## Title

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# Enhancing untargeted metabolomics using metadata-based source annotation

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## Abstract

Human untargeted metabolomics studies annotate only ~10% of molecular features. We introduce reference data–driven analysis to match metabolomics MS/MS data against metadata-annotated source data as a pseudo-MS/MS reference library. Applying this approach to food source data, we show that it increases MS/MS spectral usage 5.1-fold over conventional structural MS/MS library matches and allows empirical assessment of dietary patterns from untargeted data.

## Editorial summary:

[#]co-first author.

Author Contributions:
PCD, RK, RJD, and JMG conceptualized the idea.
MP, FDO, KCW, CMA, EB, KS, PCD, RJD, RK, NCS, ADS, KD, GA, DM, NPL, and JMG collected FoodOmics samples and performed metadata curation.
MP, FDO, FV, CMA, EB, NCS, and JMG performed FoodOmics sample processing and MS data acquisition.
AJJ, PBF, ED, QZ, DN, DM, JPS, and JMG curated Global FoodOmics metadata to match FNDDS.
JBW, BSB, BJB, RC, MGDB, MD, EOE, DG, LH, JHK, MM, CM, RK, KES, DVR, TIK, CW, KPW, MFO, RHM, DW, RT, JGA, PD, MG, DG, AKJ, BJB, RMS, KCW, ADS, FV, NPL, PKP, SMS, SLS, CMJ, NJL, KAL, SAJ, RKD and JMG provided samples, comparative dataset, and/or detailed metadata.
LMMM, TMC performed COVID-19 patient and/or food sample preparation and analysis.
PLJ was the physician responsible for the COVID-19 patients.
RDRO was the physician responsible for collecting the plasma from COVID-19 patients.
FPV was responsible for tabulation of COVID-19 patient data.
MP, JMG, TS, MB, LDG, GH prepared samples for food.
MW supported GNPS computational infrastructure used in the study.
CLW, WB, AKJ, KAW, ES, AT, NPL and JMG analyzed MS data.
CLW, WB, AKJ, KAW, CM, and JMG generated figures.
PCD, RK, RJD, ADS, and JMG supervised the work.
PCD, RK, CLW, KAW, WB, and JMG wrote the paper.
All authors have contributed feedback and edits to the manuscript.

Declaration of Interests:
BSB has a research grant from Prometheus Biosciences and has received consulting fees from Pfizer. PCD is on the scientific advisory board of Sirenas, Cybele Microbiome, Galileo and founder and scientific advisor of Ometa Labs LLC and Enveda (with approval by UC San Diego). JHK is a consultant for Medela and on the Board for Innara Health; he owns shares in Astarte Medical and Nicolette. MG has research grants from Pfizer and Novartis. PSD has received research support and/or consulting from Takeda, Pfizer, Abbvie, Janssen, Prometheus, Buhlmann, Polymedco. RJD is a consultant for and owns shares in Impossible Foods Inc, and is on the Scientific Advisory Panel of Boost Biomes. AJJ has received consulting fees from Abbott Nutrition and Corebiome. DG is a consultant for Biogen, Fujirebio, vTv Therapeutics, Esai and Amprion and serves on a DSMB for Cognition Therapeutics. KPW reports during the conduct of the study receiving research support from SomaLogic, Inc., consulting fees from or served as a paid member of scientific advisory boards for the Sleep Disorders Research Advisory Board - National Heart, Lung and Blood Institute, CurAegis Technologies, Philips, Inc, Circadian Therapeutics, LTD. and Circadian Biotherapies Ltd. RT: Research grant: Astra-Zeneca Consulting: SOBI, Selecta, Horizon, Allena, Astra-Zeneca. ADS and RK are directors at the Center for Microbiome Innovation at UC San Diego, which receives industry research funding for multiple microbiome initiatives, but no industry funding was provided for this project. MW is a co-founder of Ometa Labs LLC. Dr. Kaddurah-Daouk in an inventor on a series of patents on use of metabolomics for the diagnosis and treatment of CNS diseases and holds equity in Metabolon Inc., Chymia LLC and PsyProtix. The remaining authors declare no competing interests.

