Development of in-SPE derivatization method for GC-MS metabolome analysis

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【Introduction】

Due to complex and long-time sample preparations, including solvent extraction, centrifugal concentration, freeze-drying and derivatization, technical skills have been required in a conventional metabolome analysis for GC-MS. It causes discrepancies in the acquired data from multiple experiments, especially between in analysis batches. Furthermore, it causes a critical problem when the statistical analysis is done because a lot of samples should be handled in the metabolome analysis. The purpose of this study was to develop a rapid and robust method of sample preparation for metabolomics using solid phase extraction (SPE).

【Methods】

Extracted samples with acetonitrile were loaded on an ion-exchange cartridge SPE. Ion-exchange SPEs were used depending on what kind of chemical functional group the compounds have. The target compounds were retained in the SPE, then the cartridge was washed by acetonitrile for dehydration. Derivatization was done by methoxyamine/pridine and MSTFA which were directly added sequentially on the SPE. After derivatization, the object substances were eluted by acetone/n-hexane. The 10μL of derivatized compounds were injected in the GC-MS with a Large Volume Injection system equipped with a spiral shaped liner.

【Preliminary Results】

A cation-exchange column, CXi, was used for amino acids which have cationic amino group, and an anion-exchange column, AXi, was applied for the compounds which have carbonyl and hydroxyl group, such as organic acids and sugars. Washing with acetonitrile after loading the target compounds enabled not only to eliminate other interferences but also to dehydrate at the same time. It took several hours to dry the samples in the conventional method, while dehydration was done in a few minutes using SPE. In this method, target metabolites which were retained in the SPEs by ion-exchange interaction were methoxyaminated by methoxyamine hydrochlolide in pyridine followed by trimethlysilylation effectively. Derivatized metabolites were changed into less-polar compounds and easily eluted using organic solvent, acetone/n-hexane. The total preparation time from sample loading on the column to derivatization was within 10 minutes, and good reproducibility was obtained.

The conventional derivatization method

The derivatization processing 8～19 Hour

Extract fractionation： 400μL

Vacuum concentration（ **1～2 Hour**）

Freeze-drying（**5～16Hour**）

Added 2%Methoxyamin/pyridine 100uL

Incubation（30℃, **90min**） : Derivatization

Added MSTFA 50uL

Incubation（37℃, **30min**） : Derivatization

Centrifugal separation（16000 rpm, 4℃, 3min）

Vial

GC/MS : Injection 1μL（Split 25:1）

CX-SPE derivatization method for Amino acid

The derivatization processing 6 min

Extract fractionation： 50μL

Hybrid-SPE **CXi3**-2mg ⊖

Wash with ACN 100μL（**Dehydration**）

Added MSTFA 20μL in SPE

Derivatization in SPE

Elute Hexane 100μL

Added Hexane 400μL

Vial

GC/MS : Injection 10μL (LVI)

AX-SPE derivatization method for Organic acid

The derivatization processing 6 min

Extract fractionation： 50μL

Hybrid-SPE **AXi3**-2mg ⊕

Wash with ACN 100μL（**Dehydration**）

Added MSTFA 20μL in SPE

Derivatization in SPE

Elute Hexane 100μL

Added Hexane 400μL

Vial

GC/MS : Injection 10μL (LVI)

AX-SPE derivatization method for saccharides

The derivatization processing 10 min

Extract fractionation： 20μL

Added ACN 100μL

Hybrid-SPE **AXi3**-2mg ⊕

Wash with ACN 100μL（**Dehydration**）

Added 20%Methoxyamin/pyridine 5uL in SPE

Derivatization in SPE 3 min

Added MSTFA 25μL in SPE

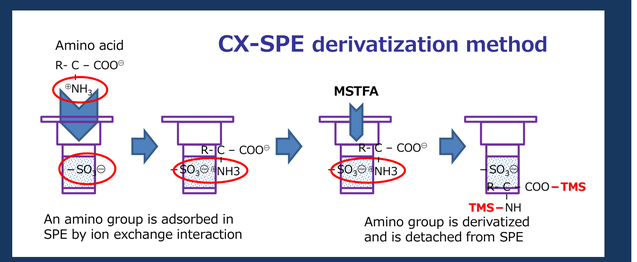
Derivatization in SPE

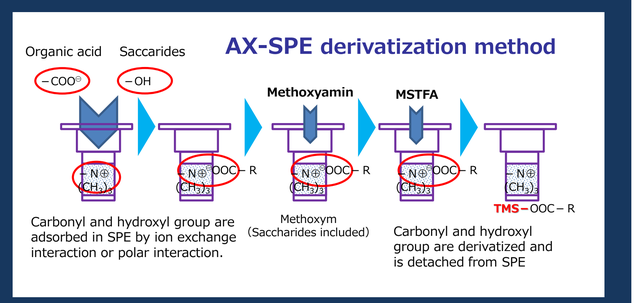
Elute Hexane 100μL

Added Hexane 400μL

Vial

GC/MS : Injection 10μL (LVI)





CX-SPE derivatization method for Amino acid

Examination of the optimum when a sample is loaded

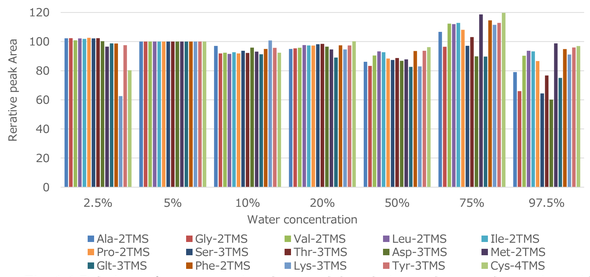


Fig. 1-1 Relations of water conc. in solvent and the relative peak area, when amino acids in solvent are loaded through the CX-SPE and adsorbed by the SPE.

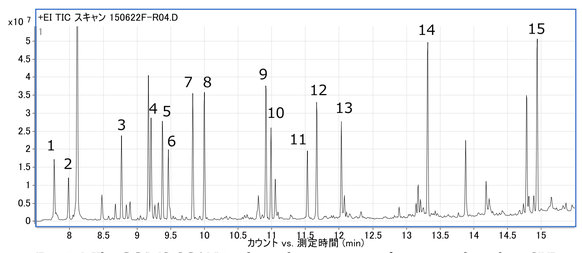


Fig. 1-2 The GC/MS-SCAN total ion chromatogram of amino acids with in-SPE derivatization method

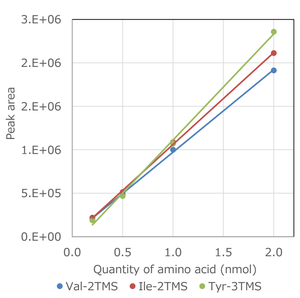


Fig. 1-3 Relations of quantity of Amino acids and the relative peak area with in-SPE derivatization method.

