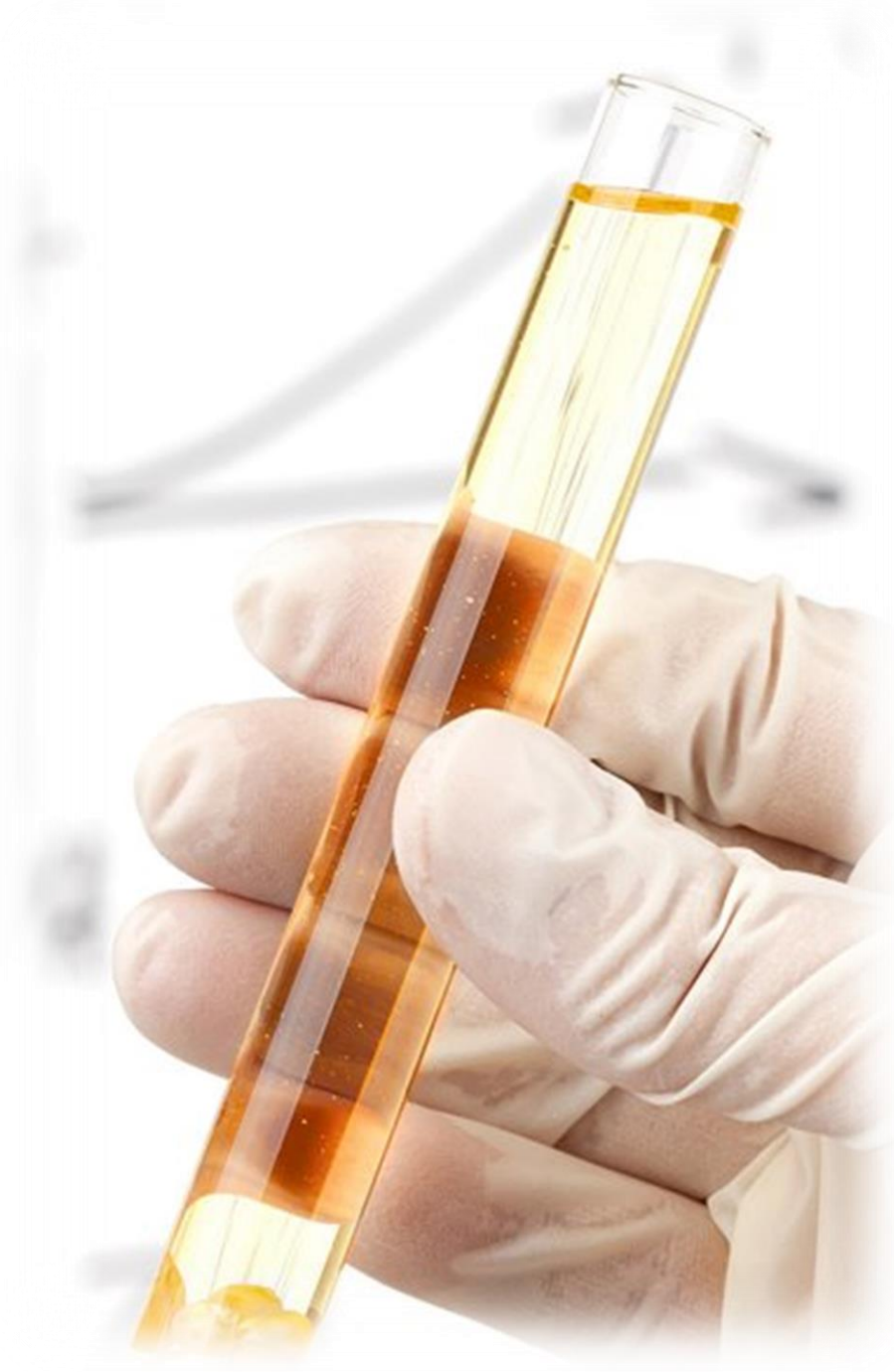
TECHNOLOGY CENTER

GC Analysis and Techniques



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Presentation Outline

1) Basic overview of GC
   - Separation Principle
   - Carrier gas
   - Maintenance and consumables
2) ASTM D5501
   - Running parameters
   - Calibrations
   - Calculations
3) CTE Process Monitoring Services
   - Profiling fusel production
   - Amino acid profile

