AccuTOF™

LC/MS: Identification of Unknowns by Combining Exact Mass Measurement with the NIST 02 Mass Spectral Database Similarity Search

Introduction: Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) tend to produce mass spectra with minimal fragmentation. Positive-ion mass spectra are dominated by protonated molecules and cation attachment, while negative-ion mass spectra usually show molecular anions or ions produced by hydride abstraction. However, by varying the potentials in the atmospheric pressure interface, collision-induced dissociation (CID) can produce mass spectra with extensive fragmentation. This is sometimes referred to as "in-source CID". Because the ionization process and ion energies are different for ESI or APCI compared to electron ionization (EI), the fragmentation is often different from the EI mass spectra in common mass spectral databases. Furthermore, the fragmentation pattern can vary depending on in-source CID conditions. This leads one to question whether there is any value to searching an EI mass spectral database for ESI or APCI mass spectra.

The NIST 02 Mass Spectral Search software provides functions for a structure similarity search. This search can be used to search the library for compounds with similar structures based on neutral losses, which is a more suitable method for searching ESI or APCI mass spectra against the library. Compounds that have mass spectra in the library database can often be identified from the search results. The similarity search can provide structural information about compounds that do not have mass spectra in the library by displaying the structures of similar compounds identified by the search.

ESI and APCI mass spectra acquired under "mild" API conditions provide information about the molecular weight of the unknown compound which can be used to eliminate unreasonable results from the library search. Because the JEOL AccuTOF mass spectrometer can easily provide elemental composition information through exact mass measurements without requiring dilute solutions or statistical corrections, the elemental composition for an unknown can provide additional information for evaluating a library search result.

Experimental: ESI or APCI mass spectra were acquired for a variety of compounds, including several flavonols, a dye and various organic acids, by using HPLC or flow injection for sample introduction. The resolving power was 6000 (FWHM) for all measurements. The automatic function-switching feature of the AccuTOF was used to alternately acquire molecular ion information (under "mild" API conditions with an orifice 1 potential of 30 V) and mass spectra with extensive fragmentation (with an orifice 1 potential of 50-80 V).   This feature provides both molecular weight and fragment information with a single sample injection.

Mass spectra were corrected for temperature drift by using a "lock mass" (palmitic acid $[M-H]^-$ at m/z 255.2324) and elemental compositions were calculated for each compound by using the Mass center elemental composition software. Peak-picked mass spectra were exported as integer-mass spectra to the NIST search program by using the library search function of the JEOL Mass Center software.   The NIST/EPA/NIH Mass Spectral library was searched by using the "MS/MS" similarity search function which searches for neutral losses over a wider m/z range than the "Neutral loss" search function.

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Recommended Search Parameters:

- **_Type of search:_** Similarity Search (MS/MS search)

- **_Precursor ion molecular weight:_** Use the <u>actual</u> molecular weight of the compound (not the $[M+H]^+$ or $[M-H]^-$ mass.

- **_Presearch:_** OFF   Note: the search will be slightly slower than a typical EI mass spectrum library search.

- **_Contraints:_** Set the maximum molecular weight to the actual molecular weight of the compound (same as precursor MW). If you are confident of the elemental composition assignment from the exact mass measurements, you can also set constraints for the presence and number of elements.

- **_Mass spectrum display range:_** Restrict the display to show the fragment ions and the protonated molecule (or the $[M+Na]^+$ or $[M-H]^-$ or equivalent ion. Avoid setting the display range high enough to show dimers and other adducts.

Example: Kaempferol

**Ionization mode:** APCI (negative-ion mode)

**Sample introduction:** HPLC analysis of a synthetic mixture of flavor components.

Elemental composition determination:

Element Limits: C 0/40   H 0/80   O 0/12
Tolerance: 5.00 mmu
Even or odd electron ion or both: EVEN
Minimum unsaturation: -1.0
Maximum unsaturation: 100.0

Meas. mass u	Abund. %	Diff. mmu	Unsat.	Compositions
285.03992	100.0	0.01	11.5	**C15 H9 O6**

Only even-electron ions (unsaturation ends with ".5") are reasonable for protonated molecules or ions due to hydride abstractions. <u>The elemental composition determination shows that the $[M-H]^-$ composition is $C_{15}H_9O_6$, or that the compound has the composition $C_{15}H_{10}O_6$ with 12 rings and double bonds.</u>