Table 1 : A coefficient of correlation and RSD

No.	Amino acid	R2	RSD n=6, %
1	Ala-2TMS	0.9996	7.4
2	Gly-2TMS	0.9958	13.1
3	Val-2TMS	0.9993	7.5
4	Leu-2TMS	0.9992	6.7
5	Ile-2TMS	0.9995	6.2
6	Pro-2TMS	0.9982	12.5
7	Ser-3TMS	0.9988	5.5
8	Thr-3TMS	0.9990	4.7
9	Asp-3TMS	0.9989	4.9
10	Met-2TMS	0.9991	7.5
11	Glt-3TMS	0.9911	8.8
12	Phe-2TMS	0.9996	5.6
13	Lys-3TMS	0.9927	32.2
14	Tyr-3TMS	0.9977	4.8
15	Cys-4TMS	0.9950	29.2

AX-SPE derivatization method for Organic acid

Examination of the optimum when a sample is loaded

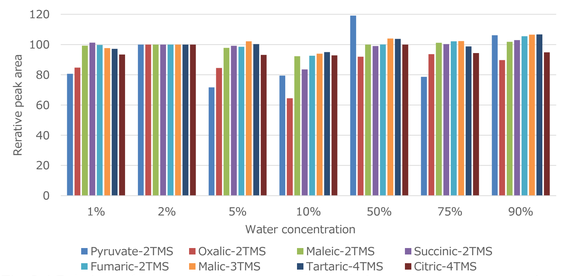


Fig. 2-1 Relations of water conc. in solvent and the relative peak area, when organic acids in solvent are loaded through the AX-SPE and adsorbed by the SPE.

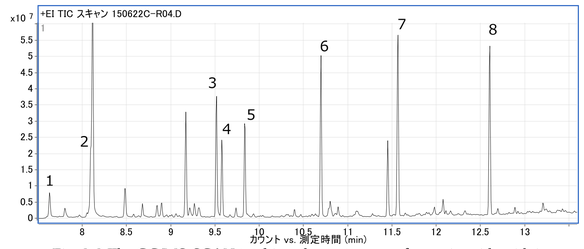


Fig. 2-2 The GC/MS-SCAN total ion chromatogram of organic acids with in-SPE derivatization method

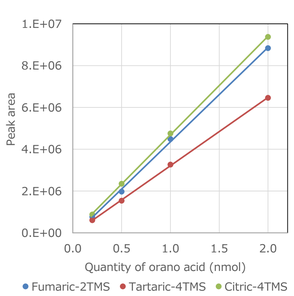


Fig. 2-3 Relations of quantity of Organic acids and the relative peak area with in-SPE derivatization method.

Table 2: A coefficient of correlation and RSD

No.	Organic acid	R2	RSD n=6, %
1	Pyruvate-2TMS	0.9998	15.1
2	Oxalic-2TMS	0.9946	6.2
3	Maleic-2TMS	0.9991	1.8
4	Succinic-2TMS	0.9588	22.0
5	Fumaric-2TMS	0.9991	2.2
6	Malic-3TMS	0.9997	2.1
7	Tartaric-4TMS	0.9998	1.9
8	Citric-4TMS	0.9998	2.5

RSD;Reproducibility parameters for the peak area with in-SPE derivatization method

AX-SPE derivatization method for saccharides

Examination of the optimum when a sample is loaded

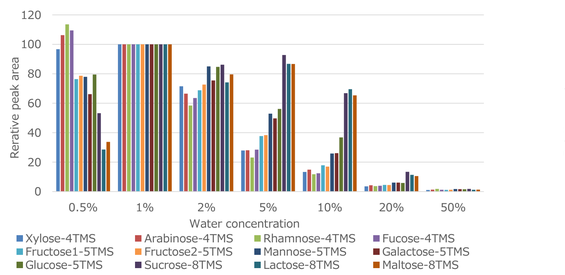


Fig. 3-1 Relations of water conc. in solvent and the relative peak area, when saccharides in solvent are loaded through the AX-SPE and are adsorbed by the SPE.

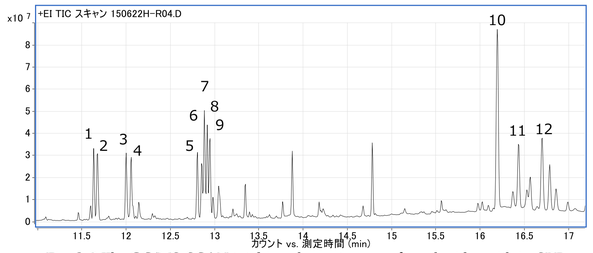


Fig. 3-2 The GC/MS-SCAN total ion chromatogram of saccharides with in-SPE derivatization method

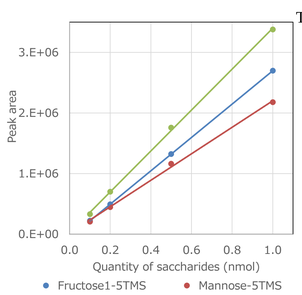


Fig. 3-3 Relations of quantity of Saccharides and the relative peak area with in-SPE derivatization method.

Table 3: A coefficient of correlation and RSD

No.	Saccharides	R2	RSD n=6, %
1	Xylose-4TMS	0.9998	5.4
2	Arabinose-4TMS	0.9996	7.5
3	Rhamnose-4TMS	0.9974	10.8
4	Fucose-4TMS	0.9988	9.8
5	Fructose1-5TMS	1.0000	3.3
6	Fructose2-5TMS	0.9998	3.2
7	Mannose-5TMS	0.9982	2.5
8	Galactose-5TMS	0.9995	1.8
9	Glucose-5TMS	0.9778	2.3
10	Sucrose-8TMS	0.9993	2.9
11	Lactose-8TMS	0.9997	7.2
12	Maltose-8TMS	0.9999	6.5

RSD;Reproducibility parameters for the peak area with in-SPE derivatization method

Examination of the optimum for methoxym derivatization

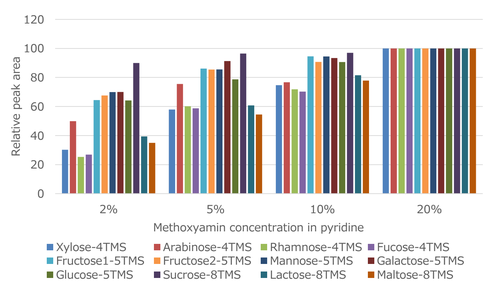


Fig. 3-4 Relations of Methoxyamin conc. in pyridine and the relative peak area.