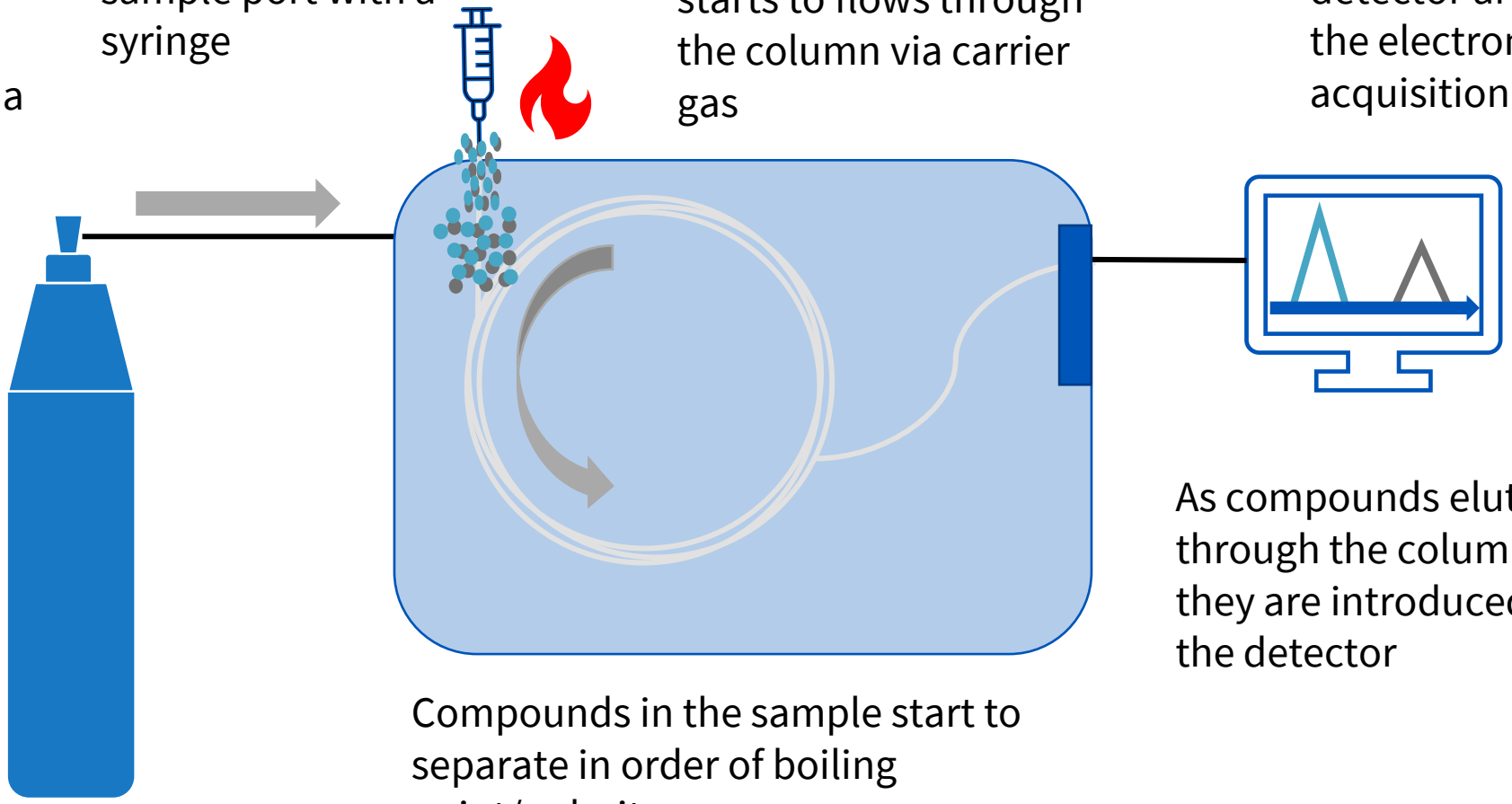
GC Basics – Separation Principle

Carrier gas: inert gas controlled by a pressure control unit/regulator

Sample injected at sample port with a syringe

Sample is vaporized and starts to flows through the column via carrier gas

Signals from the detector are sent to the electronic data acquisition system



Compounds in the sample start to separate in order of boiling point/polarity

As compounds elute through the column they are introduced to the detector

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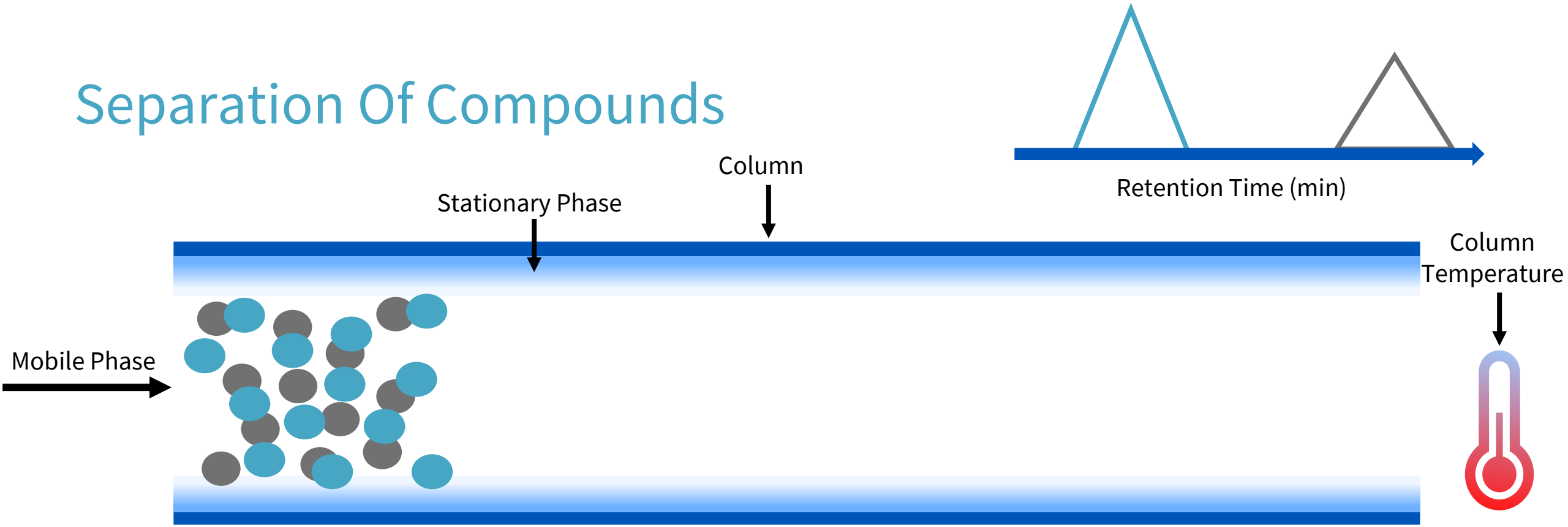
Separation Of Compounds



- Stationary phase and mobile phase are fixed throughout analysis
- Carrier gas flow rate and oven temperature are the only variables changing throughout analysis
- The separation of compounds is heavily dependent on its boiling point and its interaction with the stationary phase
- Compounds with a lower boiling point will travel through the column faster and elute first
- If boiling points are similar, then the compounds will elute with respect to their polarity – weaker interaction with the stationary phase will elute first

Separation Of Compounds

- Separation of compounds in a sample matrix depends mainly on two factors:
  - Column temperature, each compound elutes at different times based on its boiling point
  - The polarity of a components in a sample vs polarity of stationary phase

- But also depends on:
  - Carrier gas
  - Column length
  - Injection volume
  - + more

This will cause the compounds to elute at different times (retention time) and flow to the detector

Carrier Gas

To achieve good separation and reproducible chromatograms:

1. Carrier gas supply needs to be high-purity
   - Min. purity 99.95% - Reduces base line noise
2. Carrier gas flow needs to be constant
   - Using pressure and flow regulators
3. Carrier gas needs to be inert
   - so, it won't interfere with the separation and detection

Helium, hydrogen and nitrogen are some of the commonly-used carrier gases.

Carrier Gas

Carrier Gas	Advantages	Disadvantages
Helium	• Inert (safe) and non-flammable • Gives high resolution	• Expensive, not easily available
Hydrogen	• High diffusivity and linear velocities • Gets good separation efficiencies • Short analysis and run time (results in cheap operational cost)	• Flammable • Not completely inert (e.g. reacts with some compounds at high temperature)
Nitrogen	• Cheap and easily available	• Not suited for use in temperature-programmed GC analysis • Lower or poor separation resolution • Long analysis and run time

https://www.shimadzu.com/an/service-support/technical-support/analysis-basics/fundamentals/carriergas.html#anchor2

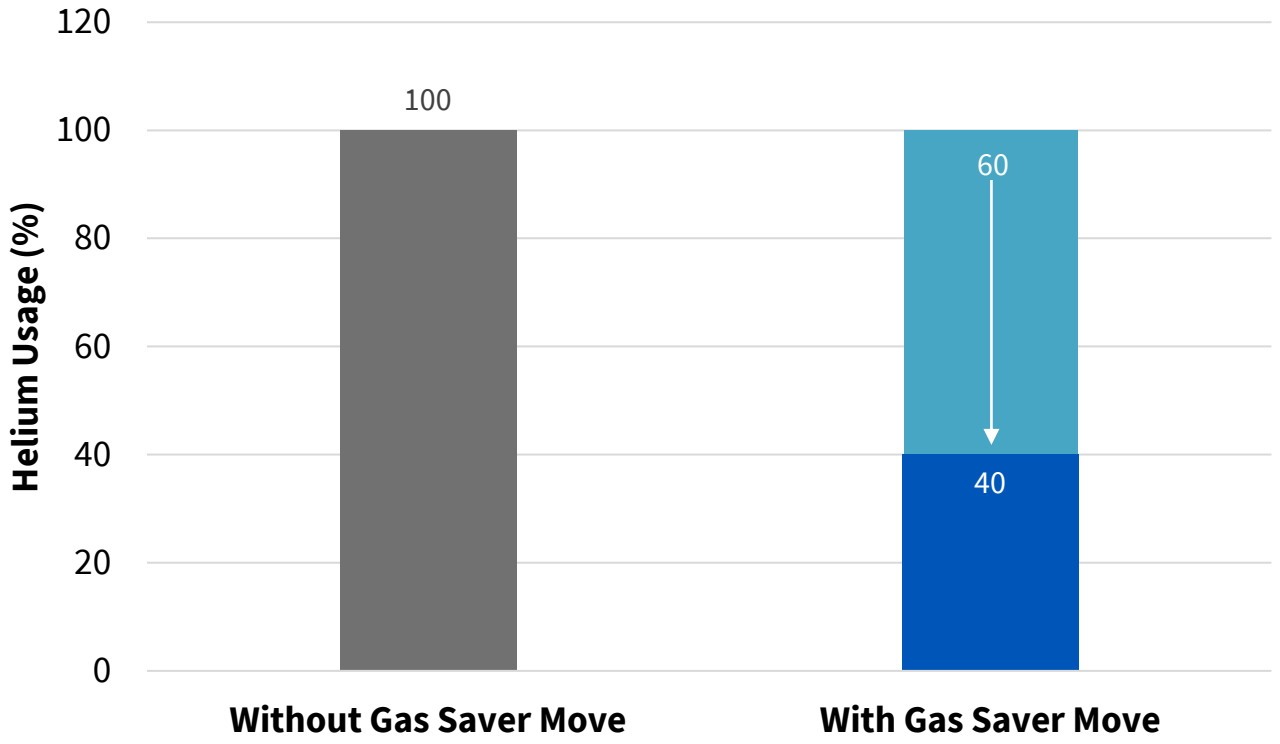
Carrier Gas – Alternatives to Helium

If using an alternative, you will need to consider:

- Safety (hydrogen requires additional safety measures)
- Purity
- Any hardware changes
- Parameter conditions

Carrier Gas

Optimize helium conservation methods through Gas Saver mode and Ecology Mode on Shimadzu GC



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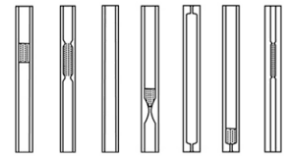
Equipment Maintenance/Consumables

- Should have yearly scheduled PM's
- Routinely maintain consumables
  1. Gas
     - Pure, make sure you have inventory
  2. Syringe
     - Both for manual and autosample
  3. Septa (after 100 injections)
     - Low bleed and optimum sealing

4. Glass Liner (after 500 injections)
   - Elect correct one for improved performance, vaporization



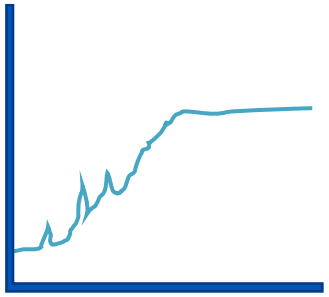
5. O-rings
   - Low bleed and optimum sealing

6. Ferrules
   - Ensures best connection is achieved

7. Columns
   - Ensure correct installation, conditioning

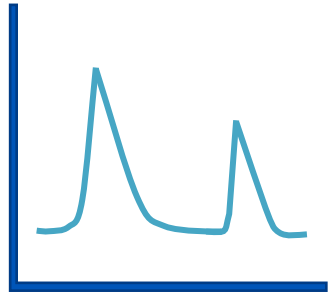
GC Peak Troubleshooting

Column Bleed
- Conditioning
- Contamination
- Leak

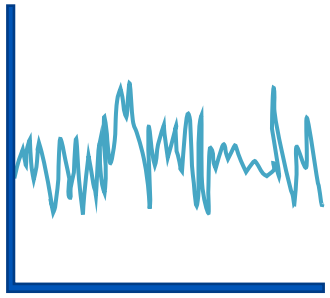


Reduced Size
- Clogged syringe
- Leak
- Split ratio
- Temp

Tailing
- Contamination
- Column Installation/cut
- Temp
- Split ratio
- Inlet overloading

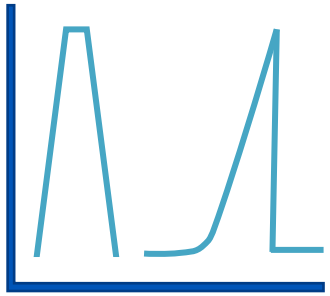


Noise
- Column installation
- Leak
- Contamination

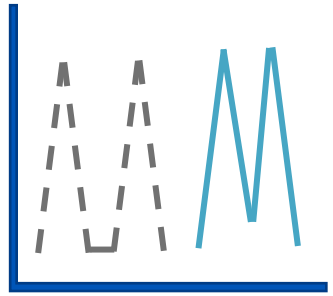


Flap Top
– Detector overloaded

Fronting
- Column overloaded



Peak Overlap
- Column dimensions
- Column trimming
- Damaged column stationary phase



