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碩士論文

Department of Chemistry
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Master Thesis

五種偵測含胺類代謝物的衍生化方法之比較

Comparison of five derivatization methods for the detection of amine-containing metabolites

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中文摘要

代謝體學是後基因體世代的新興研究領域，複雜生物樣品的代謝體分析可以反映生物系統內即時的反應狀況，目前估計人體血漿有超過 10000 種可定量的代謝物，而目前研究代謝體的分析工具主要為液相層析質譜儀。但不是每種代謝物都適合用液相層析質譜分析，而將代謝物做適當的衍生化可以增強分析物的分離效果、游離效率、並利用二次質譜碎裂的圖譜做身份鑑定。在代謝體中許多分子具有胺類官能基，其中包含胺基酸及其衍生物和胜肽，經過文獻搜尋探討，我們鎖定五種胺基衍生化試劑：OPA、Dansyl、Dabsyl、Fmoc-Cl、Marfey 試劑來進行分析比對。

在這份研究中，我們用液相層析質譜配合螢光偵測來比較不同胺基衍生化試劑的相對優劣性，藉由在相同儀器和最佳化的條件下，來找出這些試劑適合的實驗條件與實驗目的。我們比較了酸鹼性對螢光、紫外/可見光強度影響、產物疏水性、揮發性鹽類對層析分離效果及質譜游離效率的影響，還有不同衍生化試劑的二次質譜碎裂的能量及碎裂後產生的離子。在過去的文獻中，尚未建立如此系統性的研究。

在這份研究中，我們選擇三種常見的動向組成 0.1%甲酸水溶液 (pH 2.6)、2 mM 醋酸銨水溶液 (pH 5)、2 mM 碳酸銨水溶液 (pH 8)當作共同的沖提條件。從實驗結果我們觀察到下列的相對強弱，紫外光可見光吸收 (Dansyl > Fmoc > Marfey > Dabsyl > OPA)、螢光強度 (Fmoc > OPA > Dansyl)、疏水性 (Dabsyl > Fmoc > Dansyl ≈ Marfey ≈ OPA)、游離效果 (Dansyl ≈ Dabsyl > OPA ≈ Fmoc > Marfey)。Fmoc 和 Dansyl 在碰撞誘導碎裂室中都會產生固定荷質比的特徵離子，但 OPA 和

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Marfey 則是產生失去固定的碎片的特徵離子，Dabsyl 碎裂的位置太多因而產生複雜的質譜圖。

經過系統性的比較試劑間的優劣性我們發現 Dansyl 適合代謝體研究，包含螢光定量和多重反應偵測，Fmoc 和 Dansyl 一樣，都是應用潛力比較大的衍生化試劑。OPA 也是泛用的螢光試劑，OPA 反應的條件可以再優化，未來值得更深入的探討和研究，而 Marfey 試劑主要可以應用在鏡像異構物的分離。Dabsyl 的應用就比較狹隘，必須視分析目的而定，因為其分子碎裂能裂太高，從這份研究中得到的不同試劑間的比較可以當作一項參考工具，幫助未來想投入突觸代謝體學研究的人，在設計實驗上更順利。

關鍵字: 代謝體學、液相層析串聯質譜、胺基酸、衍生化、比較研究

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ABSTRACT

The study of complex metabolites in biological samples is a rapidly advancing field. Tremendous progress has been made using advanced liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques to analyze human metabolomes. It is now believed that human serum alone contains over 10,000 quantifiable metabolites. However, there is no universal LC-MS/MS condition that suits all metabolites. Instead of constantly tuning LC-MS conditions for different metabolites, a better approach is to derivatize metabolites to give them more desirable properties such as better LC separation efficiency, enhanced ionization efficiency, and favorable MS/MS fragmentation patterns.

In this study we focus on identifying optimal derivatization methods for amine-containing metabolites in LC-MS/MS analysis coupled with fluorescence detection. These metabolites may include amino acids, their derivatives, and peptides, which may function as hormones, neurotransmitters, and other signaling molecules in the body. We surveyed a wide variety of amine-derivatization regents and narrowed the candidate list down to five: o-phthlaldehyde (OPA), Dansyl-Cl, Dabsyl-Cl, Fmoc-Cl and Marfey's reagent. We compared them in terms of absorbance intensity, product hydrophobicity, fluorescence intensity and pH dependence, separation efficiency in reversed-phase LC, ionization efficiency and salt dependence, as well as MS/MS fragmentation energy and fingerprint. To our knowledge such detailed comparisons of amine derivatization methods have never been carried out before.

In this study we compared three general aqueous mobile phase compositions: 0.1% FA (pH 2.6), 2 mM AA (pH 5), 2 mM ABC (pH 8). Under respective optimal eluent conditions, we have observed these general trend in terms of absorbance intensity (Dansyl > Marfey > Fmoc > Dabsyl > OPA), fluorescence intensity (Fmoc > OPA > Dansyl),

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hydrophobicity (Dabsyl > Fmoc > Dansyl ≈ Marfey ≈ OPA), and ionization efficiency (Dansyl ≈ Dabsyl > OPA ≈ Fmoc > Marfey). Fmoc and Dansyl exhibit characteristic product ions with fixed *m/z* in collision-induced dissociation cell, while OPA and Marfey show characteristic fixed mass loss in fragment product ions. Dabsyl fragments at many positions to create a complex MS/MS spectrum.

After extensive comparisons, we found that Dansyl shows the greatest potential for a universal derivatization method for metabolomics studies, especially for quantitation by fluorescence and multiple-reaction monitoring. Fmoc is a similarly useful reagent and has the advantage of low collision energy. OPA is a versatile fluorogenic reagent and its chemistry can be fine-tuned using different thiol molecules, which is worth further investigating and optimizing. Marfey's reagent is useful for the chromatographic separation of enantiomers due to its chiral nature. Dabsyl is very difficult to fragment in MS/MS experiemnts, which may be a strength or a weakness depending on analytical goals. The performance comparisons between different reagents derived from this study can serve as a guide for designing better metabolomics experiments under different contexts.

Key word: metabolomics, liquid-chromatography tandem mass spectroscopy, amino acid, derivatization, comparative research

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Abbreviations

NMR	Nuclear Magnetic Resonance Spectroscopy
ACN	Acetonitrile
FT	Fourier transform
IR	infrared spectroscopy
LC	Liquid chromatography
MS	mass spectrometry
OPA	Ortho-phthaldialdehyde
Dansyl	5-(DimethylAmino)Naphthalene-1-Sulfonyl chloride
Fmoc-Cl	9-fluorenylmethyl chloroformate
Dabsyl	4-Dimethylaminoazobenzene-4'-sulfonyl chloride
Marfey	1- fluoro-2,4-dinitrophenyl-5-L-alanine amide
NDA	Naphthalene-2,3-dicarboxyaldehyde
IT	2-iminothiolane
PITC	Phenyl isothiocyanate
FQ	5-furoylquinoline-3-carboxaldehyde
Ninhydrin	2,2-dihydroxy-1,3-indandione
Fluorescamine	4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione
Glu	Glutamine
Ser	Serine
Gly	Glycine
Ala	Alanine
Tyr	Tyrosine

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Nva	Norvaline
Phe	Phenylalanine
M	Molar
RP	Reverse phase
UV/Vis	Ultraviolet–visible spectroscopy
LOD	Limit of detection
LOQ	Limit of quantification
FA	Formic acid
ESI	Electrospray ionization
SRM	Selected reaction monitoring
RT	Room temperature
S	Second
MRM	Multiple-reaction monitoring
MPA	3-mercaptopropionic acid
CID	Collision-induced dissociation
x g	g=9.81 ms^{-1}, relative centrifugal force
DBF	dibenzofulvene
AA	Ammonium acetate
ABC	Ammonium bicarbonate

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Chapter 1 INTRODUCTION

1.1 Introduction to metabolomics

Metabolomics is a newly emergent science in the field of omics. The original concept of metabolic could be traced back to ancient Greece, Ullrich Pinder thought out an idea that changed in tissues and biological fluids were indicative of disease. Then, he proposed diagnostic "urine chart" which were widely used in Middle Ages. These charts linked the colours, smells and tests of urine to various medical conditions[1]. (Figure 1-1)

