Polar Constituents in Crumb Rubber Extracts

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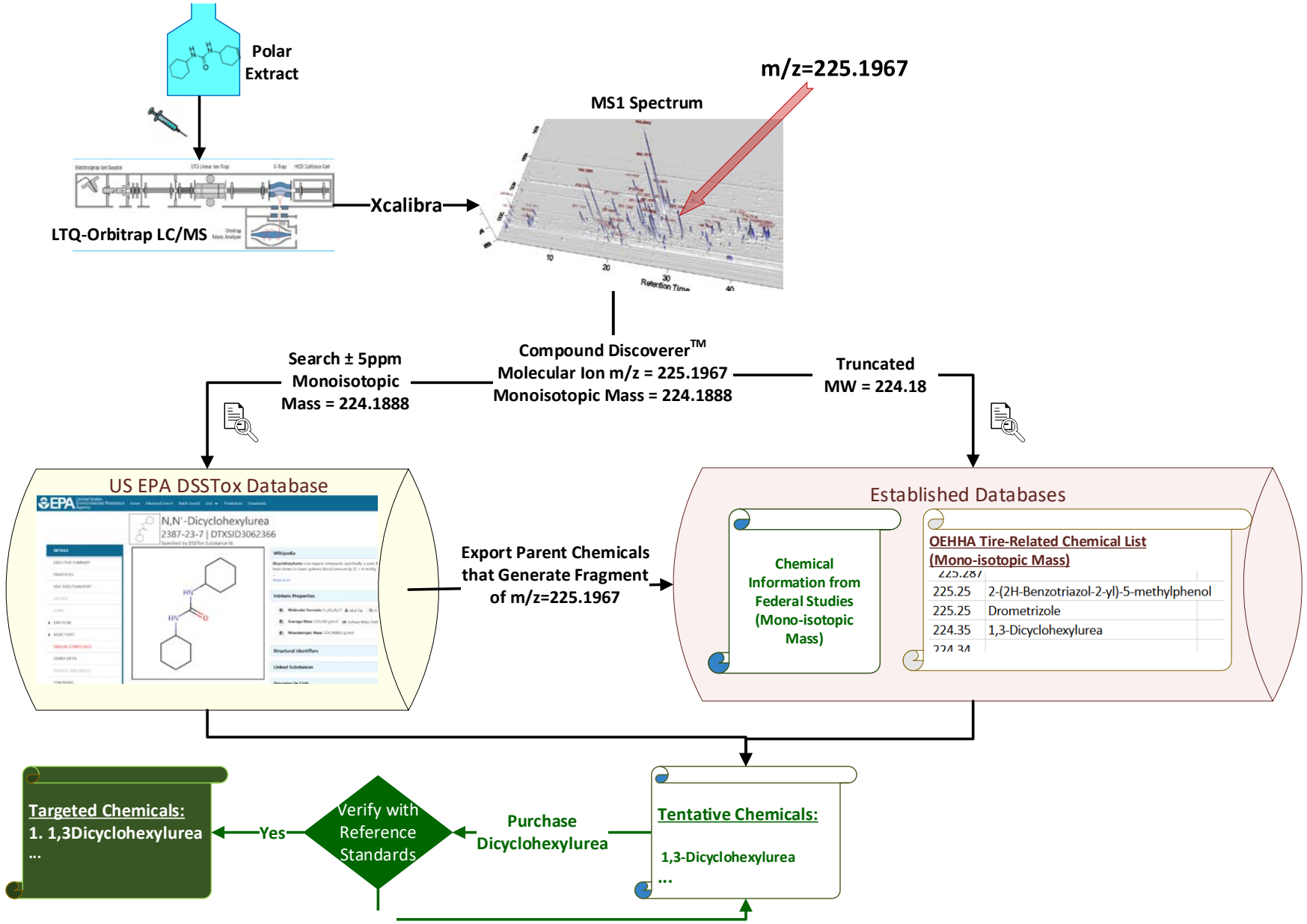
Non-Targeted Analysis of Polar Chemicals

- Most of the polar chemicals (especially those with high water solubility) are not suitable for GC/MS analysis

- Polar chemicals can be analyzed using LC/MS
  - LC/MS results will compliment GC/MS results to provide comprehensive analysis of field samples
  - Develop a 2-Tiered Non-Targeted Approach to analyze LC/MS data
  - Applying advanced computational tools may improve the success of identifying candidates for unknowns.

Tier 1: Suspect Screening Analysis – Identify chemicals for testing all fields



Tier 1. Suspect Screening Results of Manufacturer Samples – Database Search Using Molecular Mass (from MS1) of LC Peaks

Database	Screening Method/Software	No. of Chemicals in the Database	No. of Possible Chemicals	Chemical Purchased
ChemSpider	Compound Discoverer™	72 million chemical structures	743,492	-
Distributed Structure-Searchable Toxicity (DSSTox)	Web-Based Batch Search	>850,000	>80,000	-
OEHHA Tire-Related Chemicals List*	Excel	754	250	27

* A collection of tire-related chemicals reported in the literature and federal studies.