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ASTM D5501

- At ethanol plants, GC analysis is used to determine the amount of ethanol, methanol and denaturant is found in fuel blends
- The standard GC test method used is ASTM D5501
  Determination of Ethanol and Methanol Content in Fuels Containing Greater than 20 % Ethanol by Gas Chromatography
- Ensuring the GC is running at optimal conditions for this method is crucial for reproducibility and accuracy

ASTM D5501 Running Parameters

- Need a GC capable of operating at the conditions listed in the adjacent table
- Any column with good resolution and selectivity can be used
- Ensure consumables are within usage limits
- Accurate sample injection and split ratios is crucial to the precision and accuracy
- Carrier gas + detector gases should have a min purity of 99.95%

TABLE 1 Typical Operating Conditions

Column Temperature Program		
Column length	100 m	150 m
Initial temperature	15 °C	60 °C
Initial hold time	12 min	15 min
Program rate	30 °C/min	30 °C/min
Final temperature	250 °C	250 °C
Final hold time	19 min	23 min
Injector		
Temperature	300 °C	
Split ratio	200:1	
Sample size	0.1 µL to 0.5 µL	
Detector		
Type	Flame ionization	
Temperature	300 °C	
Fuel gas	Hydrogen (30 mL/min)	
Oxidizing gas	Air (300 mL/min)	
Make-up gas	Helium or Nitrogen (30 mL/min)	
Date rate	20 Hz	
Carrier Gas		
Type	Helium or Hydrogen[A]	
Average linear velocity	21 cm/s to 24 cm/s (constant flow)	

[A] Use of hydrogen carrier gas requires additional safety considerations.

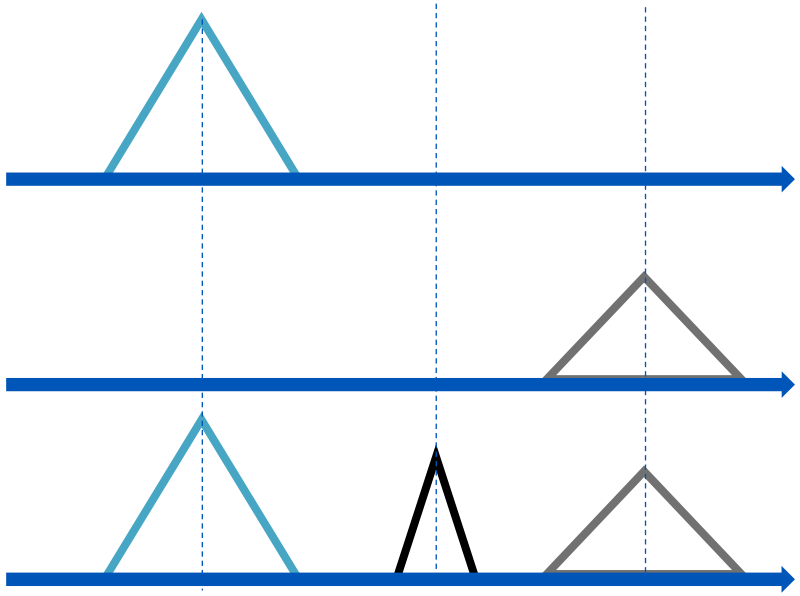
ASTM D5501 - Qualitative Analysis

- Inject ethanol and method standards
- The retention time of each standard under set parameter conditions will be used to identify the eluting compounds from your sample
- When analyzed under the same conditions, the same compound elutes at the same time

Methanol Standard

Ethanol Standard

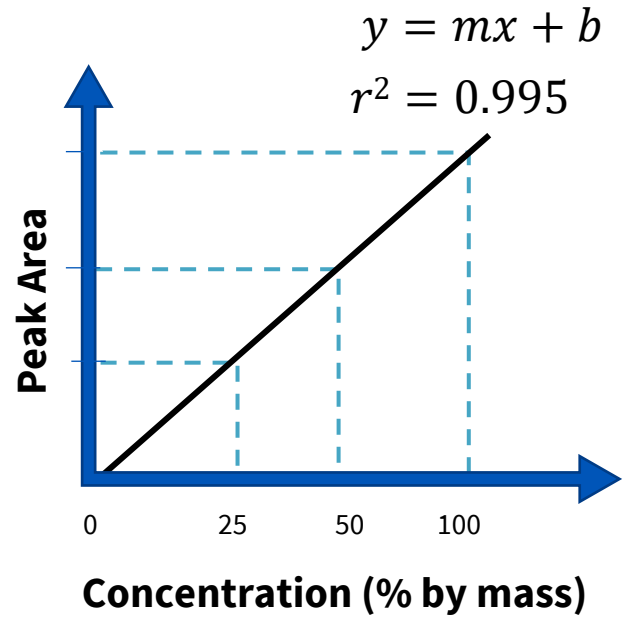
Unknown sample containing methanol, ethanol and an unknown peak



Retention Time (min)

ASTM D5501 – Calibration Linearity

- Run calibration standards that cover the expected range of ethanol and methanol
- Analyze the peak areas vs concentrations and ensure there is a linear regression with a minimum r^2=0.995
- When peak area = 0, ethanol concentration is +/-3% by mass
- If the conditions above are not met, troubleshoot until they are
  - Increase split ratio
  - Reduce injection volume
  - Correct peak integration of ethanol and methanol

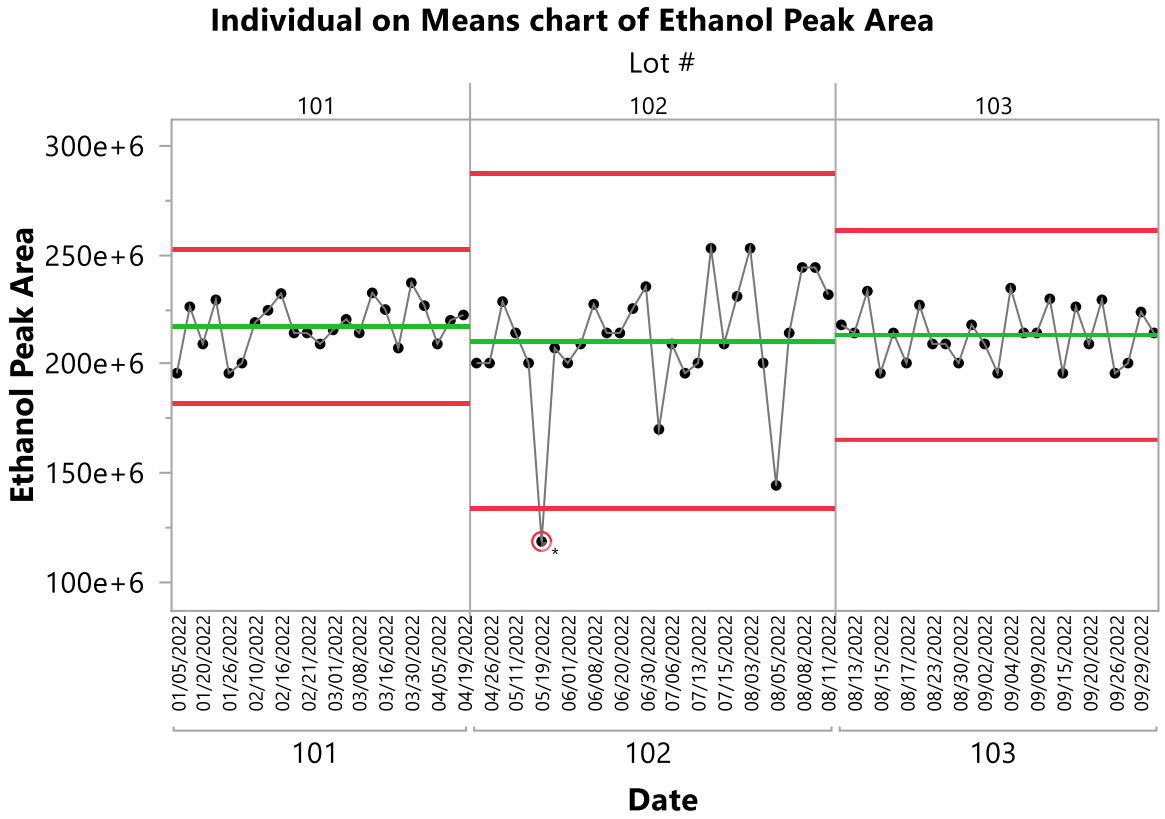


ASTM D5501 Quantitative Analysis

- Absolute Calibration
  - Single point
    - Calculate the mass response factor (MRF) of the compounds in each calibration standard
    - The average MRF is used for the calibration
  - Multi-point
    - Generate individual calibration curves for each compound in the GC software
- Relative Calibration
  - Calculate the MRF of ethanol, methanol and heptane
  - Then, calculate the mass relative response factor (MRRF) of methanol and ethanol relative to heptane
  - The average MRRF is used for the calibration

ASTM D5501 Quality Control

- Run a quality control (QC) sample daily along side samples
- Compile the data and build a control chart
- Monitor the control chart for any outliers
- Ensures precision and accuracy



