有机小分子化合物色谱-质谱联用分析介绍

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GC-IRMS，GC-MS，LC-MS

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提 纲

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1. 现代分离分析概述

分析化学已发展成为分析科学：分析化学是化学测量和表征的科学，是研究获取物质化学组成和结构信息的科学。随着科技生产和经济的不断发展，分析化学已不单纯是化学的一个分支学科，现已成为其他学科（生命科学、环境科学、能源科学、材料科学与信息科学）发展的工具，解决了这些学科中不断提出的分析化学的难题。

Good Analytical Chemists Are Good Chemists First

It is always exciting for analytical chemists, whether in academe or industry, to see the emergence of a new measurement science principle or device. One sees the frontier of analytical chemistry palpably moving. With the emergence, there is a period in which researchers probe and explore the basics of the new measurement, to find whether it can flexibly expand to a family of measurements and its experimental eccentricities.

Following the discovery phase, researchers ask, well, what kind of information can we actually gain from this new measurement? This second phase of measurement science research, and the role of analytical chemists in chemistry and their recognition within chemistry at large, deserves our attention. Attention to the chemical context within which the measurement may be applied is of crucial importance within the post-discovery phase. History tells us (me) that development of a measurement principle, without demonstration that significant chemical information can be evoked with it, can bring recognition to analytical chemists within the analytical community but much less so to those outside it. Recognition by the broader population of chemists comes, I think, mainly to those who contribute to the production of new chemical insights, reactions, materials, and processes.

Why shouldn't analytical chemists participate in the applications of the tools they develop? Measurement science and the production of new chemical knowledge should blend seamlessly, and I believe analytical chemists should strive to contribute to both—and not solely, single-mindedly, to the former. To do this, the analytical chemist must glean perceptions of where development of a chemical area or process is being limited by a lack of information, as a stage on which to perform and demonstrate the new measurement approach. Whether in industry or academe, analytical chemists make a real impact on other chemists when they do this. And from this I draw my oft-repeated admonition that good analytical chemists must first be good chemists.

Analytical chemists of various backgrounds can, I hope, see many examples of how this principle has worked and still works. Contemporary examples in which analytical chemists are contributing effectively to new chemical knowledge include the applications of electrospray ionization and MALDI to biopolymer characterization, microelectrodes and laser-induced fluorescence to observations of individual chemical reaction events and individual molecules, multivariate statistics with near-IR spectrophotometry for complex mixture analysis, biocompatibility studies with ion-selective electrodes for in vivo analysis, Raman spectroscopy to catalyst and carbon surface chemistry, HPLC in pharmacokinetic investigations, ICPMS in environmental studies, and self-assembled chemically modified electrodes in electron-transfer dynamics.

That the preceding list is long yet still incomplete is a good sign for analytical chemists' substantive contributions to the larger world of chemistry. It can only be maintained, however, by the continuing lifelong recognition by analytical chemists of the significance of reading broadly about chemical phenomena and by professors of the importance of having their graduate students sample courses outside analytical chemistry—especially those dealing with chemical reactions and properties.

This editorial originally appeared in the December 1, 1995, issue of Analytical Chemistry.

1. 现代分离分析概述

- **有机小分子化合物**：分子量小于2000（多数在1000以下）的有机化合物。如微生物初级代谢产物、次级代谢产物，酶促反应代谢产物等。

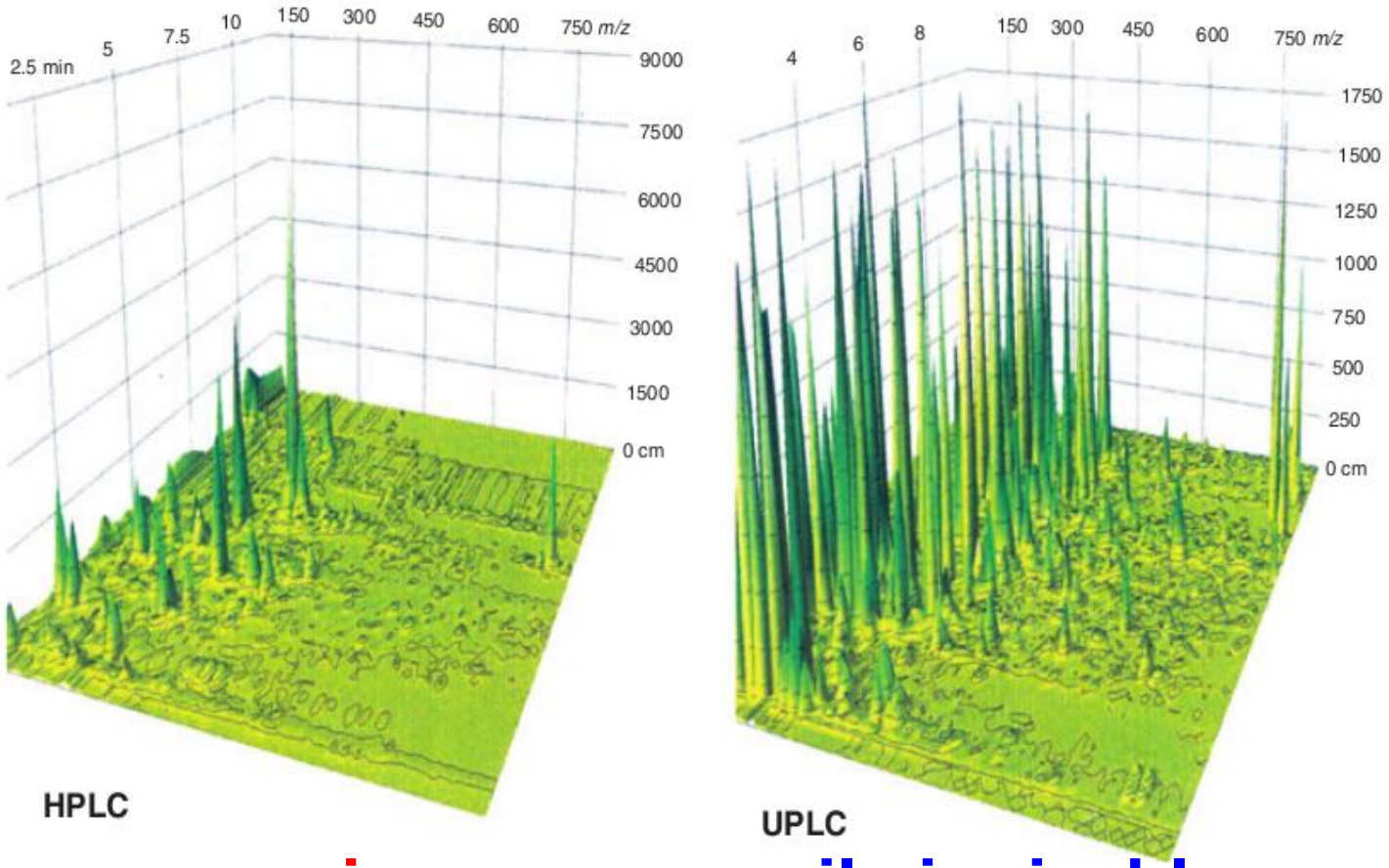
- **色谱-质谱联用分析**：色谱-质谱联用技术利用了色谱的强分离能力，以及质谱的强定性能力，是对复杂体系中目标有机小分子化合物进行定性、定量分析的强有力的工具。
- **现代分离分析**：色谱分析和高效毛细管电泳分析，目前也被统称为色谱分析。

1. 现代分离分析概述

- **现代分离分析仪器：** 主要包括气相色谱（GC）、液相色谱(HPLC)、毛细管电泳(CE)，以及这些技术与质谱，及其它各种检测器的联用。

- 现代分离是分析技术的“前沿”课题。要牢固树立“分离”的概念。

HPLC compared to UPLC



Ion suppression: more easily ionizable species masking the presences of less ionizable species.

1.1 常用的有效分离手段

- 按历史发展排序：蒸馏、离心、离子迁移、膜分离 、谱法分离 、质谱法的分离

几种主要的经典样品前处理方法（传统分离方法）

方法	原理	适用范围
物理方法		
吸附	吸附能力强弱不同	气体、液体及可溶的固体
离心	分子量或密度不同	不同相态或分子量有差别的物质
透析	渗透压不同	分子与离子或渗透压不同的物质
蒸馏	沸点或蒸汽压的不同	各种液体
过滤	颗粒或分子大小的不同	液固二相分离
液-液萃取	在二种互不相溶液体中分配系数不同	各种在二种液体中溶解度差别较大的物质
冷冻干燥	蒸汽压的不同	在常温下易于失去生物活性的物质
柱层析	溶质与固定相作用力的不同	气体、液体及可溶的固体
索氏萃取	不同溶剂中溶解度的不同	从固体、半固体中提取有用物质
真空升华	蒸汽压的不同	从固体中分离有一定蒸汽压的物质
超声振荡	不同溶剂中溶解度的不同	从固体中分离可溶物质
化学方法 衍生	通过化学反应改变溶质性质，提高灵敏度及选择性	能与衍生化试剂作用的化合物
沉淀	不同溶剂中溶度积不同	与沉淀剂发生反应生成沉淀的物质
络合	使干扰物生成络合物，除去对被测组分测定的干扰	各种与配位体反应的金属离子或其它物质

SPE 固相萃取

SPME 固相微萃取

1.2 质谱分析法

- 质谱分析法主要是通过对样品离子的质荷比的分析而实现对样品来进行定性和定量分析的方法。

质谱仪的工作过程

- 样品导入→离子源→质量分析器→检测器
  →数据系统→质量图谱
- 样品导入→离子形成→离子分离→离子检测→数据输出

质谱仪的种类

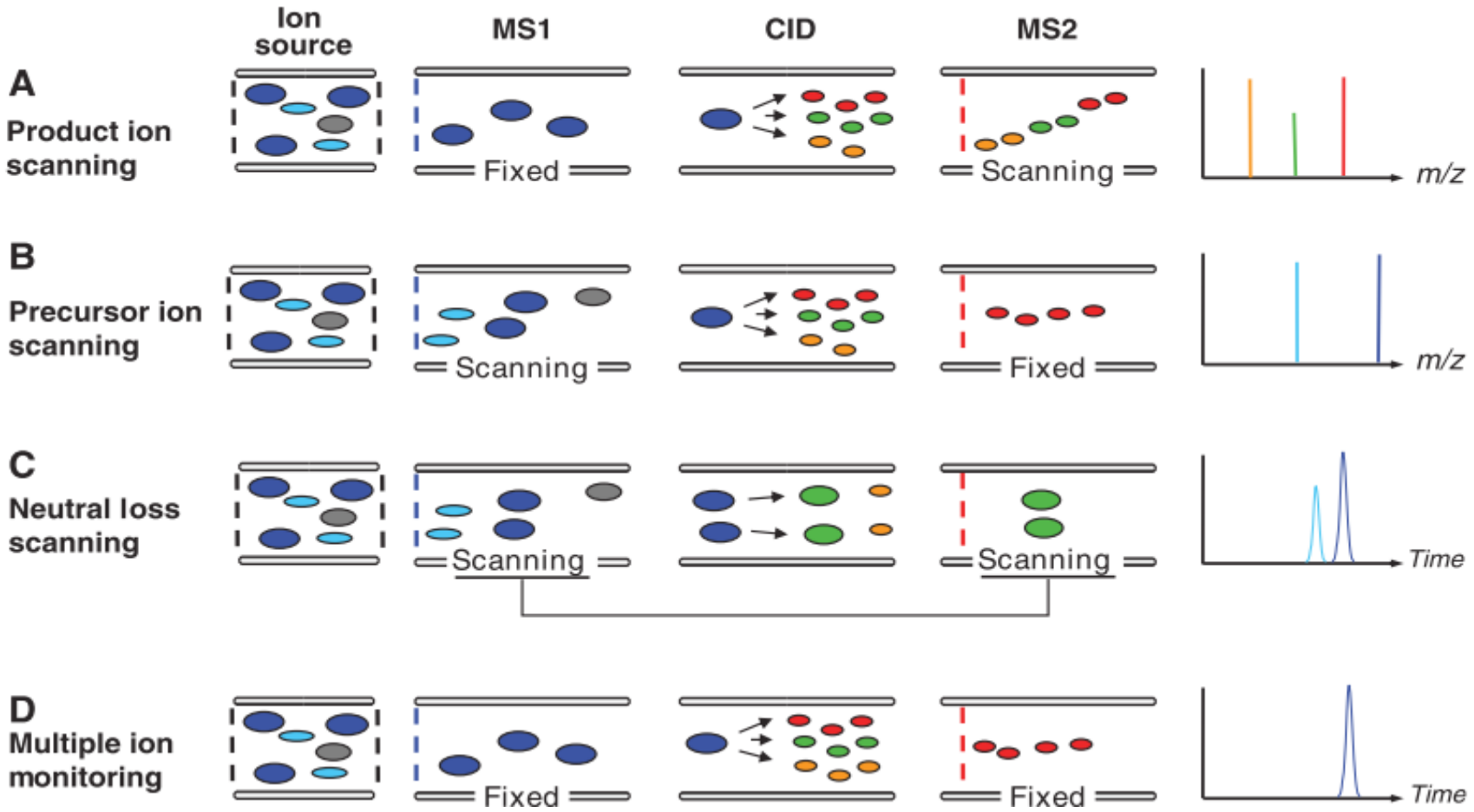
- 有机质谱、生物质谱、无机质谱
- 质量分析器：四极杆质谱仪(**Quadrupole, Q**)、离子阱质谱仪(**Ion Trap, IT**)、飞行时间质谱仪(**Time of Flight, TOF**)、傅立叶变换离子回旋共振质谱仪(**Fourier transform ion cyclotron resonance, FTICR**)
- 无机质谱：气体稳定同位素比质谱**IRMS**，电感耦合等离子体质谱仪ICP-MS，二次离子质谱仪SIMS

串联质谱的性能

Table 1. Characteristics and performances of commonly used types of mass spectrometers. Check marks indicate available, check marks in parentheses indicate optional. +, ++, and +++ indicate possible or moderate, good or high, and excellent or very high, respectively. Seq., sequential.

	IT-LIT	Q-Q-ToF	ToF-ToF	FT-ICR	Q-Q-Q	QQ-LIT
Mass accuracy	Low	Good	Good	Excellent	Medium	Medium
Resolving power	Low	Good	High	Very high	Low	Low
Sensitivity (LOD)	Good		High	Medium	High	High
Dynamic range	Low	Medium	Medium	Medium	High	High
ESI	✓	✓		✓	✓	✓
MALDI	(✓)	(✓)	✓			
MS/MS capabilities	✓	✓	✓	✓	✓	✓
Additional capabilities	Seq. MS/MS			Precursor, Neutral loss, MRM		
Identification	++	++	++	+++	+	+
Quantification	+	+++	++	++	+++	+++
Throughput	+++	++	+++	++	++	++
Detection of modifications	+	+	+	+		+++

串联质谱的工作方式


Ion source
MS1
CID
MS2
A
Product ion scanning
Fixed
Scanning
m/z
B
Precursor ion scanning
Scanning
Fixed
m/z
C
Neutral loss scanning
Scanning
Scanning
Time
D
Multiple ion monitoring
Fixed
Fixed
Time

1.3 基于质谱的代谢组学研究

- 代谢组学（Metabolomics）是考察生物体受刺激或扰动后（如基因变异或环境变化）其所有小分子代谢产物的变化或其随着时间的变化，来研究生物体系代谢途径，认识生物或细胞功能的一种研究方式。
  - **Comprehensive and simultaneous systematic determination of metabolite levels in the metabolome and their changes over time as a consequence of stimuli**

MASS SPECTROMETRY-BASED METABOLOMICS

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This review presents an overview of the dynamically developing field of mass spectrometry-based metabolomics. Metabolomics aims at the comprehensive and quantitative analysis of wide arrays of metabolites in biological samples. These numerous analytes have very diverse physico-chemical properties and occur at different abundance levels. Consequently, comprehensive metabolomics investigations are primarily a challenge for analytical chemistry and specifically mass spectrometry has vast potential as a tool for this type of investigation. Metabolomics require special approaches for sample preparation, separation, and mass spectrometric analysis. Current examples of those approaches are described in this review. It primarily focuses on metabolic fingerprinting, a technique that analyzes all detectable analytes in a given sample with subsequent classification of samples and identification of differentially expressed metabolites, which define the sample classes. To perform this complex task, data analysis tools, metabolite libraries, and databases are required. Therefore, recent advances in metabolomics bioinformatics are also discussed. © 2006 Wiley Periodicals, Inc., Mass Spec Rev 26:51–78, 2007

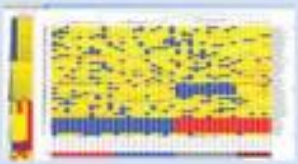
Keywords: *metabolomics; metabolic fingerprinting; metabolic profiling; lipidomics; mass spectrometry*

central role in this new science (see Fig. 1). The integrative analysis of an organism's response to a perturbation on the transcriptome, proteome, and metabolome levels will lead to a better understanding of the biochemical and biological mechanisms in complex systems. However, whereas genomics, transcriptomics, and proteomics have made significant strides in technology development, the tools for the comprehensive examination of the metabolome are still emerging (Bino et al., 2004). Although metabolomics is the endpoint of the "omics cascade" and is the closest to phenotype, there is no single-instrument platform that currently can analyze all metabolites. Possibly, because there is at least the perception that the other "omic" approaches can be handled by a single platform, metabolomics has lagged behind the other technologies. This is illustrated in Figure 2, showing the bibliographic search containing the words metabolomics, metabonomics, and proteomics in Chemical Abstracts Plus (SciFinder Scholar). While in 1999 three articles containing the keywords metabolomics or metabonomics were published, the number increased to 147 articles in 2003 and 203 in 2004. Moreover, the journal *Metabolomics* (Springer) was recently launched, which is dedicated to publish research results related to metabolomics technology development, data analysis and storage, integrated

- 主要流程：代谢指纹图谱、代谢谱差异统计分析、潜在生物标记物结构鉴定及代谢通路分析
- 非靶标代谢组学（Untargeted Metabolomics）与靶标代谢组学（Targeted Metabolomics）
- 至今没有任何一种分析技术能对代谢过程中的所有的小分子化合物进行无歧视分析。
- 现今代谢组学采用的方法是对不同的实际样品以及不同的类型的代谢产物分别采用不同的分析方法。

Discovery metabolomics

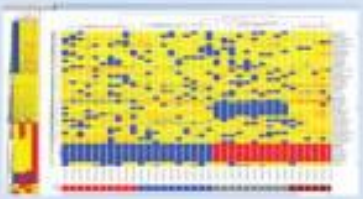
Discovery metabolomics experiments involve examining an untargeted suite of metabolites, finding the ones with statistically significant variations in abundance within a set of experimental versus control samples, and determining their chemical structure. An interpretation step allows the researcher to connect the metabolite with the biological process or condition.