3 possible methods of analysis:
1. GC/MS with chemical-specific detection method
2. LC/MS with positive ionization
3. LC/MS with negative mode

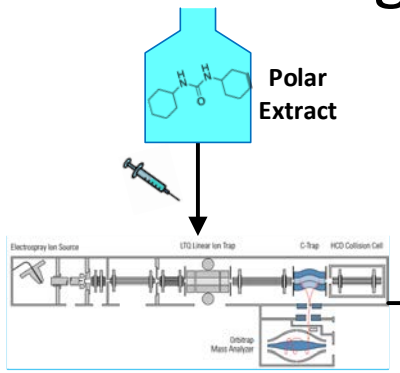
18 Tire-Related Targets Confirmed by Reference Standards*

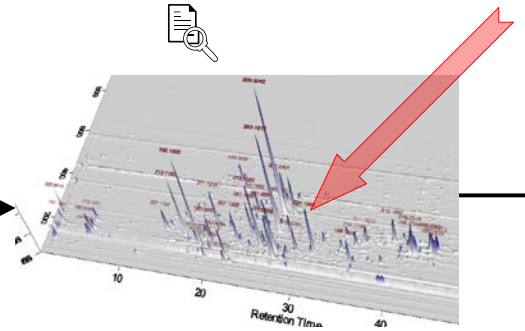
Chemical Name	Formula	Chemical Name	Formula
1,3-Benzothiazole-2-thiol	C7H5NS2	13-cis-Retinoic acid	C20H28O2
2-(Methylthio)benzothiazole	C8H7NS2	Linoleic acid	C18H32O2
Benzothiazolone	C7H5NOS	Oleic acid	C18H34O2
Benzothiazole	C7H5NS	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	C15H22O2
1,3-Diphenylguanidine	C13H13N3	Phenoxazine	C12H9NO
N,N-Diethyl-meta-toluamide (DEET)	C12H17NO	1,3-Benzothiazol-2-amine	C7H6N2S
N,N'-Dicyclohexylurea	C13H24N2O	N-Cyclohexyl-N-methylcyclohexanamine	C13H25N
N-Cyclohexylformamide	C7H13NO	N,N'-Diphenyl-p-phenylenediamine - 2H	C18H14N2
Diphenylurea	C13H12N2O	Dicyclohexylamine	C12H23N

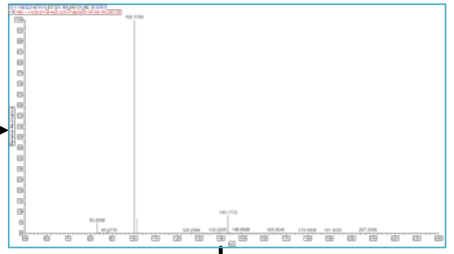
*Chemicals were analyzed by LC/MS with positive ionization mode.

Tier 2: Non-Targeted Analysis with the Aid of Cheminformatics

e.g., 1,3-Dicyclohexylurea

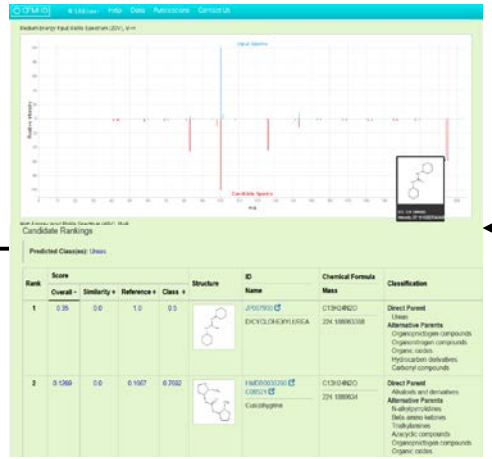






$[M+H]^+$ = 225.1961
RT: 31.38

m/z	Rel Int
83.2686	4.53
91.0791	0.18
100.1413	100
101.2622	6.19
125.1556	0.23
143.2128	8.19
144.2437	0.72
147.0886	0.35
148.2509	0.17
161.8341	0.29
177.0316	0.39
179.8776	0.16
180.9882	0.20
183.171	0.18
192.5676	0.24
195.2134	0.21
197.2946	0.24
205.1281	0.3
206.8469	0.18
208.0789	0.24



Cheminformatics Tools Prediction

Tentative Chemicals:
1. N,N'-Dicyclohexylurea
2. Cuscohygrine
...

Purchase Dicyclohexylurea

Verify with Reference Standards

Targeted Chemicals:
1. N,N' Dicyclohexylurea
...

Cheminformatic Algorithms

- MetFrag (Mass Spectroscopy and Bioinformatics, Bioinformatics Center, Gatersleben-Halle, Germany)
- Global Natural Products Social Molecular Networking (GNPS, UCSD)
- XCMS (The Scripps Research Institute, La Jolla, CA)
- Compound Discoverer™ (version 3.0, ThermoFisher Scientific, Waltham, MA)
- Competitive Fragmentation Modeling-ID (CFM-ID, version 3.0, Wishart Research Group, University of Alberta, Edmonton, Canada). Trained and searched 11 diverse database: CASMI2016, ContaminantDB, DrugBank, FiehnLib, HMDB, KEGG, MassBank, MetaboBASE, NIST, PhytoHub, iTree

Algorithm Validation Using 18 Standard References

Validation Steps:

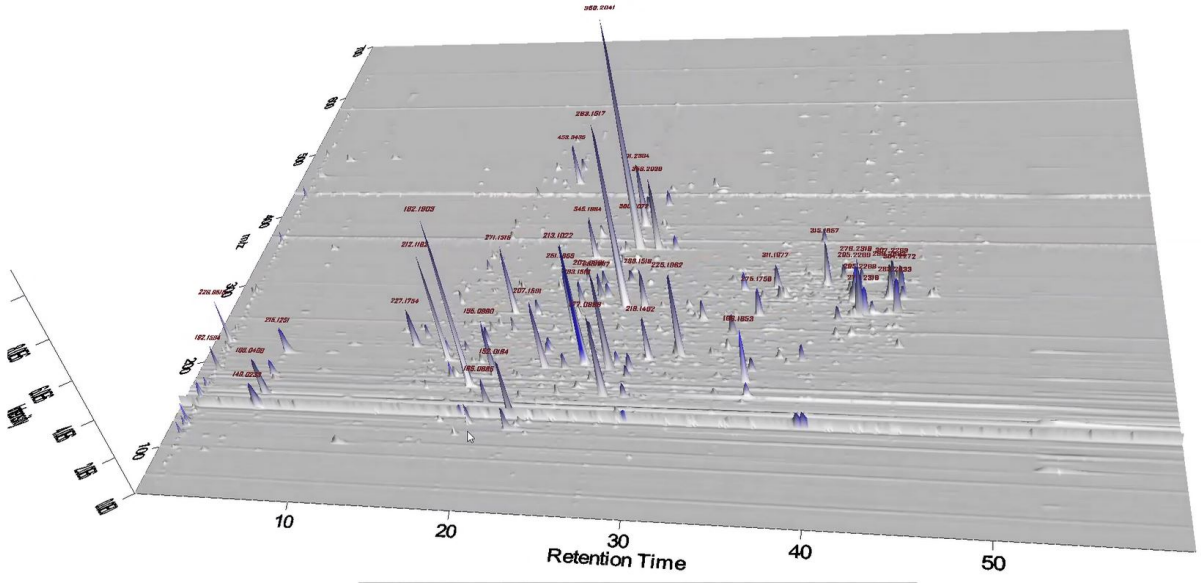
1. Analysis standard reference using HRAM-LC/MS
2. Extract M2 data
3. Input data into each algorithm for chemical matching
4. Check if the standard chemical is matched as top candidate

Algorithm	Position of Correctly Matched Chemical	No. of Correctly Matched Candidate
CFM-ID	Top 1	11
	Top 2	2
	Not on the candidate list	5
MetFrag	Not on the top 10 candidate	0

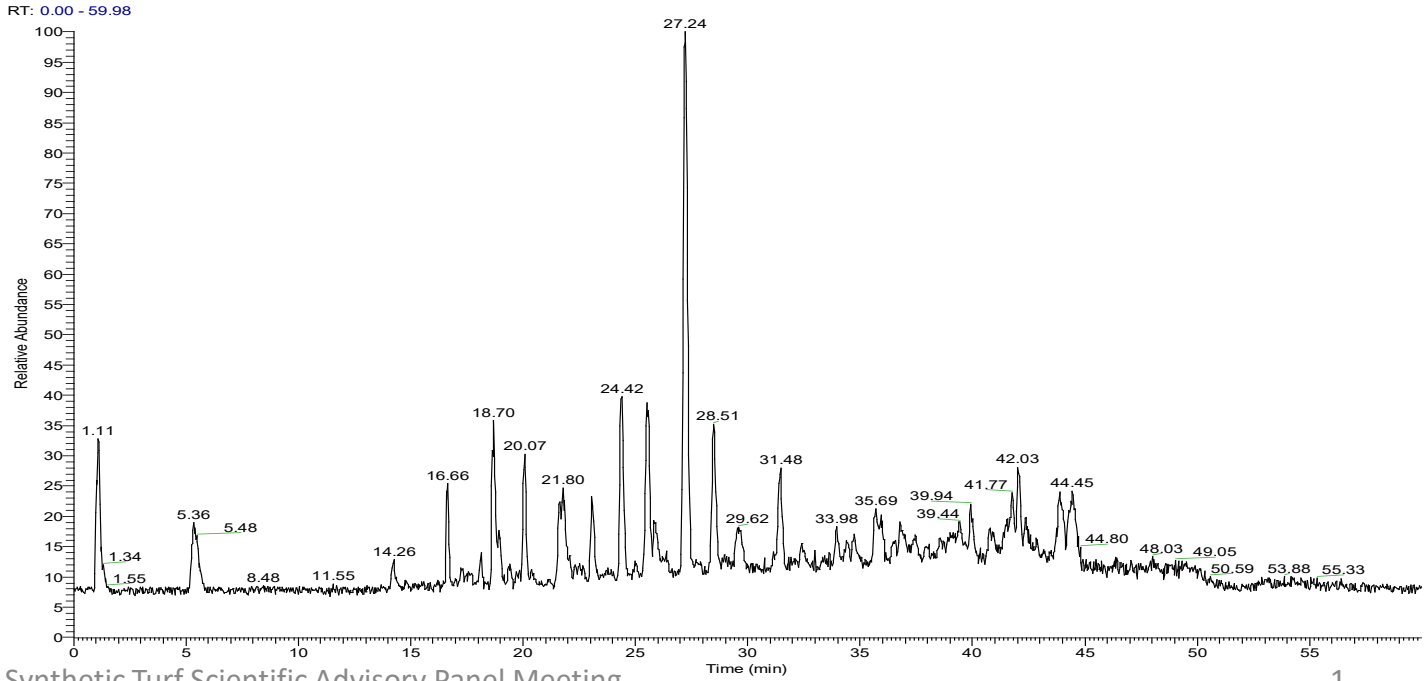
High-Resolution Accurate-Mass (HRAM)-LC/MS Data Applied to Tier 2 Analysis Using Cheminformatics (CFM-ID) Tool

Manufacturer Sample (MS1 Spectrum)