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ASTM D5501 Calculations

- From the standard chromatograms, obtain the peak area and calculate the response factor using mass %
- Calculate the MRRF
- From the denatured ethanol chromatographs, obtain the peak area
- Calculate for corrected peak area then normalize mass % and correct for moisture (ASTM D1364)
- Report results in volume % using compound density and sample density (ASTM D1298 or D4052)

Process Monitoring Services

Corn	Liquefaction	Fermentation	Production
Starch	Starch Solubility	Starch	Residual Starch
Fermentation Potential	FAN	FAN	Fusels *
	Fusels *	Fusels *	Impurities *
	Viscosity	Amino Acid Profiling *	Corn Oil tracing
	HPLC	HPLC	
	Liquefactions	Residual Trehalose	
	Phytate	Residual Phytate	
		Flask/Bioreactor Fermentation	

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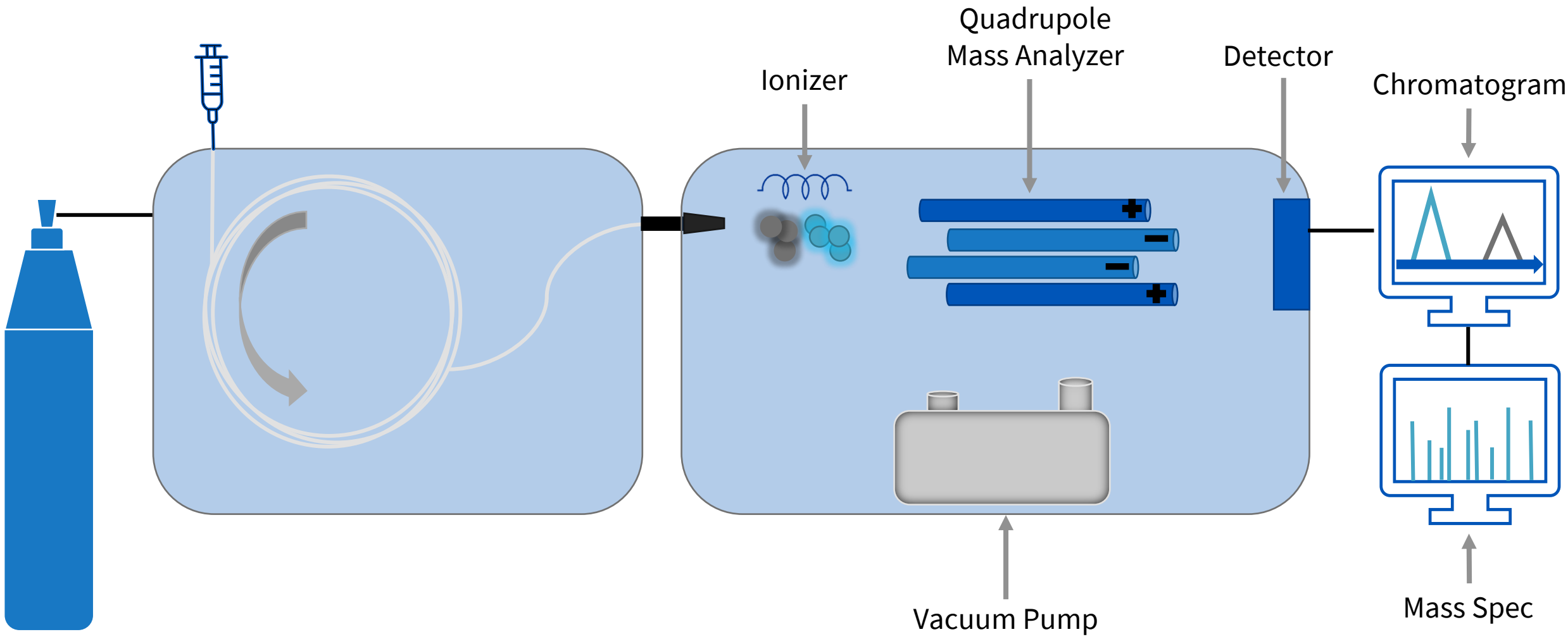
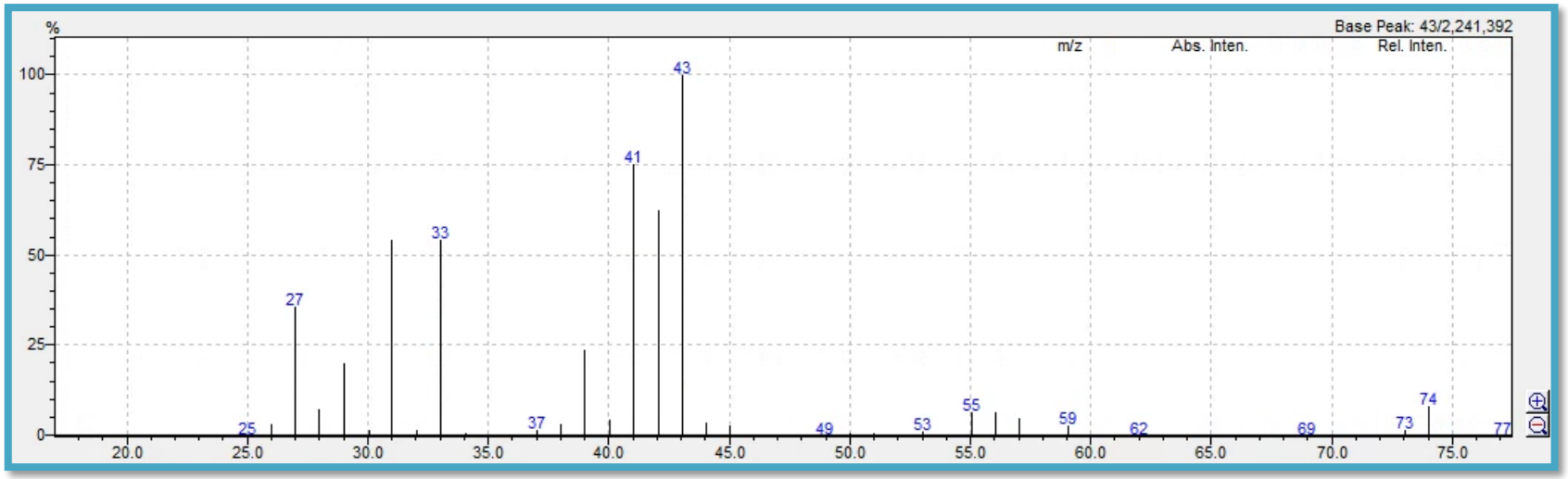
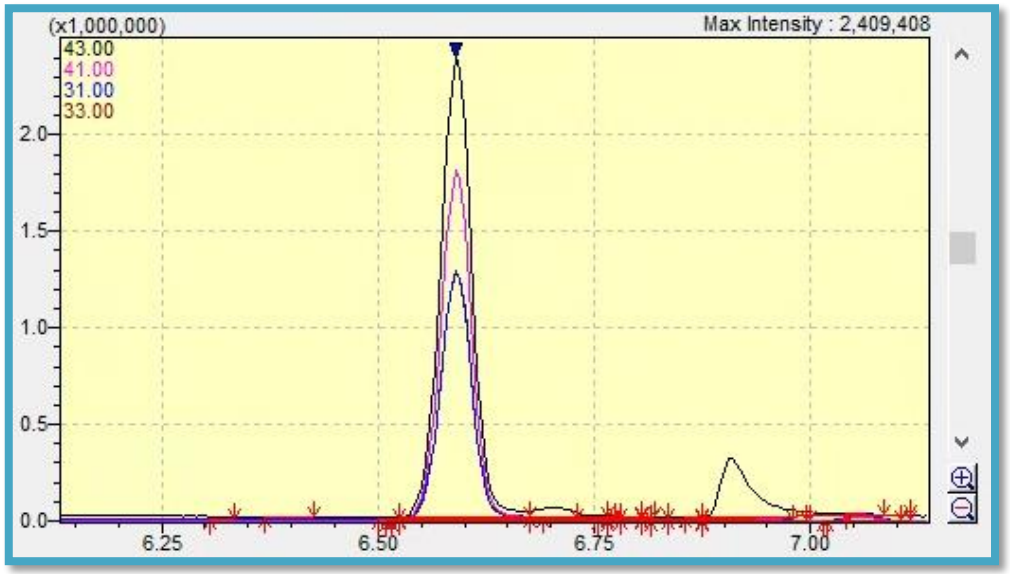
GC-MS