Table 4: GC/MS condition

PTV Injector	**LVI-S200 (AiSTI Science) : Spiral Insert**
Injectoin Temp.	**70℃(0.3min)-120℃/min-240℃(0.5min)-50℃/min-280℃(35min)**
Solvent Purge Time	**0.25min**
Injection Volume	**10, 25μL**
GC	**Agilent 7890N**
Pre-column	**Deactivated silica capillary tube 0.25mm×0.3m**
Column	**BPX5, 0.25mm i.d.× 30m, df0.25μm (SGE)**
Column Oven Temp.	**60℃(4min)-20℃/min-310℃(4min)**
Splitpurge Flow	**150mL/min(0.25min)-0mL/mion(3.75min)-50mL/min(6min)-20mL/min**
Splitless Time	**4min**
MS	**Agilent 5975C**
Detector Temp.	**280℃**
MS Method	**SCAN : 50-450**

【Conclusion】

By means of development of in-SPE derivarization method, we can achieve rapid and robust method of sample preparation for GC/MS metabolome analysis.

By using this solid phase derivatization ,

1. With holding targeted compounds into solid phase and washing with ACN, we could achieve dehydration process become quite easy and quickly.
2. As Methoxyamine and MSTFA directly added into the strong ionic exchange solid phase which hold the targeted compounds, the derivatization reaction was proceeding promptly on the solid phase.
3. The operating time of dehydration and derivatization was greatly reduced from 19 hours (conventional method) to 10 minutes .

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GC/MS対象メタボローム分析のための固相誘導体化法の開発

2015年7月1日
株式会社アイスティサイエンス
佐々野僚一

Beyond your Imagination

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従来の誘導体化前処理法

誘導体化前処理時間　**9～19時間**

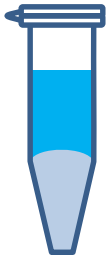
減圧濃縮遠心分離（1600rcf, 4℃, 3min）

凍結：液体窒素

凍結乾燥（一晩：16時間）

誘導体化試薬添加
メトキシアミン/ピリジン溶液 20mg/mL 100uL

インキュベーション（30℃, 90min）：誘導体化反応



誘導体化試薬添加：MSTFA 50uL

インキュベーション（37℃, 30min）：誘導体化反応

遠心分離（16000 rcf, 4℃, 3min）

分取：上澄み 100μL　→　バイアル瓶

測定：GC/MS：注入1μL（スプリット 25:1)