	Separation and detection	Feature finding and quantitation	Alignment and statistical analysis	Identification	Pathway analysis
		MassHunter software	MPP software		
GC/MS			 Analysis and visualization	 Compound identification	 Pathway visualization
LC/MS					

Agilent solutions for discovery metabolomics

Targeted metabolomics

Targeted metabolomics experiments focus on validation, and use a large number of samples to accurately measure the abundance of previously identified metabolites. It is highly quantitative and usually requires the use of analytical standards.

	Separation and detection	MRM quantitation	Statistical analysis	Pathway analysis
		MassHunter software	MPP software	
GC/MS		 Compound identification	 Analysis and visualization	 Pathway visualization
LC/MS				

Agilent solutions for targeted metabolomics research

Chemical classes suitable for GC/MS versus LC/MS

Owing to the wide range of phsiochemical properties and concentration there is no one method that can separate, detect, and identify all known metabolites.

代谢组学研究中的样品制备

A universal quenching and extraction protocol for micobles does not yet exist.

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intracellular or extracellular metabolites?

Sampling for Metabolome Analysis of Microorganisms

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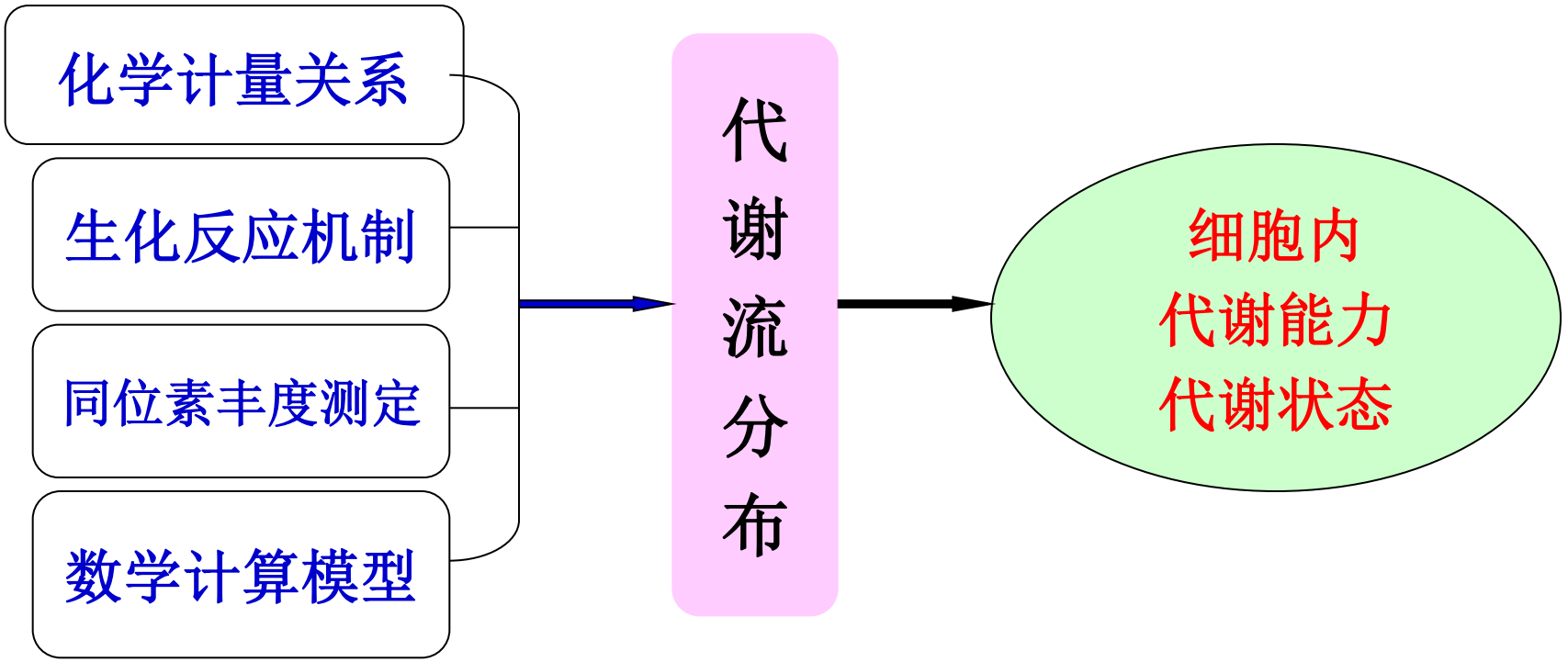
In the present work we investigated the most commonly applied methods used for sampling of microorganisms in the field of metabolomics in order to unravel potential sources of error previously ignored but of utmost importance for accurate metabolome analysis. To broaden the significance of our study, we investigated different Gram-negative and Gram-positive bacteria, i.e., *Bacillus subtilis*, *Corynebacterium glutamicum*, *Escherichia coli*, *Gluconobacter oxydans*, *Pseudomonas putida*, and *Zymononas mobilis*, and analyzed metabolites from different catabolic and anabolic intracellular pathways.

tion of development. They have borne new analytical techniques for the various metabolites, as well as new data-mining and modeling tools to handle and interpret the large metabolome data sets. With the use of these novel tools metabolome analysis has been applied to different biological systems involving different microorganisms, plants, or mammalian cells. Sampling is especially critical in metabolome analysis due to high exchange rates and small pool sizes of the metabolites of interest.[1] Due to this, quenching of the cells during sampling is usually applied. The most popular method for microbial cells is quenching with cold methanol, maintaining the sample temperature below −20 °C,

1.4 代谢流分析 (MFA, Metabolic Flux Analysis)



^{13}C-MFA是代谢工程、系统生物学、合成生物学研究的基础

现有色谱-质谱联用分析仪

Agilent
7890A/5975C
气相色谱-质谱联用仪
（GC-MS）

ThermoFisher
Trace GC/Delta V Advantage
气相色谱-同位素比质谱
（GC-IRMS）

Agilent
1260/6460
液相色谱/三重四极杆串联质谱联用仪
（Triple-quadrupole LC-MS）

2. 气相色谱-质谱联用分析

- 主要配置：7890A气相色谱仪，5975C单四极杆质谱仪（配备EI及CI离子源）

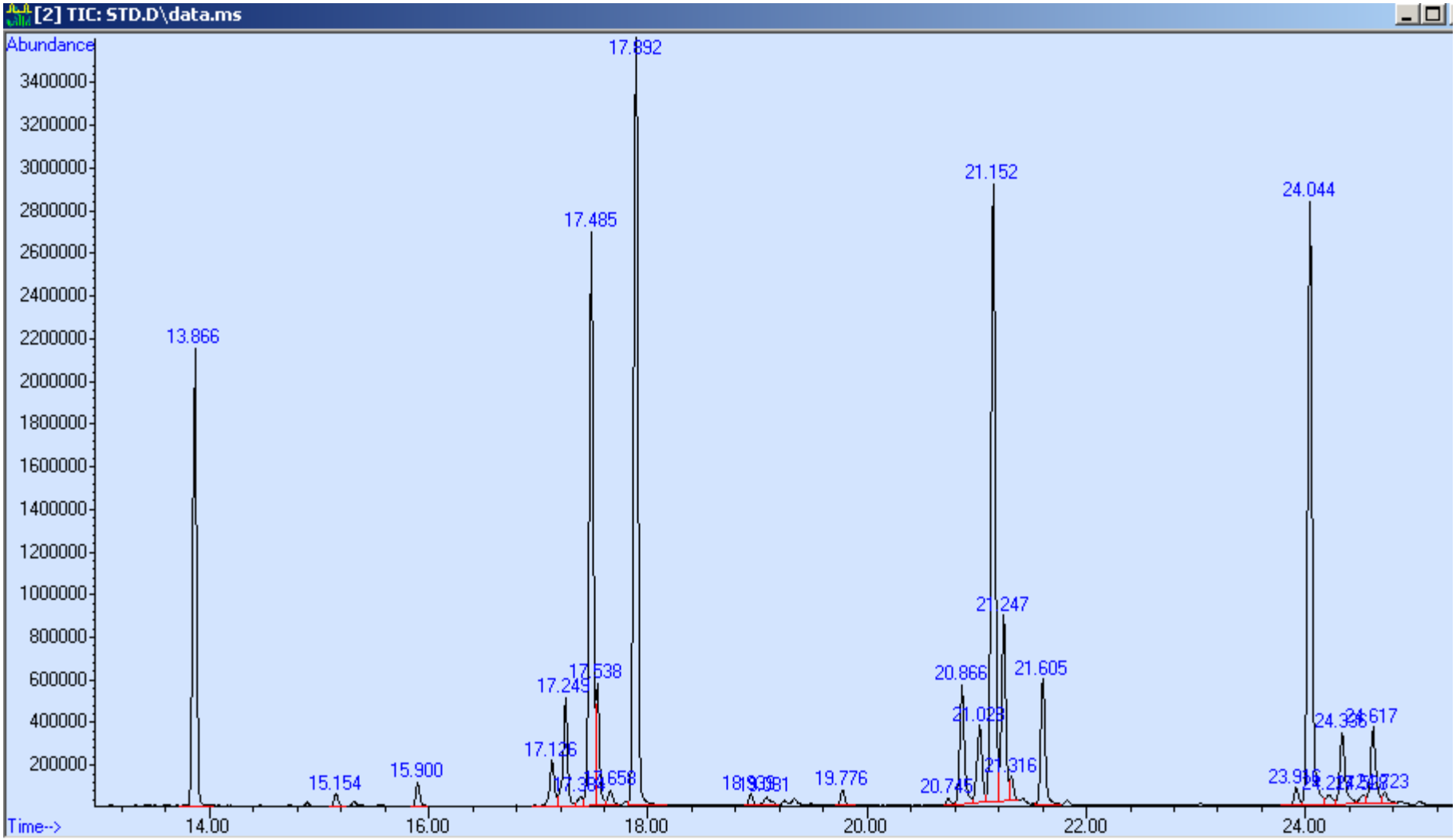
- 主要功能：主要用于挥发性、半挥发性，且热稳定的有机小分子化合物的定性、定量以及化学结构分析，适用于对这些化合物进行高灵敏度、高准确性的直接GC-MS分离分析，或衍生化后的GC-MS分离分析。

- 适合挥发性（沸点＜350℃）有机小分子化合物的定性、定量分析（成分分析），如烃类、酮类、醛类、酯类等。
- 常见的生物活性物质如氨基酸、糖、长链脂肪酸的分析需要经合适的衍生化制备方法。
- 如果分析对象为蛋白质、脂肪、多糖等高分子化合物，则需经合适的水解方法制备其小分子水解代谢产物，并经合适的衍生化制备方法。

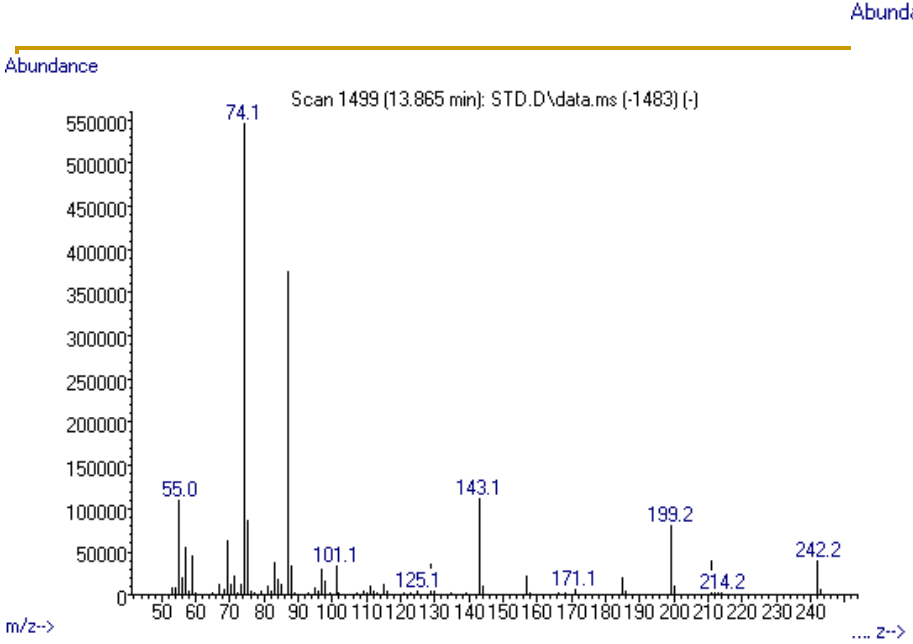
- ***样品信息***：送样时应提供样品来源、制备方法、溶剂、可能混有成分、目标化合物类型及化学结构、分子量范围、样品沸点，气相色谱分析条件等。

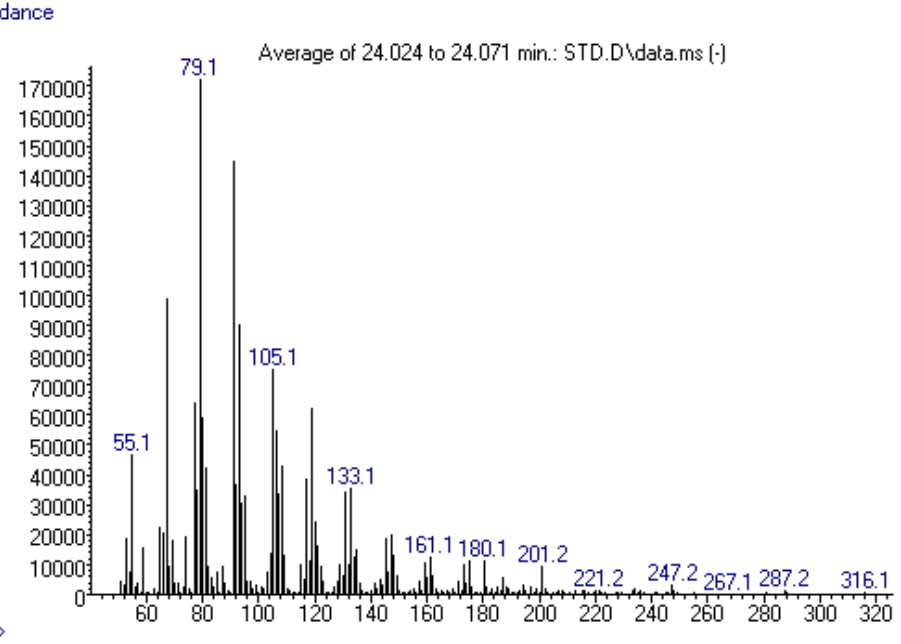
- ***样品要求*：1.样品需用有机溶剂溶解。2.样品中不含无机盐、强酸、强碱等化合物。3.样品中不含难挥发（沸点＞450℃）的组分。4.样品（包括所含杂质）须稀释到合适的浓度范围，最好有气相色谱分析的资料。5.样品须经微孔滤膜（≤0.45 um）过滤。6.提供尽可能全的样品信息。**

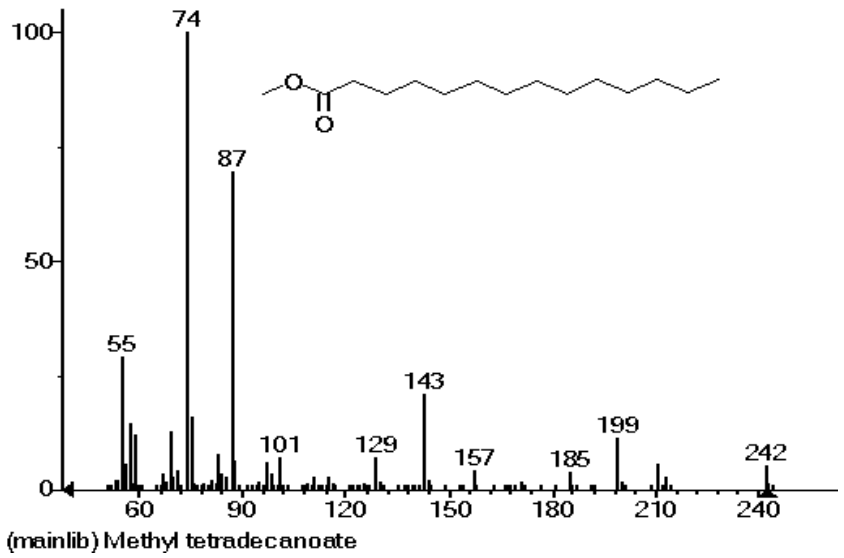
在定性定量分析中的应用

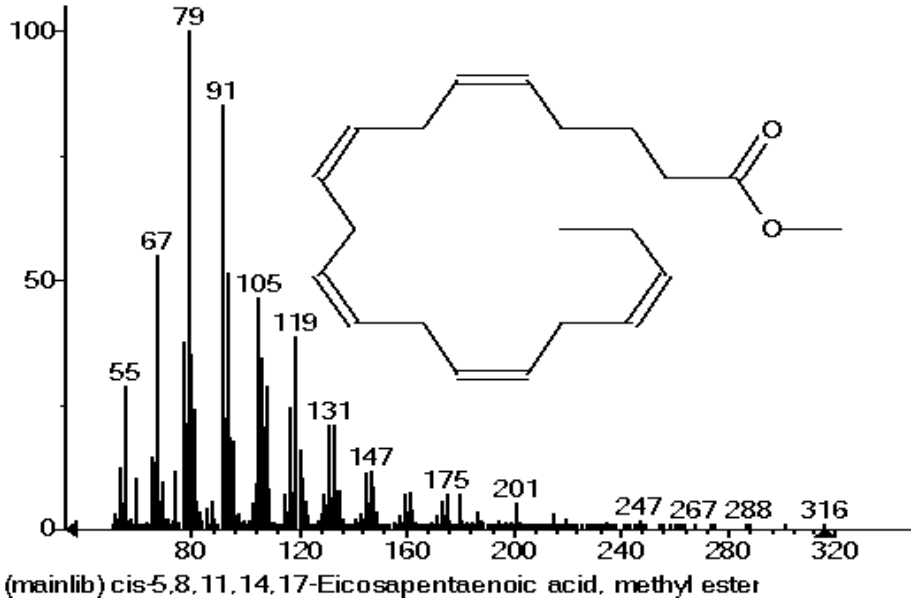


脂肪酸甲酯标准品GC/MS分析总离子流图


Abundance
Scan 1499 (13.865 min): STD.D\data.ms (-1483) (-)
74.1
55.0
143.1
199.2
101.1
125.1
171.1
214.2
242.2
m/z-->


Abundance
Average of 24.024 to 24.071 min.: STD.D\data.ms (-)
79.1
105.1
55.1
133.1
161.1
180.1
201.2
221.2
247.2
267.1
287.2
316.1
.... z-->


74
87
55
143
101
129
157
185
199
242
(mainlib) Methyl tetradecanoate


79
91
67
105
119
55
131
147
175
201
247
267
288
316
(mainlib) cis-5,8,11,14,17-Eicosapentaenoic acid, methyl ester

在代谢途径、代谢组学、代谢流研究中的应用

- 适用于高浓度标记代谢产物及代谢途径的确认
- 适用于已知代谢途径、已知代谢产物的代谢组学、代谢流学研究
- 是目前用于代谢流（稳态）研究的重要分析技术（另一分析技术是核磁共振）。
- 目前用于代谢组学研究的主流气质联用仪是气相色谱-高分辨质谱联用仪

Microbial Metabolomics with Gas Chromatography/ Mass Spectrometry

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An analytical method was set up suitable for the analysis of microbial metabolomes, consisting of an oximation and silylation derivatization reaction and subsequent analysis by gas chromatography coupled to mass spectrometry. Microbial matrixes contain many compounds that potentially interfere with either the derivatization procedure or analysis, such as high concentrations of salts, complex media or buffer components, or extremely high substrate and product concentrations. The developed method was extensively validated using different microorganisms, i.e.,

compounds, MW < 1000) in the cell (the metabolome), body fluids, or tissue.[3] As the biochemical level of the metabolome is closest to that of the function of a cell (the phenotype), the study of the metabolome is key in understanding biological functioning.[1] By analyzing differences between metabolomes using biostatistics (multivariate data analysis; pattern recognition), metabolites relevant to a specific phenotypic characteristic can be identified. By using such a nontargeted, holistic approach instead of the traditional hypothesis-driven approach, metabolomics studies can lead to new insights in cellular behavior.[1,2]

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Standardizing GC–MS metabolomics☆

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Data correction and normalization

ABSTRACT

Metabolomics being the most recently introduced "omic" analytical platform is currently at its development phase. For the metabolomics to be broadly deployed to biological and clinical research and practice, issues regarding data validation and reproducibility need to be resolved. Gas chromatography–mass spectrometry (GC–MS) will remain integral part of the metabolomics laboratory. In this paper, the sources of biases in GC–MS metabolomics are discussed and experimental evidence for their occurrence and impact on the final results is provided. When available, methods to correct or account for these biases are presented towards the standardization of a systematic methodology for quantitative GC–MS metabolomics.