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Screen Dump of NIST Search
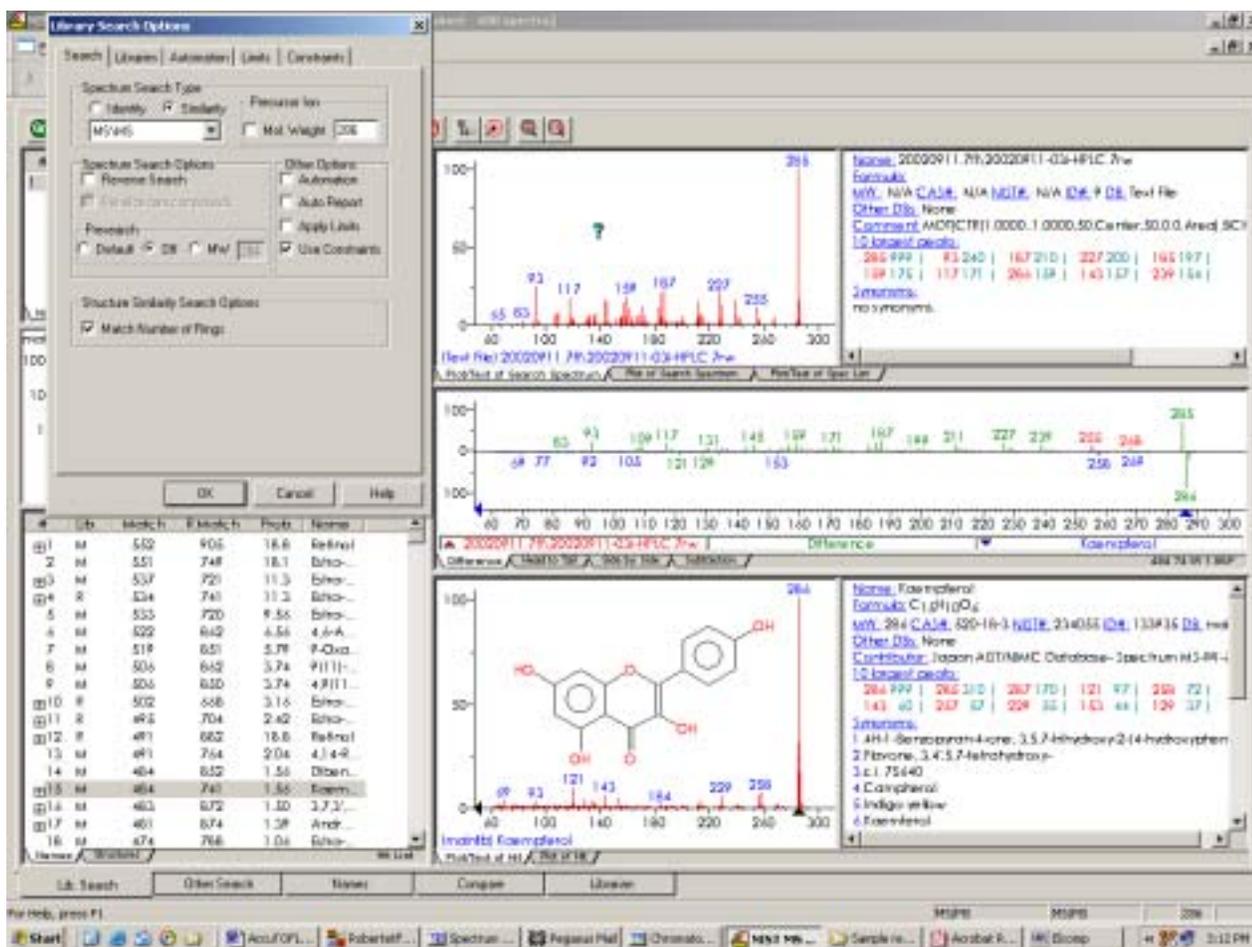
If no constraints are placed on the elements, a large number of similar compounds are reported. The correct composition is the 15th compound in the list. However, the correct composition is the first compound in the list that has the correct elemental composition. The other 14 compounds have elemental compositions that would produce an [M-H][-] with a measured m/z that would be very different from the measured m/z of 285.03992.

If the elemental composition is used to place constraints on the elements present, then only nine compounds are returned by the search and the first match in the list is kaempferol. The fifth match is also kaempferol from the replicate library.