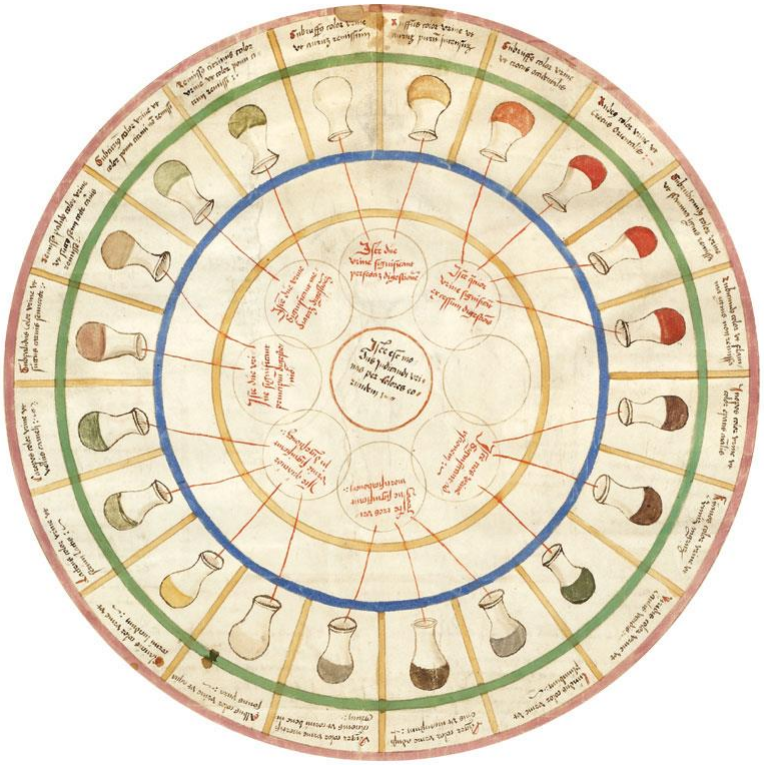

Figure 1-1. This urine wheel was published in 1506 by Ullrich Pinder, in his book *Epiphanie Medicorum*[1]. It describes the possible colours, smells and tastes of urine, and uses them to diagnose disease.

The contemporary metabolomics concept came out very late[2] (1997). As it was an end point of system biology of the "omics" cascade, namely genomics, transcriptomics

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and proteomoics. Metabolomics is the closest to phenotype. (Figure 1-2)

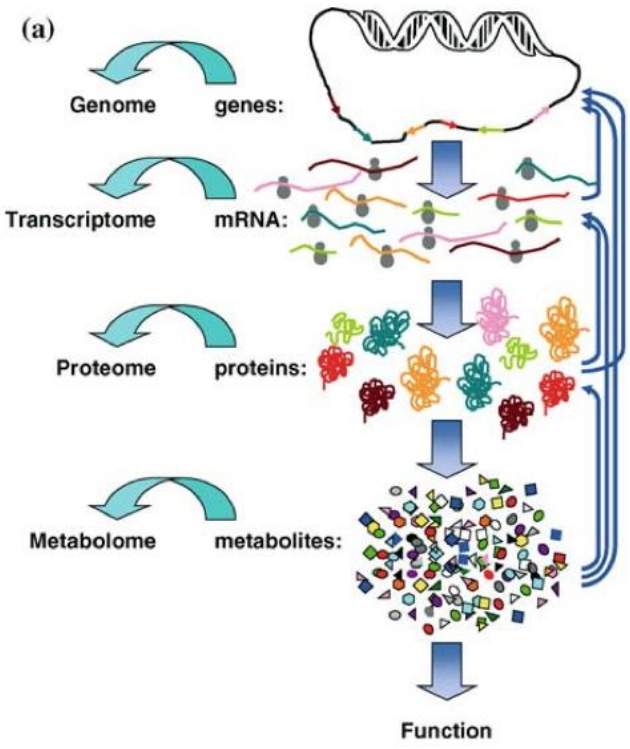

Figure 1-2. Show the general schematic of the omics cascade. The general flow of information is from genes to transcripts to proteins to metabolites to function; the blue vertical arrows indicate interactions regulating respective omics expression.[2]

Nowaday, the integrative analysis of an organism's response to a perturbation on the transcriptome, proteome, and metabolome will give us a better understanding of the biochemical and biological mechanism in complicate system.

In the beginning, there are two term that is metabonomics[3] and metabolomics[4]. Metabonomics broadly aims to measure the global, dynamic metabolic response of living systems to biological stimuli or genetic manipulation. The focus is on understanding systemic change through time in complex multi cellular systems. Metabolomics seeks an analytical description of complex biological samples, and aims to characterize and quantify all the small molecules in such a sample. In practice, the terms are often used

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interchangeably, and the analytical and modelling procedures are the same. (Table 1-1)

Currently, there are two complementary approaches used for metabolomics investigations: metabolic profiling and metabolic fingerprinting. (Figure 1-3)

The application of metabonomics is quite broad[1]. There are three areas that might benefit from metabonomics including individual profiling, population profiling and identifying biological targets. Metabolic profiling of individuals could be used in personalized health care to work out patients' susceptibilities to disease or their responses to medicines, and to tailor their lifestyles and drug therapies accordingly. Metabolic profiling of populations could allow the development of "molecular epidemiology"—the ability to work out the susceptibilities of specific groups to disease. This might allow metabolites to be identified as risk identifiers (biomarkers) for diseases, with implications for health screening programs. Finally, by identifying biochemical pathways for disease, metabonomics could uncover new targets for drug discovery. (Figure 1-4)

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Table 1-1. Summary metabolomics-related definitions[5].

Metabolite	Small molecules that participate in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell.
Metabolome	The complete set of metabolites in an organism.
Metabolic profiling	Identification and quantification of a selected number of pre-defined metabolites, generally related to a specific metabolic pathway(s).
Metabolic fingerprinting	High-throughput, rapid, global analysis of samples to provide sample classification. Quantification and metabolic identification are generally not employed. A screening tool to discriminate between samples of different biological status or origin.
Metabolite target analysis	Qualitative and quantitative analysis of one or a few metabolites related to a specific metabolic reaction.

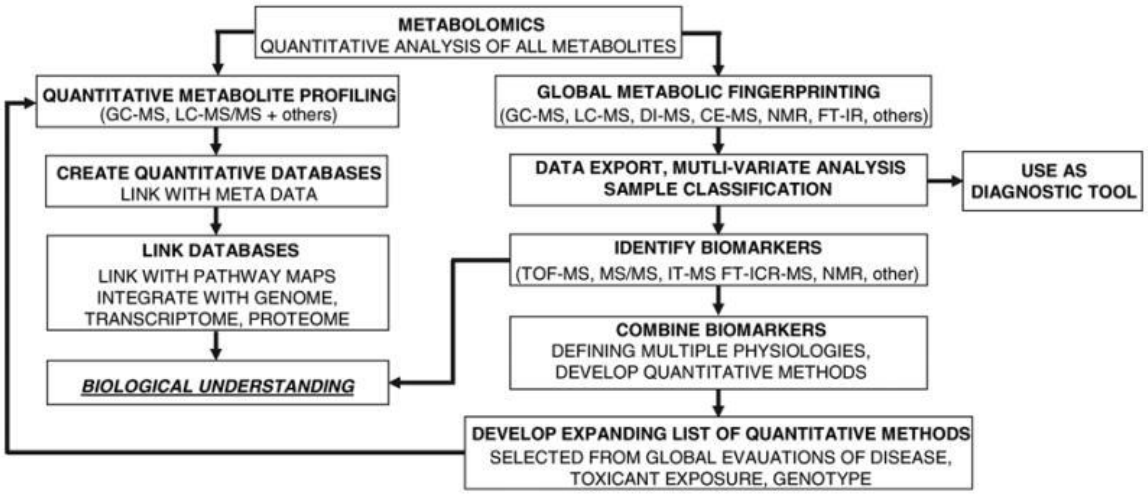

Figure 1-3. The work flow for metabolic profiling and metabolic fingerprinting.[5]

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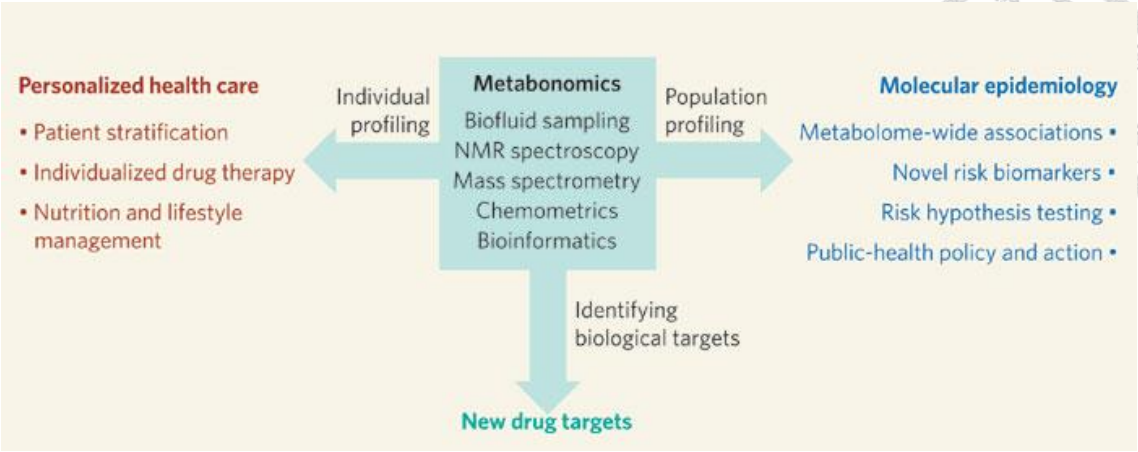

Figure 1-4. The application of metabonomics[1].