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Metabolomics is improved by using a reference library of both known and unknown molecules.

Complex sequence data from metagenomic or metatranscriptomic experiments require for interpretation both databases of curated genes and reference data, such as whole genomes or other sequence data with carefully curated metadata (developmental stage, tissue location, phenotype, etc.).[1–4] Such reference data-driven (RDD) analysis increases understanding of complex communities by using matches between genes or transcripts of known and unknown origin. The RDD strategy is essential for the successful analysis of most metatranscriptomics or metagenomics data. By analogy, interpreting LC-MS/MS-based untargeted metabolomics data is performed by searching structural MS/MS libraries. However, leveraging reference data with curated and structured controlled vocabulary metadata to improve insights obtainable from untargeted MS/MS-based metabolomics is not yet done.

RDD analysis uses not only annotated MS/MS-spectra but also all unannotated spectra. The GC-MS BinBase resource has made a step in the direction of RDD. With BinBase one can annotate if a spectrum match has been observed in a non-public GC-MS dataset. However, the metadata is not well controlled and lacks the ability to add contextualized metadata.[18,19] In addition, as we have previously demonstrated, using structural annotations, the source can be determined by literature mining.[20] However, due to above mentioned limitations and/or inability to link related spectra in the case of metabolism, the above strategies to annotate unknowns cannot be used to systematically to interpret the source information at the data set level. We therefore introduce the RDD approach for metabolomics (Figure 1), followed by a use case demonstrating empirical food readouts from untargeted human data (Figure 2).

Untargeted MS/MS-based metabolomics experiments involve searching MS/MS structural libraries since the late 1970’s [5,6], or, more recently, for investigating the distribution of a MS/MS spectrum across public untargeted data.[21] Instead of only leveraging a single MS/MS spectrum to obtain an annotation, RDD metabolomics uses all MS/MS spectra from untargeted metabolomics files, which contain hundreds to thousands of MS/MS spectra, for metadata-based source annotation. The key differences are that the output reports contextualized information from source reference datasets. For successful RDD analysis, it is critical that the contextualized data are curated using controlled vocabularies or the results will not be amenable to downstream analysis. In the presented application for RDD, we investigated which food compositions could be recovered from data acquired from human biospecimens. Answering this question required a resource of reference food MS/MS source data and associated curated metadata. The source data includes MS/MS spectra of multiple ion forms of known and unknown molecules, isotopes, adducts, in-source fragments, and multimers.[7,8] The curated reference dataset can be matched in human biospecimens via direct matching of the MS/MS spectra or by molecular networking. Unlike static libraries, RDD analysis retains flexibility by enabling custom addition of files or metadata, and also gives the user control on how the reference data is processed. We created a step-by-step tutorial for RDD analysis using GNPS (https://ccms-ucsd.github.io/GNPSDocumentation/tutorials/rdd/ and corresponding video tutorial https://www.youtube.com/watch?v=2-XsifrUY0Y).[9]

To exemplify RDD metabolomics, and because food is critical for health, we created a food metabolomics reference data set. There is an unmet need to retrospectively and empirically read out food and beverage information from human metabolomics data, complementing current state-of-the-art mass spectrometry nutrition readout approaches targeting up to ~150–200 metabolites, food frequency and abundance questionnaires, diet records, 24-hr recalls, which can be self-monitored or assisted by a nutritional specialist. [10,11] The food reference data set consists of untargeted metabolomics and detailed and structured metadata for ~3500 foods (157 different food-specific metadata fields, Table S1). It contains 107,968 unique MS/MS spectra merged from 1,907,765 spectra. The food source data can be easily expanded by creating and depositing additional data sets and metadata in GNPS/MassIVE.