#	Lib.	Match	R.Match	Prob.	Name
⊞1	M	484	741	50.4	Kaempferol
⊞2	M	483	872	48.5	3,7,3',4'-Tetrahydroxyflavone
⊞3	R	445	889	48.5	3,7,3',4'-Tetrahydroxyflavone
4	M	312	673	1.04	5,7,3',4'-tetrahydroxyflavone
⊞5	R	284	501	50.4	Kaempferol
6	M	139	377	0.02	2,2'-Biphenyldicarboxylic acid, 4,5-methylenedioxy-
7	M	139	174	0.02	Anthraquinone, 1,4,5,7-tetrahydroxy-2-methyl-
8	M	60	154	0.00	7,8,2',4'-Tetrahydroxy-isoflavone
9	M	50	132	0.00	5,7,2',4'-Tetrahydroxy-isoflavone

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Other compounds in the flavor component mixture (quercitin, myricetin, syringaldehyde) gave similar results. That is, the first compound in the list with the correct elemental composition was the correct compound. The compound syringic acid was not identified among the top matches in the search, but the first two compounds with a matching elemental composition were isomers. Although two compounds with the same nominal molecular weight, quercitin (m/z 301.0348) and ellagic acid (m/z 300.9984) were added to the mixture, only one (quercitin) was identified by its exact mass. It was later learned that the ellagic acid had precipitated out of the solution before the sample was shipped to our laboratory.

A search for the hormones testosterone and epitestosterone returned a large list of isomers of these compounds including the correct compounds within the top 25 matches. This is not unexpected, because isomers will often produce mass spectra with similar masses, but different relative peak abundances. One cannot expect to compare the very different peak abundances produced by a negative-ion APCI mass spectrum against a database of EI mass spectra of a large number of isomers and expect to return the correct structure as the best match.

Of course, if a compound is not in the library, a similarity search cannot return the correct compound. However, the search can be used to suggest possible structural features or provide confirming evidence for an assignment. Among the matches returned from a search for an unknown ionic dye compound was a nonionic compound with a substructure that was believed to be present in the dye based on information about the origin of the sample and elemental composition determination from the exact mass measurement. This provided supporting evidence that the assignments of structures to fragment ions in the dye mass spectrum were consistent with fragmentation pathways observed in an EI mass spectrum of a compound with similar structure.

Supporting information from fragment-ion compositions

It can be difficult to interpret fragmentation observed in the in-source CID mass spectrum of a complete unknown. However, the similarity search can suggest structural features and exact mass measurements for fragment ions can provide valuable information about elemental compositions.

Two of the compounds (kaempferol and syringaldehyde) in the flavor component mixture showed a neutral loss of 30 in the in-source CID mass spectra. Without more information, one might assume that both compounds fragmented in the same way. However, the AccuTOF provided exact mass measurements for the fragment ions for both compounds, which showed that the two compounds fragment differently.

Kaempferol

The [M-H]⁻ ion from kaempferol ($C_{15}H_9O_6$ at m/z 285.0399) produces a fragment ion at m/z 255.0299, which is assigned the elemental composition $C_{14}H_7O_5$ (loss of H_2CO).

Meas. mass u	Abund. %	Diff. mmu	Unsat.	Compositions
255.02992	0.00	0.57	11.5	C14 H7 O5
285.03992	0.00	0.01	11.5	C15 H9 O6 (loss of H_2CO)

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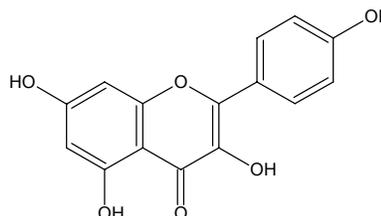
Syringaldehyde

The loss of m/z 30 from syringaldehyde is assigned to loss of C_2H_6 (probably two methyl group losses from the two methoxy substituents).

Meas. mass u	Abund. %	Diff. mmu	Unsat.	Compositions
181.050079	0.00	-0.01	5.5	C9 H9 O4
151.001526	0.00	-1.61	6.5	C7 H3 O4
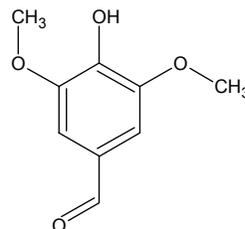
Conclusion: APCI or ESI mass spectra obtained under in-source fragmentation conditions can be searched against an EI mass spectral database by using the NIST 02 similarity search with the MS/MS function. Compounds that have EI mass spectra or their isomers can be identified by using the AccuTOF's exact mass and elemental composition capability to restrict the search. Compounds with similar substructures can often be identified when attempting to identify a compound whose mass spectrum is not in the database. The ability of the AccuTOF to assign elemental compositions to fragment ions can greatly facilitate the interpretation of in-source CID mass spectra.

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