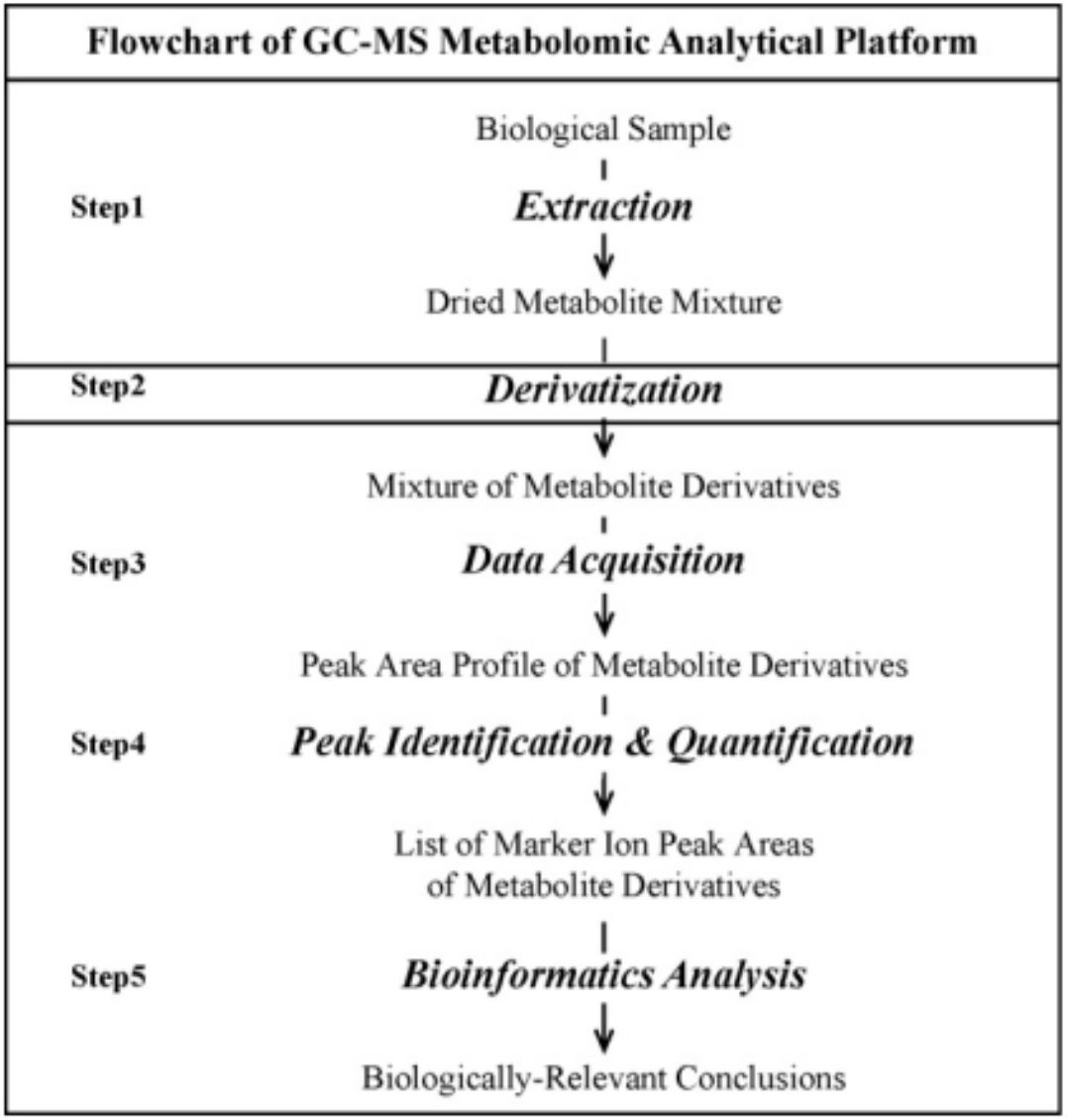

Fig. 1. Schematic diagram of the GC–MS metabolomics analytical platform.

analytical chemistry

A dramatically improved peak separation capacity and an increase in sensitivity and selectivity

ARTICLE

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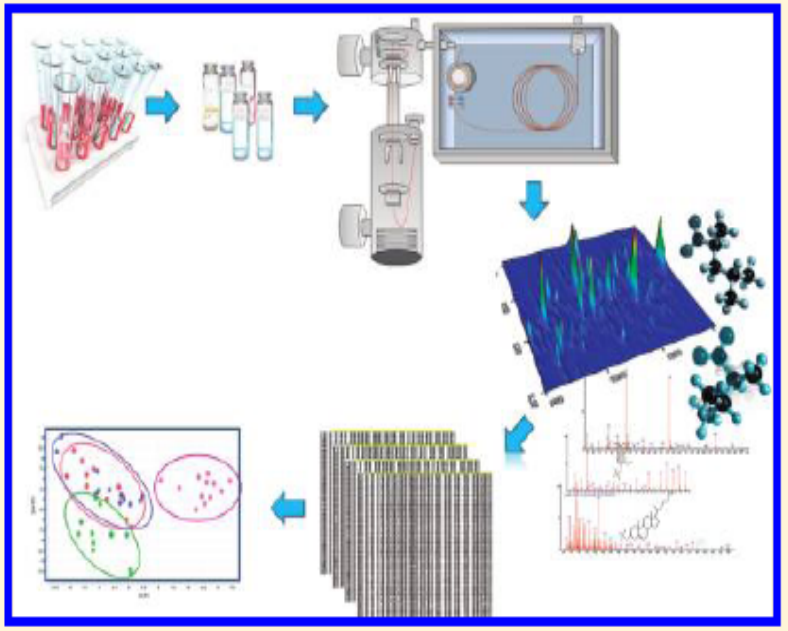
Data Analysis Tool for Comprehensive Two-Dimensional Gas Chromatography/Time-of-Flight Mass Spectrometry

Sandra Castillo, Ismo Mattila, Jarkko Miettinen, Matej Orešič, and Tuulia Hyötyläinen*

VTT Technical Research Centre of Finland, Espoo, FI-02044 VTT, Finland

ABSTRACT: Data processing and identification of unknown compounds in comprehensive two-dimensional gas chromatography combined with time-of-flight mass spectrometry (GC×GC/TOFMS) analysis is a major challenge, particularly when large sample sets are analyzed. Herein, we present a method for efficient treatment of large data sets produced by GC×GC/TOFMS implemented as a freely available open source software package, Guineu. To handle large data sets and to efficiently utilize all the features available in the vendor software (baseline correction, mass spectral deconvolution, peak picking, integration, library search, and signal-to-noise filtering), data preprocessed by instrument software are used as a starting point for further processing. Our software affords alignment of the data, normalization, data filtering, and utilization of retention indexes in the verification of identification as well as a novel tool for



GC/MS的不足

- 需要衍生化
- 副产物
- 不完全衍生化
- 高温分析条件下化合物易分解
- 目标分析化合物的化学转变
- 衍生化产物的分解（如水解等）

PROTOCOL

GC/MS

^{13}C-based metabolic flux analysis

Nicola Zamboni[1], Sarah-Maria Fendt[1,2], Martin Rühl[1,3] & Uwe Sauer[1]

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Published online 21 May 2009; doi:10.1038/nprot.2009.58

Stable isotope, and in particular ^{13}C-based flux analysis, is the exclusive approach to experimentally quantify the integrated responses of metabolic networks. Here we describe a protocol that is based on growing microbes on ^{13}C-labeled glucose and subsequent gas chromatography mass spectrometric detection of ^{13}C-patterns in protein-bound amino acids. Relying on publicly available software packages, we then describe two complementary mathematical approaches to estimate either local ratios of converging fluxes or absolute fluxes through different pathways. As amino acids in cell protein are abundant and stable, this protocol requires a minimum of equipment and analytical expertise. Most other flux methods are variants of the principles presented here. A true alternative is the analytically more demanding dynamic flux analysis that relies on ^{13}C-pattern in free intracellular metabolites. The presented protocols take 5–10 d, have been used extensively in the past decade and are exemplified here for the central metabolism of *Escherichia coli*.

GC/MS/MS

Metabolic Engineering 13 (2011) 225–233

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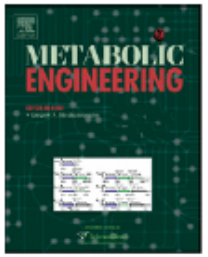

Tandem mass spectrometry: A novel approach for metabolic flux analysis

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Department of Chemical Engineering, Metabolic Engineering and Systems Biology Laboratory, University of Delaware, 150 Academy St, Newark, DE 19716, USA

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ABSTRACT

The goal of metabolic flux analysis (MFA) is the accurate estimation of intracellular fluxes in metabolic networks. Here, we introduce a new method for MFA based on tandem mass spectrometry (MS) and stable-isotope tracer experiments. We demonstrate that tandem MS provides more labeling information than can be obtained from traditional full scan MS analysis and allows estimation of fluxes with better precision. We present a modeling framework that takes full advantage of the additional labeling information obtained from tandem MS for MFA. We show that tandem MS data can be computed for any

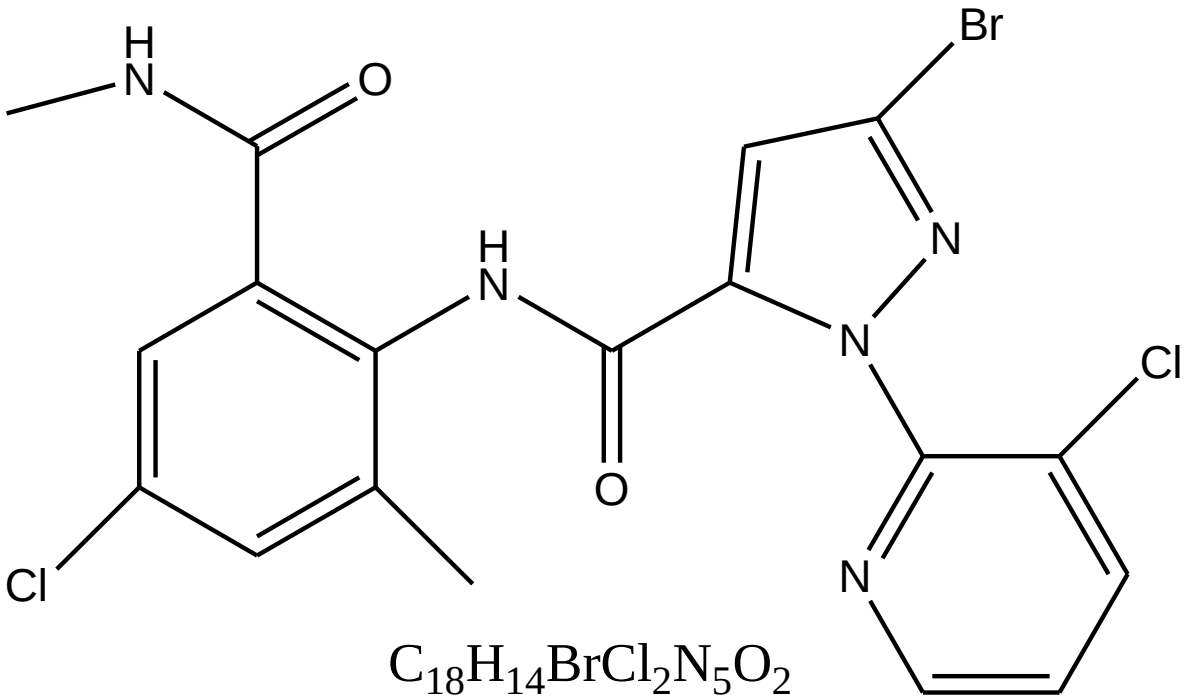
串联质谱：提高灵敏度，获得更多的同位素标记信息

3. 液相色谱/质谱联用分析

- 主要配置：1260液相色谱仪，6460三重四极杆串联质谱仪（配备ESI及APCI离子源），DAD检测器
- 主要功能：主要用于中等极性至强极性的有机小分子化合物的定性、定量以及化学结构分析，特别适用于有化学结构信息背景的化合物的结构鉴定，以及对复杂基质中已知化合物进行高灵敏度的定量分析。

- 适合热不稳定的、中等极性以上的有机小分子化合物的定性、定量分析（成分分析），如微生物初级代谢产物、次级代谢产物、微生物对外源化合物的生物转化等研究中有关有机小分子化合物的化学结构确认及定量分析。

样品信息：送样时应提供样品来源、制备方法、溶剂、可能混有成分、目标化合物类型及化学结构、单同位素精确质量数、分子量范围、**HPLC**分析条件等。

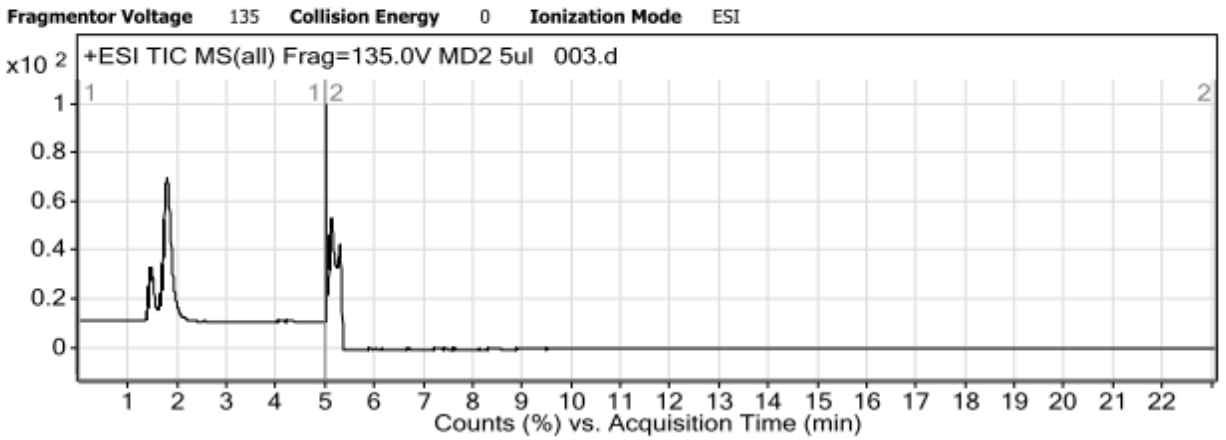


$C_{18}H_{14}BrCl_2N_5O_2$
Exact Mass: 480.97079
Mol. Wt.: 483.14606

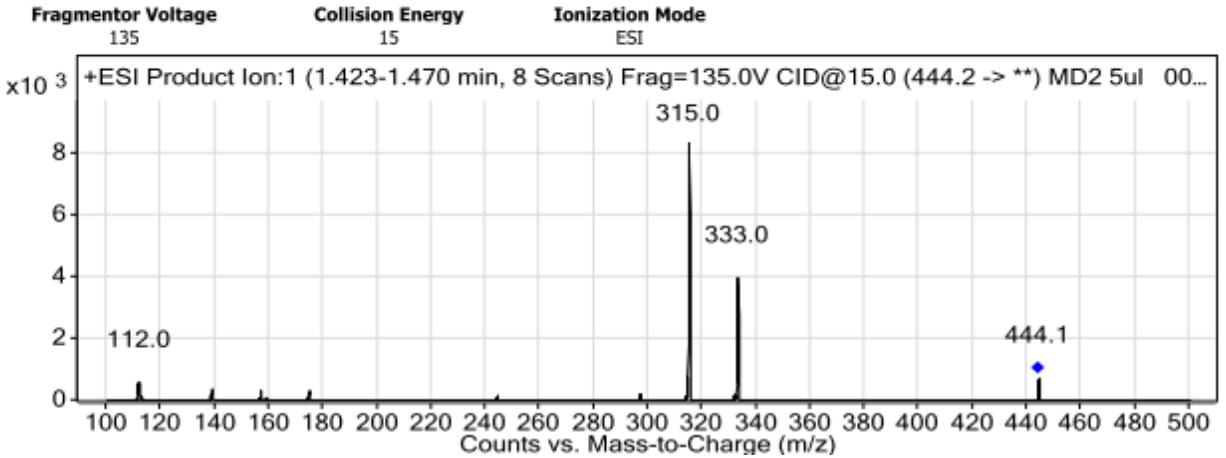
- ***样品要求***：1. 样品需用极性的色谱纯溶剂（或流动相）溶解, 并经微孔滤膜（≤**0.45 um**）过滤。2. 样品中不含难挥发性磷酸盐等无机盐；高浓度的表面活性剂；离子对试剂；强酸、强碱等化合物。3. 样品（包括所含杂质）须稀释到合适的浓度范围，并最好提供**HPLC**分析的资料。4. 提供尽可能全的样品信息。