For RDD, food source data is subjected to GNPS based molecular networking[14,15] together with human metabolomics datasets (Figure 2a). Using information on the controlled research diets of participants of a sleep and circadian study we assessed if RDD recovers food known to be consumed[12]. In this study, the participants were housed for four weeks and were given a controlled diet, therefore we know if the results agreed with the known diet from that study (Figure 2b). Of the 15 food categories, eleven represented direct matches to foods provided to the participants. Of those eleven matches, three matched to fermented versions of the non-fermented foods consumed such as fermented grapes instead of grapes, apple cider instead of apple, yogurt instead of milk, and four categories were not documented as consumed during the study, three of which could be explained. Evidence of caffeinated beverage consumption was observed only in two individuals — in the first 48hrs in one volunteer and once in a second volunteer in the middle of the study — that there were few matches to caffeinated beverages is consistent with the elimination of caffeinated beverages in the controlled diet. Although not always written on the ingredient list of packages, rosemary is a common ingredient added to ground meat to slow oxidation and spoilage. The source of the matches to soda are unknown. This demonstrates that RDD can successfully obtain the correct diet information from untargeted metabolomics data but also be used to monitor diet adherence in controlled-diet studies.

We also tested mismatched food inventories by cross-matching US or Italian foods (different diets) and clinical cohorts. Crossover revealed that MS/MS spectral usage rates —the percentage of MS/MS spectra interpreted by the analysis— were 5–6% in reciprocal tests, versus 15–30% when the correct regional foods were used (Figure 2c, p=0.019). These observations show that RDD analysis is selective based on the foods that are consumed but also that it is important to continue to grow the food reference database as generic food databases have considerable value. Efforts, such as the Periodic Table of Food Initiative, and linking of Metabolights and Metabolomics workbench repositories with GNPS/MassIVE will aid the expansion of the food reference data.

We next assessed if RDD analysis could recover a reference food spiked into human biospecimen extracts. We therefore analyzed mixtures of two human fecal samples or the NIST 1950 plasma reference extract with a tomato seedling extract in different proportions.[22] In all three biospecimens, the proportion of spectral matches relative to the tomato seedling extract increased linearly with the spiked-in proportion ($p=2.32E^{-31}$, SI Figure 1).

Because RDD analysis can be performed retrospectively, we co-analyzed the food reference dataset with 28 additional public human datasets (Table S2, SI Figure 2). 10.1±4.4% of MS/MS spectra matched to spectral structural libraries. RDD increased MS/MS spectral usage 5.1±3.3-fold over structural MS/MS library matches. With molecular networking, which can capture metabolized versions of molecules, spectral data usage increased 6.8±3.5-fold. Inclusion of connected nodes, representing potential metabolism via molecular transformations, resulted in a total increase of 43.7±3.1% (fecal; $P$=6.9e-10), 51.2±6.9% (plasma; $P$=2.8e-06), and 58.0±4.2% (other; $P$=1.4e-06) of MS/MS spectra that can be leveraged as empirical readout of diet (SI Figure 2).

To validate the food consumption read-outs obtained via RDD analysis from these 28 datasets, direct spectral library matches in the molecular networks created by the food-based RDD analyses (1% FDR, and level 2/3 according to the metabolomics standards initiative [13, 17]) were evaluated to verify whether they make sense in the context of food. An InChIKey is available for 4,586 of 5,455 spectral matches against the reference libraries, which yielded 1,492 unique structures upon consideration of planar structures. For 415 out of 1492 planar structures that had lifestyle tags associated in GNPS [20,21], “food consumption” was the most frequently reported tag (357 entries; 86%). Additionally, other matches are related to the food production chain, such as feed additives to promote animal growth that are tagged as “drug”, such as the antimicrobial agents monensin, enilconazole, kanamycin and other agricultural additives or environmental toxins such as domoic acid.[25]