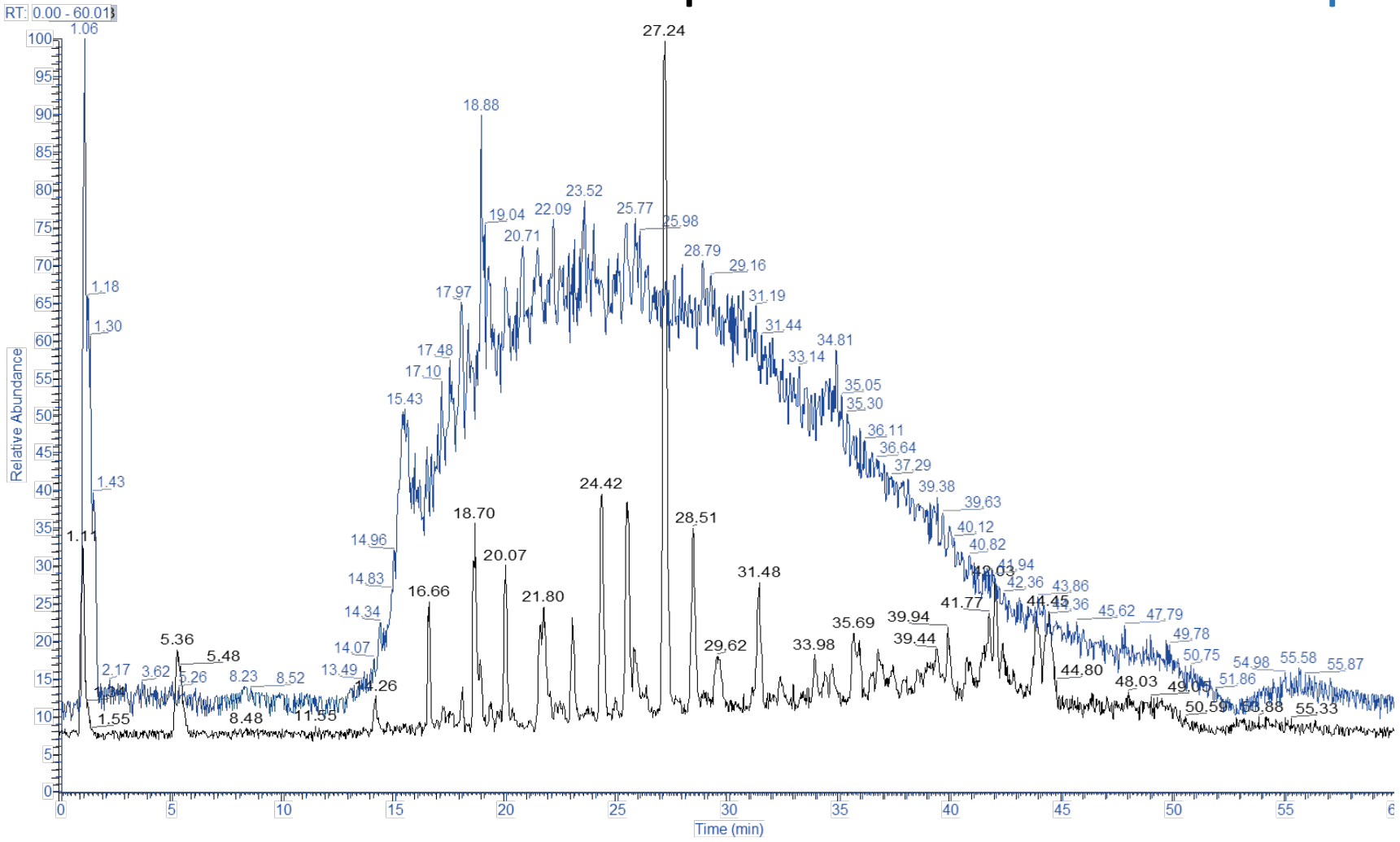
3D-LC/MS Chromatogram



2D-LC Chromatogram



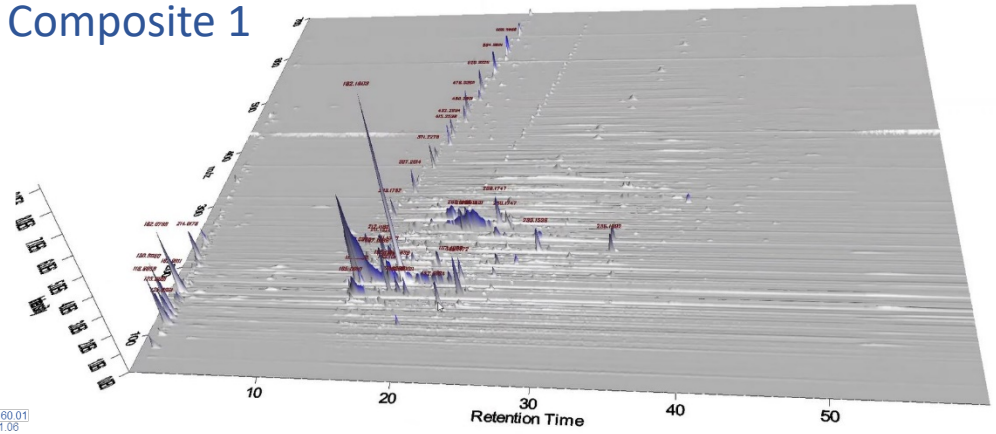
LC Chromatogram: Manufacturer Sample vs. Field Composite 1