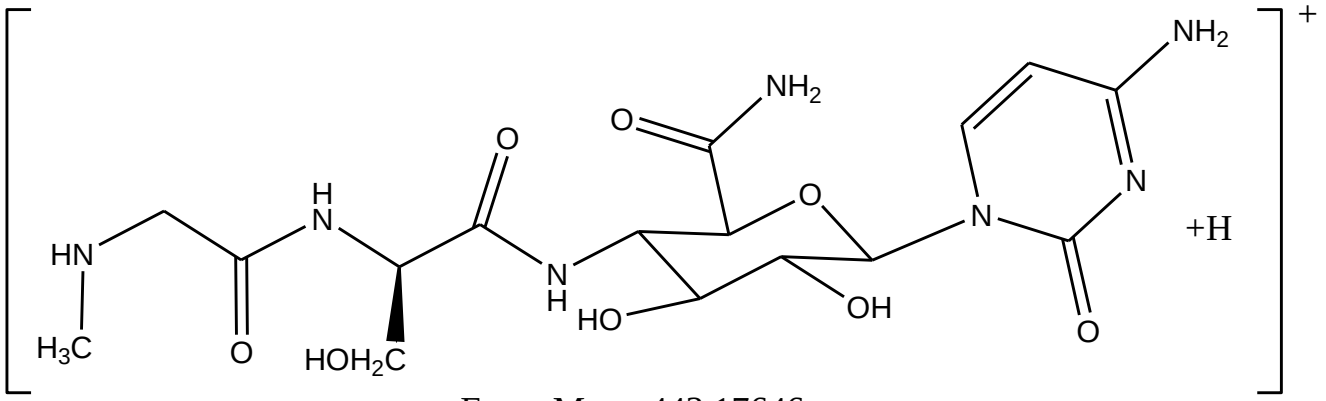
有机小分子化合物结构鉴定

User Chromatograms



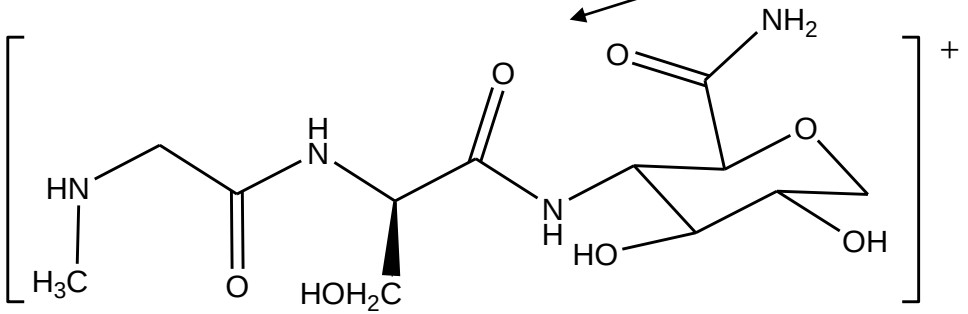
User Spectra





Exact Mass: 443.17646

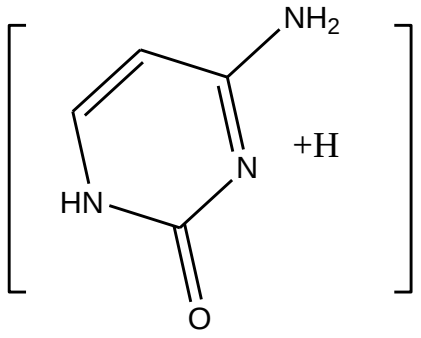
M/Z 444.1



M/Z 333

-H_2O

M/Z 315



M/Z 112

在代谢途径、代谢组学、代谢流研究中的应用

- 适用于高浓度标记代谢产物及代谢途径的确认
- 适用于已知代谢途径、已知代谢产物的代谢组学、代谢流学研究
- 是动态代谢流研究的重要工具。
- 目前用于代谢组学研究、动态代谢流分析的主流液质联用仪是液相色谱-高分辨质谱联用仪

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Journal of BIOTECHNOLOGY
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Determination of metabolic flux changes during fed-batch cultivation from measurements of intracellular amino acids by LC-MS/MS

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Abstract

Metabolic flux analysis using ^{13}C-labeled substrates is a well-developed method for investigating cellular behavior in steady-state culture condition. To extend its application, in particular to typical industrial conditions, such as batch and fed-batch cultivations, a novel method of ^{13}C metabolic flux analysis is proposed. An isotopomer balancing model was developed to elucidate flux distributions in the central metabolism and all amino acids synthetic pathways. A lysine-producing strain of *Escherichia coli* was cultivated by fed-batch mode in a growth medium containing yeast extract. Mass distribution data was derived from both intracellular free amino acids and proteinogenic amino acids measured by LC-MS/MS, and a correction parameter for the protein turnover effect on the mass distributions of intracellular amino acids was introduced. Metabolic flux distributions were determined in both exponential and stationary phases. Using this new approach, a culture phase-dependent metabolic shift was detected in the fed-batch culture. The approach presented here has great potential for investigating cellular behavior in industrial processes, independent of cultivation modes, metabolic phase and growth medium.

- 目前更多的代谢组、代谢流分析中的液质联用仪为液相色谱–高分辨质谱，如**TOF, Q-TOF, LTQ Orbitrap**等。

Demonstration of the ethylmalonyl-CoA pathway by using ^{13}C metabolomics

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Edited by Hans Kornberg, Boston University, Boston, MA, and approved January 8, 2009 (received for review October 31, 2008)

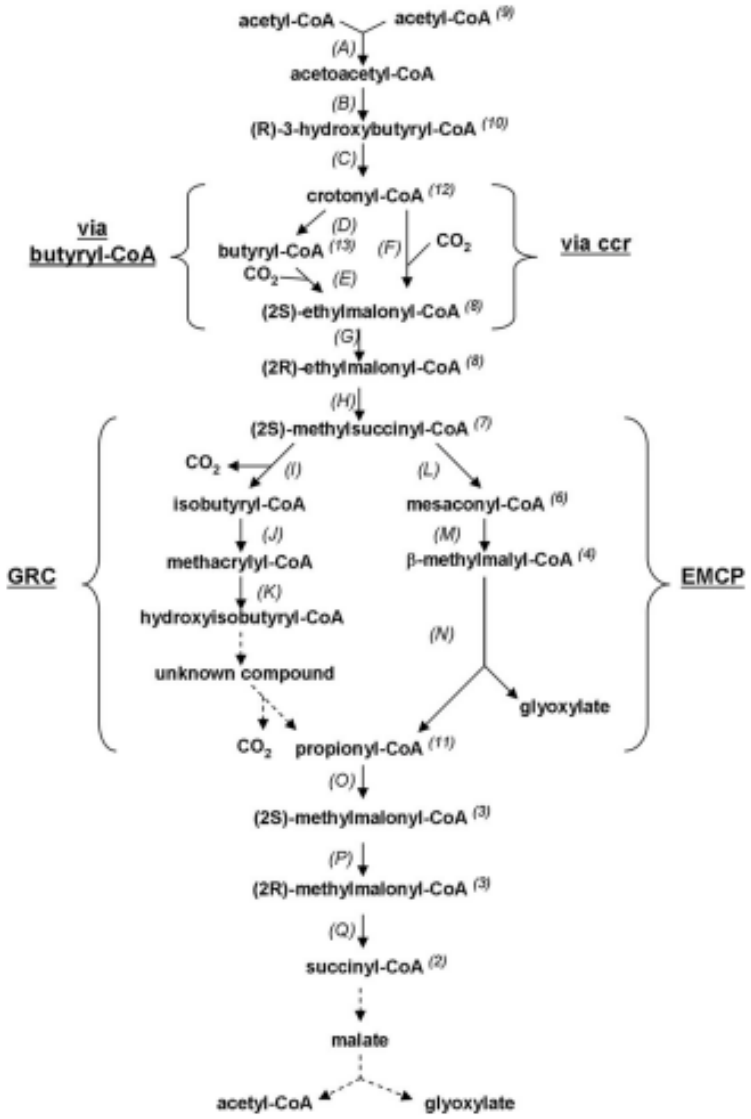
The assimilation of one-carbon (C1) compounds, such as methanol, by serine cycle methylotrophs requires the continuous regeneration of glyoxylate. Instead of the glyoxylate cycle, this process is achieved by a not yet established pathway where CoA thioesters are known to play a key role. We applied state-of-the-art metabolomics and ^{13}C metabolomics strategies to demonstrate how glyoxylate is generated during methylotrophic growth in the isocitrate lyase-negative methylotroph *Methylobacterium extorquens* AM1. High-resolution mass spectrometry showed the presence of CoA thioesters specific to the recently proposed ethylmalonyl-CoA pathway. The operation of this pathway was demonstrated by short-term ^{13}C-labeling experiments, which allowed determination of the sequence of reactions from the order of label incorporation into the different CoA derivatives. Analysis of ^{13}C positional enrichment in glycine by NMR was consistent with the predicted labeling pattern as a result of the operation of the ethylmalonyl-CoA pathway and the unique operation of the latter for glyoxylate generation during growth on methanol. The results also revealed that 2 molecules of glyoxylate were regenerated in this process. This work provides a complete pathway for methanol assimilation in the model methylotroph *M. extorquens* AM1 and represents an important step toward the determination of the overall topology of its metabolic network. The operation of the ethylmalonyl-CoA pathway in *M. extorquens* AM1 has major implications for the physiology of these methylotrophs and their role in nature, and it also provides a common ground for C1 and C2 compound assimilation in isocitrate lyase-negative bacteria.

^{13}C labeling | CoA ester | methylotroph | one-carbon metabolism | glyoxylate regeneration

Recent studies, including mutant analyses, gene predictions, enzyme assays, and metabolite studies in *M. extorquens* AM1, have led to the observation that a complex sequence of CoA thioester derivatives is involved in glyoxylate regeneration, resulting in the hypothesis of the so-called glyoxylate regeneration cycle (GRC) (19, 20) [Fig. 1 and supporting information (SI) Table S1]. According to this pathway, a C5 compound, methylsuccinyl-CoA, is formed from the condensation of 2 acetyl-CoA molecules plus 1 CO_2 and is decarboxylated twice in a process similar to valine degradation. The specific intermediates of the GRC are isobutyryl-CoA, metacrylyl-CoA, and hydroxylisobutyryl-CoA, and the result is the formation of propionyl-CoA. Subsequently, propionyl-CoA is transformed to malate, from which 1 glyoxylate and 1 acetyl-CoA are generated (20). More recently, a second hypothesis, the ethylmalonyl-CoA pathway (EMCP), was proposed from studies of C2 assimilation pathways in *R. sphaeroides* (21–23). This pathway (Fig. 1 and Table S1) includes the formation of methylsuccinyl-CoA, which is further converted to methylmalyl-CoA, from which both glyoxylate and propionyl-CoA are released by cleavage (22). The propionyl-CoA can then be converted to C4 compounds and assimilated as cell material (23).

The 2 pathways mentioned above are still hypothetical, and none has been firmly demonstrated to operate in vivo. They differ strikingly in terms of carbon balance and, therefore, overall carbon yield for methylotrophic growth. The GRC includes a net decarboxylation step, whereas the ethylmalonyl-CoA pathway includes net carboxylation steps. This makes the second pathway more efficient in terms of carbon assimilation and has important implications with regard to the physiology of these methylotrophs and their actual biotechnological potential.

In this work, we combined state-of-the-art metabolomics and ^{13}C



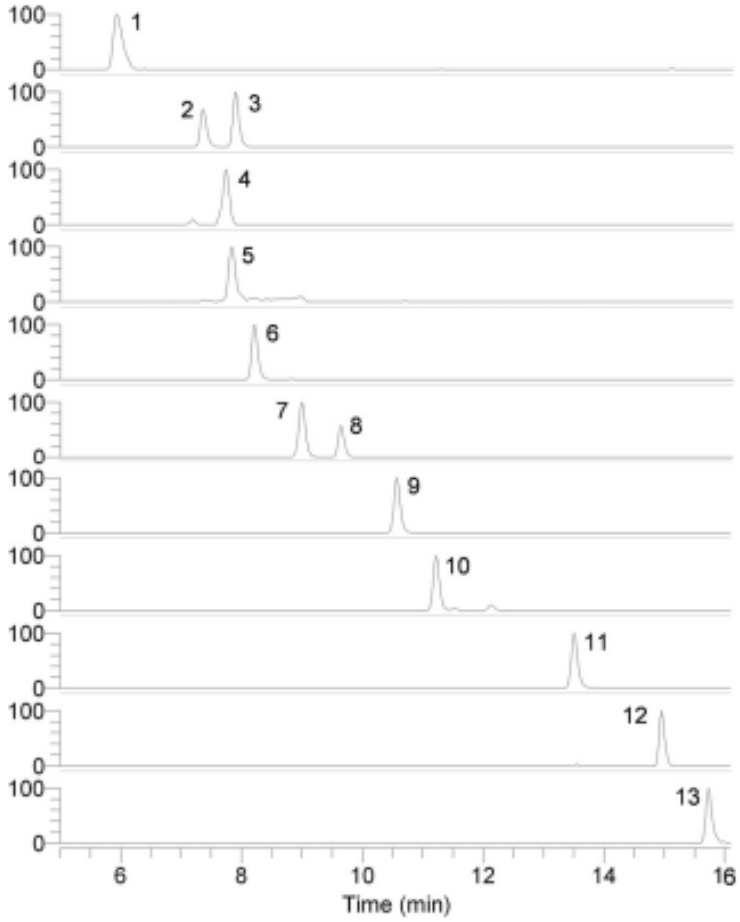


Fig. 2. LC-MS analysis of CoA thioesters occurring in cell extracts of *M. extorquens* AM1 during growth on methanol. 1, malyl-CoA; 2, succinyl-CoA; 3, methylmalonyl-CoA; 4, β-methylmalyl-CoA; 5, CoA; 6, mesaconyl-CoA; 7, methylsuccinyl-CoA; 8, ethylmalonyl-CoA; 9, acetyl-CoA; 10, 3-hydroxybutyryl-CoA; 11, propionyl-CoA; 12, crotonyl-CoA; and 13, butyryl-CoA.

MICROBIOLOGY

4. 气相色谱/同位素比质谱分析

- 气相色谱与同位素比质谱联用(非直接联用)

  Gas chromatography coupled to isotope ratio mass spectrometry via *GC Isolink*

- 混合物中经GC分离的单个化合物的同位素比分析

  (High Precise) Compound-specific isotope analysis, CSIA

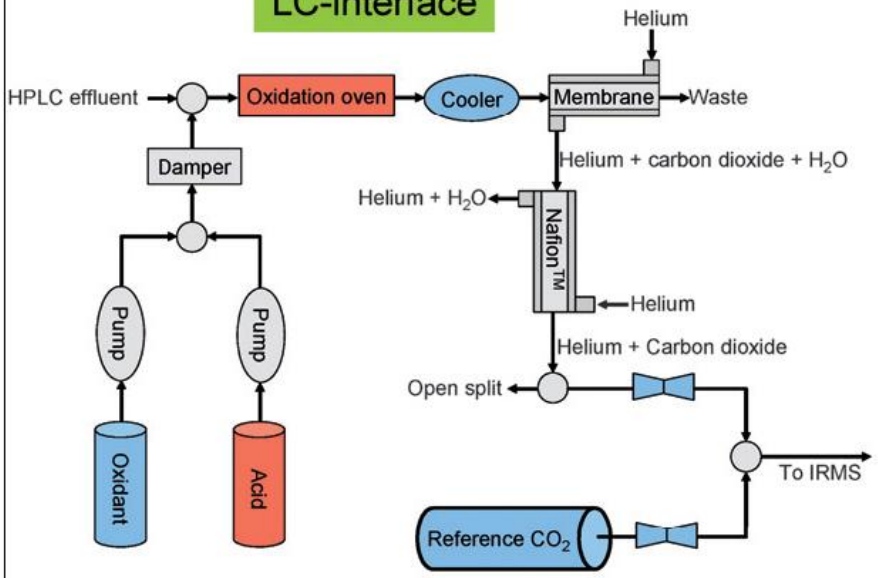
GC-C-IRMS for $^{13}C/^{12}C$ and $^{15}N/^{14}N$

GC-TC-IRMS for D/H and $^{18}O/^{16}O$

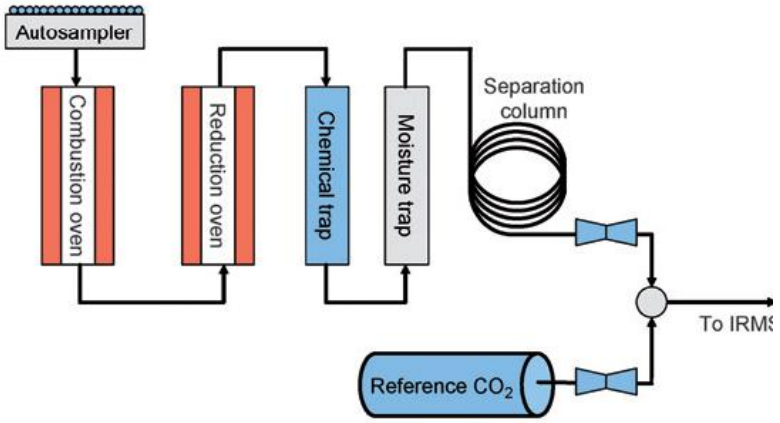
GC/IRMS 主要应用

- IRMS能够对低浓度标记，以及天然丰度的同位素比进行精确的测定， 其在地球化学、考古学、法庭科学、环境科学、食品认证、医学、生物学等科学上有广泛而成熟的应用。
- 同位素分馏效应研究
- 同位素示踪研究（代谢途径，代谢流）
- 同位素指纹研究