Ionizer
Quadrupole
Mass Analyzer
Detector
Chromatogram
Vacuum Pump
Mass Spec

Mass Spec Results





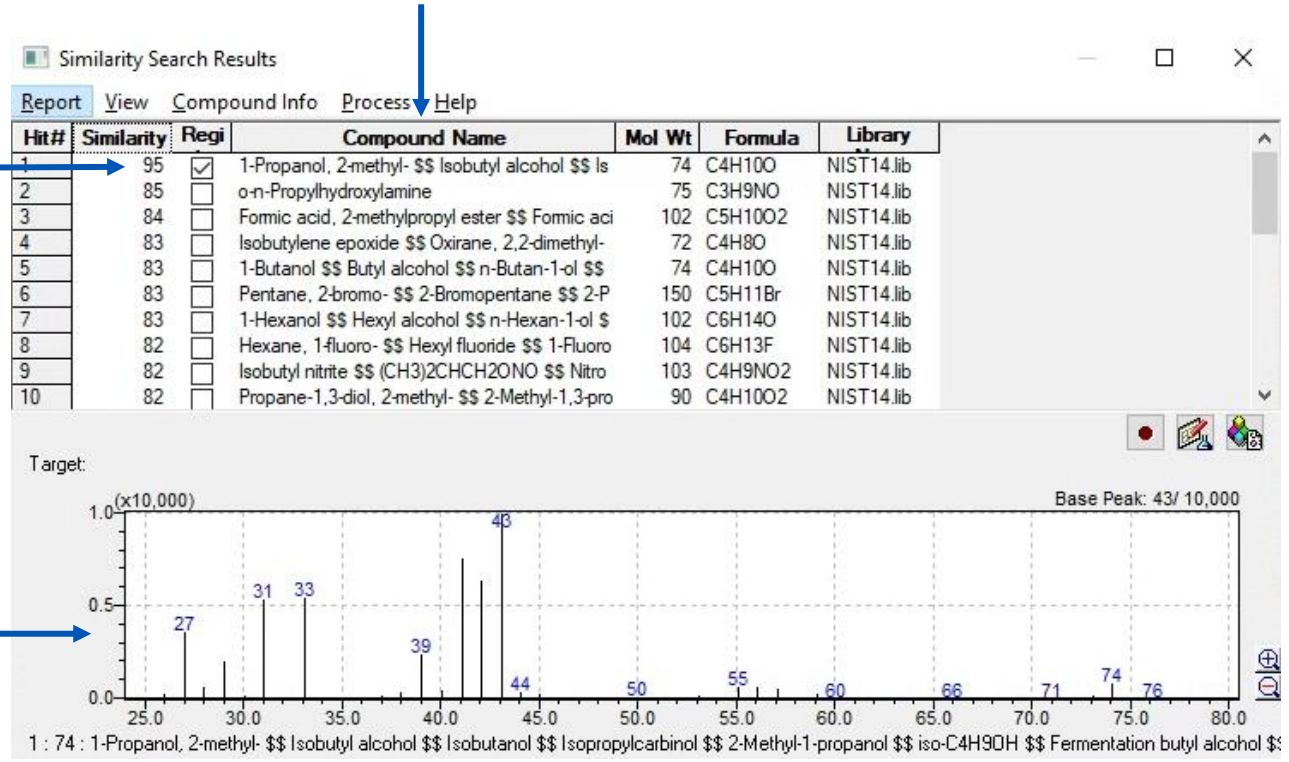
Type	m/z	Area	Set %	Act. %	Ref. Band
Target	43.00	6035490	100.00	100.00	---
Ref. Ion1	41.00	4642068	81.64	76.91	30
Ref. Ion2	31.00	3390469	79.87	56.18	30
Ref. Ion3	33.00	3362711	67.20	55.72	30

- Ions are separated based on their mass-to-charge ratio (m/z)
- The relative intensity of each ion is measured and then recorded to produce a mass spec
- The result displays the relative ion intensity against their m/z
- Compounds are identified by a fingerprint of mass fragments measured by mass spec

NIST Library

- Each fragment is logged into an online database (NIST library) that can identify the compound
  - The library will produce a % match, the higher the percentage, the more closely it relates to the compound being identified

- In this example, the mass fragment fingerprint matches up very well with the expected fingerprint for isobutanol



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Profiling Fusel Production

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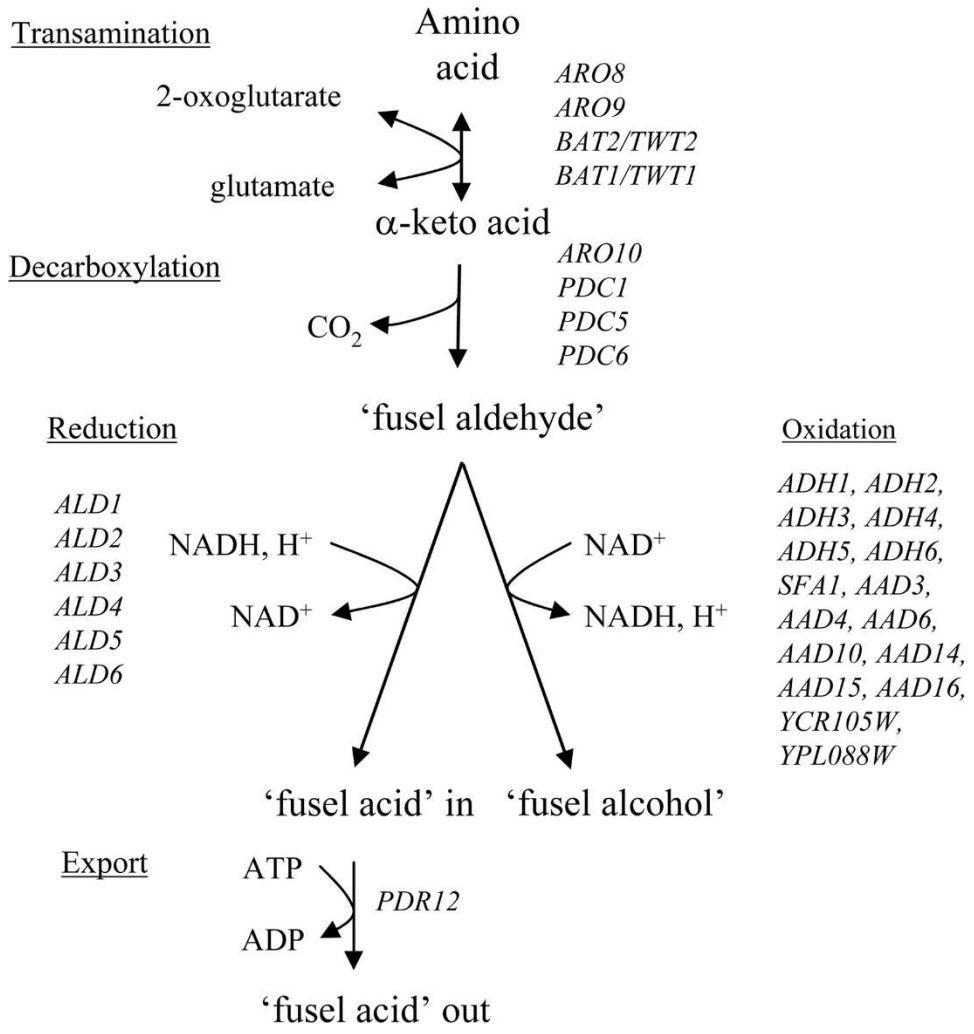
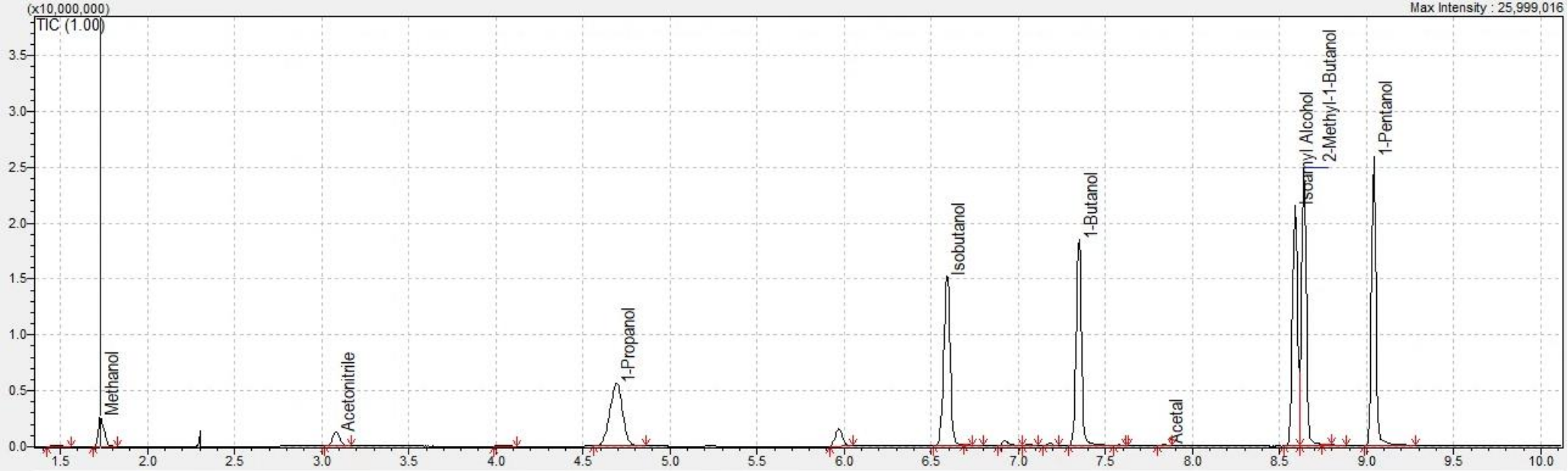
The Ehrlich Pathway

- Fusels are normal metabolites produced by yeast and bacteria, but can be about 15 times more inhibitory to yeast than ethanol
- Depending on several factors, inhibition may be evident even in the 100-500 ppm range
- The carbon skeletons of some amino acids are not incorporated into central metabolism, and are instead metabolized to fusels through the Ehrlich Pathway
- The Ehrlich pathway describes the harvesting of the nitrogen through transamination, followed by decarboxylation and oxidation to produce a fusel alcohol



Lucie A. Hazelwood et al. Appl. Environ. Microbiol. 2008;74:2259-2266

Fusel Chromatograph



Compounds Identified:

- Acetaldehyde
- Methanol
- Isobutanol
- Butanol
- 3-methyl-1-butanol (Isoamyl alcohol)
- 2-methyl-1-butanol
- Pentanol
- 2,3-Butanediol
- Phenylethyl Alcohol
- Acetal
  - Can add any additional compounds upon request.