1.2 LC-MS in metabolomics research

Currently, there are four analytical platforms in metabolomics researches, such as NMR[6], Fourier transform-infrared spectroscopy (FT-IR)[7], LC-MS, or direct flow injection into MS instrument. However, only medium to high concentration metabolites will be detected with NMR. FT-IR is also not suitable for metabolomics research due to less information content present in the spectrum. Therefore, it is hard to show difference between classes. Matrix effects, salt and isobaric substances make direct flow injection inadequate to metabolomics research.

LC-MS is a novel technique that combines the physical separation of HPLC with the mass analysis of MS. The emergence of LC-MS meets the increasing needs for quicker, more sensitive, and selective analytical measurements. Nevertheless, not every compound is suitable for LC-MS analysis, for instance, compounds which are unstable, low in ionization efficiency, difficult to fragment, highly polarity, or low in abundance. Therefore, derivatization plus LC-MS has proved its value. It provides an alternative strategy to cope with some difficult analytical problems[8,9,10].

There are many advantages of this hyphenated techniques include: (1) improve

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analyte stability during sampling, storage, preparation, and analysis[11]; (2) improve extraction efficency and selectivity.[12]; (3) increase retention time of polar compounds on reverse-phase columns.[13]; (4) increase HPLC separation selectivity.[14]; (5) Enhance analyte ionization efficiency.[15]; (6) increase molecular weight and improve selectivity.[16]; (7) facilitate structure elucidation of a specific chemical group.[17]; (8) assisting fragmentation for compounds which are difficult to fragment.[18]; (9) help quantitative assessment using a stable isotope-labeling or fluorescent-labeling reagent.[19]; (10) extend the linear dynamic range of calibration curve[20].

In derivatization-based chromatographic method, it could be categorized into four topics included pre-column, post-column, online and offline by characterized when the derivatization procedure conducted and whether the reaction is hyphenated with LC-MS directly. There are four combination groups included offline pre-column, offline post-column, online pre-column, online post-column. Among these, offline pre-column derivatization is the most frequently performed mode. (Table 1-2)

Typically, metabolites are categorized into four sub-groups by their specific functional group including amines, phenols, carbonyl, carboxylic group. Table 1-3 is the summary of common reagent in derivatization procedure.

Biological amines are a large and crucial subset of organic molecules including amino acids, other biogenic amines, and important signaling molecules, such as the catecholamine and tryptamine neurotransmitters. As mentioned above, biological amines have some shortcoming that results in difficult analysis including unstable, difficult to fragment, high polarity, or low abundance. Thus, derivatization of biological amines is essential before conducting LC-MS experiments.

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Table 1-2. Summary advantages and disadvantages of pre-column and post-column derivatization.[21]

	Advantage	disadvantage
Post-column	The reaction is reproducible without the need to form a single derivative.	Presence of interferences due to excess of reagent or degradation products. Loss of resolution caused by the widening of the chromatographic bands in the reactor where the reaction is performed. It is not allowed to use very long retention times. Reaction solvents must be miscible with the mobile phase used for separation.
Pre-column	The only limitation to the conditions of the reaction is that it must be completed in a reasonable time and quantitative. The reaction can be performed in a solvent not compatible with the mobile phase used in chromatographic separation. The secondary product formed in the column or before chromatographic separation can be separated.	Presence of interfering peaks in the chromatograms due to the reagent, reaction or degradation products or impurities of the reagents. Hence, it is convenient to remove the excess of reagent, solvents or other components of the reaction mixture prior to injection into the chromatograph.

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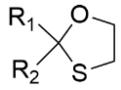
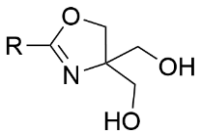
Table 1-3. Summary of common function group and corresponding group in derivatization procedures[22,23,8]. (X denotes leaving group)

Functional group (Target compound)	Reaction group (Derivative reagent)	Derivatization reaction	Product structures
Primary and secondary amines: $-NH_2$ -NRH	X-CO-R	Acylation	-N-CO-R
	X-CO-Ph	Benzoylation /Nitrophenylation	-N-CO-R
	X-SO_2-R	Sulphonamide formation	-N-SO_2-R
	S=C=N-R	Forming thiourea	-N-CS-N-R
	O=C=N-R	Forming urea	-N-CO-N-R
Phenlos:	Cl-SO_2-R	Sulfonation /dansylation	Ph-O-SO_2-R
	X-CO-R	Benzoylation	Ph-O-CO-Ph
	HO-CO-R	Esterification with organic acid or anhydrides.	Ph-O-CO-R
Ketones and aldehydes:	NH_2-R	Forming imine	-C=N-R
	NH_2-NH-R	Forming hydrazone	-C=N-NH-R
	NH_2-OH	Forming oxime	-C=N-OH
	NH_2-R + $NaCNBH_3$	Amide-reduction	-C-N-R
	SH-R	Forming oxathiolane	
Carboxylic acid: -COOH	NH_2-R	Forming amide	-CO-NH-R
	NH_2-NH-R	Forming acyl-hydrazine	-CO-NH-NH-R
	HO-R or N_2-R	Esterification	-COO-R
	Tris(hydroxymethyl) aminomethane	Forming oxazoline	

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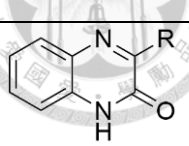
Functional group (Target compound)	Reaction group (Derivative reagent)	Derivatization reaction	Product structures
α-keto acid -CO-COOH	1,2-diaminobenzene	cyclization	

Commonly, pre-column derivatization reagents for amino group include OPA, Marfey's reagent, Fmoc-Cl, Dabsyl-Cl, Dansyl-Cl, PITC, NDA, FQ, IT, N-alkyl-nicotinic acid N-hydroxysuccinimide ester. On the other hand, in post-column derivatization reagent for amino group including Ninhydrin and fluorescamine.

1.3 Pre-column derivatization method

OPA (Ortho-phthaldialdehyde)

OPA's history could be traced back to 1971 when Roth first showed the reactivity of OPA and amino acids in the presence of 2-mercapatoethanol in aqueous alkaline solution[24]. And the best excitation and emission wavelengths for fluorescent detection were 340 and 450 nm, respectively. The investigation of the reaction was well examined by Simons & Johnson in 1976, they first confirmed the result proposed by Roth, but they could not crystallize the adducts of OPA, 2-mercapatoethanol and α-amino acid[25]. After that, they turn the model to the reaction of OPA, t-butyl mercaptan and propylamine in 95% of ethanol, the result was successful that they crystallized the adduct. A serial property of structure was performed by thin-layer chromatography, NMR, IR and Mass spectral analysis that showed it to be a 1-alkylthio-2-alkyl-substituted isoindole[26]. Degradation of this compound yielded a non-fluorescent product. Simon indicated the mechanism was an intramolecular sulfur to oxygen rearrangement to generate an ethylene sulfur polymer and the 2,3-dihydro-1H-isoindol-1-one. Jacob et al[27] made a deep

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investigation in the degradation of the adduct of OPA. He researched the several experiment parameters included thiol structure, thiol concentration, amine structure, solvent composition and pH. They found that increasing side chain and branching α to the thiol group could enhance the stability of the adduct. They proposed that it may concern with steric effect. The result of the effect of structural in amine moiety was also showed the same trend. In the effect of thiol concentration, he found a nonlinear decay curve with increasing thiol concentration. When the concentration of thiol is beyond 0.12M, the effect was quite small. The degradation undergoes quickly when the content of water in the solution increased or the pH of the solution was too acidic, below pH 5. Recently, OPA plus 3-mercapatopropionic acid was proposed[28]. The stability of the product was better than the adduct of 2-mercapatoethanol and ethanethiol. The reaction of OPA/3-MPA with α-amino acid is shown below.