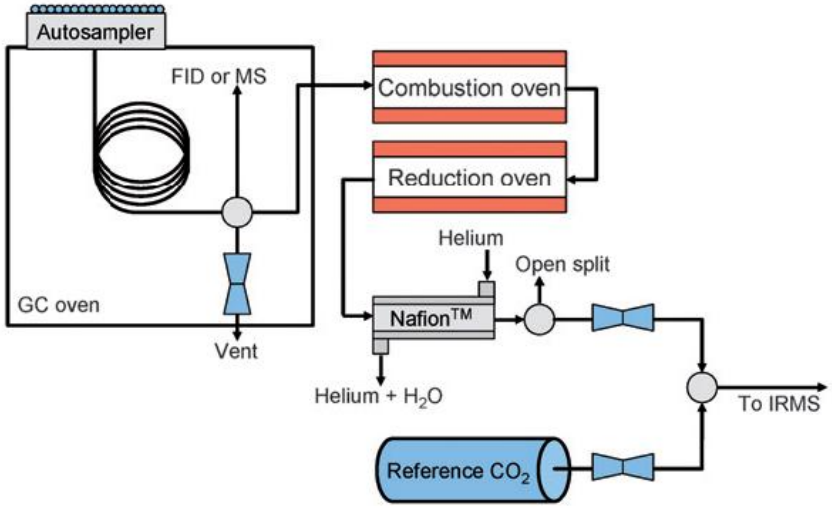
LC-interface



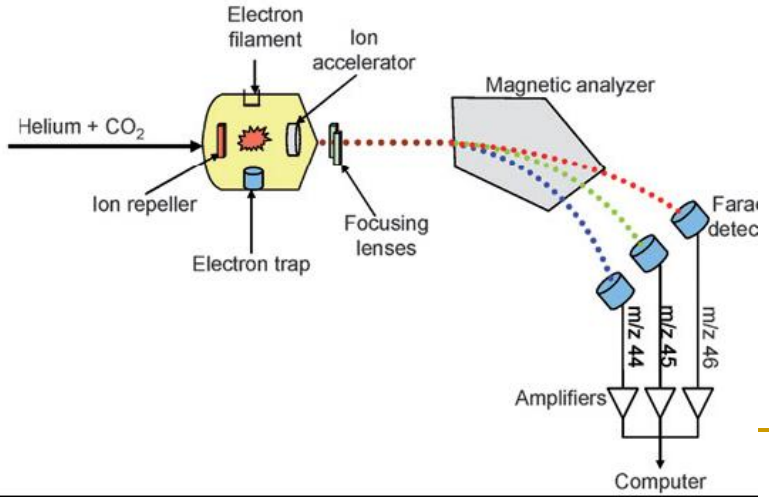
EA-interface



GC-interface



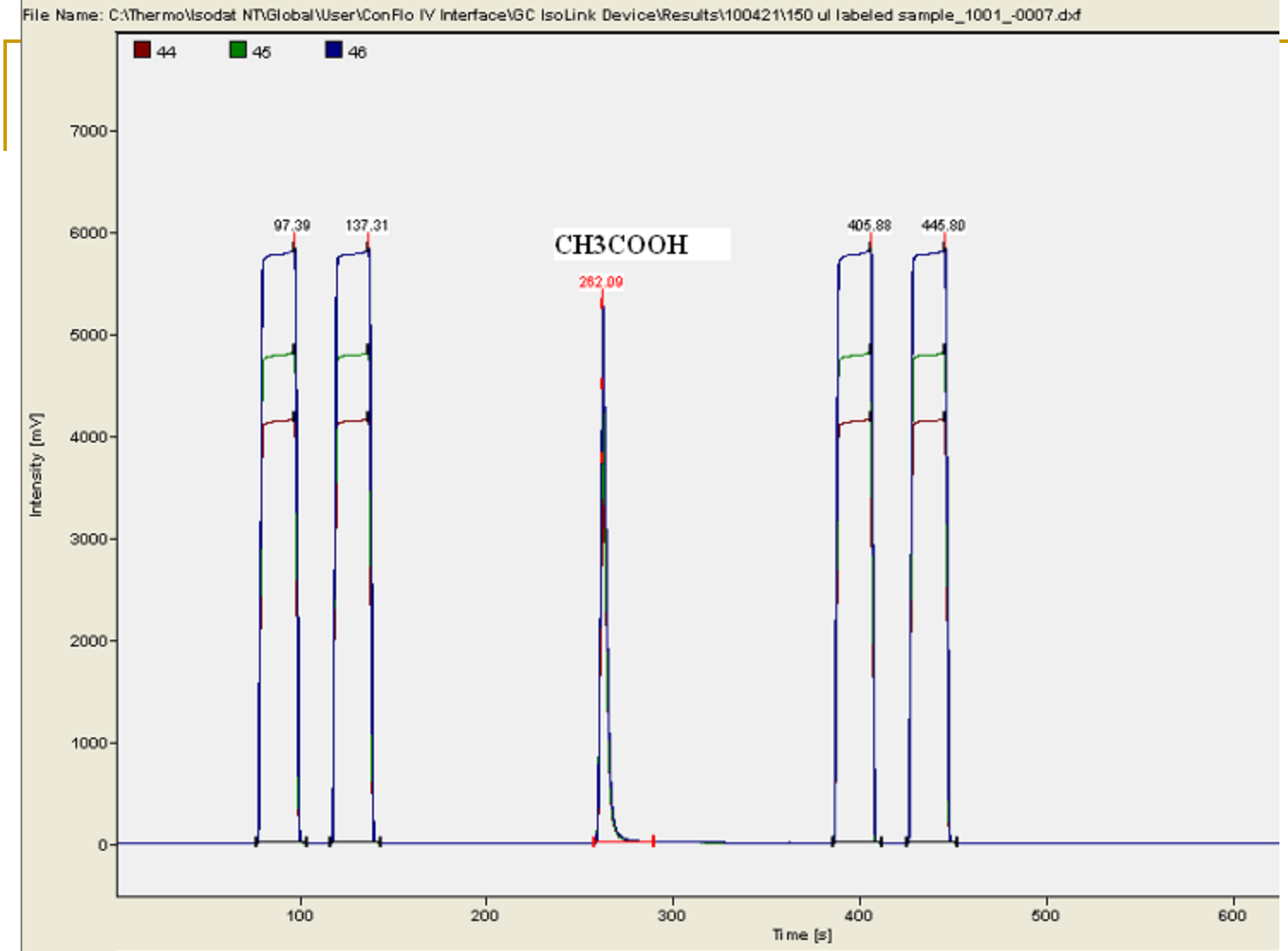
Isotope Ratio Mass Spectrometer (IRMS)



GC-C-IRMS的分析过程

- 制备得到的适宜于GC分析的混合物样品，经适宜的色谱柱分离，分离的组分依次进入高温氧化炉，组分转变为CO_2、NOx、H_2O蒸汽（H_2O经半透膜溢出）等混合气体。对于$\delta^{15}N$的测定，NOx经还原为N_2。得到的混合气体经Conflow Ⅳ得到稳定的气流进入同位素比质谱仪。
- $^{13}C/^{12}C(\delta^{13}C)$:$CO_2$-44 ($^{12}C^{16}O_2$)，45 ($^{13}C^{16}O_2$,$^{12}C^{16}O^{17}O$) ，46($^{12}C^{16}O^{18}O$)
- $^{15}N/^{14}N(\delta^{15}N)$：$N_2$- 28($^{14}N_2$)，29($^{14}N^{15}N$)，30($^{15}N_2$)

样品制备 → 进样 → GC 分离 → 高温燃烧 → IRMS 分析



CO2 | Channel 1 | Infos | Errors | Sequence Line

Peak Nr.	Start [s]	Rt [s]	Width [s]	Ampl. 44 [mV]	Ampl. 45 [mV]	Ampl. 46 [mV]	BGD 44 [mV]	BGD 45 [mV]	BGD 46 [mV]	Area All [Vs]	d 45CO2/44CO2 [per mil] vs. VPDB	d 46CO2/44CO2 [per mil] vs. VPDB	d 13C/12C [per mil] vs. VPDB	d 18O/16O [per mil] vs. VPDB
1	77.1	97	26.8	4183	4838	5842	3.7	2.4	2.9	82.1	-28.656	-14.755	-30.194	-14.705
2*	117.(	13	26.8	4184	4840	5842	4.6	3.5	4.3	82.2	-28.762	-14.873	-30.303	-14.823
3	257.(	26	31.8	3774	4496	5295	4.2	3.0	3.5	14.3	-25.454	-7.979	-27.015	-7.928
4	385.(	40	26.8	4181	4837	5839	3.8	2.5	2.9	82.0	-28.592	-14.709	-30.127	-14.659
5	425.(	44	26.8	4183	4839	5841	4.6	3.5	4.3	82.2	-28.722	-14.835	-30.262	-14.784

底物同位素比的变化暗示着某种反应的产生

- The isotopic composition of MTBE steadily changed from the source regions along the major contaminant plume (**−26.4‰ to +40.0‰** (carbon); **−73.1‰ to +60.3‰** (hydrogen)) indicating substantial biodegradation.

- Constant carbon isotopic signatures of TBA suggest the absence of TBA degradation at the site.

1. New Evaluation Scheme for Two-Dimensional Isotope Analysis to Decipher Biodegradation Processes: Application to Groundwater Contamination by MTBE. ***Environmental Science & Technology*** 39 (2005) 1018-1029.

2. An Indicator of Biodegradation at a Petroleum Hydrocarbon Contaminated Field Site. ***Environmental Science & Technology*** 36 (2002) 2464-2470.

微生物代谢途径的发现与确认

- Conversion of consumed hexadecane to CH_4 and CO_2 was verified in subsequent growth experiments with ^{13}C-labelled substrate.
- The ^{13}C-content in CH_4 and CO_2 after 158 days of incubation was 10.1 (±0.8) and 1.85 (±0.001) atom%, respectively. In a control experiment using unlabelled hexadecane, the ^{13}C-content in CH_4 and CO_2 was 1.07 and 1.09 atom%, respectively.
- However, in the case of our enrichment culture we could exclude the possibility that aerobic bacteria initiated alkane degradation by traces of oxygen that might diffuse slowly through the stoppers.

Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* 1999, 401, (6750), 266-269.

1. 气相色谱-同位素比质谱（GC-IRMS）能够对天然丰度、低浓度^{13}C标记物的$^{13}C/^{12}C$比值进行高精密度测定。

2. GC-IRMS在生命科学领域常用于单个或数个代谢途径的确认、新代谢途径的发现等。

3. 国际上刚开始有科学家应用GC-IRMS进行代谢流分析

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journal homepage: www.elsevier.com/locate/ymben

Regular Article

^{13}C metabolic flux analysis for larger scale cultivation using gas chromatography-combustion-isotope ratio mass spectrometry

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^{13}C-labeling
Proteinogenic amino acid
Corynebacterium glutamicum

ABSTRACT

^{13}C-based metabolic flux analysis (^{13}CMFA) is limited to smaller scale experiments due to very high costs of labeled substrates. We measured ^{13}C enrichment in proteinogenic amino acid hydrolyzates using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) from a series of parallel batch cultivations of *Corynebacterium glutamicum* utilizing mixtures of natural glucose and [1-^{13}C] glucose, containing 0%, 0.5%, 1%, 2%, and 10% [1-^{13}C] glucose. Decreasing the [1-^{13}C] glucose content, kinetic isotope effects played an increasing role but could be corrected. From the corrected ^{13}C enrichments *in vivo* fluxes in the central metabolism were determined by numerical optimization. The obtained flux distribution was very similar to those obtained from parallel labeling experiments using conventional high labeling GC-MS method and to published results. The GC-C-IRMS-based method involving low labeling degree of expensive tracer substrate, e.g. 1%, is well suited for larger laboratory and industrial pilot scale fermentations.

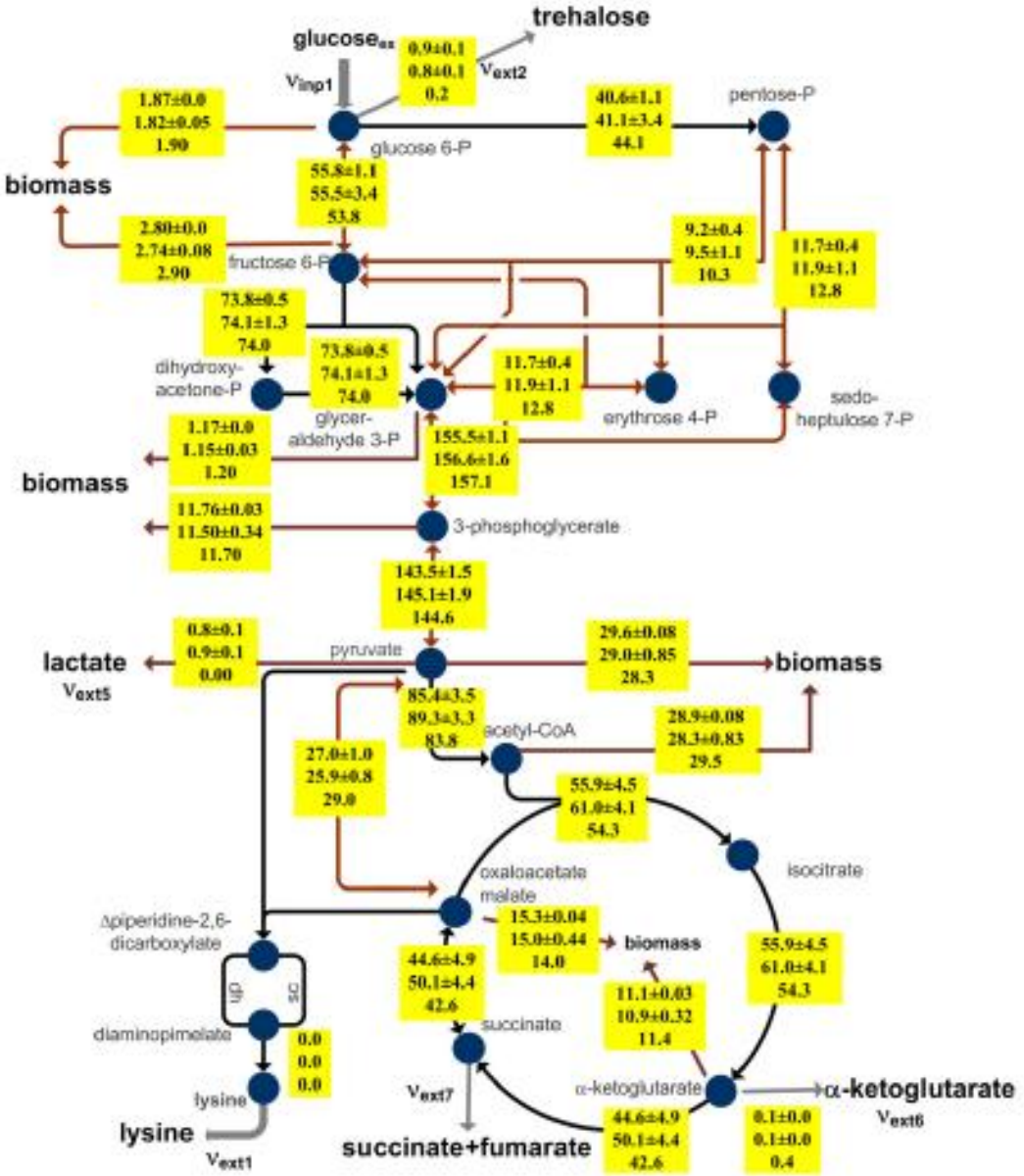
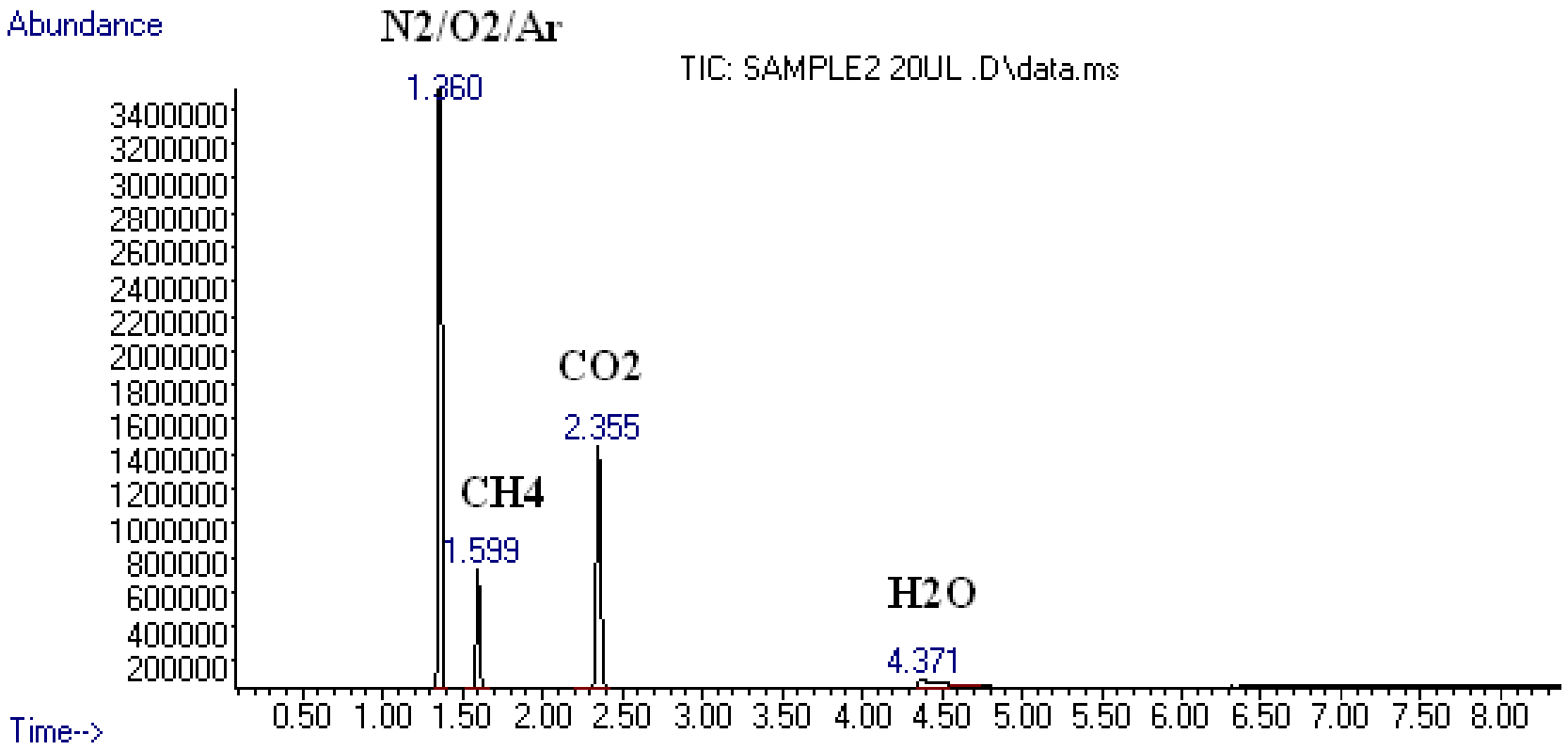


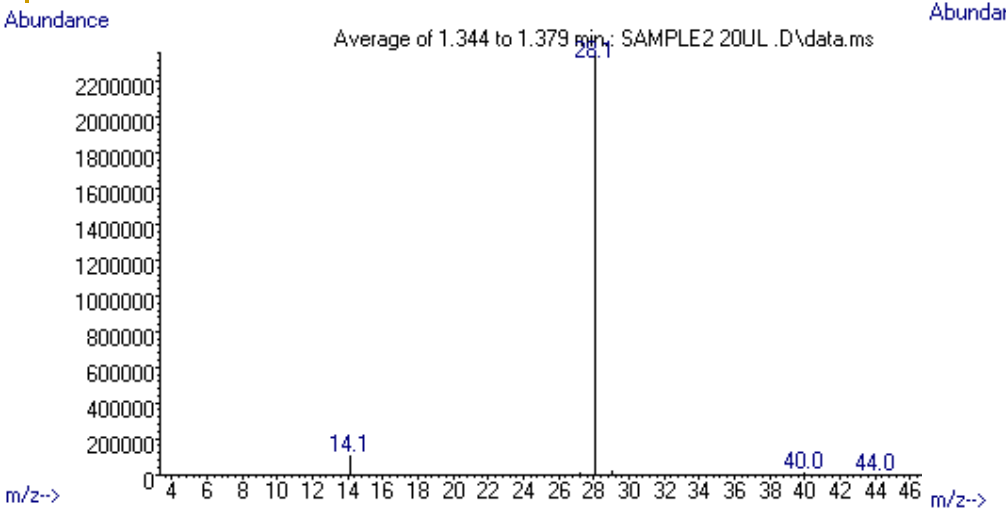
Fig. 2. *In vivo* carbon fluxes estimation in the central metabolism of *C. glutamicum* ATCC 13032. Fluxes in molar % of glucose uptake flux with estimated standard deviations are given in square boxes. Upper values are estimated fluxes based on GC-C-IRMS method using combined labeling experiments with 0.5%, 1% and 2% [1-^{13}C] glucose; middle values are estimated fluxes based on GC-MS method using 99% [1-^{13}C] glucose; bottom values are estimated fluxes from literature (Kim et al., 2006).