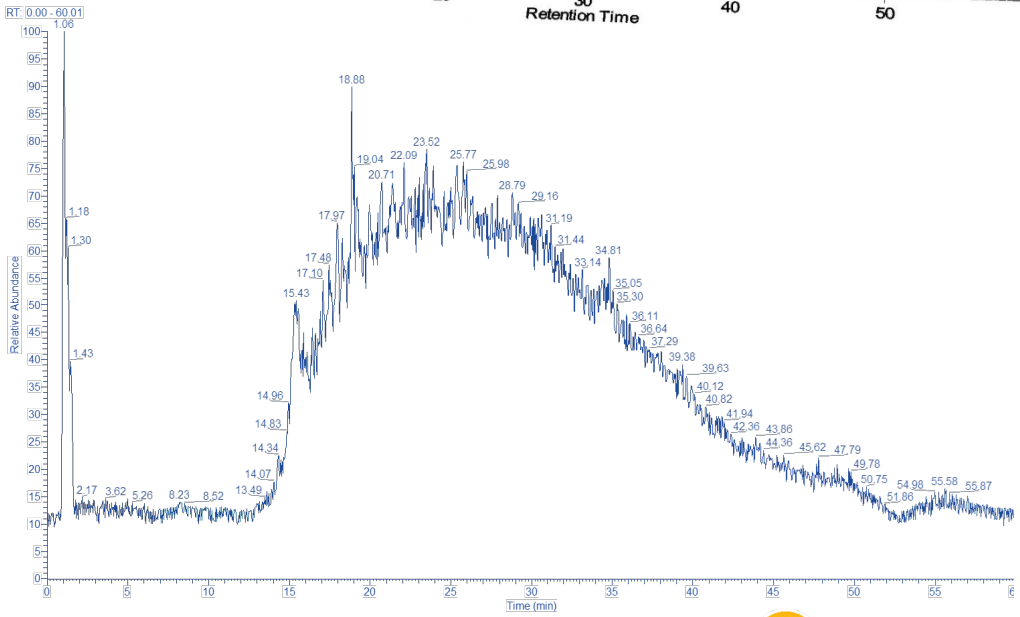


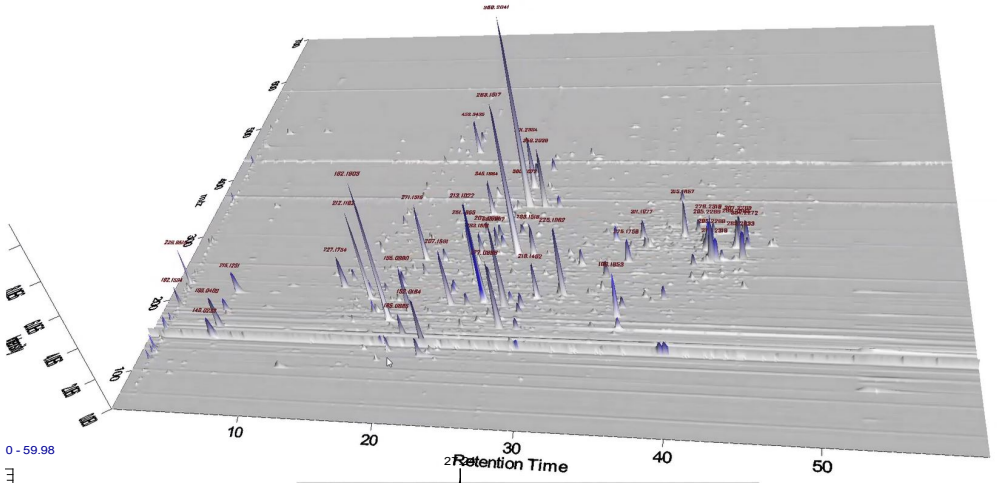
3-D LC/MS Chromatogram (MS1)

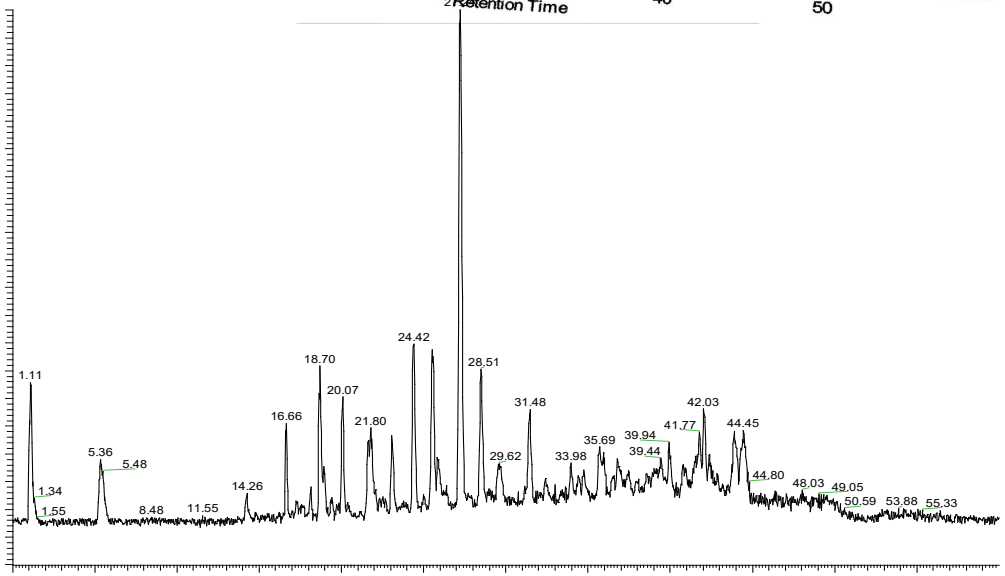
- 3-D chromatogram shows most peaks are resolved
- Drastic difference between field and manufacturer samples