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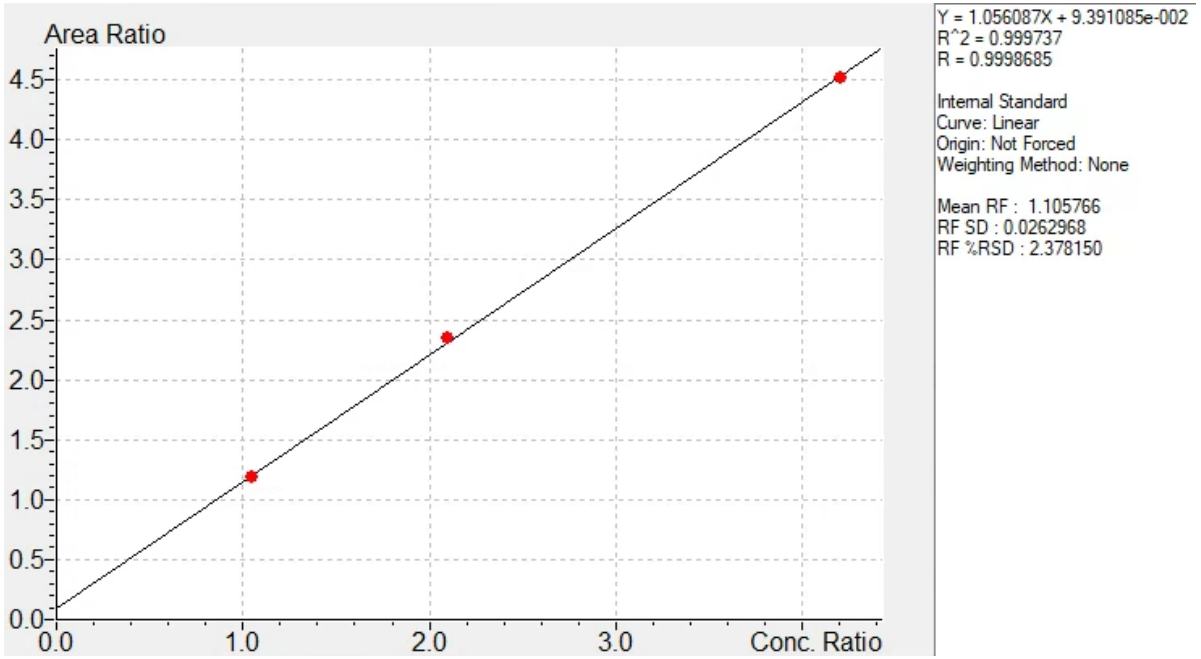
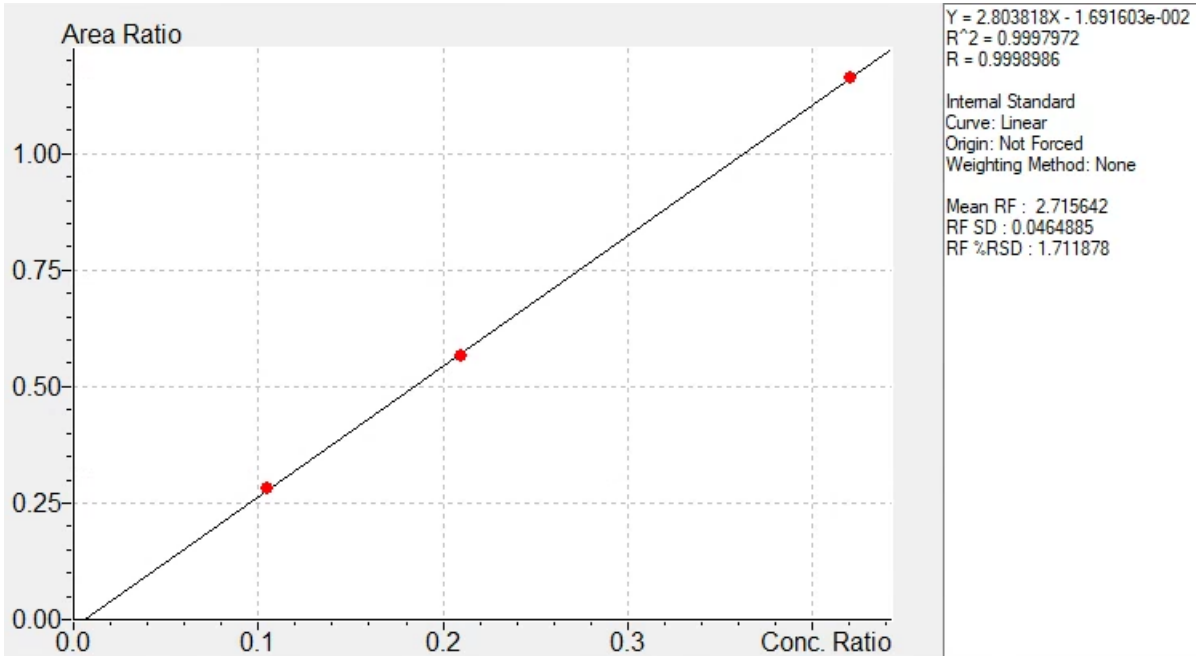
Excellent linearity

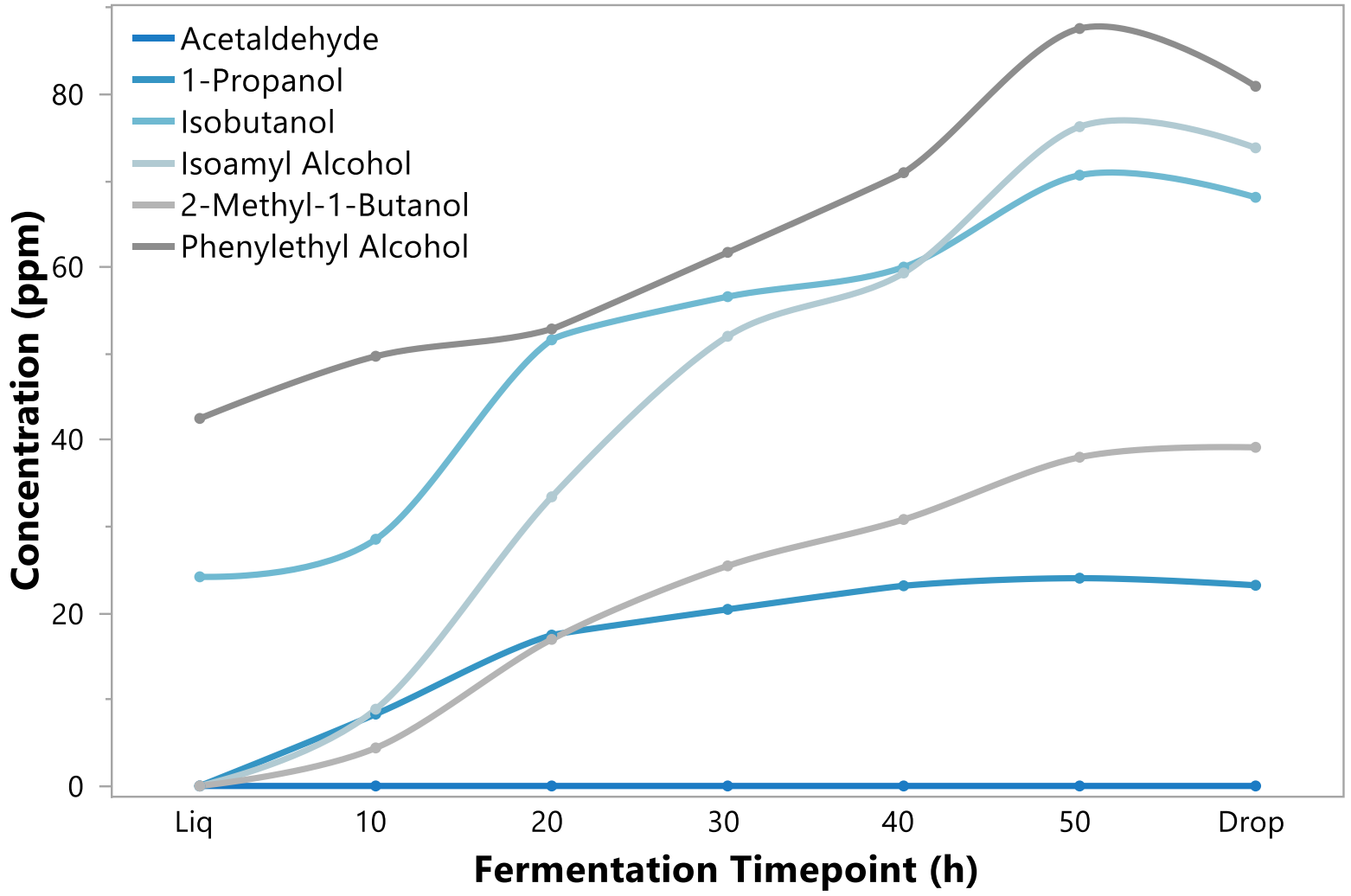
Isobutanol



Phenylethyl Alcohol



Fusels & Organic Impurities Profile in Fermentation



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Summary: Fusel Analysis

- Assess fusels production throughout fermentation
- Optimize fusels removal during distillation
- Evaluate the potential for fusels recycling in your process
- Ultimately, prevent fusels inhibition at your plant