CX固相誘導体化前処理法（アミノ酸）

誘導体化前処理時間 **6分**

試料（抽出液）負荷時の
水-アセトニトリルの比率を検討

抽出液分取　50μL

コンディショニング
水　100μL
ACN　100μL

Hybrid-SPE **CXi3**-2mg

洗浄：ACN 100μL（**脱水効果**）

誘導体化試薬を含浸　MSTFA 20μL

固相誘導体化反応

溶出：ヘキサン　100μL

添加：ヘキサン　400μL

検液

GC/MS：大量注入10μL

AX固相誘導体化前処理法（有機酸）

誘導体化前処理時間 **6分**

試料（抽出液）負荷時の
水-アセトニトリルの比率を検討

抽出液分取　50μL

コンディショニング
水　100μL
ACN　100μL

Hybrid-SPE　A**Xi3**-2mg

洗浄：ACN 100μL（**脱水効果**）

誘導体化試薬を含浸　MSTFA 20μL

固相誘導体化反応

溶出：ヘキサン　100μL

添加：ヘキサン　400μL

検液

GC/MS：大量注入10μL

AX固相誘導体化前処理法（糖類）

誘導体化前処理時間 **10分**

抽出液分取　20μL

試料（抽出液）負荷時の
水-アセトニトリルの比率を検討

添加 ACN 100μL

コンディショニング
水　100μL
ACN　100μL

Hybrid-SPE **AXi3**-2mg

洗浄：ACN 100μL （**脱水効果**）

メトキシム化時の
メトキシアミン濃度の検討

誘導体化試薬を含浸
20%メトキシアミン溶液5μL

固相上誘導体化反応：3min

誘導体化試薬を添加　MSTFA 25μL

固相誘導体化反応

溶出：ヘキサン　100μL

添加：ヘキサン　400μL

検液

GC/MS：大量注入10μL

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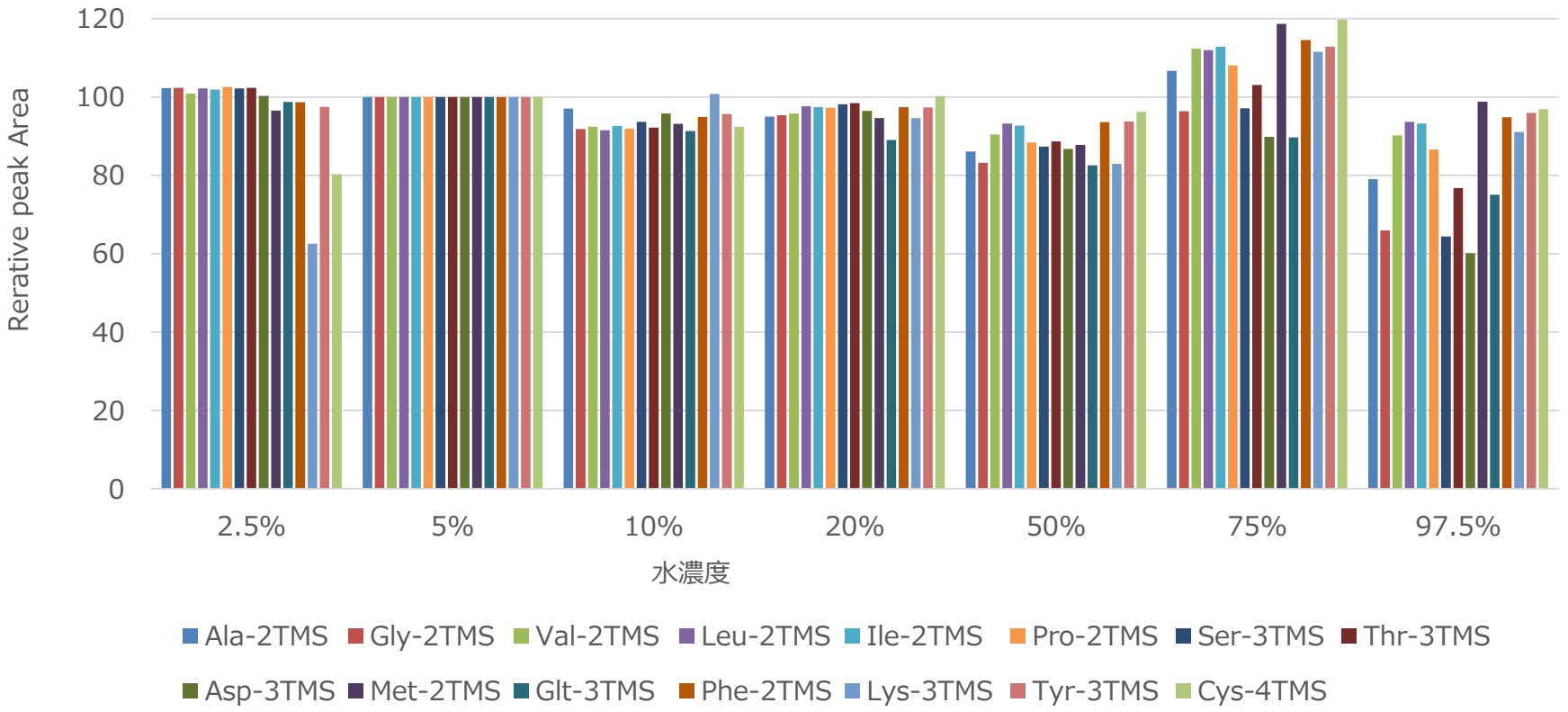
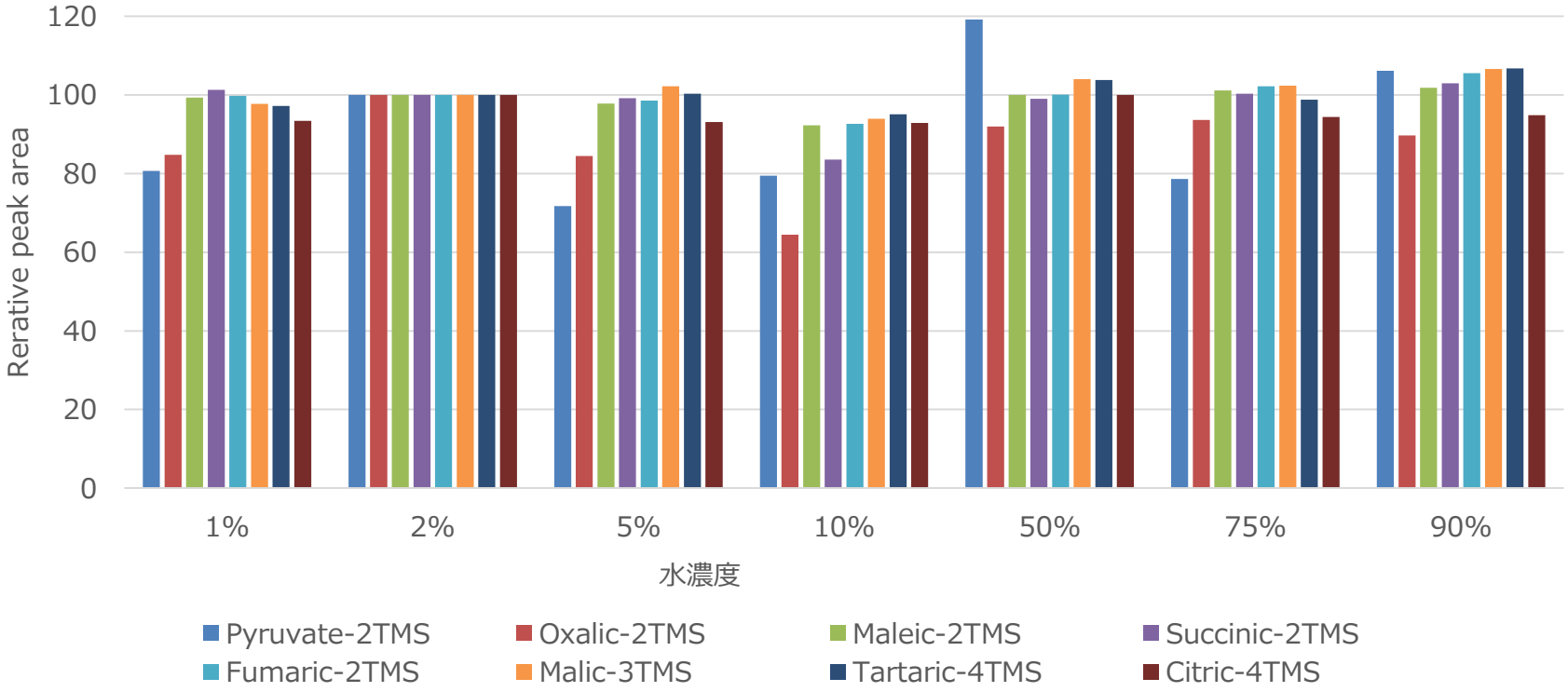
試料負荷時の水濃度とアミノ酸のCX固相への保持について