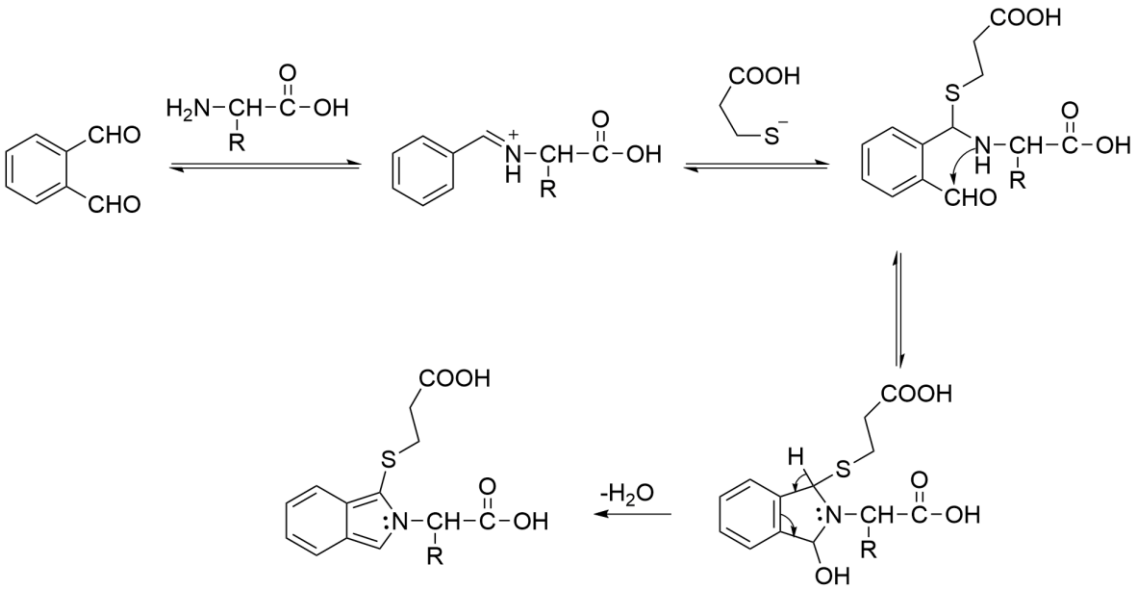

Figure 1-5. The reaction mechanism of OPA/3-MPA with α-amino acid.

The application of OPA method included amino acid[29], peptide[30], and biological sample[31] analysis. Drescher et al[29] applies OPA to analyze the amino acid composition of

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hydrolysates of purified protein which is at nanogram sample levels. Benson et al[30] utilizes OPA to detect tryptic digestion peptides and its sensitivity reach picomole range. Yongqing et al[31] use OPA to analyze content of L-Homoarginine in biological sample, and combine with HPLC within 23 min. The limit of detection was 188 fmol hArg.
The advantages of OPA method are high sensitivity, fast reaction, no reactivity toward ammonia, low cost, and ease of simple preparation. But there are some disadvantages of this method that the product is unstable and could not react with secondary amine (like proline or hydroxylproline).

Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide)

The prototype of Marfey's reagent could be dated back to the Sanger's reagent. In 1945, Sanger proposed a novel reagent that used 1-fluoro-2,4-dinitrobenzene (FDNB) to mask the free amino acid group within insulin[32]. The attribute of Sanger's reagent is that the DNP-AAs releasd from acidic total hydrolysis are separable chromatographically and comparable with the authentic DNP-AAs derivatized from standard amino acids. In 1959, Sanger finished whole sequence determination of insulin by means of FDNB reagent. After Sanger's discovery, Zahn and Meienhofer[33] synthesized a bifunctional modification version of Sanger's reagent, namely 1,5-difluoro-2,4-dinitrobenzene (DFDNB), for the crossing-linking of proteins included wool, silk, and insulin. In detail, Zahn and Meienhofer prepared the product of DFDNB with amino acid ester monofunctional, bifunctional and mixed bifunctional derivatives which were analyzed by paper chromatography. In addition, they also tried to separate certain diastereomeric derivatives by fractional crystallization. In 1965, Marfey et al[34,35] utilized DFDNB for the intramolecular cross-linking of bovine pancreatic ribonuclease A. Then, in 1984, he found the replacement of one of fluoro atom of DFDNB by L-alanine amide could generate a

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chiral version of Sanger's reagent[36]. In this paper, he successfully separated and quantified alanine, aspartic acid, glutamic acid, methionine, and phenylalanine derivative diastereomers by reverse-phase HPLC. After this milestone, Bruckner et al[37] synthesized the other "Sanger's type" chiral reagent using L-valine amide, L-valine ester, L-phenylalanine amide, and other substituents. After Bruckner's trials, he found FDNP-valine-NH_2 gave the best resolving power of derivative amino acid.

The mechanism of Marfey's reagent is that the nucleophilic attack of α-amino group of amino acid on the carbon-fluoro bond activated by the two nitro groups on the aromatic ring. After the reaction, it yields diastereomeric aniline derivatives which could be detected by UV region. The reaction summary below.

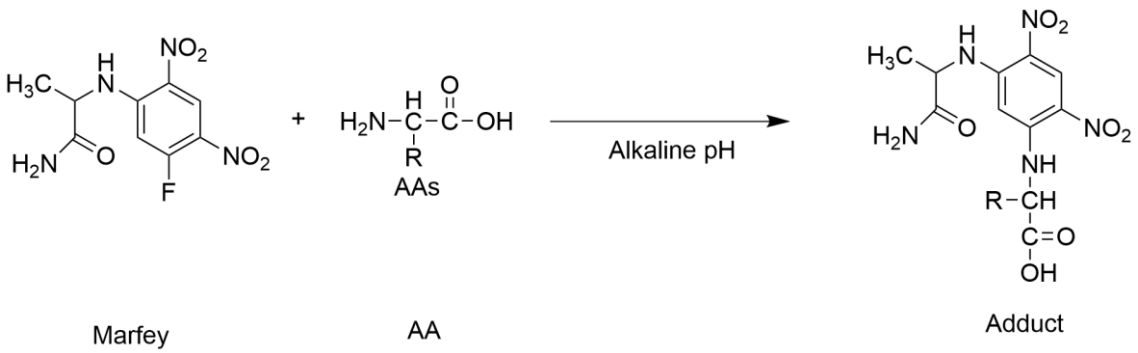

Figure 1-6. The reaction of Marfey reagent with α-amino acid.

Marfey's reagent has been widely used to analyze amino acid[38], peptide, protein and biological sample. Kochhar et al[38] successfully separated 20 amino acids including proline and cysteine within a linear 110-minute gradient. They also analyzed the protein hydrolysate of aldolase, aspartate aminotransferase, and lysozyme. The amino acid composition of three protein fitted with other reports which obtained from ion exchange chromatography.

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Fmoc-Cl (9-fluorenylmethyl chloroformate)

Fmoc was first described by Carpino and Han[39] in 1972 as an amino protecting group during peptide synthesis. By this technique the longer peptides could be obtained. Then in 1979, Anson Moye and Boning[40] showed that primary and secondary amines may be derivatizated with Fmoc-Cl which was suitable for analytical purposes with fluorescence detection. The excessive Fmoc-Cl was removed by extraction with diethyl ether before HPLC separation.

Fmoc-Cl reacts with both primary and secondary amino groups under alkaline pH, amino group replaces chloride ion then generates carbamate products. Figure 1-7 show this reaction.

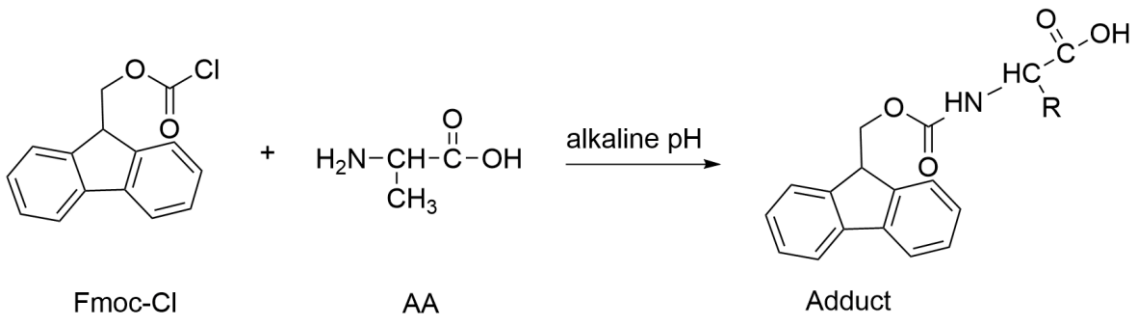

Figure 1-7. The reaction of Fmoc reagent with α-amino acid.

Fmoc has been extended to many field[41,15]. Kushnir et al[41] utilized Fmoc-Cl method and combine with ESI-MS/MS to determine the urinary catecholamines which is important for clinical diagnosis of pheochromocytoma, paraganglioma, and neuroblastoma. Quantification limits were 2.5 μg/L for Epinephrine and dopamine and 10 μg/L for norepinephrine. The total imprecision (CV) was ≤ 9.6%; extraction recoveries were 71% ± 12%. Xianlin et al[15] derivatized ethanolamine glycerophospholipid (PE) and lysoPE extracted from biological sample. Then analyzing the corresponding carbamate products by directly infused into ESI-MS. The detection limit reached

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attomoles per microliter for PE and lysoPE with a > 15,000-fold dynamic range.