To assess if RDD can reveal dietary preferences, we analyzed a data set of omnivores and vegans. Principal component analysis (PCA) of the spectral match relative proportions to reference foods revealed distinct patterns between dietary preferences (Figure 2d). Omnivores had more MS/MS matches to dairy, meat, and seafood ($P$=0.0021, 2.2e-10, and 7.7e-7 respectively), while vegans had more MS/MS matches to legumes, fleshy fruit, and vegetables ($P$=2.2e-10, 0.0096, and 0.029, respectively, Figure 2e). Because many MS/MS spectra from foods may overlap, using only MS/MS spectra unique to each food can provide additional specificity (Figure 2f). RDD analysis on an elderly population[16] found that individuals with lower diet diversity had more spectral matches to dairy, soda, and coffee, and this diet type was more prevalent in the Alzheimer’s Disease group than those with normal cognition (SI Figure 3). This demonstrates that RDD analysis can be used to retrospectively stratify clinical studies based on empirical readout of diet composition for each sample.

RDD thus enables readout of dietary patterns (e.g. vegan versus omnivore) and consumption of specific food items, and, more generally, can be used to match against any curated and ontology - aware reference database of sources, including environmental or microbial sources. RDD metabolomics is currently unique to GNPS, as it requires highly scalable molecular networking and incorporation of detailed metadata. However, as other analysis ecosystems add molecular networking capabilities, or that make RDD compatible with other spectral alignment algorithms, it will become possible to use other resources for RDD metabolomics. As scalable molecular networking for GC-MS is also possible[24], specialized resources, such as BinBase[18,19], may eventually be leveraged for RDD analysis of specific applications or questions. To expand the scope of RDD metabolomics beyond food readout, well curated datasets of personal care products, medications (not just active ingredients but also formulations), microbial isolates, country of origin, biological sex, age, etc. might also be used as source reference data and requires careful curation with controlled vocabularies and structuring of metadata. Potential applications of RDD metabolomics include understanding diet and nutritional intake, exposure risks, medication use, consumption of illegal substances, environmental allergens, pollution studies, microbiome investigations, food ingredients/adulteration, forensics, and personal care product tracing to inform of potential exposures and health implications.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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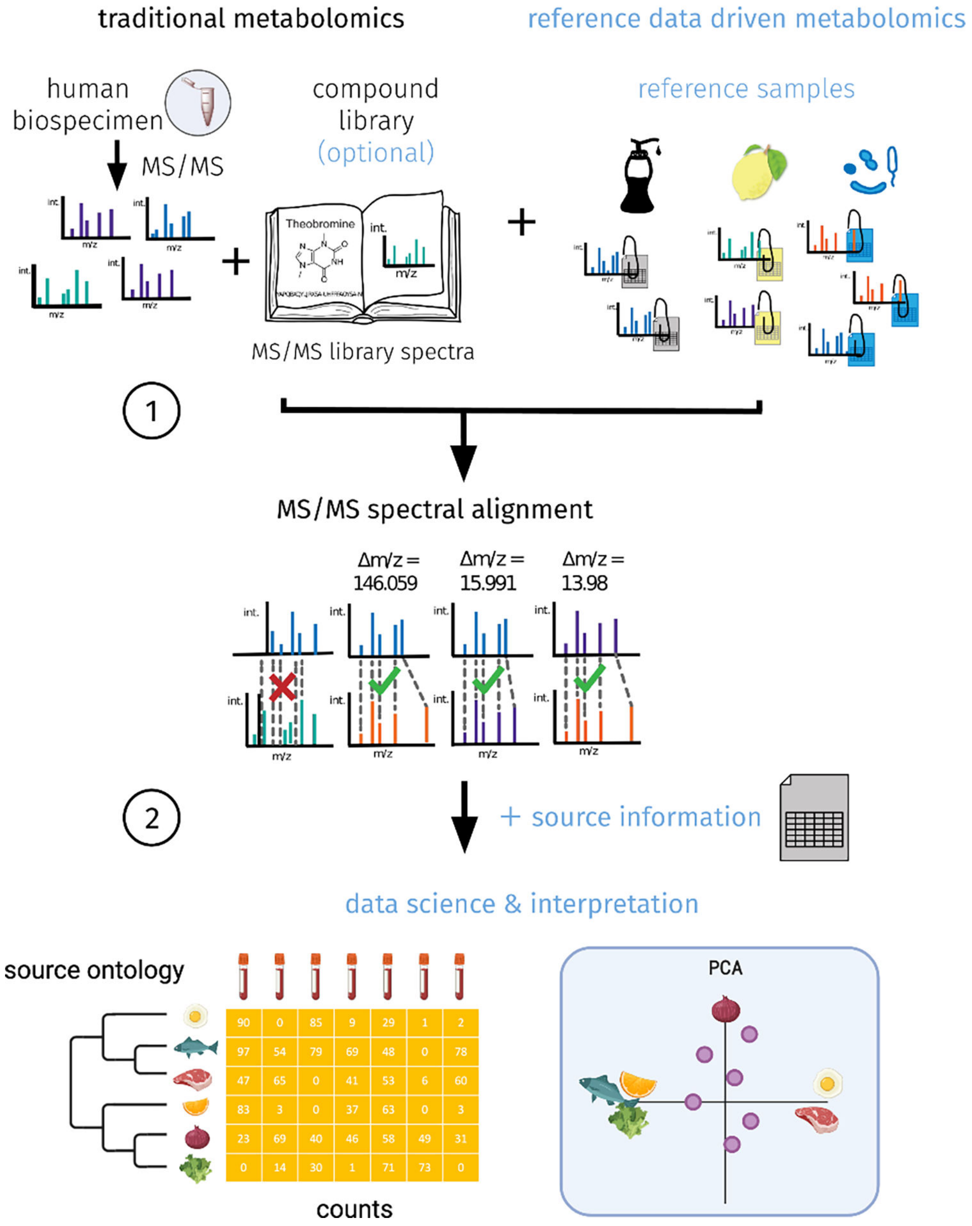