Field Composite 1

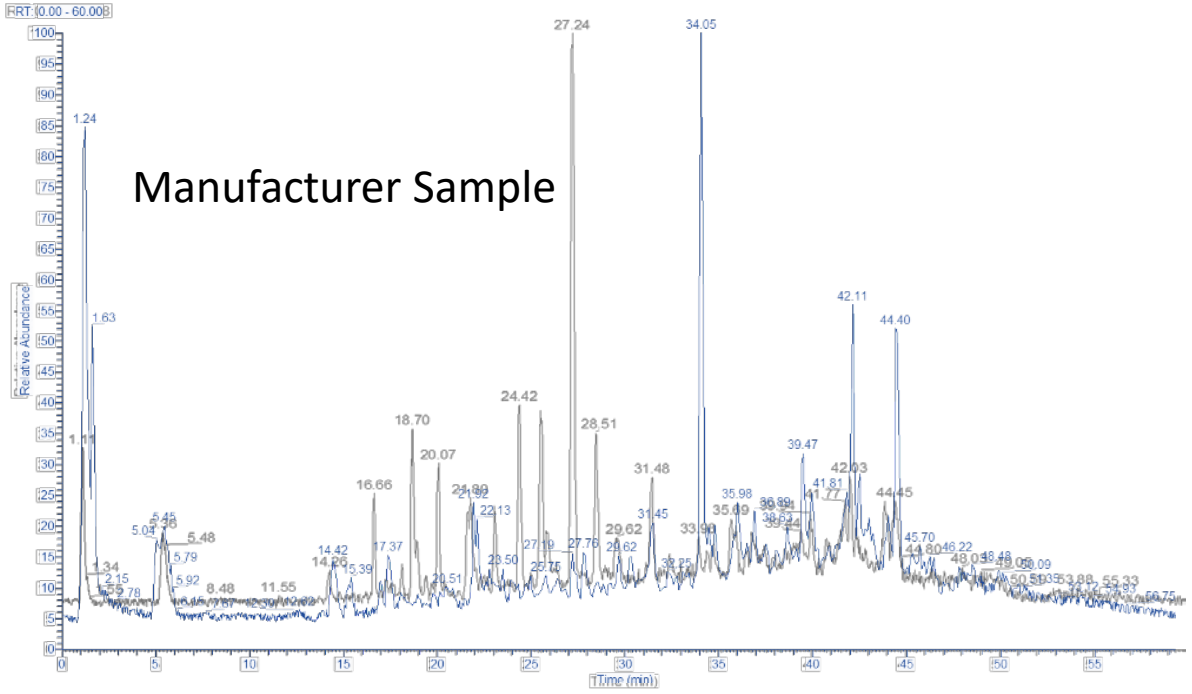


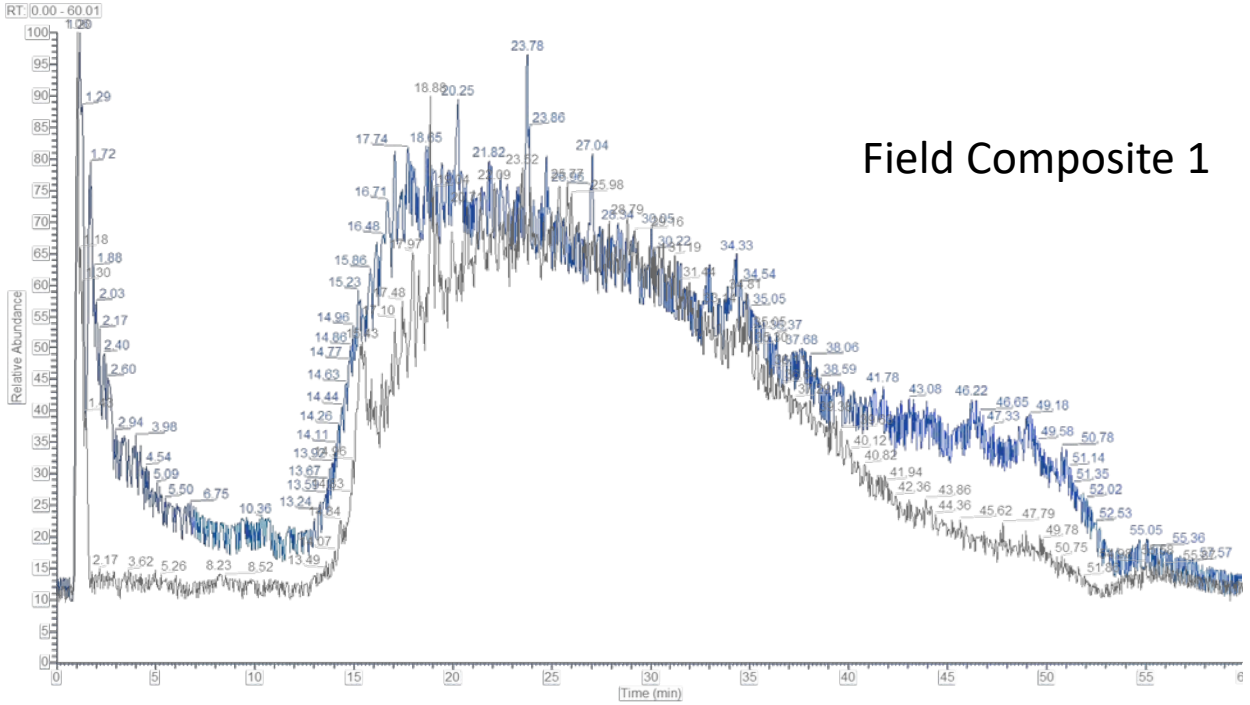


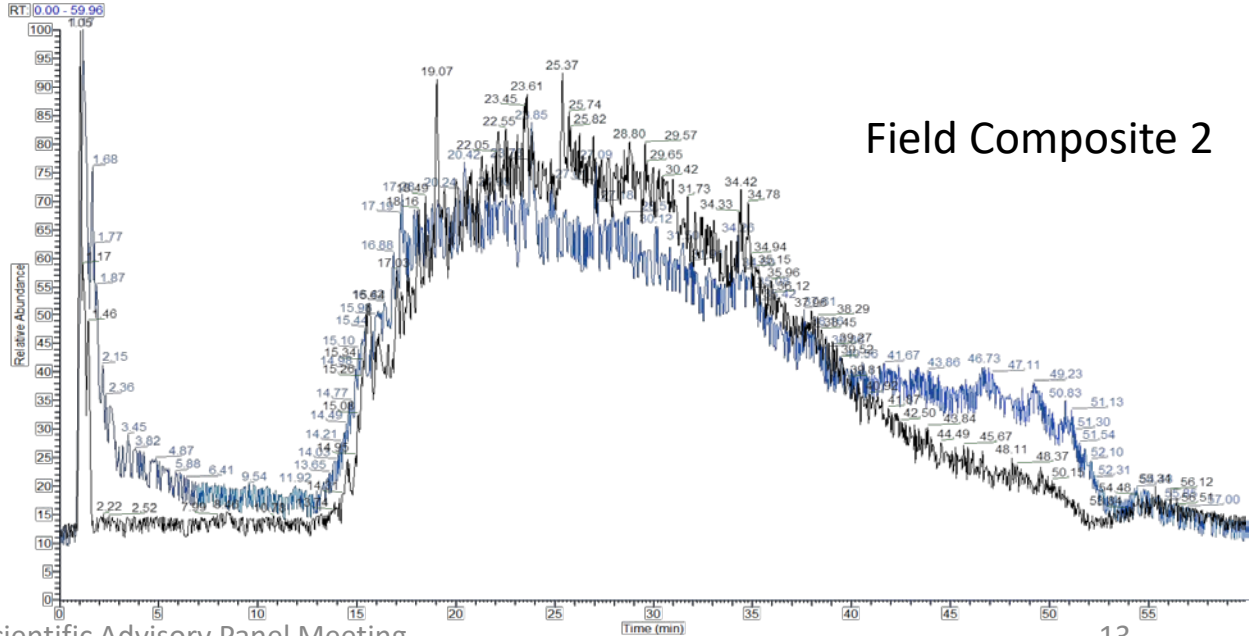




Positive vs. Negative Ionization Mode



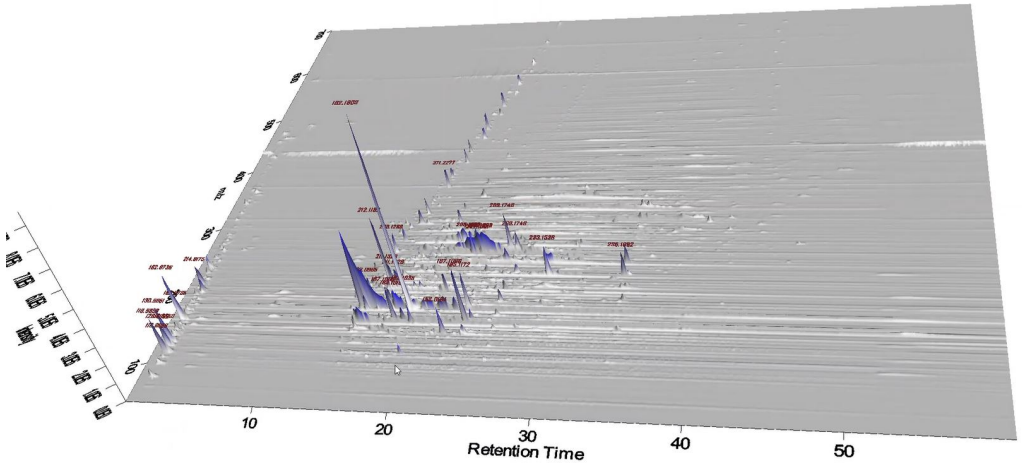


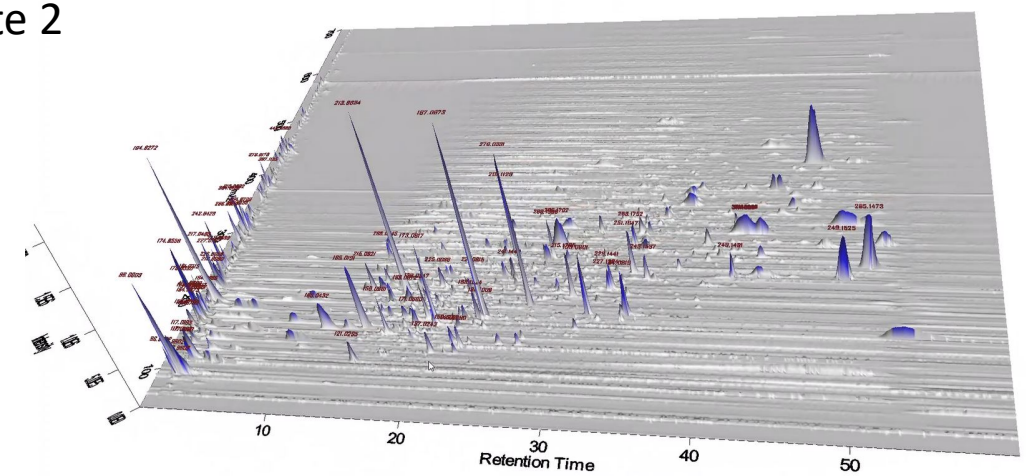


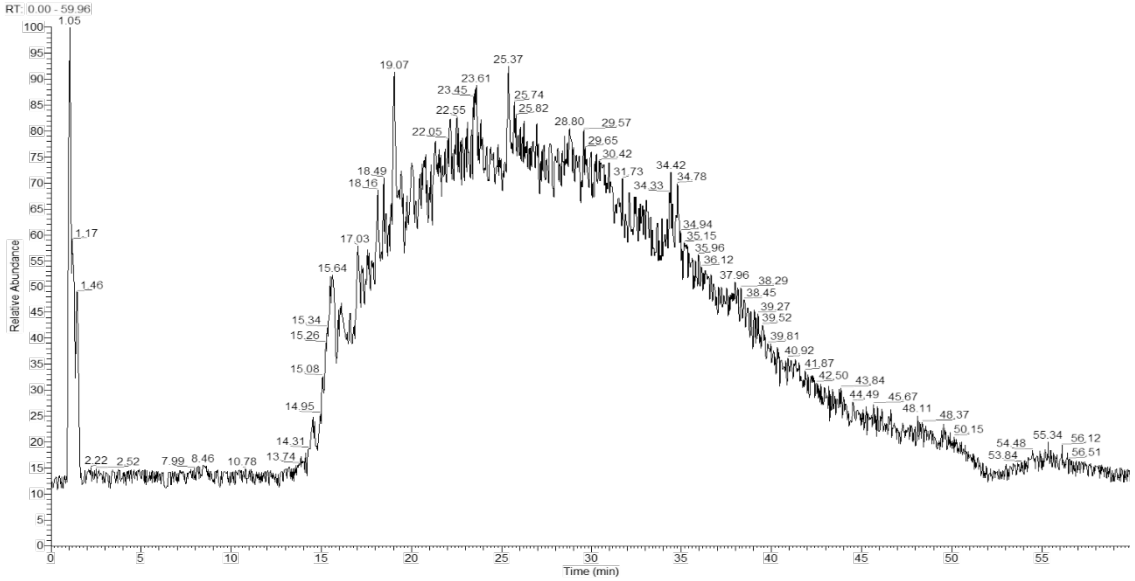
- Spectra generated under positive and negative ionization look different!
- Spectra between samples look different!

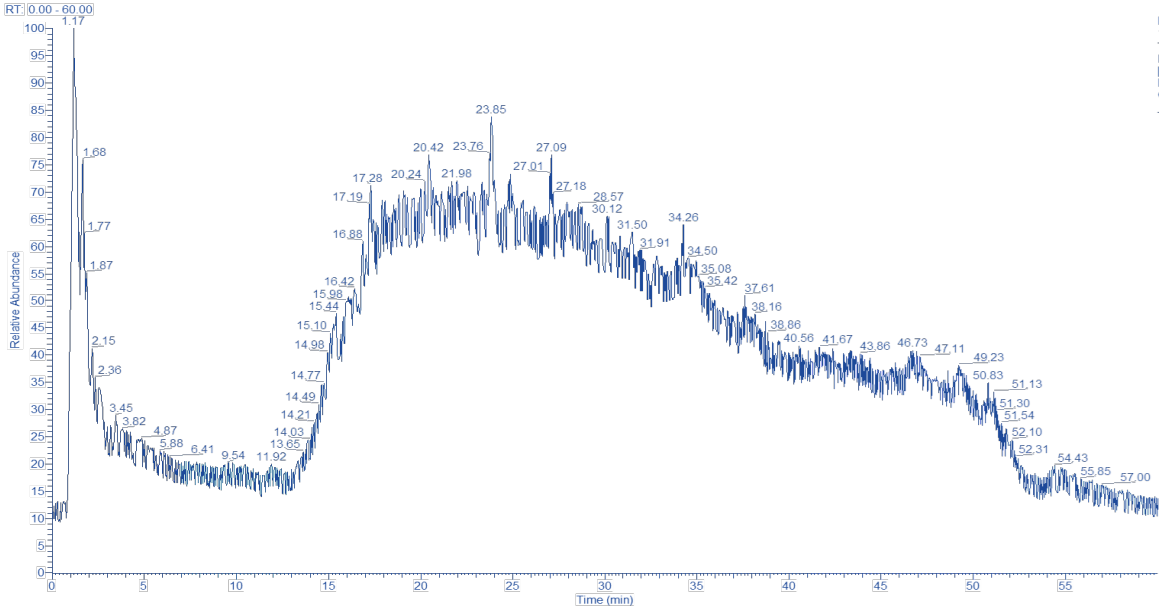
3-D LC/MS Chromatograms from Positive & Negative Ionization Modes

Field Composite 2









Chemical Analysis Results So Far: Chemicals to be tested in all field samples

Chemical Class/Matrix	Instrumental Analysis	No. of Confirmed Chemicals*	No. of Tentatively Identified Chemicals[ŧ]