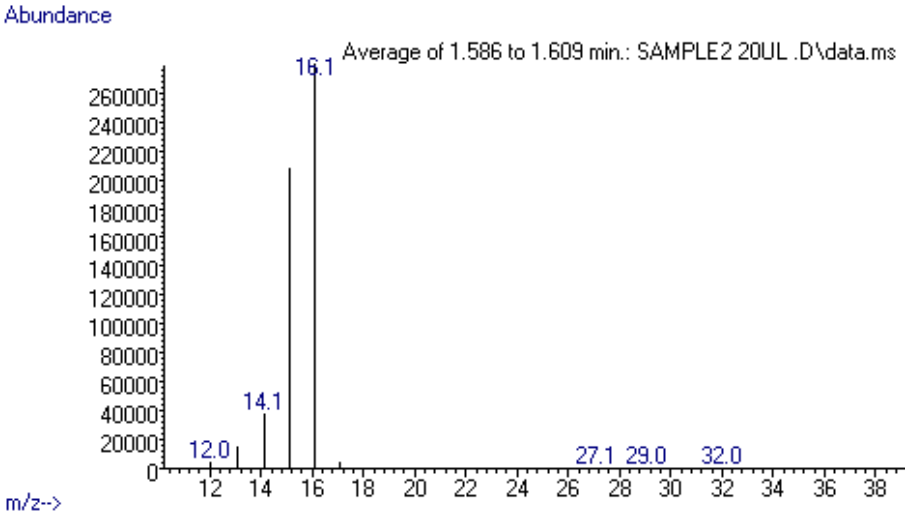
4.1 GC-IRMS在甲烷菌代谢研究中的应用

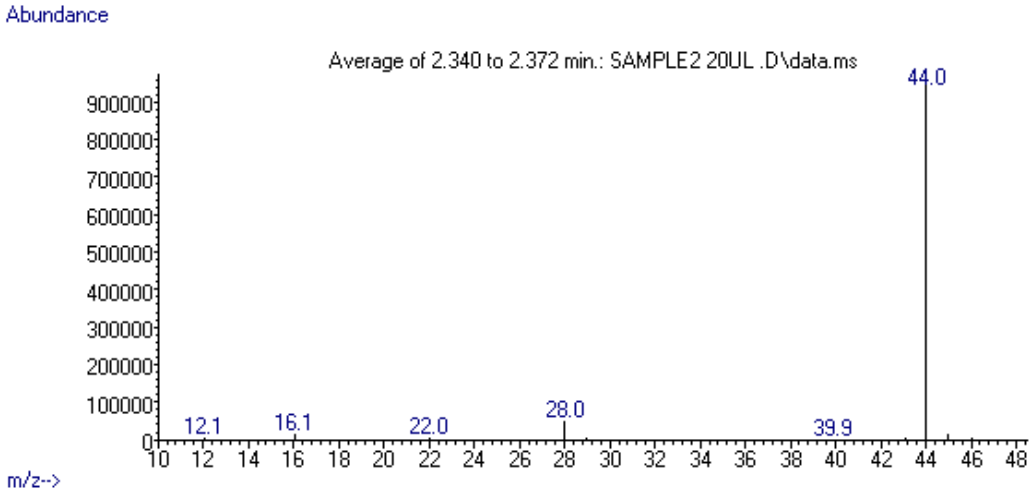
4.1.1 甲烷菌代谢产物的GC-MS鉴定

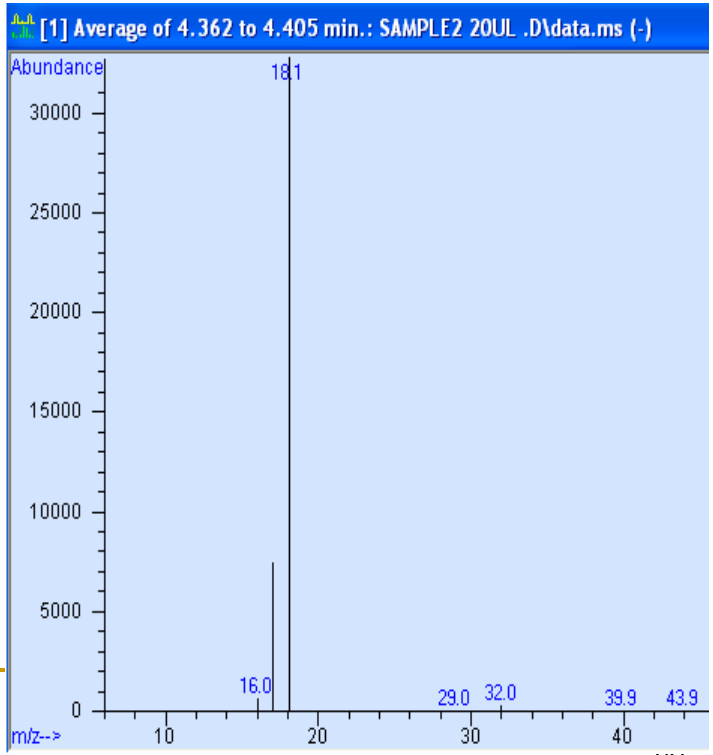


气体代谢产物GC-MS分析的总离子流图





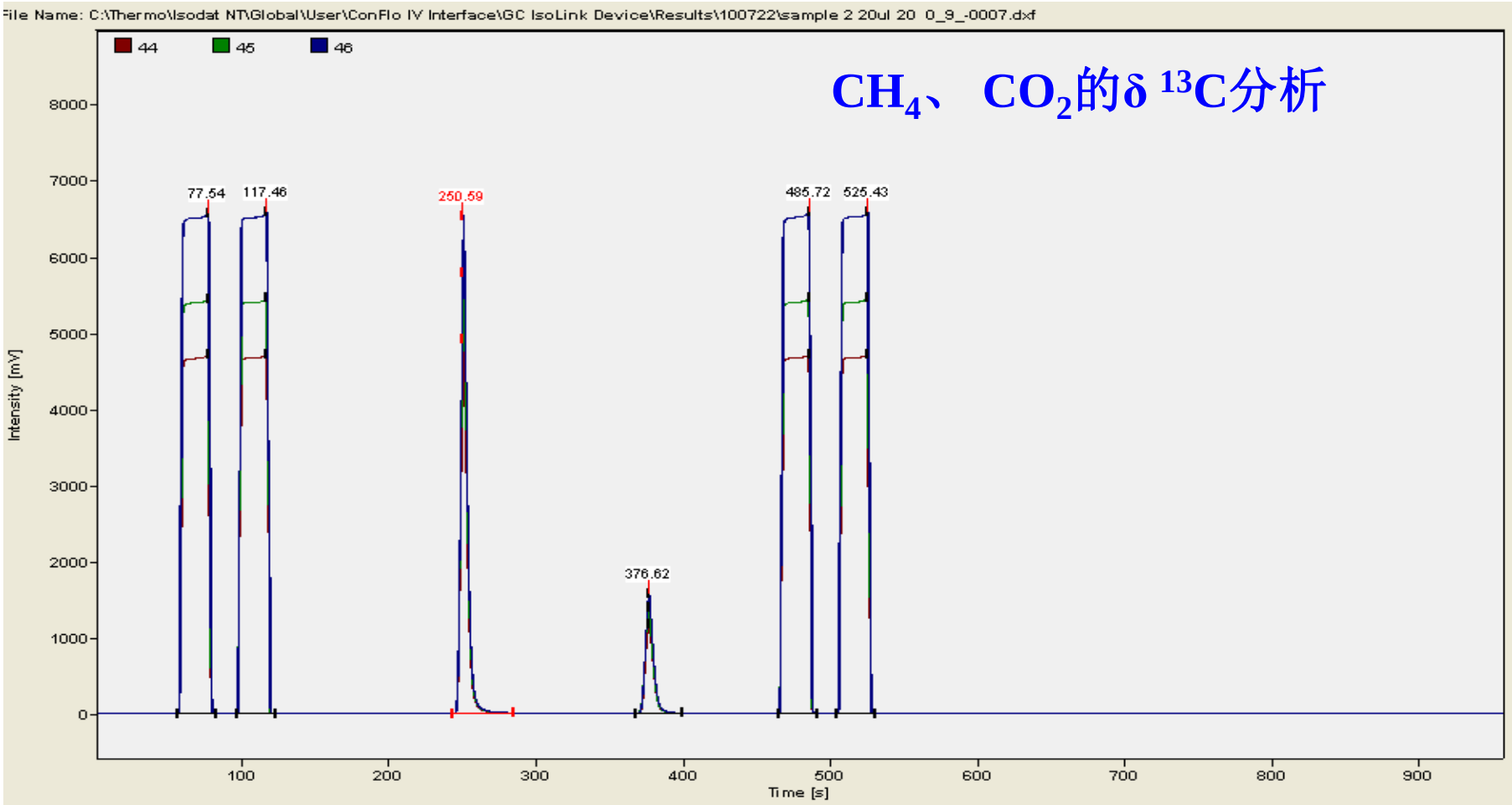




气体代谢产物化学结构的GC-MS确认

4.1.2 甲烷菌气体代谢产物的GC-IRMS分析

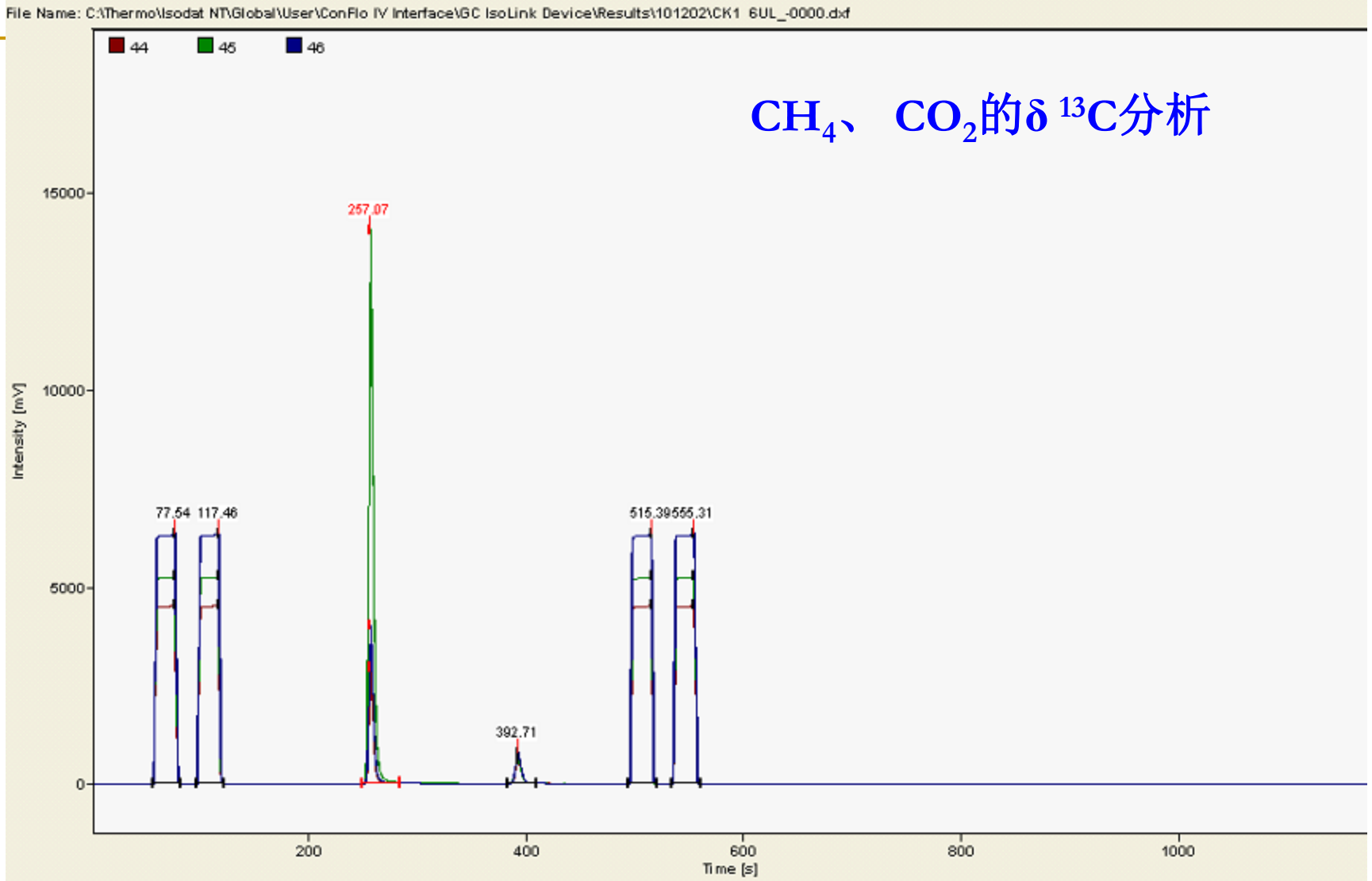
CH_4、 CO_2的$\delta^{13}C$分析



CO2 | Channel 1 | Infos | Errors | Sequence Line

BGD 46 [mV]	Area All [Vs]	Area 44 [Vs]	Area 45 [Vs]	Area 46 [Vs]	R 45CO2/44CO2	rR 45CO2/44CO2	d 45CO2/44CO2 [per mil] vs. VPDB	R 46CO2/44CO2	d 46CO2/44CO2 [per mil] vs. VPDB	R 13C/12C	d 13C/12C [per mil] vs. VPDB	AT% 13C/12C [%]
1.4	92.1	91.156	0.580	0.382	0.0116535	1.1567745	-28.680	0.0040824	-14.791	0.010842	-30.219	1.072605
2.6	92.2	91.333	0.581	0.382	0.0116518	1.1566099	-28.818	0.0040819	-14.913	0.010840	-30.363	1.072448
4.3	24.3	24.079	0.156	0.096	0.0118644	1.1777089	-11.102	0.0039070	-57.124	0.011071	-9.727	1.095022
4.9	7.42	7.351	0.047	0.029	0.0117642	1.1677646	-19.452	0.0039053	-57.535	0.010971	-18.672	1.085238
1.5	92.1	91.228	0.580	0.382	0.0116525	1.1566778	-28.761	0.0040821	-14.874	0.010841	-30.303	1.072513
2.6	92.3	91.410	0.581	0.383	0.0116507	1.1565012	-28.910	0.0040816	-14.983	0.010839	-30.458	1.072343

CH_4、 CO_2的$\delta^{13}C$分析



CO2 | Channel 1 | Infos | Errors | Sequence Line

BGD 46 [mV]	Area All [Vs]	Area 44 [Vs]	Area 45 [Vs]	Area 46 [Vs]	R 45CO2/44CO2	rR 45CO2/44CO2	d 45CO2/44CO2 [per mil] vs. VPDB	R 46CO2/44CO2	d 46CO2/44CO2 [per mil] vs. VPDB	R 13C/12C	d 13C/12C [per mil] vs. VPDB	AT% 13C/12C [%]
2.2	88.8	87.967	0.561	0.370	0.0116443	1.1595776	-29.444	0.0040777	-15.935	0.0108337	-30.995	1.071756
3.8	13.9	13.496	0.353	0.055	0.0477322	4.7533175	2978.484	0.0039686	-42.255	0.0469358	3198.121	4.483163
4.1	3.63	3.598	0.023	0.015	0.0116906	1.1641872	-25.586	0.0039356	-50.213	0.0108947	-25.539	1.077726
1.0	88.5	87.587	0.559	0.368	0.0116532	1.1604574	-28.708	0.0040821	-14.868	0.0108420	-30.246	1.072576
2.1	88.7	87.807	0.560	0.369	0.0116525	1.1603932	-28.761	0.0040821	-14.874	0.0108414	-30.303	1.072513

一株甲烷菌的代谢途径

- $^{13}CH_3COONa$：产生一定量的CO_2；
- $^{13}CH_3OH$：产生一定量的CH_4，而不产生CO_2；
- $^{13}CH4$：产生一定量的CO_2，而不产生乙酸。

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RESEARCH PAPER

Isotopic Confirmation of Occurrence of Microbial Denitrification Based on N_2 and N_2O Production Monitored by Gas Chromatography/Isotope Ratio Mass Spectrometry and Gas Chromatography/Mass Spectrometry