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Amino Acid Profile For Best Performance

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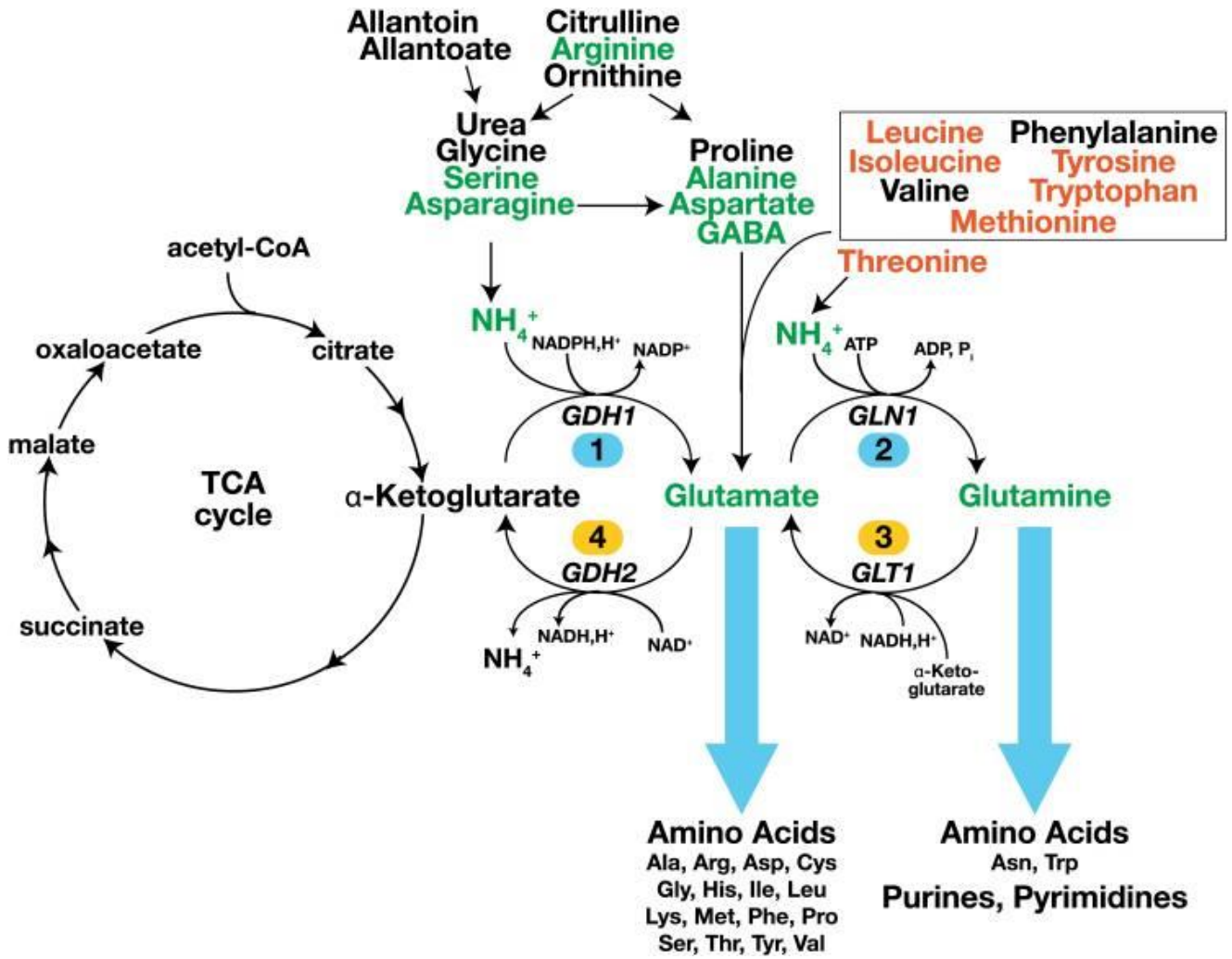
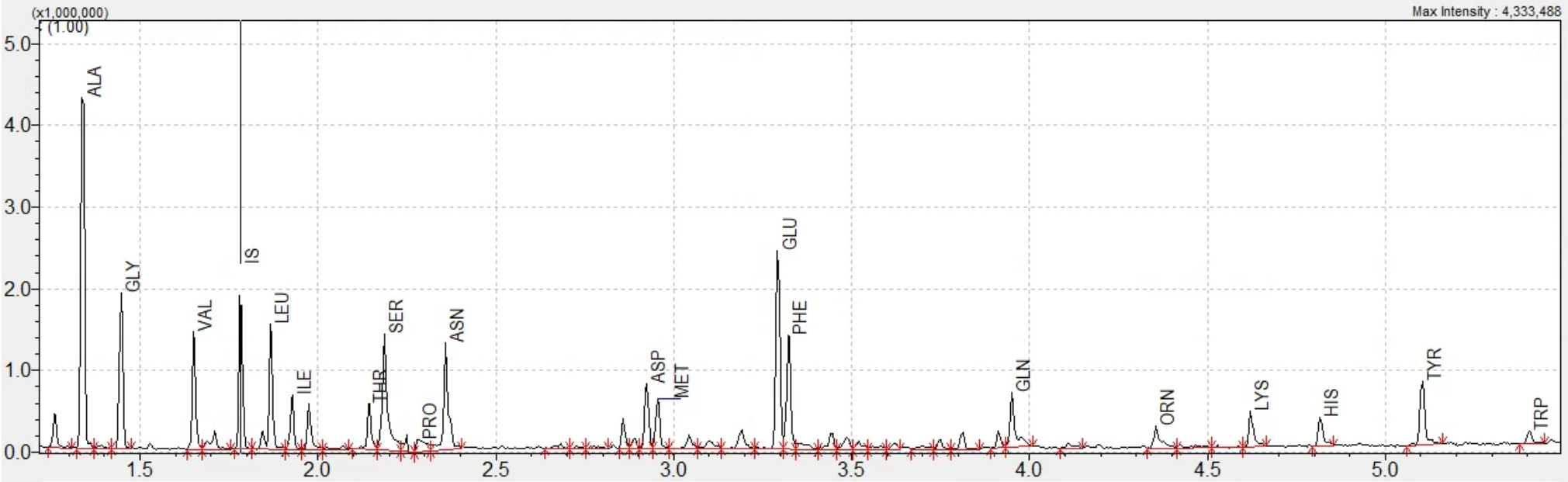
Nitrogen metabolism in yeast

- Research literature shows that not all nitrogen sources are the same
- Each nitrogen source can be categorized based on their ability to support growth and activate different metabolic pathways
- The diagram on the right shows the preferred nitrogen sources in green, intermediately preferred in black, and non-preferred amino acids in red



Ljungdahl, P.O. and Daignan-Fornier, B. Regulation of Amino Acid, Nucleotide, and Phosphate Metabolism in *Saccharomyces cerevisiae*. *Genetics* 190 (2012) 885-929

Amino acid analysis by GC-MS



- GC-MS analysis of derivatized samples resolves 21 primary amino acids and many others
- Sample prep in 8 minutes and GC-MS runs are 30 minutes
- Understand the kinetics of amino acid production and consumption in fermentations/liquefactions treated with protease
- Important insights of effects of protease dosing and urea/ammonia reductions on fusel alcohol production

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Amino acid profile: Optimize performance

*Preferred amino acids: alanine, asparagine, aspartate, glutamine, glutamate, and serine

*Intermediately preferred amino acids: glycine, ornithine, phenylalanine, and valine

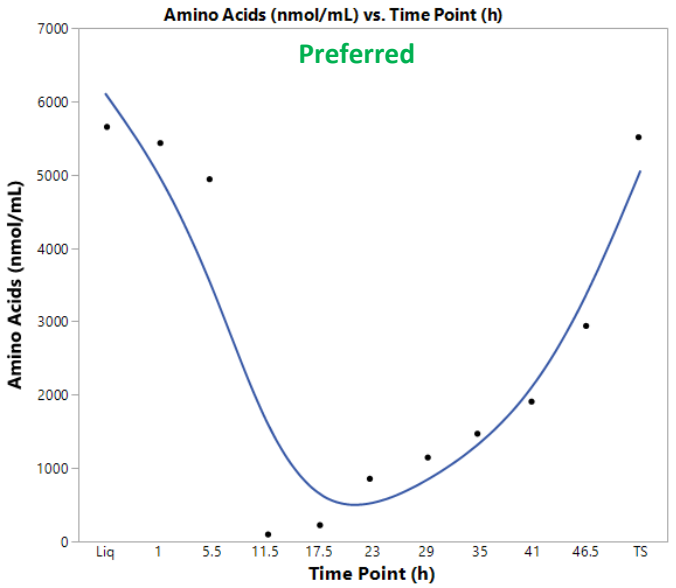
*Non-preferred amino acids: methionine, isoleucine, leucine, threonine, tryptophan, and tyrosine