Polar Extract	LC/MS	18	47 (ongoing)
Non-Polar Extract	GC/MS	32	182
PAHs	SIM-GC/MS	20	182
Aldehydes/Ketones in Field Air	HPLC	11	0
VOCs in Field Air	GC/MS	67	0
This cell was purposefully left empty	**Total**	**122 (unique chemicals)**	**228 (unique chemicals)**

*Some of these chemicals were detected in multiple matrices or by different instruments.
[#]Chemicals tentatively identified in polar extracts analyzed by HR-AM LC/MS under positive ionization mode. Data collected under negative ion mode are being analyzed.
[ŧ]One chemical was detected in both polar and non-polar extracts of crumb rubber

Questions for Discussion

1. Do you have any comments or concerns on the general approaches for the non-targeted analysis?
2. Do you agree with the approach of the non-targeted analysis for the Polar Extracts? Do you have any comments or concerns?
3. We are doing the non-targeted analysis in both ionization modes. Do you have any comments on this approach?
4. Do have any comments regarding the following factors to prioritize tentatively identified chemicals? Any additions or suggestions? Do you have any suggestions on how to weigh these factors?

   Factors to Prioritize Chemicals for Confirmation Process:

   - Chemicals with Toxicity Criteria
   - Chemicals associated with Tall Peaks
   - Chemicals known to be Tire Related
   - Chemicals detected in multiple samples (manufacturer's sample and field composite samples)
   - Others?
5. Any additional comments?