mistakes in ion type interpretation were also
 83 found during the reanalysis. The most commonly observed mistake was the reporting of in-source
 84 fragment ions, isotopic peaks, or other ion types instead of the protonated molecule $[M+H]^+$ ion
 85 (Fig. 1e, Supplementary Fig 12-16).

86 This reanalysis of published metabolomics studies was far from exhaustive. The newly reported
 87 lists of ions for omitted compounds (Supplementary Data 1) are incomplete, may contain mistakes
 88 as well, and additional unreported compounds are very likely present in the raw data. The selected
 89 metabolomics studies have impressive quality of the raw data as well as original data analysis
 90 results which must have required significant effort and insight. And yet the results of this simple
 91 reanalysis point to an additional unexplored potential of current as well as legacy metabolomics
 92 data. Hopefully, these results will strengthen the appreciation for the complexities of the data

93 analysis process and further motivate improvements in computational tools and knowledgebases for
94 metabolomics data analysis.

95 **Conflict of interest**

96 The author's company Baran Bioscience, LLC provides data analysis services for metabolomics
97 and small molecule mass spectrometry.

98 **References**

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135 opportunity for harmonisation through Galaxy. *Metabolomics*, 13(2), 12.

136 Tables

137 **Table 1 - Untargeted metabolomics studies selected for reanalysis**

Study Identifier	ST000403	ST000326	ST000220	MTBLS214	ST000259
Instrument	Thermo Scientific Q-Exactive Orbitrap	Agilent 6530 QTOF	Waters Synapt-G2 Si	AB Sciex TripleTOF 5600	Bruker MicrOTOF II
Sample Layout	6 groups of 3 replicates ^a	19 individual samples	3 groups of 7 replicates	3 groups of 4-5 replicates	14 groups of 5-6 replicates
Compounds /features (+)	590	962	1259	18	857
50+ Omissions	+	+	+	+	+
Mistakes	+	+	+	+	+
Study URL	http://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Study&StudyID=ST000403	http://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Study&StudyID=ST000326	http://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Study&StudyID=ST000220	http://www.ebi.ac.uk/metabolights/MTBLS214	http://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Study&StudyID=ST000259

^aFour of the six groups contained added stable isotope labels. Peaks corresponding to clear stable isotope labeling signals or peaks absent from the two control groups without stable isotope labeling were not considered as possible compound omissions.

141 Figure Legends

142 **Figure 1 - Examples of omissions and mistakes in results from study ST000403. (a)**

143 Visualization of a part of one of the raw datafiles. Gray labels correspond to annotations from
 144 original results accompanying the study data. Magenta labels correspond to omissions or mistakes.
 145 **(b-d)** Mass spectra and extracted ion chromatograms for examples of omissions. **(e)** A mass
 146 spectrum and extracted ion chromatograms for examples of mistakes. An in-source fragment ion
 147 and an isotopic peak of a multimer of HEPES were incorrectly identified as different compounds.
 148 Peaks of related ions for a given compound in plots of mass spectra are highlighted in magenta.
 149 Color coding for groups of replicate samples in extracted ion chromatograms is the same as in
 150 Supplementary Figure 6.