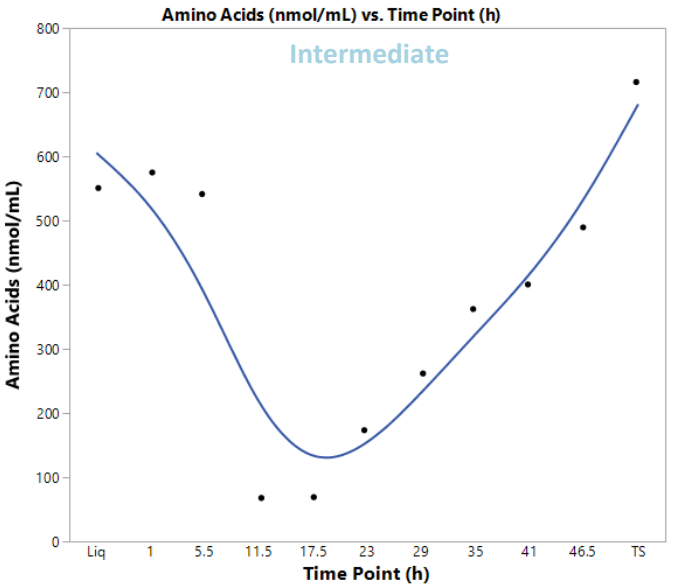
*Not involved in growth support: histidine and lysine

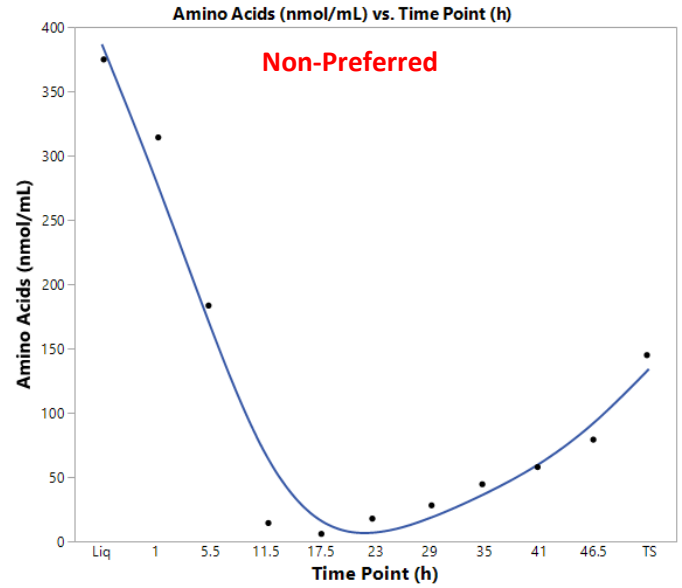
Amino Acid	Table 1. Amino acid profile at each sample point (nmol/mL)										
	Liq	1hr	5.5hr	11.5hr	17.5hr	23 hr	29hr	35hr	41hr	46.5hr	TS
ALA*	1189	1539	1610	29	52	190	307	543	755	1255	2375
ASN*	1378	1145	853	14	19	56	103	141	166	210	389
ASP*	1040	867	928	15	21	37	49	49	54	59	71
GLN*	41	81	56			57		70	103	194	268
GLU*	1527	1298	1129	38	131	328	475	455	555	820	1770
SER*	475	501	362			187	211	210	275	400	637
GLY*	330	368	416	43	63	166	242	328	352	425	547
ORN*	34	27	26								63
PHE*	47	47	17				7	11	13	16	24
VAL*	140	134	82	25	6	8	12	23	35	49	81
HIS*	26		21								18
LYS*	100	108	35						9	15	19
MET*	31	10								7	20
ILE*	49	40	20					5	8	10	19
LEU*	86	81	31	13	6	6	8	14	21	29	60
THR*	98	86	56			11	20	20	21	26	32
TRP*	11	10	7								2
TYR*	99	88	70	1				5	7	7	12

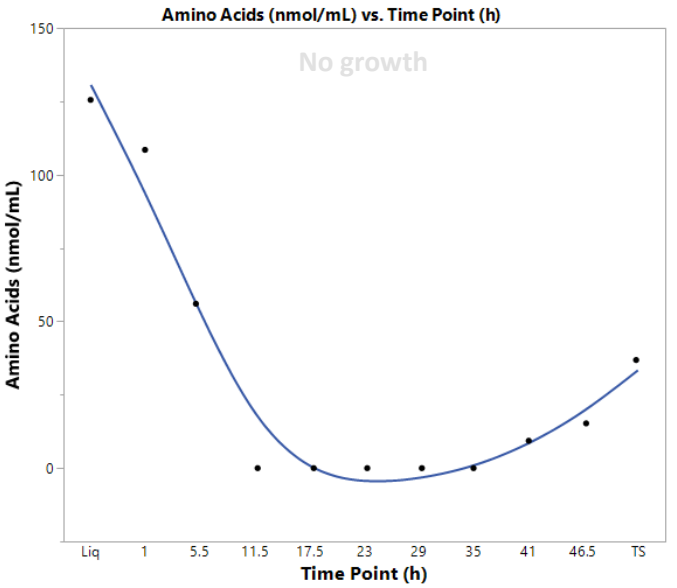
Amino acid profile: Optimize performance

- In this process, preferred amino acids were the most abundant category; quickly consumed by the 11.5-hour time point to support robust growth
- Intermediately preferred and non-preferred amino acids were lower in abundance, but also consumed early in the ferm
- All amino acids increase as the yeast population growth slows and lyses, while protease continues to produce more amino acids
- Enriching the preferred amino acid category by manipulating nitrogen sources improves performance









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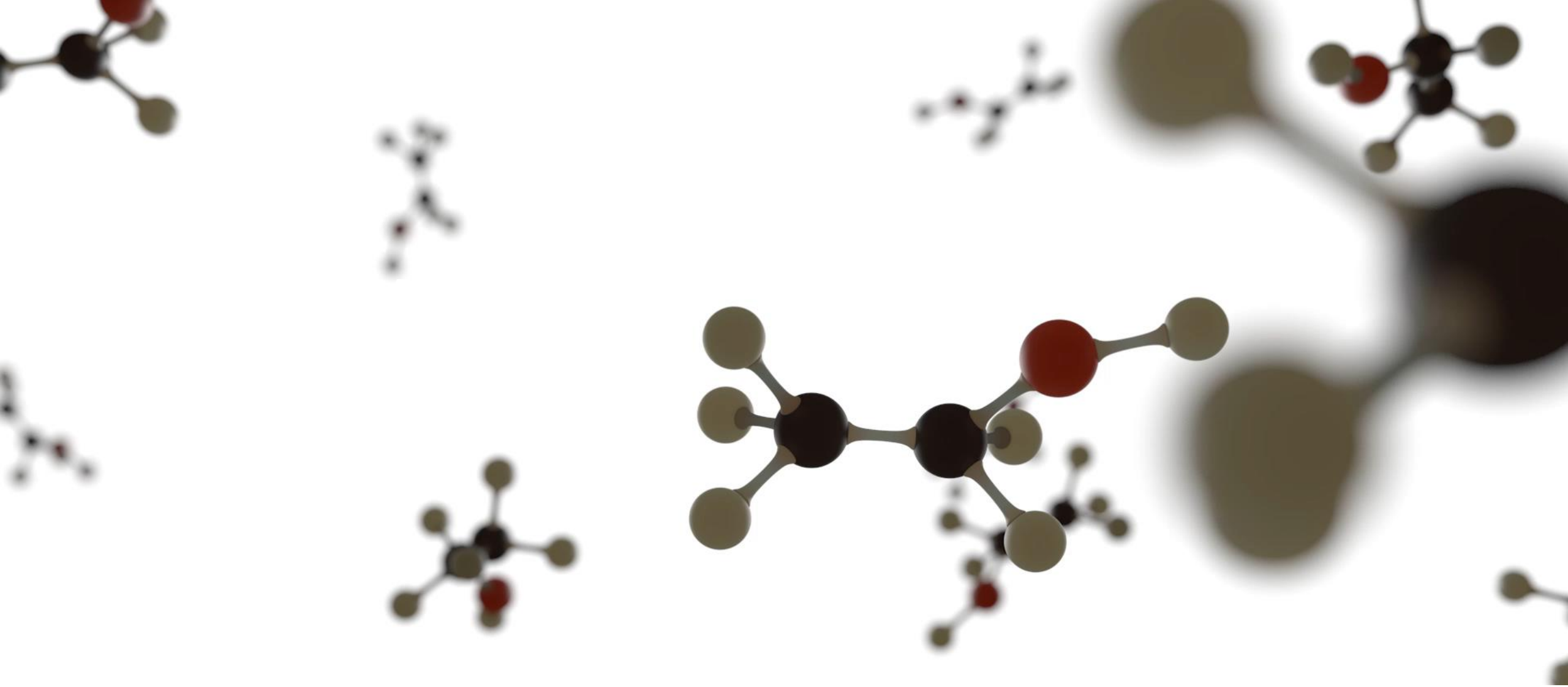
Summary: Nitrogen Optimization Strategies

CTE Global provides a comprehensive evaluation of nitrogen in fermentation

- We analyze FAN levels for each contributing source of nitrogen: amino acids, ammonia, and urea
- With our next generation of testing, we will provide amino acid profiling to go beyond FAN to a higher level of performance improvement

Our ultimate goal is to provide our customers with detailed information to **improve their process**



We can help—contact us today.

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