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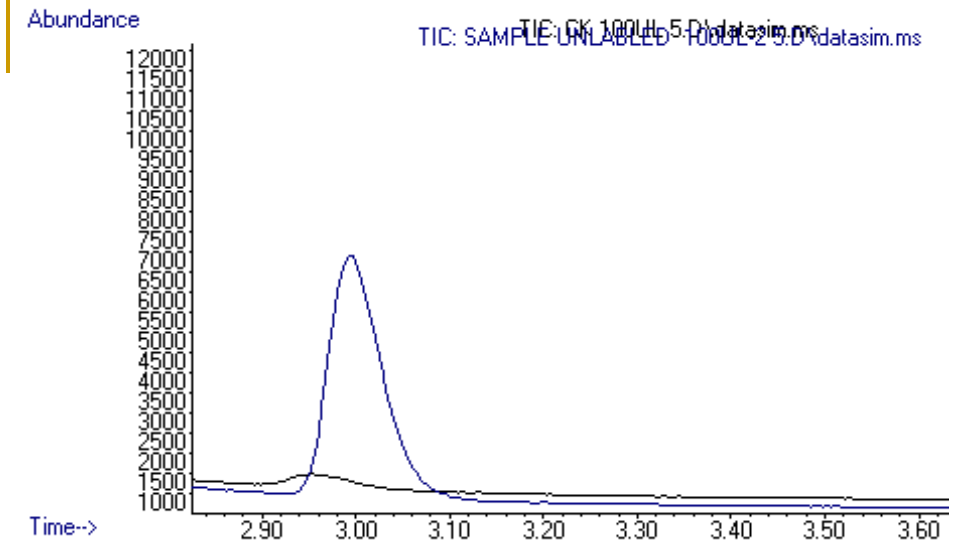
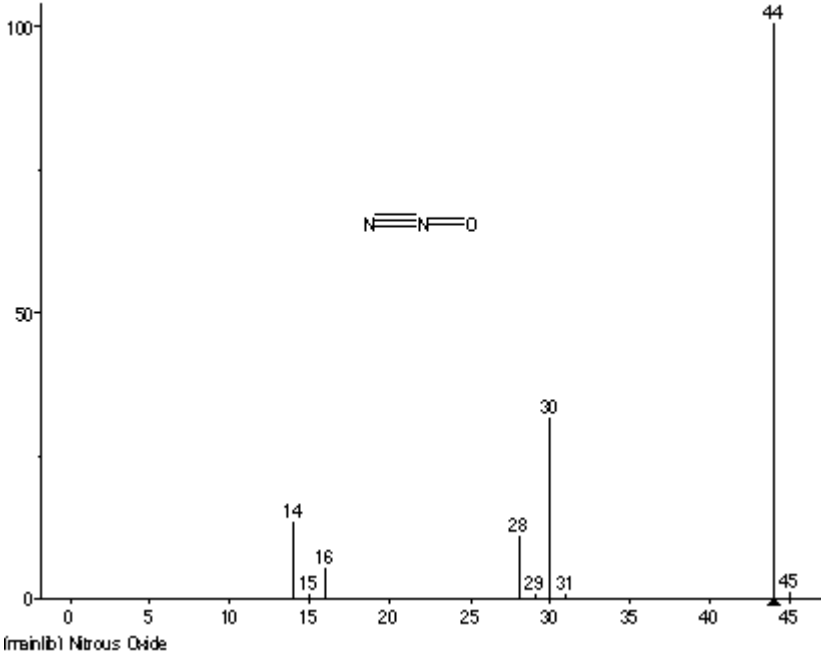
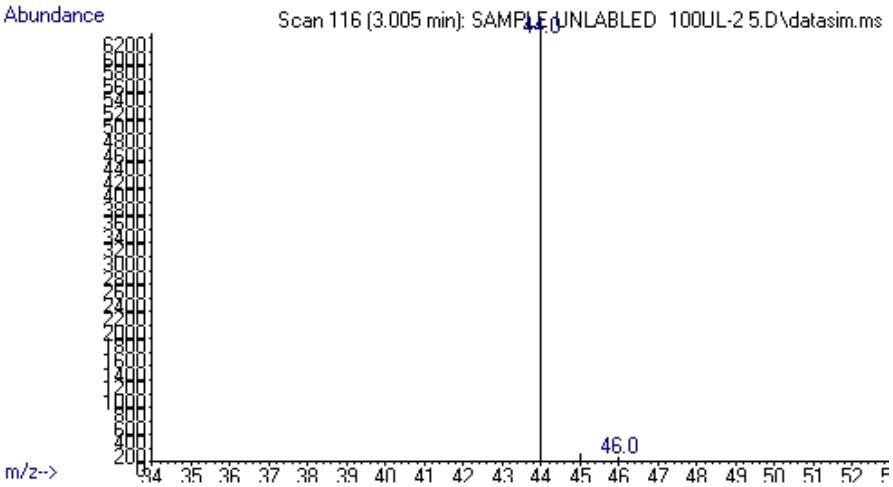
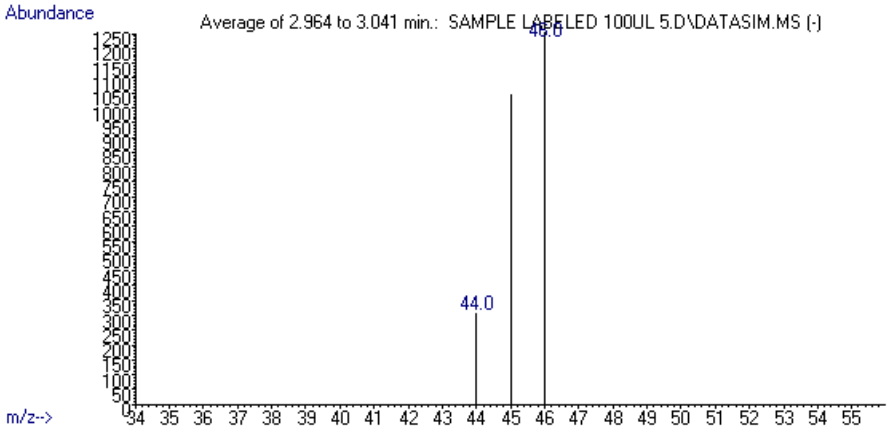
Abstract: In this study, a new ^{15}N-labeled procedure based on isotopic ratio monitoring of N_2 by gas chromatography/isolink/isotope ratio mass spectrometry with great precision and of N_2O by gas chromatography/mass spectrometry in SIM mode with high sensitivity was developed and proposed for the identification and confirmation of *in vitro* microbial denitrification. The mixture of gaseous metabolites produced by *Alcaligenes faecalis* and atmospheric gases in the confined cultivation tube was analyzed on a GS-CarbonPlot column. A baseline separation of N_2/O_2, CO_2, N_2O and water vapour was obtained in a single run, which eliminated CO_2 and H_2O interference with isotopic analysis of N_2 and N_2O. In $\delta^{15}N$ analysis of N_2, combustion oven/interface in GC isolink can remove all of the O_2 in the sample gases, thereby providing accurate $\delta^{15}N$ measurement. The $\delta^{15}N$ value of N_2 in ^{15}N-labled sample, ^{15}N-natural abundance control and ^{15}N-KNO_3 blank control were 2.394‰ ± 0.261‰, 0.022‰ ± 0.044‰ and 0.315‰ ± 0.045‰, respectively. Besides, significant increases in isotopic abundance of $^{14,15}N_2O$ and $^{15,15}N_2O$ (R_T = 2.99 min) relative to $^{14,14}N_2O$ were observed, indicating N_2 and N_2O production from denitrificaiton by *A. faecalis*. This procedure provides isotopic evidence of N_2 and/or N_2O production based on the marked increase in the ^{15}N isotope abundance, and is rapid, sensitive and accurate to indentify and confirm the occurrence of microbial denitrification. We have confirmed the denitrifying activity of several strains of microorganisms screened from the environment using this procedure. This procedure has also been applied to confirm the N_2 formation by nitrifier denitrification under defined conditions.

4.2 GC-IRMS与GC-MS结合鉴定反硝化作用







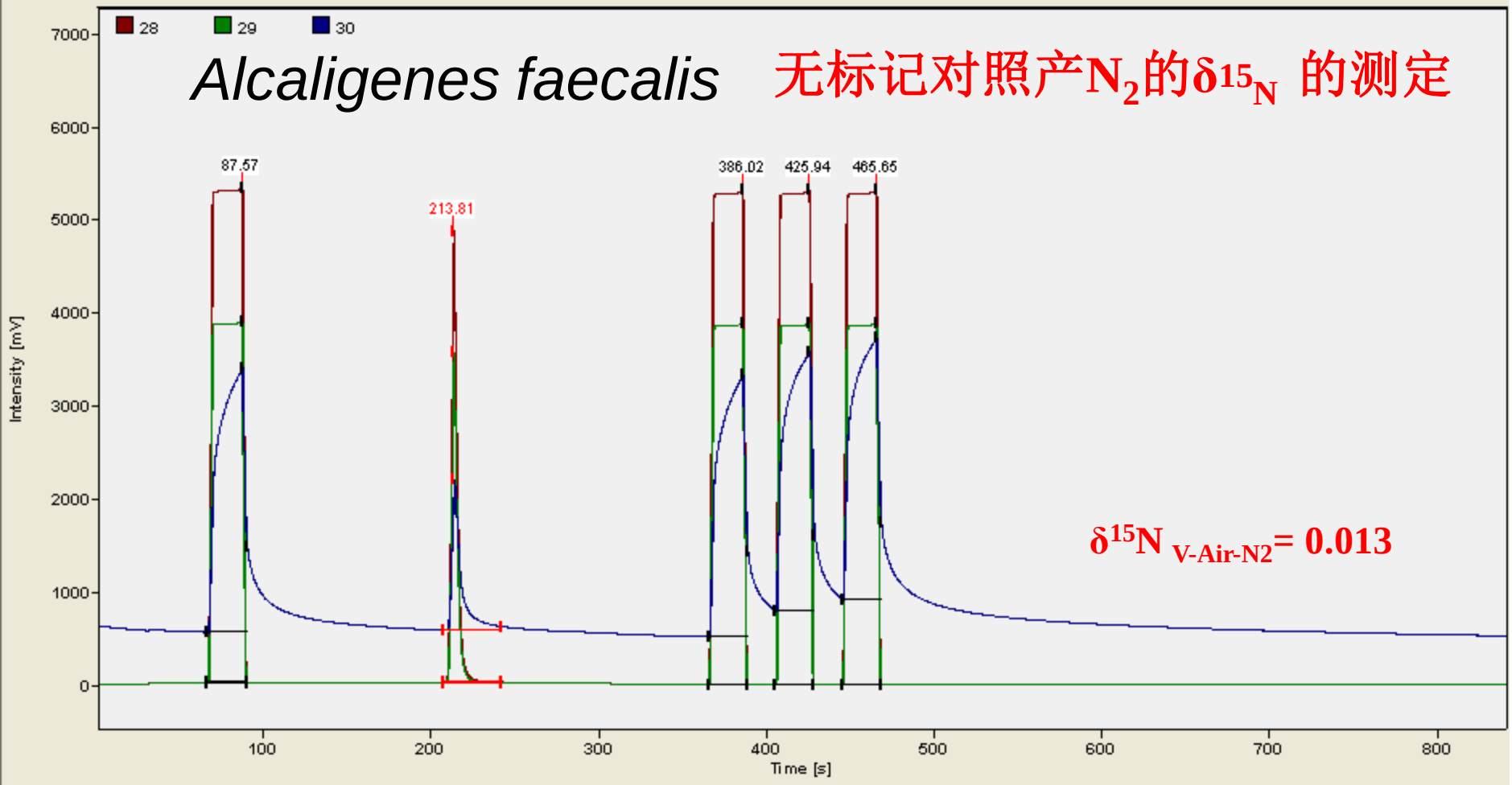


Unlabled sample-*m/z* 44, 45, 46 **^{15}N-labled sample-*m/z* 44,45,46**

环境微生物气体代谢产物的GC-MS分析（N_2O筛查）

环境微生物气体代谢产物中N_2的 GC-IRMS分析

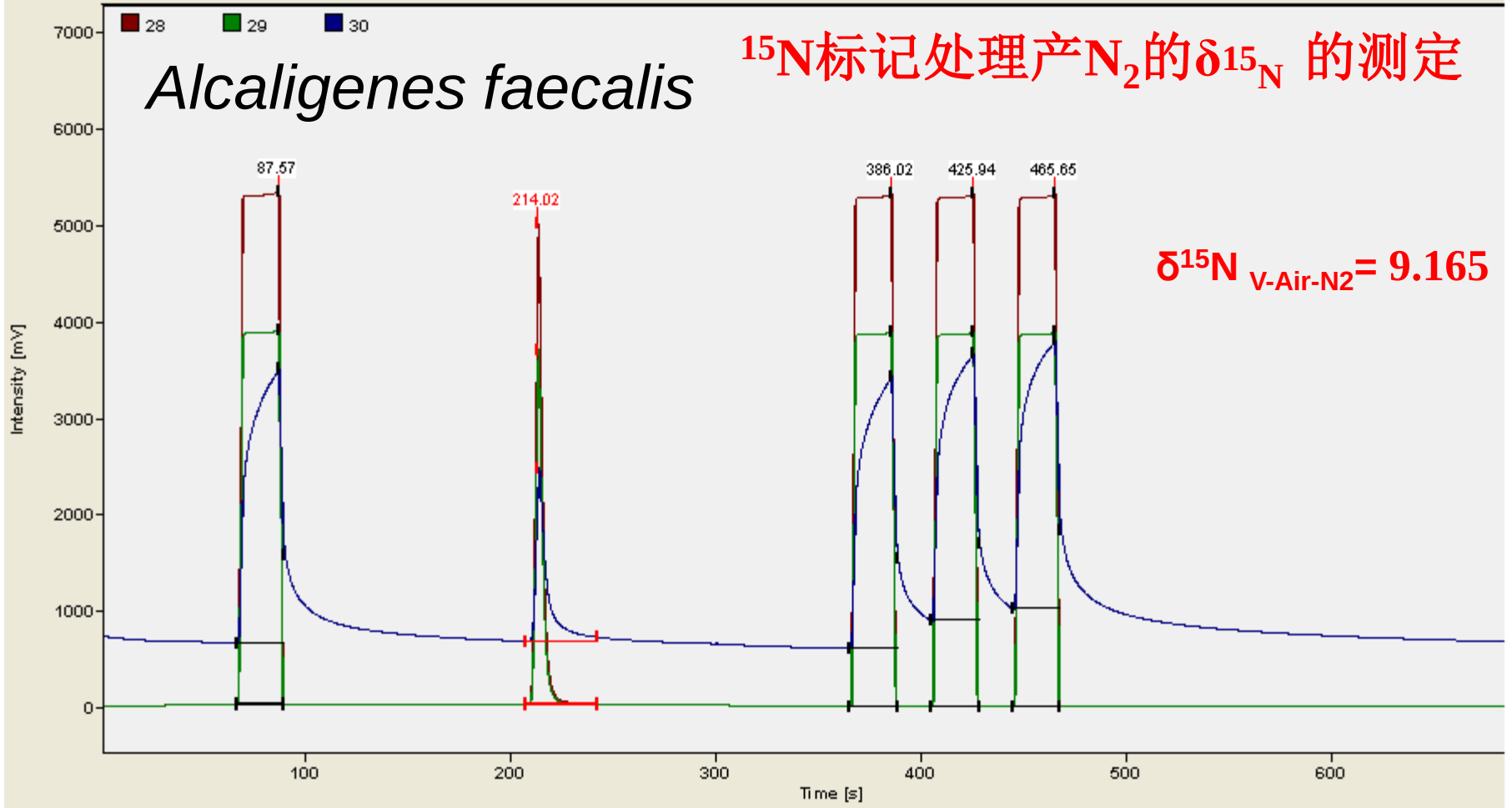
File Name: C:\Thermo\Isodat NT\Global\User\ConFlo IV Interface\GC IsoLink Device\Results\100722\unlabled sample 5 ul-1030_-0010.dxf



N2 | N2+ | Channel 1 | Infos | Errors | Sequence Line

Peak Nr.	Start [s]	Rt [s]	Width [s]	Sample Dilution [%]	Ampl. 28 [mV]	Ampl. 29 [mV]	BGD 28 [mV]	BGD 29 [mV]	Area All [Vs]	R 29N2/28N2	rR 29N2/28N2	rd 29N2/28N2 [per mil] vs. N2 Lab.Tank	d 29N2/28N2 [per mil] vs. Air-N2	DeltaDelta 29N2/28N2 [per mil]	R 15N/14N	d 15N/14N [per mil] vs. Air-N2	AT% 15N/14N [%]
1	67.1	87	23.4	0.000	5307	3880	34.2	25.3	103.4	0.0073472	0.7310842	0.002	-1.248	-	0.003673E	-1.248	0.366016
2	208.1	21	34.5	0.000	4848	3552	28.8	21.4	18.8E	0.0073565	0.7320071	1.264	0.013	-	0.003678:	0.013	0.366477
3	365.:	3E	23.2	0.000	5307	3880	6.7	6.2	103.3	0.0073480	0.7311586	0.104	-1.147	-	0.003674(	-1.147	0.366053
4*	405.:	42	23.4	0.000	5306	3879	6.7	6.4	103.4	0.0073472	0.7310828	0.000	-1.250	-	0.003673E	-1.250	0.366016
5	445.4	4E	23.2	0.000	5309	3881	6.8	6.4	103.4	0.0073470	0.7310674	-0.021	-1.271	-	0.003673E	-1.271	0.366008

File Name: C:\Thermo\Isodat NT\Global\User\ConFlo IV Interface\GC IsoLink Device\Results\100722\abled sample 5 ul-1030_-0011.dxf



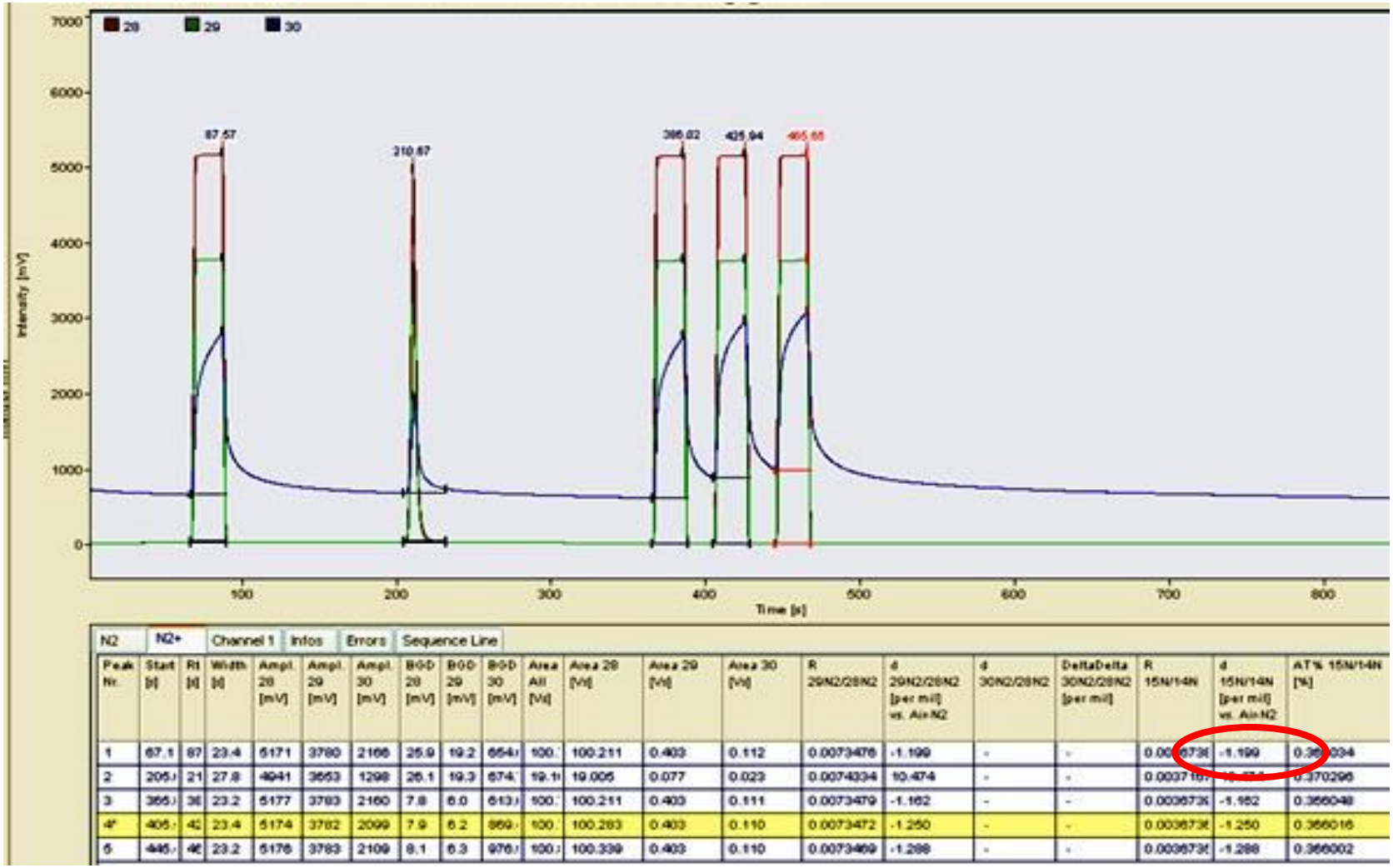
^{15}N标记处理产N_2的δ^{15}_N 的测定

$\delta^{15}N_{V\text{-}Air\text{-}N2} = 9.165$

N2 | N2+ | Channel 1 | Infos | Errors | Sequence Line

Peak Nr.	Start [s]	Rt [s]	Width [s]	Sample Dilution [%]	Ampl. 28 [mV]	Ampl. 29 [mV]	BGD 28 [mV]	BGD 29 [mV]	Area All [Vs]	R 29N2/28N2	rR 29N2/28N2	rd 29N2/28N2 [per mil] vs. N2 Lab.Tank	d 29N2/28N2 [per mil] vs. Air-N2	DeltaDelta 29N2/28N2 [per mil]	R 15N/14N	d 15N/14N [per mil] vs. Air-N2	AT% 15N/ [%]
1	67.1	87	23.4	0.000	5319	3886	33.1	24.5	103.6	0.0073471	0.7306424	-0.018	-1.268	-	0.003673	-1.268	0.366009
2	208.:	21	34.5	0.000	4988	3686	28.0	20.8	19.4	0.0074238	0.7382748	10.428	9.165	-	0.003711	9.165	0.369818
3	365.:	3	23.2	0.000	5321	3887	6.6	6.2	103.6	0.0073479	0.7307267	0.097	-1.153	-	0.003674	-1.153	0.366051
4*	405.:	42	23.4	0.000	5318	3885	6.7	6.2	103.6	0.0073472	0.7306555	0.000	-1.250	-	0.003673	-1.250	0.366016
5	445.:	4	23.2	0.000	5318	3885	6.7	6.3	103.6	0.0073470	0.7306319	-0.032	-1.282	-	0.003673	-1.282	0.366004

