**KHMN Scripts User Manual**

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**Part 1: Introduction and Installation**

KHMN is used to assist in screening and annotating defined secondary metabolites.

To use KHMN, it is recommended to install python3.6.4 or above.

**Part 2: Feature Table Input and Alignment**

Multiple .xlsx files from different tissues can be dealt with at the same time. Tissue\_ID is needed to provide in the feature table. Considering the emergence of redundancy, the features in different tissues will be aligned to generate a uniform Align\_ID for later redundancy removal.

**Part 3: MS2 Input and Filtering**

Every MS/MS spectrum in the newly generated .mgf MS2 files corresponds to an independent feature. The MSCluster algorithm doesn’t need to be activated during the molecular network construction. There are two situations in the MS/MS acquisition, including the same acquisition condition contains multiple MS2 files or only one MS2 file. We separate the two situations into simple conversion and batch conversion. The ID of initial feature table must start with tissue\_0, such as Silk\_0.

**3.1: Simple Conversion**

A single acquisition condition contains multiple MS2 files.

**3.2: Batch Conversion**

Every acquisition condition includes a single MS2 file.

**Part 4: New MS2 Loading**

Each MS/MS spectrum in the newly generated .mgf files contains a specific Tissue\_ID and Align\_ID.

**Part 5: Seed Metabolites MS2 Input**

Once the class of metabolites is selected, the .mgf MS2 file of seed metabolites will be imported, which is from the seed metabolites LC-MS/MS database, containing the predicted retention time.

**Part 6: Seed Metabolites Identification and Marking**

The seed metabolites from non-targeted data are identified and marked based on the seed metabolites LC-MS/MS database.

**Part 7: MS2 Similarity and Molecular Network Generation**

A heterogeneous molecular network of seed metabolites and non-targeted data is constructed. The MS/MS similarity algorithm used here refers to gnpsalignment (Wang M, et al. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nat. Biotechnol.* **34**, 828-837 (2016). https://www.nature.com/articles/nbt.3597). There are many MS2 similarity algorithms currently available (https://github.com/mwang87/GNPS\_SpectralSimilarityHub). Users could select them according to requirements. However, the computing power of personal computers is limited. It is recommended to complete the molecular network construction on the powerful GNPS (https://gnps.ucsd.edu/) platform.

**Part 8: Candidates Features Screening**

The features are retained existing in the same molecular cluster with the seed metabolites, regardless of the seed metabolites from databases or from non-targeted data.

**Part 9: Nodes and Edges Annotation**

For HCAAs (hydroxycinnamic acid amides), the nodes are searched for the characteristic ions and neutral losses from the given list. For CGFs (C-glycosylflavones), the nodes are searched for the Motifs and glycosyls from the given lists. The positive searching results for each node will be returned to a node table. The edges of the three classes of secondary metabolites are searched for a given list of enzyme-catalyzed reactions. The positive searching results were recorded to an edge table for each edge. If you search for compounds other than the three types of secondary metabolites, you can modify the corresponding knowledge list.

**9.1: Nodes and Edges Annotation of HCAAs**

**9.2 Nodes and Edges Annotation of CGFs**

**9.3 Edges Annotation of BOAs (benzoxazinoids)**

**Part 10: Redundancy Removal**

The nodes with the same Align\_ID will be merged, which refers to redundant nodes in the same acquisition condition but different tissues. There are some small changes for the connection of the network clusters. But it does not affect the final result.