The disadvantage of Fmoc-Cl is that Fmoc-Cl is fluorescent itself and needs to be removed before chromatography. Or it will interfere with separation of amino acid derivatives and be detrimental to column performance. Generally, it removed by hexane extraction. While its advantages are that the femtomole sensitivity reaches by using detection of fluorescent derivatives. Derivatives are stable and its reaction is fast.

Dabsyl-Cl

Dabsyl-Cl was first proposed by Chang in 1975, in this paper he described how to synthesize Dabsyl-Cl and prepare Dabsyl-amino acid, peptide and proteins. In addition, he also provided stability, results of TLC and IR data of Dabsyl-amino acid, in detail.
The reaction of Dabsyl-Cl is that it undergoes a substitution of chloride ion by attacking amino group under alkaline pH then form a stable sulfonamide product. The reaction is indicated below.

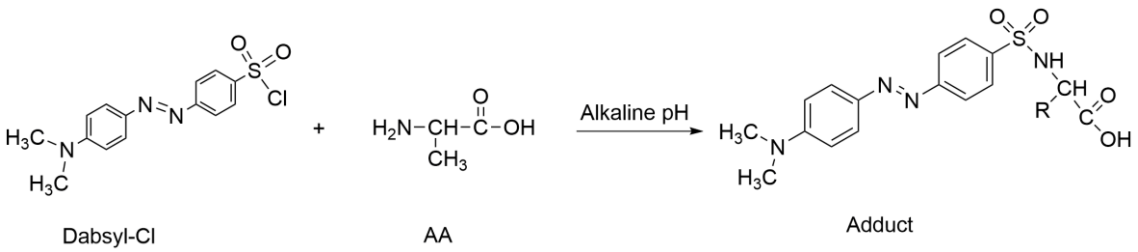

Figure 1-8. The reaction of Dabsyl reagent with α-amino acid.

The Dabsyl-Cl pre-column derivatization technique has been shown to be very useful for micro-analysis of amino acids[42], proteins[43] and amino acid neurotransmitters[44]. Chang[42] successfully separated dabsyl-amino acids by reverse-phase liquid chromatography and reached picomole range thus it could analyze polypeptide in the nanogram scale. Tzeng et al[43] reported the use of Dabsyl-Cl to label proteins prior to

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electrophoresis so that the proteins bands were visible during the run. This pre-staining method has two advantages: (1) monitoring the separation process immediately (2) Saving much time because results are known during the run and the gel could be stop at the desired time and photographed simultaneously. Chang and Martin[44] reported application of Dabsyl-Cl in the analysis of the amino acid neurotransmitters including taurine, GABA, glutamate, glycine and β-alaine in the mouse brain. They also separated these neurotransmitters from other common amino acid within 30 min by a gradient system.

The disadvantage of Dabsyl method is that Dabsyl-dervatives are concentration dependent and easily generate multiple derivatives with several amino acids (Lys, Trp, His, Cys, Arg). Excess salt (urea or phosphate) and detergents will interfere with the reaction. Howerer, Dabsyl-Cl could react with primary and secondary amines. Derivatives are stable. The detection limit reaches pmol range.

Dansyl-Cl

Dansyl was first proposed by Weber in 1952, he react Dansyl-Cl with proteins (ovalbumin, serum albumin and α-chymotrypsin) to give fluorescent compounds. After conjugation, the proteins lost its activity so he thought it might react with core of these proteins and then inhibit the enzyme[45]. In 1956, Hartley and Massey utilized Dansyl-Cl to label proteins (α-chymotrypsin, chymotrypsin, DIP- chymotrypsin) and 20 amino acids.[46] In this paper, they first obtained same result with Weber's discovery. Furthermore, they reported the TLC and absorption result of 20 amino acids.

The reaction of Dansyl-Cl typically undergoes substitution where amino group substitutes chloride ion then forms sulfonamide adduct. The product could be detected by either UV/Vis or fluorescence. Figure 1-9 show this reaction.

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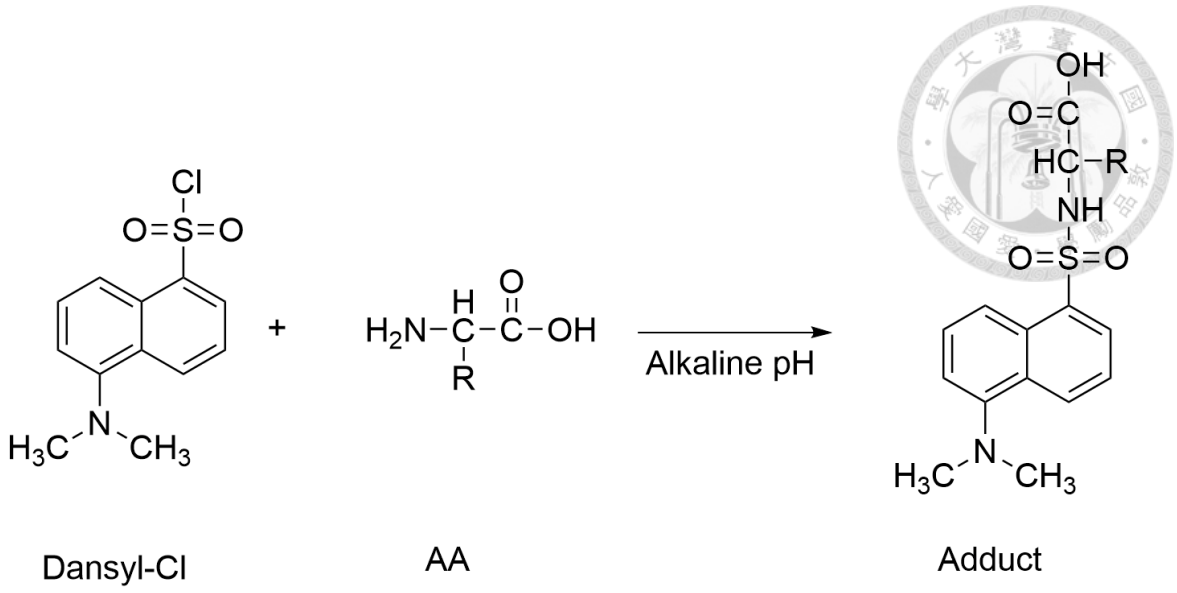

Figure 1-9. The reaction of Dansyl reagent with α-amino acid.

Liang Li et al[47] reported $^{13}C_2$- and $^{12}C_2$-dansylation method for profiling amine- and phenol-containing metabolites. They first synthesized heavy Dansyl-Cl by following Bergmann and Pfleiderer's method[48]. In short, the $^{13}C_2$-dimethyl sulfate was reacted with 5-aminonaphthalene-1-sulfonic acid in alkaline medium to form 5-($^{13}C_2$-dimethylamino)-naphthalene-1-sulfonic acid. The resulting residues were dried and then chlorinated by phosphorus pentachloride. Then introducing $^{12}C_2$- and $^{13}C_2$- to target analytes in a sample and a comparative control, respectively. After mixing the labeled sample and control, the mixture is analyzed by LC-MS. Because there is no isotopic effect in reverse-phase LC, they will coeluted simultaneously as a pair of peaks with defined mass difference ($^{12}C_2$- and $^{13}C_2$-). The intensity ratio of the peak pair can be determined to provide relative quantification of the metabolite in the sample versus the control. Absolute quantification is also possible if the concentration of the metabolite in the control is known.

The advantage of Dansyl-Cl is nonspecific since it reacts with amino group of both aliphatic and aromatic amines or phenol, thiol and imidazole. The detection of low quantities of amine-containing compounds (pmol or fmol range).

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The disadvantage of Dansyl-Cl is reaction time relatively lengthy. Dansyl derivatives are photosensitive. Dansyl-derivatives are concentration dependent and may form multiple derivatives with several certain amino acids (Lys, Trp, His, Cys, Arg).

PITC (Phenyl isothiocyanate)

PITC was first described by Pehr Edman in 1950[49], it allowed determination of extended sequences of peptides or whole proteins. In 1982, this reagent was extended to amino acid analysis of purified proteins[50,51,52]. Then it was developed commercially by Waters (Milford, MA) with the name of Pico-Tag.[51]

Amino group react with electrophilic carbon of isothiocyanate group to generate its corresponding phenylthiocarbamyl (PTC) derivatives (in the presence of base). Figure 1-10 shows the reaction.

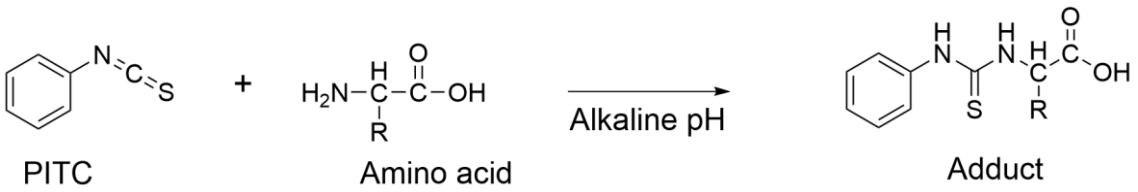

Figure 1-10. The reaction of PITC reagent with α-amino acid.