試料負荷時の水濃度と相対ピーク面積値の関係

アミノ酸とCX固相において、試料負荷時は水濃度が75%以下であれば保持できることがわかった。イオン交換相互作用により保持されていると考えられる。

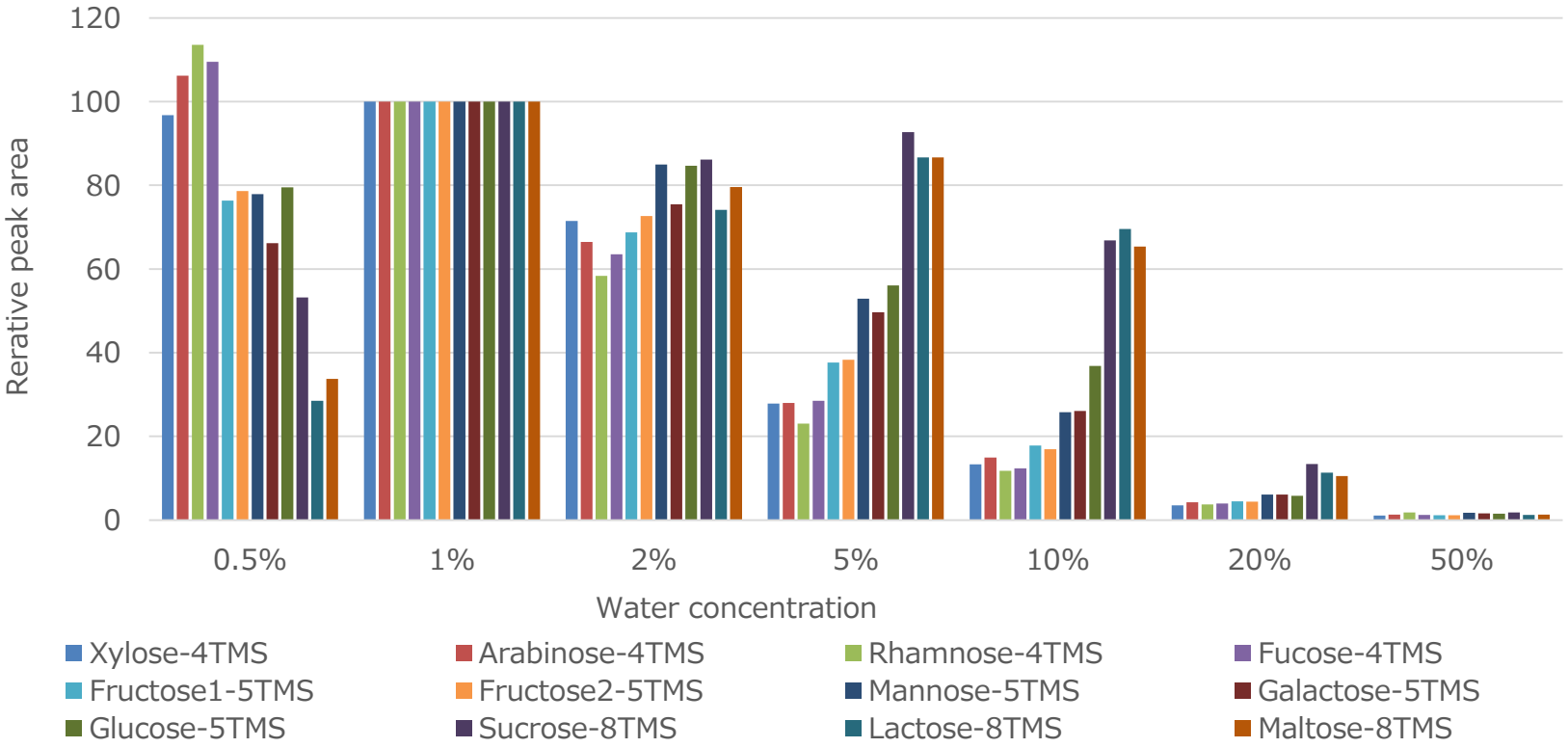
試料負荷時の水濃度と有機酸のAX固相への保持について



試料負荷時の水濃度と相対ピーク面積値の関係

有機酸とAX固相において、試料負荷時は水濃度に依存することなく保持できることがわかった。イオン交換相互作用により保持されていると考えられる。

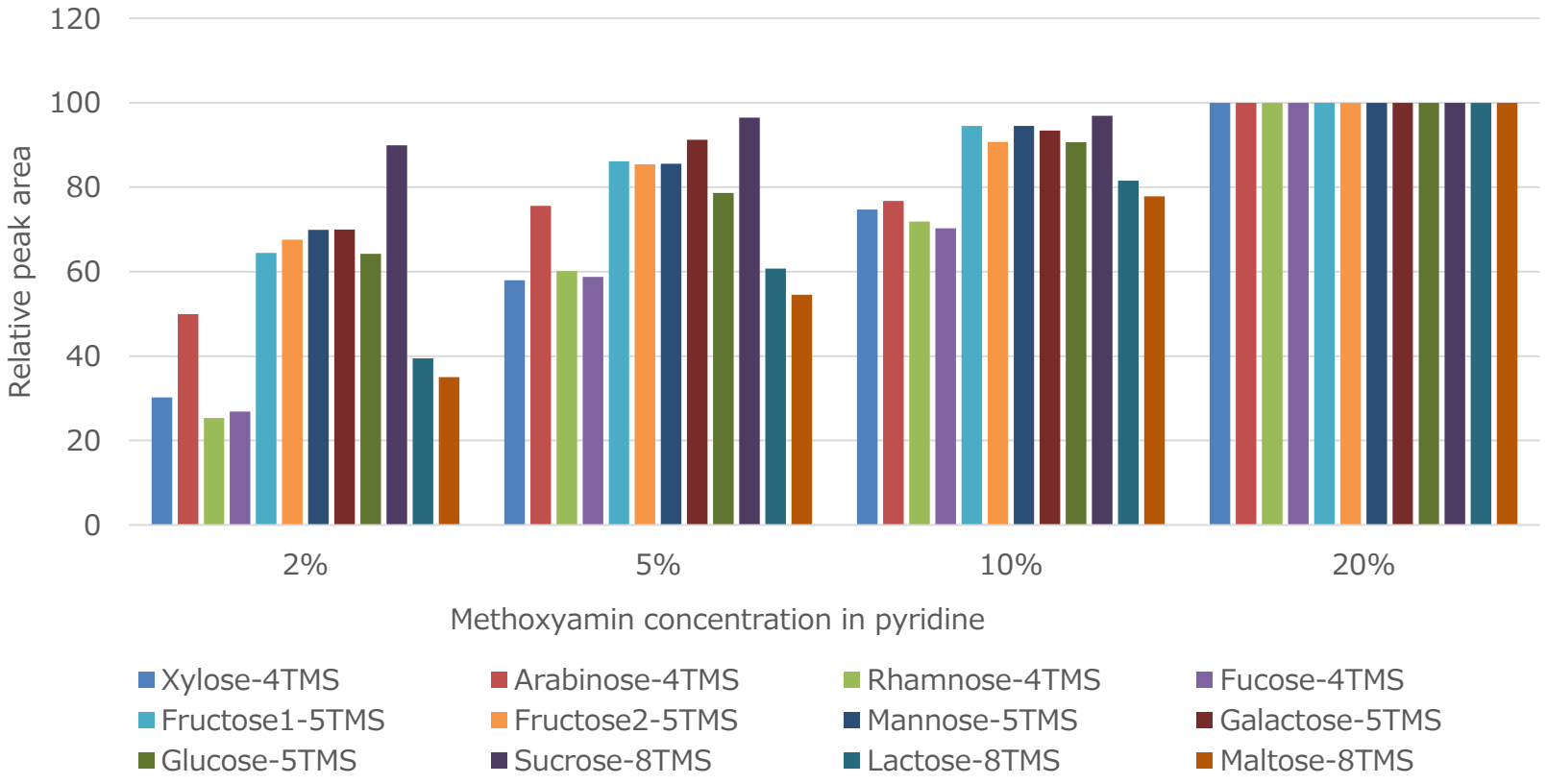
試料負荷時の水濃度と糖類のAX固相への保持について



試料負荷時の水濃度と相対ピーク面積値の関係

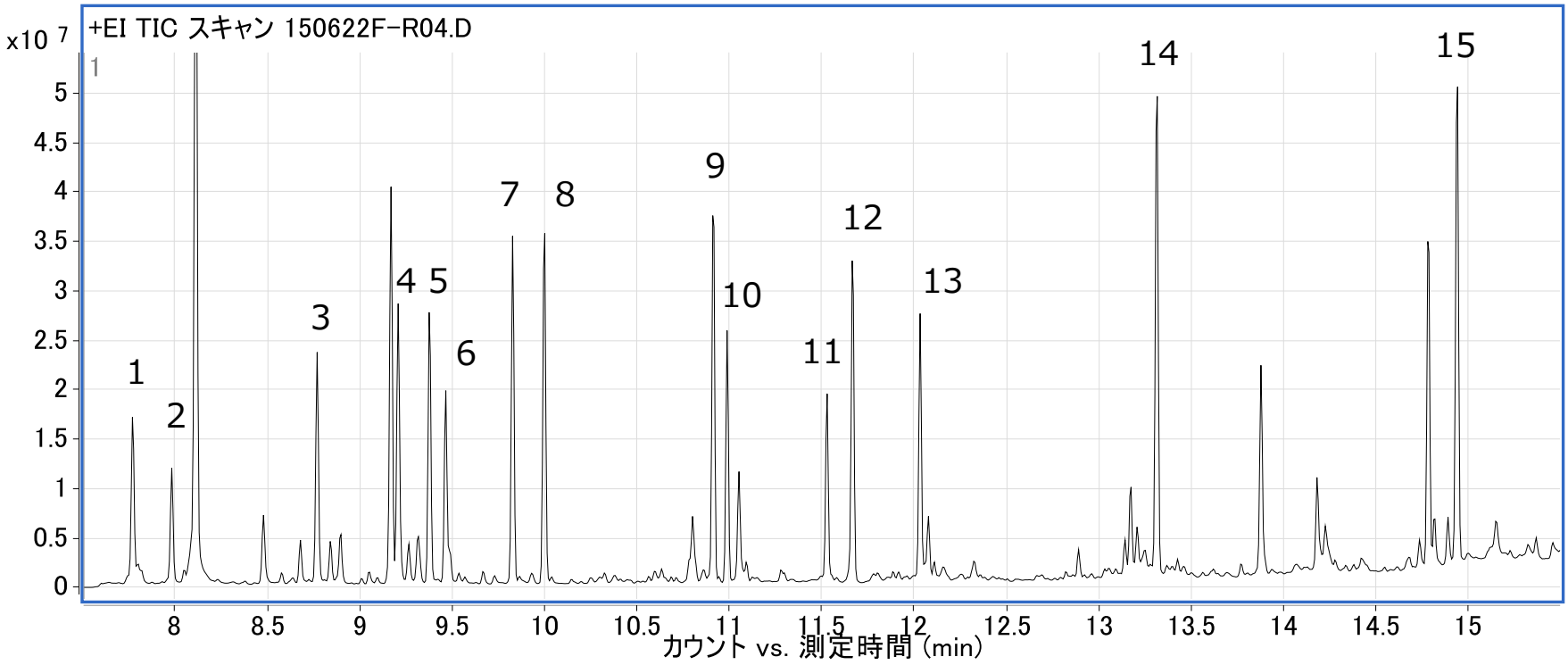
糖類とAX固相において、試料負荷時は水濃度が1%が最適な保持条件であることがわかった。極性相互作用により保持されていると考えられる。
また、0.5%以下になるとSucrose,Lactose,Maltoseは溶液に溶けきれなくなり、カートリッジ壁面等に析出していることが考えられる。

メトキシアミン濃度と固相メトキシム化について



固相誘導体化時のメトキシアミン濃度と相対ピーク面積値の関係

CX固相誘導体化前処理法（アミノ酸）



1. Alaninse-2TMS
2. Gliycine-2TMS
3. Valine-2TMS
4. Leucine-2TMS
5. Isoleucine-2TMS
6. Proline-2TMS
7. Serine-3TMS
8. Threonine-3TMS
9. Aspartic acid-3TMS
10. Methionine-2TMS
11. Glutamic acid-3TMS
12. Phenylalanine-2TMS
13. Lysine-3TMS
14. Tyrosine-3TMS
15. Cystine-4TMS