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| 97 | 54 | 79 | 69 | 48 | 0 | 78 |
| 47 | 65 | 0 | 41 | 53 | 6 | 60 |
| 83 | 3 | 0 | 37 | 63 | 0 | 3 |
| 23 | 69 | 40 | 46 | 58 | 49 | 31 |
| 0 | 14 | 30 | 1 | 71 | 73 | 0 |

**Figure 1. The concept of reference data-driven based analysis workflow.**

1 - Perform spectral alignment of the MS/MS based untargeted metabolomics data from human biospecimens with data from reference samples that have controlled vocabularies for metadata. This can, optionally, be combined with MS/MS libraries. 2 - link the spectral matches to the source information from the metadata from the reference samples. Create a data table of source ontology, human biospecimen and counts to enable data science and interpretation.

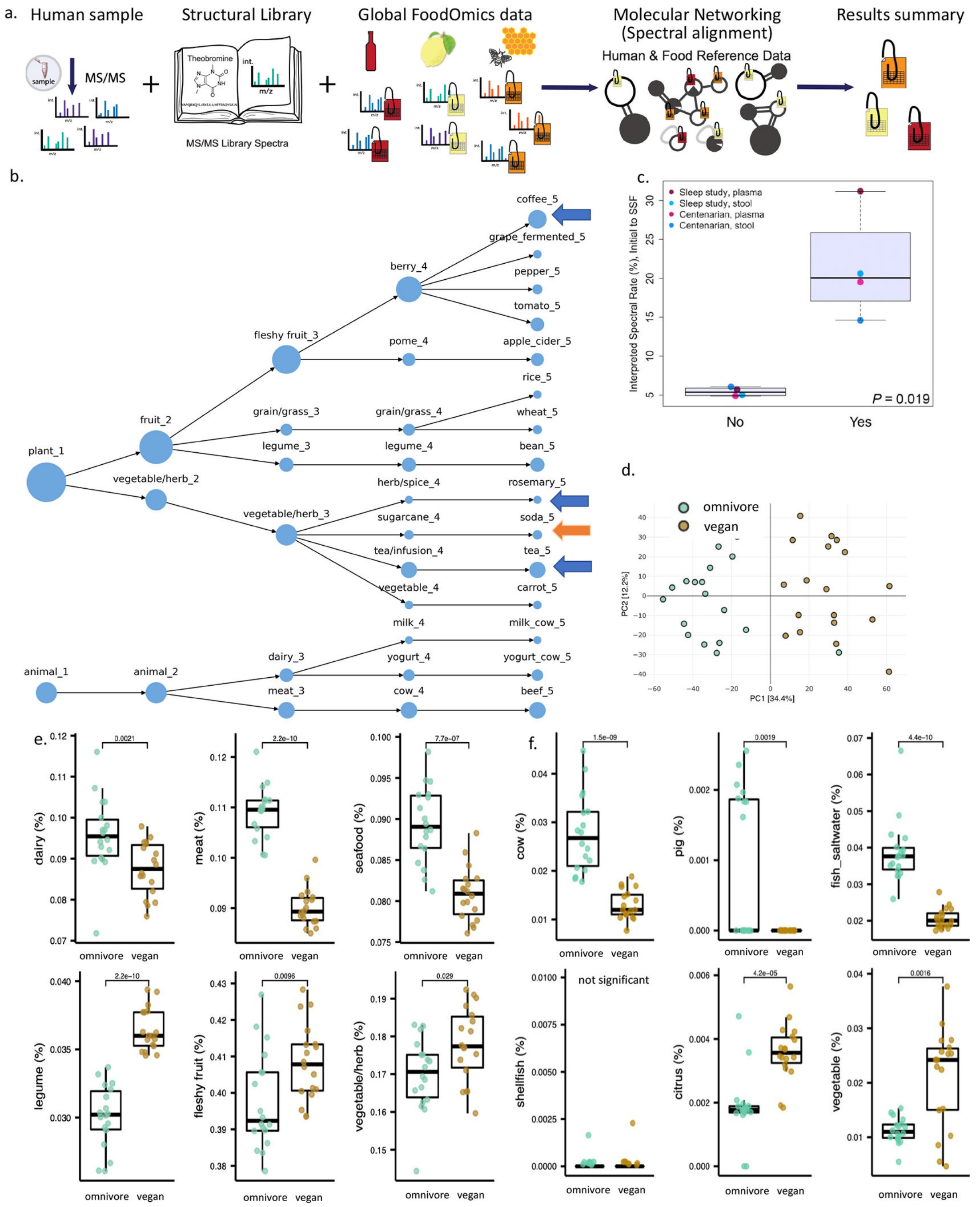

**Figure 2. RDD with food reference data.**

a. Food RDD analysis schema. b. Food spectral counts (1% FDR[13]) observed in plasma from a sleep restriction and circadian misalignment study that controlled the diet of the participants (n=371 samples from 20 healthy adults).[12] The size of node represents the relative number of spectral matches at each food level. Blue arrow - foods that could be explained based although they were not provided in the study, orange arrow– source is not known. c. A crossover experiment between centenarian data from Italy and a sleep and circadian study from the US, for both fecal and plasma samples. Study region specific foods consumed by those individuals (yes) vs a different set of study region specific foods (no), (one way Welch's t-test, thick line is the mean, range within the box is the interquartile range, from the 25 to 75 quartile, min / max are the whiskers). d. PCA of food counts color coded by vegan (brown) vs omnivore data (green). e. Statistical analysis for the food counts at level 3 of the ontology, in relation to omnivore and vegan data (Wilcoxon test, n=36, 19 are vegan and 19 are omnivore). f. Same as e. but level 4 ontology using unique spectral counts (spectral usage is the percentage of MS/MS spectra used in the analysis. Since they are unnamed ontologies as one would find in microorganism phylogeny in microbiome science - e.g. kingdom, genus, species we have denoted these as layers, Table S1). For e-f, The boxes represent the interquartile range (IQR). Lower limit (Q1) is 25th percentile, median (Q2), upper limit (Q3) is 75th percentile. Bars show Q3+1.5xIQR and Q1–1.5xIQR.