Detection of the amount of misfolded prion protein in blood is a challenging analytical problem. Onisko et al[53] utilize PITC method that successfully detect the VVEQMCTTQYQK peptide obtained from prion protein. This method will further develop to diagnose the early stage of transmissible spongiform encephalopathies (TSEs) and assure a safer food supply.

The PITC method has several disadvantages: the derivatization procedure is lengthy; a vacuum system is required to evaporate excess coupling reagent before HPLC analysis or it would cause rapid deterioration of the columns; There will be multiple spurious

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peaks in RP-LC chromatogram and PITC is highly toxic. It reacts with primary and secondary amines. Derivatives are stable. There is no evidence for the formation of any disubstituted derivatives with Tyr and His.

NDA (Naphthalene-2,3-dicarboxyaldehyde)

NDA was first proposed by Montigny[54] in 1987, The first part of the study mentioned how to synthesize NDA compound and how is its kinetic behavior. The other part is the use of NDA method in amino acid analysis and peptide hydrolysates investigation. After this paper, there had been considerable work for the optimization of the derivatization reaction[55]. The cyanide ion was found to be superior to the traditional thiol, yielding more stable derivatives with a higher quantum yield[55].

Typically, the reaction of NDA with primary amines and cyanide ion (or a thiol) under alkaline pH produces a highly fluorescent N-2-substituted-1-cyanobenz-[f]-isoindole (CBI) product which is relatively stable. (Figure 1-11)

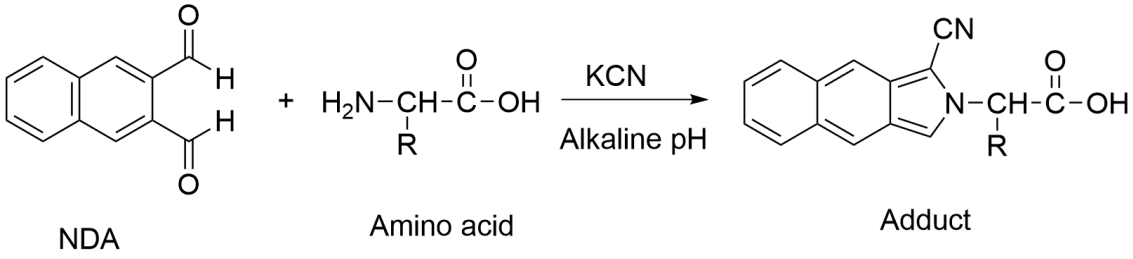

Figure 1-11. The reaction of NDA reagent with α-amino acid.

NDA was widely used in the determination of amino acids[56], catecholamine[57,58], chiral amino acids[59].

The advantages of this method are: (1) little influence of the by-product; (2) quick derivatization reaction; (3) Stable products were obtained relative to the use of OPA. Whereas there are four limitations (1) because CN^- accelerates the degradation of NDA's

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product, the components of the NDA/CN^- reagent must be prepared as separate solutions; (2) It needs to control concentration of NDA/CN^- due to the formation of bis-derivatives of lysine. This bis-derivatives display a reduced quantum yield of fluorescence (Φ_f= 0.02) compared to other NDA derivatives. (3) It only reacts with primary amines.

FQ (5-furoylquinoline-3-carboxaldehyde)

FQ was first described by Novotny et[60] al in 1990, and he described the synthesis of FQ, liquid chromatography of adducts of FQ-labeled amino acid and fluorescence measurements (quantum yield) of each adduct.

The reaction of FQ with primary amines and cyanide ion (or a thiol) under alkaline pH produces a highly fluorescent isoindole adduct. (Figure 1-12)

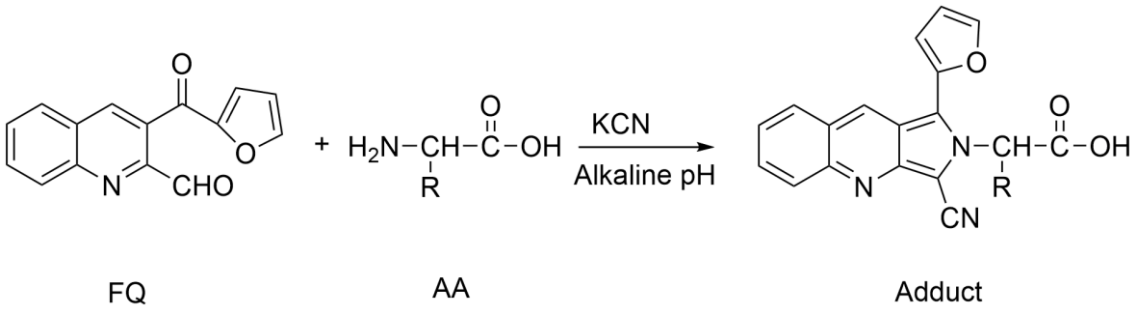

Figure 1-12. The reaction of FQ reagent with α-amino acid.

FQ was widely used in the determination of proteins[61,62], biogenic amines[63], tissue homogenates[64]. The disadvantage of FQ is that (1) it is quite expensive. (2) it reacts with primary amines only. (3) relatively long reaction time. Whereas its advantage is that the stability of its adduct is relatively better than OPA's products.

IT (2-iminothiolane)

IT was first proved by Traut et al[65] in 1953, the paper not only described how to

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synthesis IT but also showed its application to the *Escherichia coli* 30s ribosome.

IT converts the primary amine group and the terminal amino group of a peptide into a sulfhydryl group. (Figure 1-13)

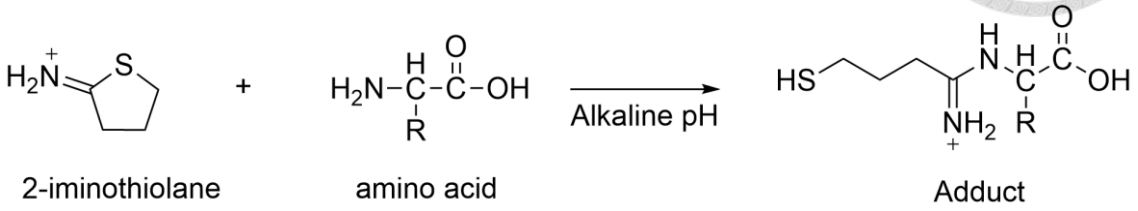

Figure 1-13. The reaction of IT reagent with α-amino acid.

sulfhydryl groups can be used in a condensation reaction to produce cross-linked dimers between neighboring proteins. For example, formation of disulfide bonds within a protein could be used to investigate the spatial arrangement of ribosomal proteins[66]. Sara et al utilized IT to immobilize β-galactosidase on the crystalline cell surface layer (S-layer) of *Bacillus stearothermophilis* PV72.

The drawback of IT is that it reacts with primary amine only, it is not suitable for quantification of certain amino acid due to formation of bis-derivatives (lysine and cysteine) and it may aggregate with itself, the products or compounds with sulfhydryl group. While the advantage of this method is that (1) The cleaved protomeric proteins retain their characteristic behavior which are available for two-dimensional gel electrophoresis or other analytical method (FAB or LSIMS). (2) the presence of the sulfhydryl group allows further derivatization using fluorophores such as N-pyrenyl maleimide (NPM).

N-alkyl-nicotinic acid N-hydroxysuccinimide ester

N-Alkylnicotinic acid reagents was first proposed by Yang et al[67] in 2006 for a novel

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amino acid derivatization reagent and an alternative tool in the relative quantification research of comparative metabolomics.

N-Alkylnicotinic acid was activated with NHS ester first, then it undergoes a quick substitution by primary or secondary amine.

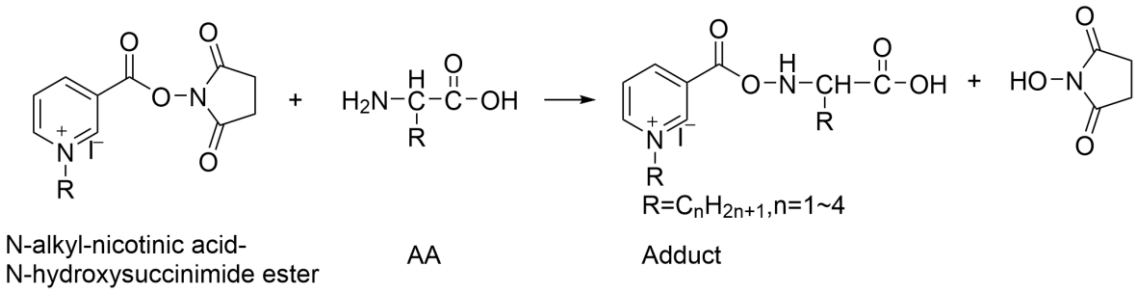

Figure 1-14. The reaction of N-alkyl-nicotinic acid N-hydroxysuccinimide ester reagent with α-amino acid.