F6菌株所产气体中N_2的同位素比测定



N2 | N2+ | Channel 1 | Infos | Errors | Sequence Line

Peak Nr.	Start [s]	Rt [s]	Width [s]	Ampl. 28 [mV]	Ampl. 29 [mV]	Ampl. 30 [mV]	BGD 28 [mV]	BGD 29 [mV]	BGD 30 [mV]	Area All [Vs]	Area 28 [Vs]	Area 29 [Vs]	Area 30 [Vs]	R 29N2/28N2	d 29N2/28N2 [per mil] vs. Air-N2	d 30N2/28N2	DeltaDelta 30N2/28N2 [per mil]	R 15N/14N	d 15N/14N [per mil] vs. Air-N2	AT% 15N/14N [%]
1	67.1	87	23.4	5171	3780	2166	25.9	19.2	664.4	100.	100.211	0.403	0.112	0.0073476	-1.199	-	-	0.0036738	-1.199	0.366034
2	205.4	21	27.8	4941	3653	1298	26.1	19.3	674.	19.1	19.005	0.077	0.023	0.0074034	10.474	-	-	0.0037167	10.474	0.370296
3	366.4	38	23.2	5177	3783	2160	7.8	6.0	613.4	100.	100.211	0.403	0.111	0.0073479	-1.162	-	-	0.0036738	-1.162	0.366048
4*	406.4	42	23.4	5174	3782	2099	7.9	6.2	869.4	100.	100.283	0.403	0.110	0.0073472	-1.250	-	-	0.0036738	-1.250	0.366016
5	446.4	46	23.2	5176	3783	2109	8.1	6.3	976.4	100.4	100.339	0.403	0.110	0.0073469	-1.288	-	-	0.0036738	-1.288	0.366002

$\delta^{15}N_{V\text{-}Air\text{-}N2} = 10.474$

5. 样品制备中的化学衍生化

- **To permit analysis of compounds with inadequate volatility or stability**

- **To improve chromatographic behavior or detectability**

- **BY derivatizing the functional group (e.g., O-H, COOH, N-H, and S-H) to promote the use of chromatographic analysis.**

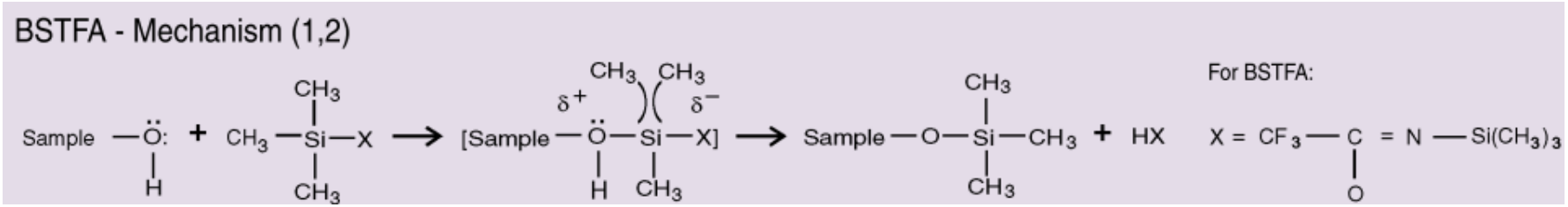
- **These groups are difficult to analyze by GC because they are not sufficiently volatile, show excessive tailing, can be too strongly attracted to the stationary phase or are thermally unstable.**

- **For small and volatile compounds excessive volatility may also pose problems during analysis. Chemical derivatization increases the molecular weight of very volatile compounds which can minimize losses in sample handling and help separate the gas chromatographic sample peak(s) from the solvent front.**

5.1 GC Derivatization

- **Silylation**
- **Acylation**
- **Alkylation**

Silylation and perfluoroacylation employed in GC-MS



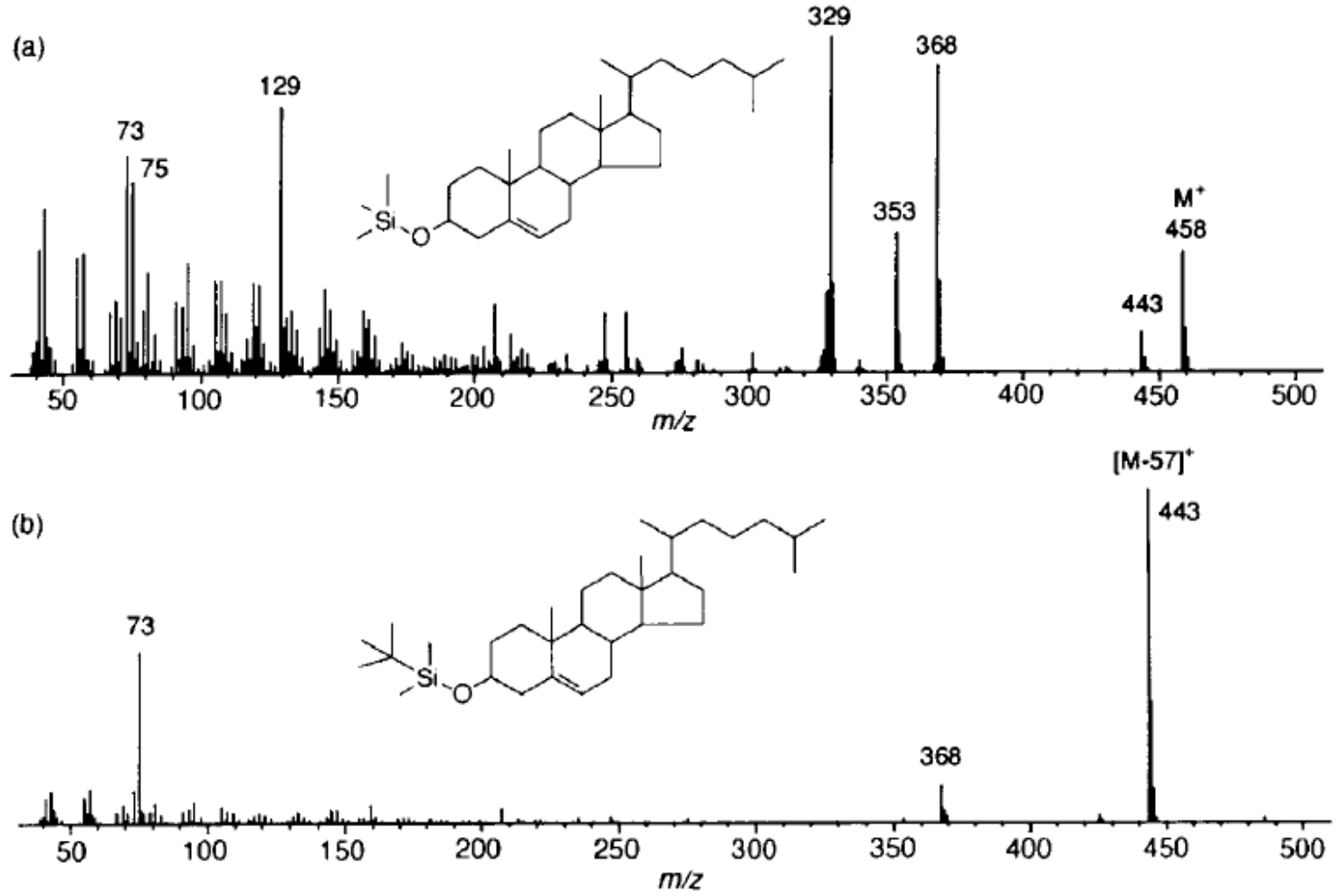


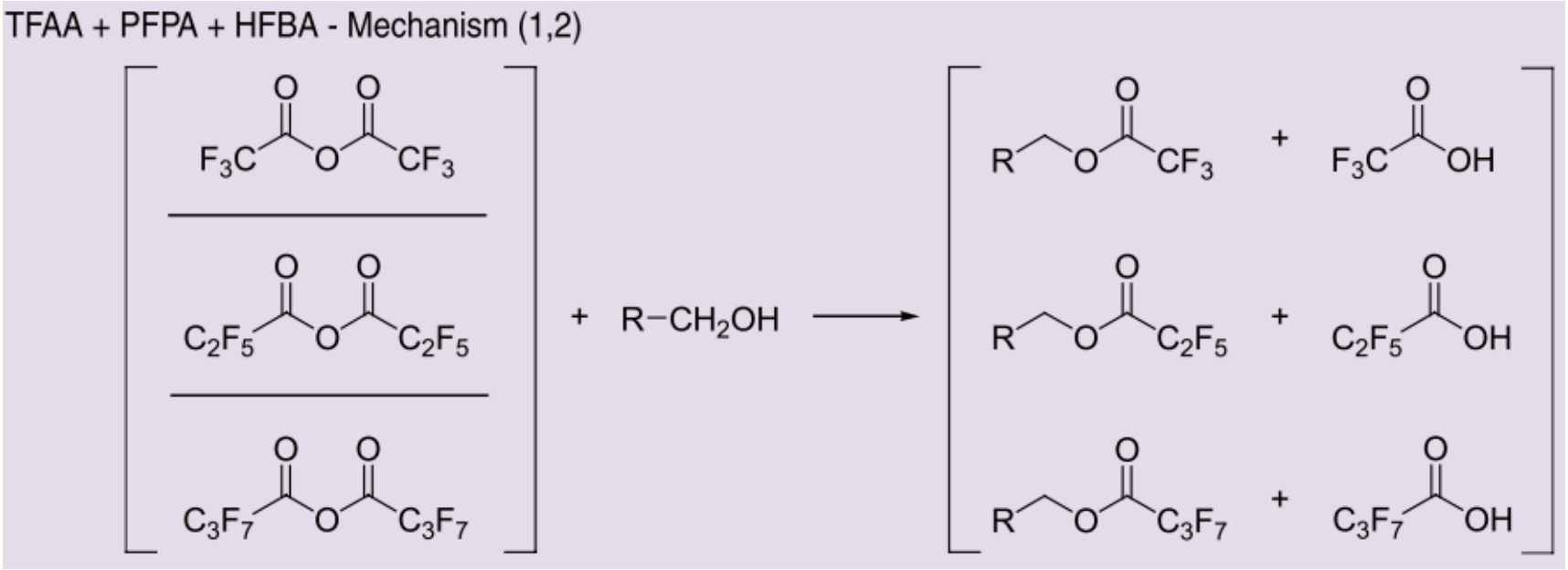
Figure 5. Electron ionization mass spectra of (a) the trimethylsilyl (TMS) ether and (b) the t-butyldimethylsilyl (TBDMS) ether derivatives of cholesterol.

Acylation

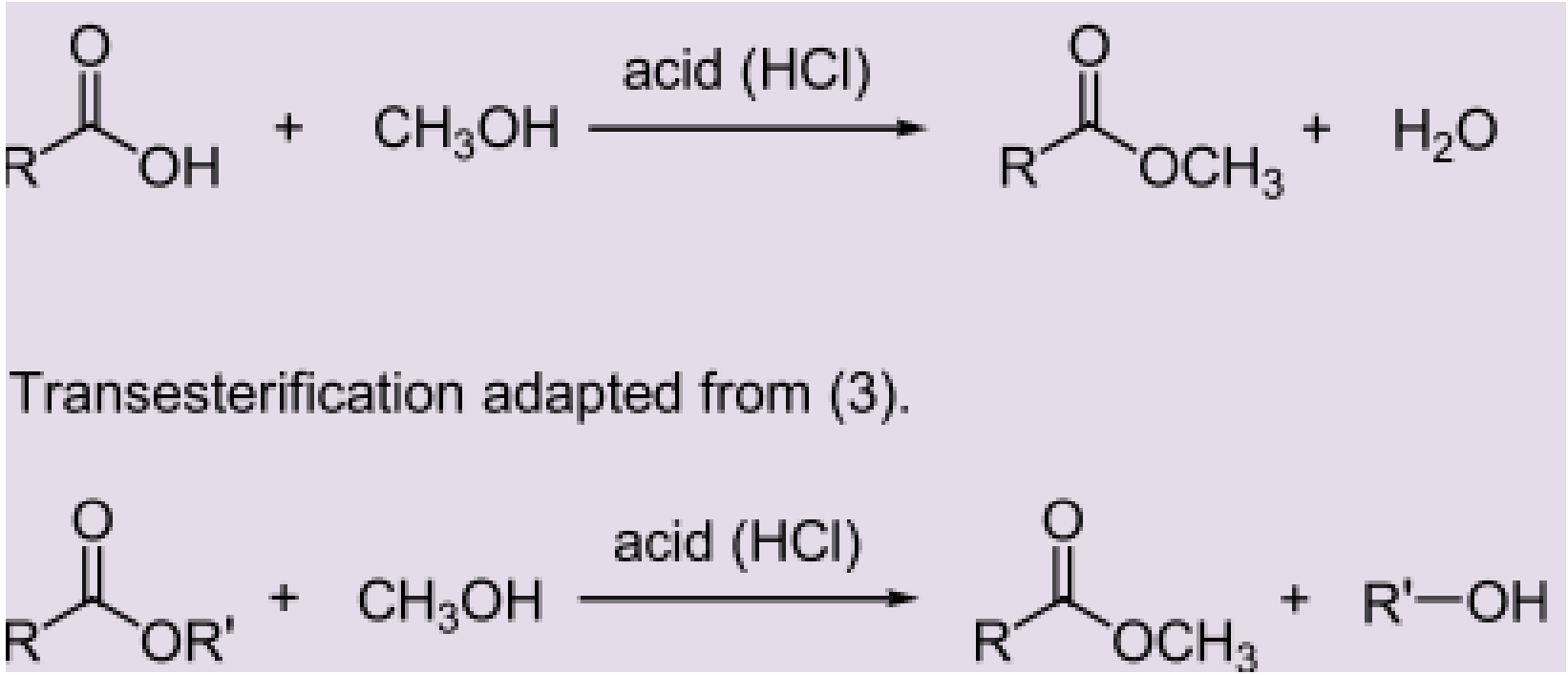
- **Acylation, an alternative to silylation, is the conversion of compounds with active hydrogen such as –OH, -SH, and –NH into esters, thioesters and amides.**

Common Reactive Functional Groups

- **Alcohols, phenols, carbohydrates and amines.**



Esterification Reaction



Transesterification adapted from (3).

Hydrogen chloride is the favored catalyst because of its acid strength and because it is readily removed.

Indirect alkylation via chloroformates

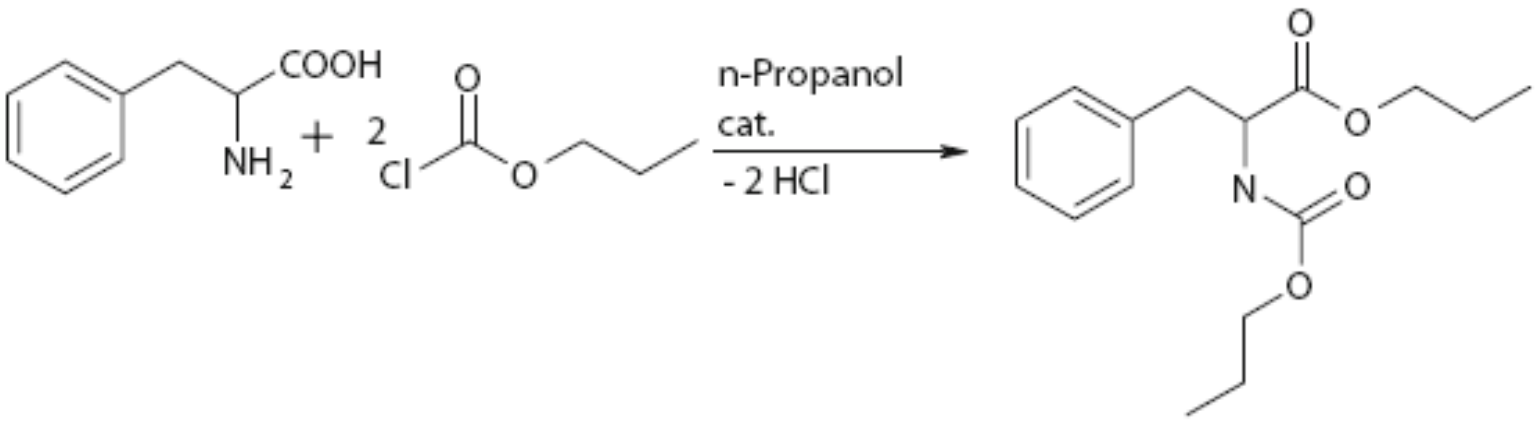
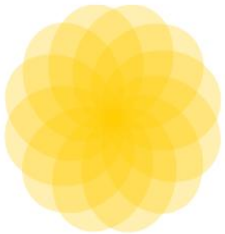


Fig. 1 Reaction scheme for the derivatization of phenylalanine with propyl chloroformate

5.2 Analyte Derivatization in HPLC and LC/MS Analyses (略)

- **Derivatization is often required to alter retention characteristics, increase response to various detection techniques and/or provide selective response for analytes in complex matrices.**



谢谢！