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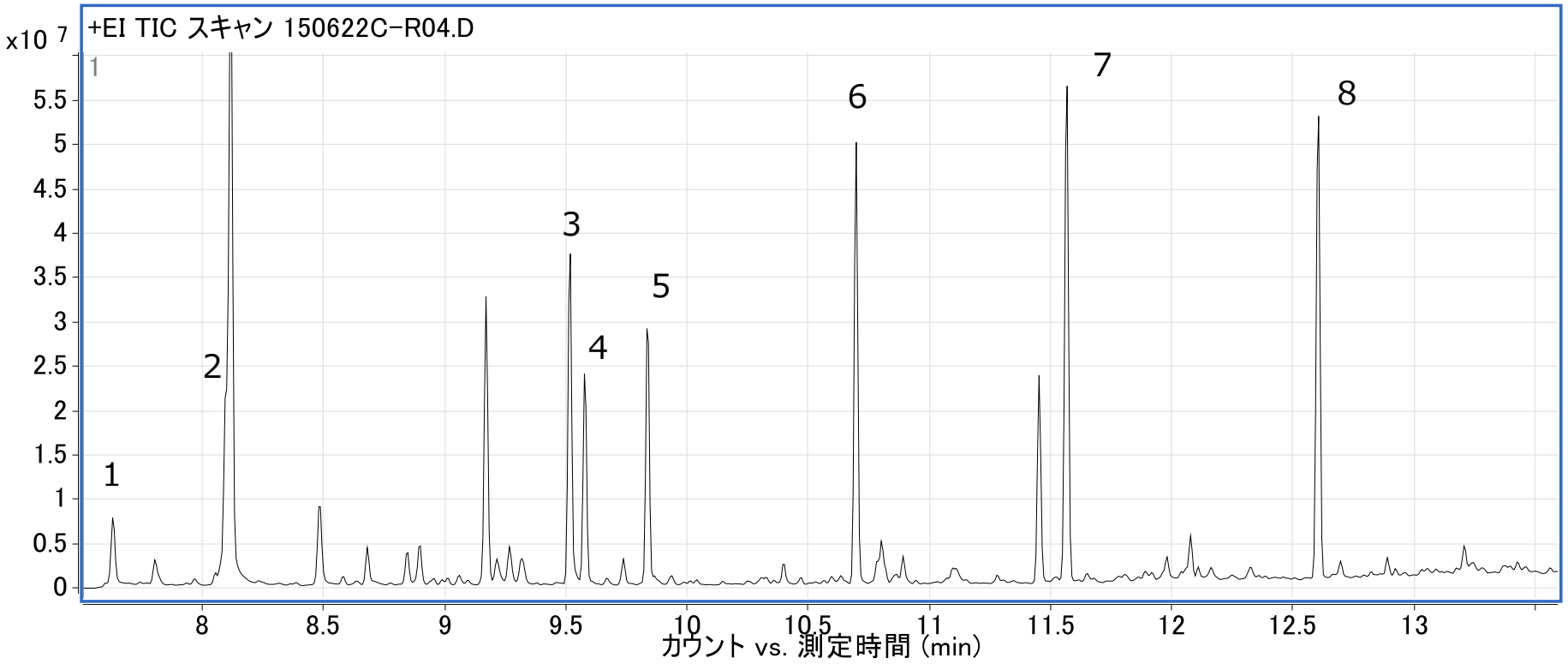
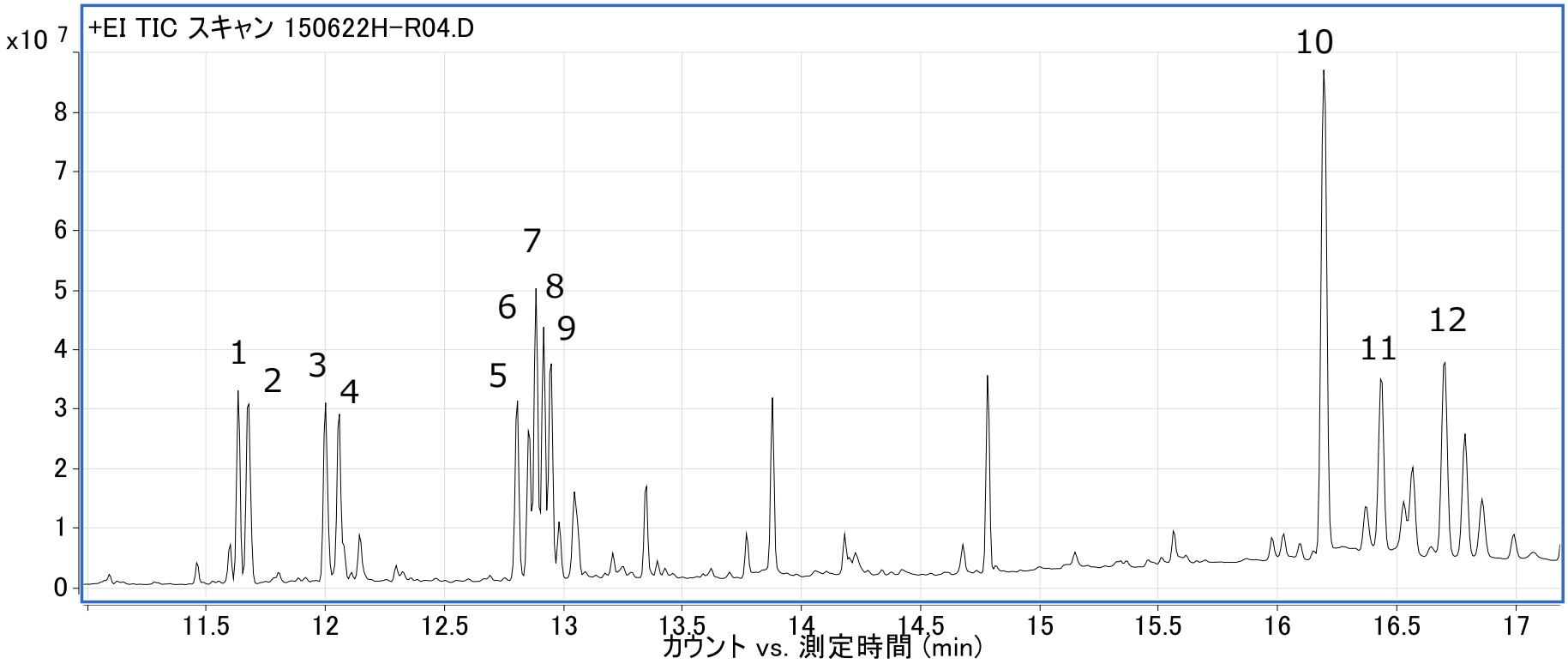
AX固相誘導体化前処理法（有機酸）



1. Pyruvate acid-2TMS　2. Oxalic acid-2TMS　3. Maleic acid-2TMS　4. Succinic acid-2TMS
5. Fumaric acid-2TMS　6. Malic acid-3TMS　7. Tartaric acid-4TMS　8. Citric acid-4TMS

SCANトータルイオンクロマトグラム

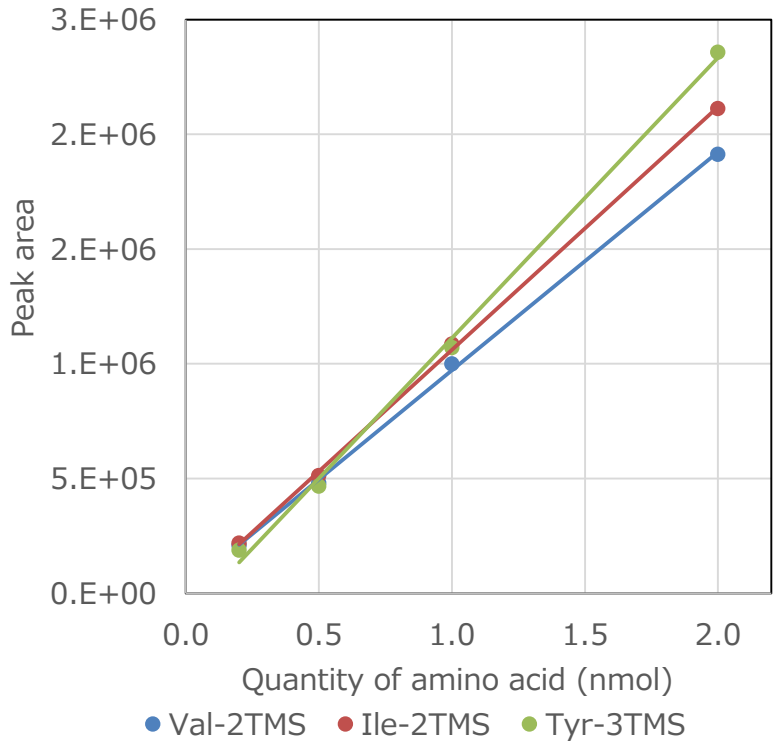
AX固相誘導体化前処理法（糖類）



1. Xylose-4TMS
2. Arabinose-4TMS
3. Rhamnose-4TMS
4. Fucose-4TMS
5. Fructose1-5TMS
6. Fructose2-5TMS
7. Mannose-5TMS
8. Galactose-5TMS
9. Glucose-5TMS
10. Sucrose-8TMS
11. Lactose-8TMS
12. Maltose-8TMS