This reagent has two character groups (1) an adjustable hydrophobicity via increasing or decreasing its alkyl chain; (2) a charged quaternary amino group. Alkyl chain help add sufficient hydrophobicity to lengthen retention time. A quaternary amino group would increase ionization efficiency.

This method has been used to sequencing the proteins[68] or comparative metabolomics[69]. One drawback of this method is the rapid hydrolysis of NHS ester.

1.4 Post-column derivatization method

In general, there are two post-derivatization reagent ninhydrin and fluorescamine. Both reagent have emerged for several decades, and extended for numerous topic such as food, drug, and environment analysis.

4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione (fluorescamine) was first described by McCaman and Robins in 1962. Fluorescamine react with primary amine to

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give highly fluorescent pyrrolinones.

Typical procedure is that primary amine buffers at alkaline pH in a test tube, followed by adding fluorescamine which dissolves in acetone. After 1 hour reacts at room temperature, the reaction mixture is ready for analysis by RP-HPLC.[70] Fluorescent detection is monitored at 390 nm excitation, 475 nm emission.

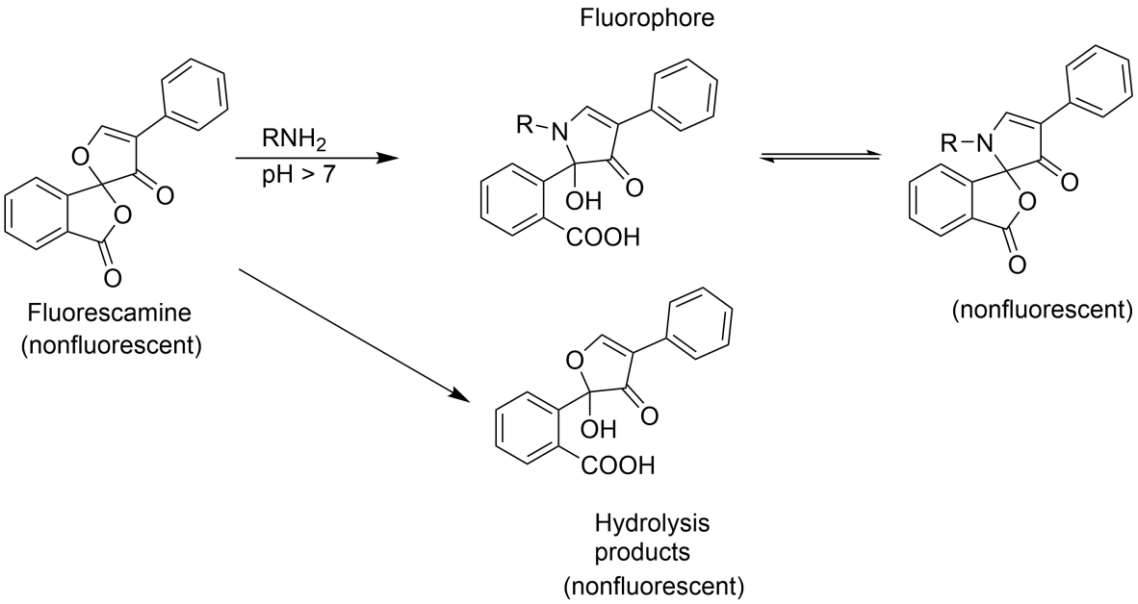

Figure 1-15. Summary of reaction of fluoresamine including hydrolyzed product of the reagent and dynamic balance of fluorescamine product[71].

Fluorescamine have found wide application in analytical chemistry such as electrophoresis[72], liquid[73] and thin-layer chromatography[74]. Fluorescamine was successfully used to anchor proteins after electrophoresis in starch gel and on paper. The latex protein hevein is hard to stain by conventional method, but was detected by fluorescamine quickly in starch gel and on paper[72]. Miedel utilized fluorescamine and combined with HPLC to purify and characterize proteins and peptides[73]. Felix reported the TLC results of fluorescmaine, exposing to the trimethylamine would improve sensitivity and stability[74]. Fluorescamine act as fluorescent labels for amino acids[75],

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peptides[75], proteins[76], and modified nucleosides[77]. Udenfriend analyzed amino acid and peptides from tryptic digest of hemoglobin in the picomole range[75]. Shu-I Tu utilize sodium dodecyl sulfate and fluorescamine for the fluorescent labeling of BSA successfully[76]. Sprinzl introduce fluorescamine to the 3'-end of $tRNA^{Phe}$-C-C-A(3'NH_2) from yeast and to the monitor nucleoside X in E. coli $tRNA^{Arg}$, $tRNA^{lys}$, $tRNA^{Met}$ and $tRNA^{Phe}$[77].

The shortcoming of fluorescamine is the non-reactivity toward secondary amine. Furthermore, it will form multiple derivatives resulted in less reliable quantitative analysis.

Ninhydrin reaction was discovered by Siegfried Ruhemann in an attempt to oxidize 1-hydridone to 1,2-diketohydridene with p-nitrosodimethylaniline at Cambridge unversity in 1910. He did not obtain expected product, but received disubstituted hydrindone which was hydrolyzed to ninhydrin. Then in 1948, the first automated chromatography is assessed successfully by Sanford Moore and William H. Stein at Rockefeller University[78,79,80]. It provides fast analysis and excellent sensitivity which down to nanomole levels. At the same time, Den[81] establishes a vast assay that includes all the common amino acid, synthetic substances (α-aminoisobutyric acid), nature product (collidine). Aiming to present a "map of the spots", he tested numerous compounds which could give ninhydrin-positive indication on thin layer chromatograms. After that, many applications emerged in different targets such as cross-linked amino acids[82], biogenic amines[83], amino sugars[84]. As time went on, Boppana[85] and Wimalasena[86] use ninhydrin reaction for the HPLC-based determination of biogenic amine. Furthermore, Laporte[87] extends ninhydrin reaction to forensic sciences for detection of fingerprint on thermal papers and other surfaces. He found that complexation of Ruhemann's purple with

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sodium ion (or potassium ion) enhances the sensitivity of analyses.

Typically, reaction mechanism is that ninhydrin tautomerize to 1,2,3-indantrione first then form Schiff's base with amino acid. After ketimine was formed, it bears decarboxylation producing an aldehyde and 2-amino-1,3-indandione. Then this intermediate amine was condensed with another molecule of ninhydrin and did form an intense chromophore Ruhemann's purple. The mechanism is summarized below.

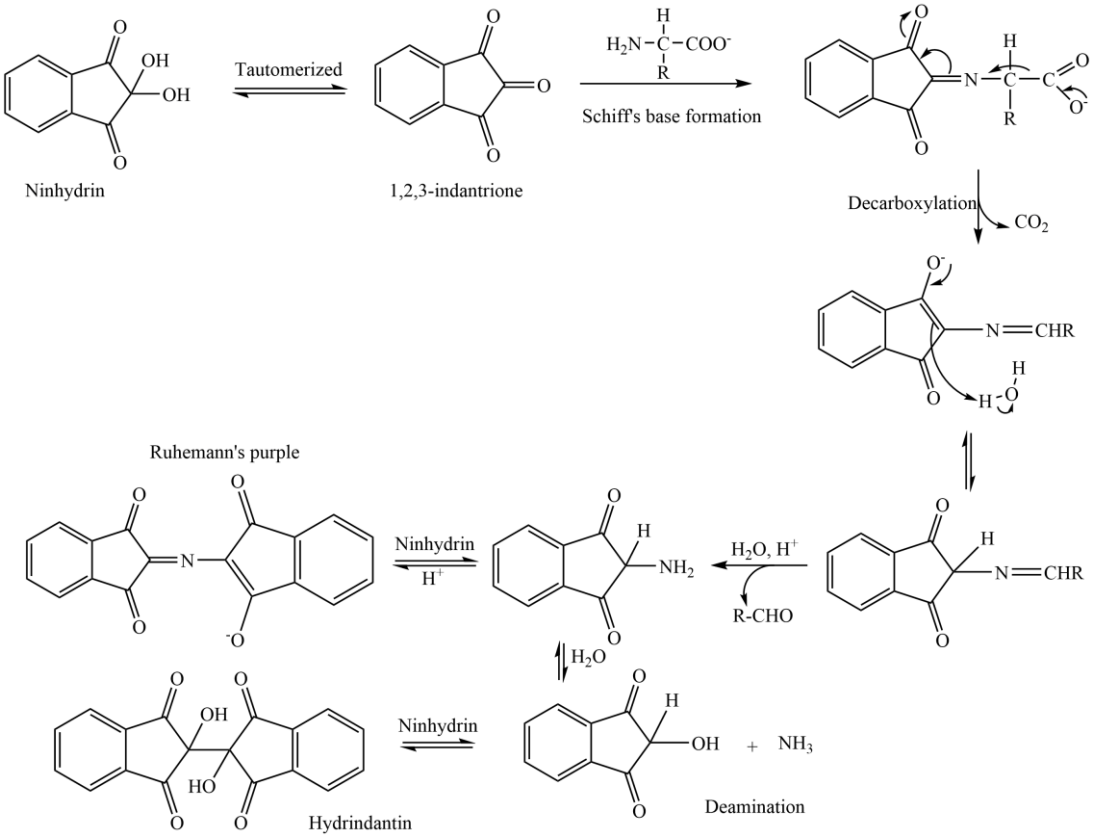

Figure 1-16. The reaction mechanism of ninhydrin reagent with α-amino acid.