SCANトータルイオンクロマトグラム

CX固相誘導体化前処理法（アミノ酸）の評価

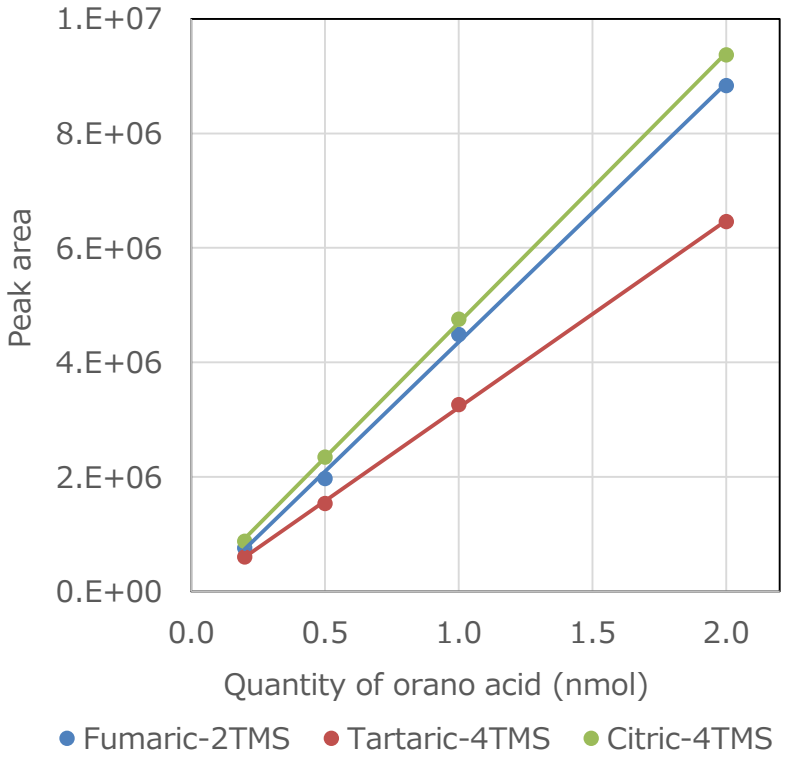


アミノ酸量とピーク面積値の関係

直線性（R2）と再現性（RSD, n=6）

No.	Amino acid	R2	RSD n=6, %
1	Ala-2TMS	0.9996	7.4
2	Gly-2TMS	0.9958	13.1
3	Val-2TMS	0.9993	7.5
4	Leu-2TMS	0.9992	6.7
5	Ile-2TMS	0.9995	6.2
6	Pro-2TMS	0.9982	12.5
7	Ser-3TMS	0.9988	5.5
8	Thr-3TMS	0.9990	4.7
9	Asp-3TMS	0.9989	4.9
10	Met-2TMS	0.9991	7.5
11	Glt-3TMS	0.9911	8.8
12	Phe-2TMS	0.9996	5.6
13	Lys-3TMS	0.9927	32.2
14	Tyr-3TMS	0.9977	4.8
15	Cys-4TMS	0.9950	29.2

AX固相誘導体化前処理法（有機酸）の評価

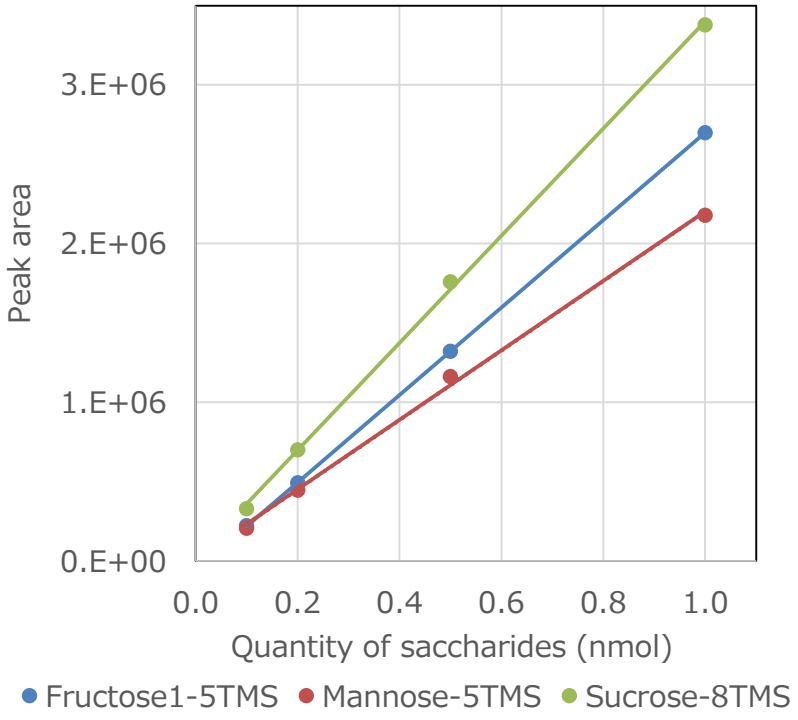


有機酸量とピーク面積値の関係

直線性（R2）と再現性（RSD, n=6）

No.	Organic acid	R2	RSD n=6, %
1	Pyruvate-2TMS	0.9998	15.1
2	Oxalic-2TMS	0.9946	6.2
3	Maleic-2TMS	0.9991	1.8
4	Succinic-2TMS	0.9588	22.0
5	Fumaric-2TMS	0.9991	2.2
6	Malic-3TMS	0.9997	2.1
7	Tartaric-4TMS	0.9998	1.9
8	Citric-4TMS	0.9998	2.5

AX固相誘導体化前処理法（糖類）の評価



糖類量とピーク面積値の関係

直線性（R2）と再現性（RSD, n=6）

No.	Saccharides	R2	RSD n=6, %
1	Xylose-4TMS	0.9998	5.4
2	Arabinose-4TMS	0.9996	7.5
3	Rhamnose-4TMS	0.9974	10.8
4	Fucose-4TMS	0.9988	9.8
5	Fructose1-5TMS	1.0000	3.3
6	Fructose2-5TMS	0.9998	3.2
7	Mannose-5TMS	0.9982	2.5
8	Galactose-5TMS	0.9995	1.8
9	Glucose-5TMS	0.9778	2.3
10	Sucrose-8TMS	0.9993	2.9
11	Lactose-8TMS	0.9997	7.2
12	Maltose-8TMS	0.9999	6.5