The advantage of ninhydrin method is that it could reacts with primary and secondary amino acid. It is able to detect pmol of amino compounds. In contrast, the disadvantage is that it is rarely reproducible below 100 pmol and interferences with the matrix. Also, it is sensitive to light, O_2, temperature changes and pH.

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1.5 Derivatization-reagent and amino acids selection

1.5.1 Derivatization-reagent selection criteria

Herein, we do no choose PITC because multiple product peaks present in HPLC chromatogram and it is highly toxic. Cyanide (CN^-) is used as co-reagent in NDA method, which is highly toxic. The cost of FQ is prohibitive for general use (1485 USD/25 mg in sigma catalog). The disadvantage of IT is that it forms dimer with itself or thiol-active molecule. The drawback of N-alkyl-nicotinic acid is ease of hydrolysis; it should be stored in desiccator until used for amino acid analysis. Ninhydrin also shows multiple product peak in HPLC chromatogram. The products of Fluorescamine have two form, which are a kinetic balance. The list narrow down to five derivatization reagent: OPA, Dansyl, Fmoc, Marfey and Dabsyl. We chose them because of low reagent costs, ease of use, non-toxic, high reaction yield and quick reaction time.

1.5.2 Amino acids selection criteria

For comparison, 7 amino acids were chosen glutamine (Glu), serine (Ser), glycine (Gly), alanine (Ala), tyrosine (Tyr), norvaline (Nva), phenylalanine (Phe) so that their properties would represent the variability in amino acid structures. Choice of amino acids was mainly based on their chromatographic behavior (according to a hydrophobicity scale[88] in which each amino acid has a value reflecting its relative hydrophobicity and hydrophilicity): Glu and Ser are ionic/polar and elute faster, Nva and Phe are hydrophobic and elute more slowly, the rest covering the intermediates range. Hydrophobicity index: Phe > Nva > Tyr > Ala > Gly > Ser > Glu.

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1.6 Aim of this study

Although there are multiple useful derivatization reagents commercially available for amine-containing metabolite, but we cannot find any extensive study that compares to their relative strengths and weaknesses under optimized conditions. Specifically, we wanted to compare these five reagents: OPA, Fmoc, Dansyl, Marfey and Dabsyl. In the present study, we will compare them using LC-MS/MS with absorbance/fluorescence detections setup for the following properties (1) UV/Vis absorbance and fluorescence (2) hydrophobicity and separation (3) ionization efficiency (4) fragmentation energy and fingerprints in tandem MS, aiming to understand which reagent is suitable for which types of experiments. Because absorbance, fluorescence, ionization, separation could be affected by the mobile phase composition, we chose three standard gradient elution conditions for comparison. In solvent A (H_2O), we added 0.1% formic acid (pH 2.6), 2 mM ammonium acetate (pH 5), or 2 mM ammonium bicarbonate (pH 8). Solvent B is 100% ACN. The relative strengths and weaknesses of each reagent were studied and compared, and we wish to identify the optimal operating condition for each derivatization method and further research on synapse metabolomics experiments.

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Chapter 2 RESULTS AND DISUSSION

2.1 UV/Vis absorbance and Fluorescence intensity assay

2.1.1 UV/Vis absorbance assay

Most amino acids are not optically active, lacking both UV/Vis absorbance and fluorescence. Although aromatic amino acids can absorb UV light, it is still much better to derivatize them to bestow stronger chromophores for sensitive detection and quantification.

The chemistry of the five derivatization reagents have been described in Introduction, and their absorption maxime are listed below OPA 340 nm, Fmoc 262 nm, Dnsyl 254 nm, Marfey 340 nm and Dabsyl 436 nm.

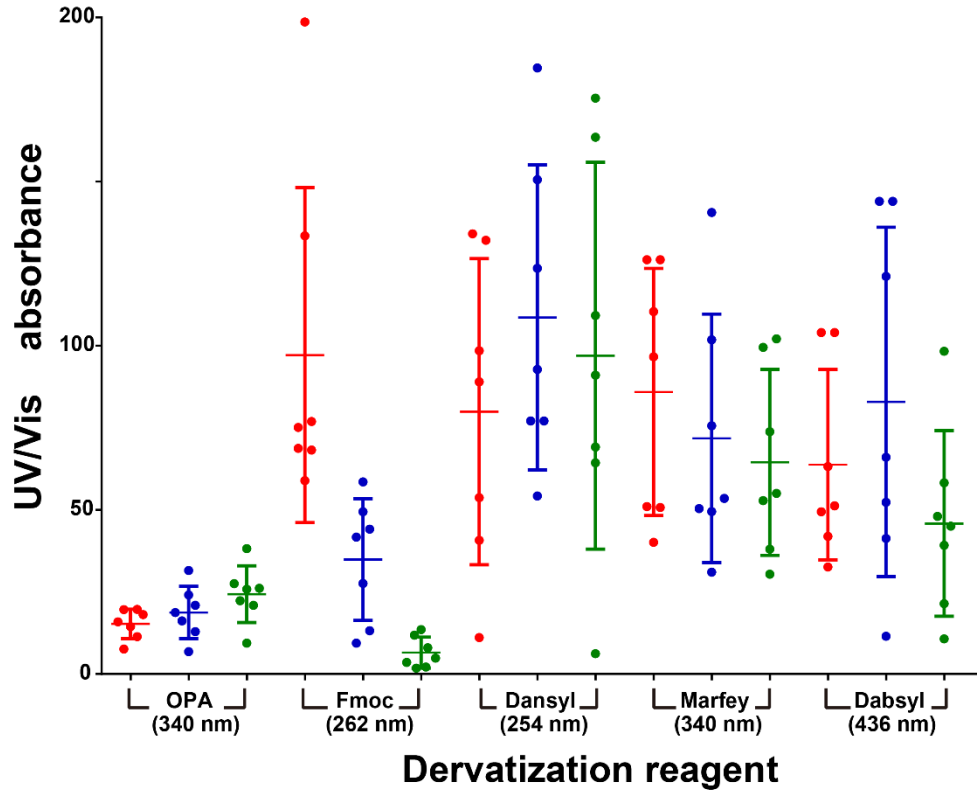

Figure 2-1 UV/Vis absorbance of different derivatization reagent at three pH condition.

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Red color indicates pH 2.6 condition, blue color is pH 5 and green color is pH 8. The middle horizontal bar indicates mean of UV/Vis absorbance intensity of seven amino acids, while upper bar is mean plus two standard deviations and lower bar is mean minus two standard deviations.

For each reagent, we measured absorbance under three different conditions with HPLC: 0.1% FA pH 2.6, AA 2 mM pH 5, ABC 2 mM pH 8. As shown in Figure 2-1 Fmoc product absorbance is highly pH dependent, but OPA, Dansyl, Marfey and Dabsyl are not. In Fmoc method, acidic condition would be the best environment for UV/Vis absorbance. The low intensity at pH 8 may arise from deprotonation on fluorine ring. Deprotonation on 9-position of fluorene ring causes the absorbance declining quickly[89,90]. In OPA method, pH has no significant difference on UV/Vis intensity performance, although its absorbance is generally weak. In Dansyl, Marfey and Dabsyl method, their absorbance is little influence by pH changes. The relative strength of intensity is Dansyl > Marfey > Dabsyl. On the other hand, at pH 2.6 Fmoc, Dansyl, Fmoc and Dabsyl display higher intensity than OPA method. At pH 5 and pH 8, Dansyl has the highest performance over the four reagents.

2.1.2 Fluorescence intensity assay

The excited wavelength and emission wavelength of each derivatized product are summarized below. OPA is excited at 340 nm and emitted at 450 nm; Fmoc is excited at 262 nm and emitted at 325 nm; Dansyl is excited at 320 nm and emitted at 525 nm.

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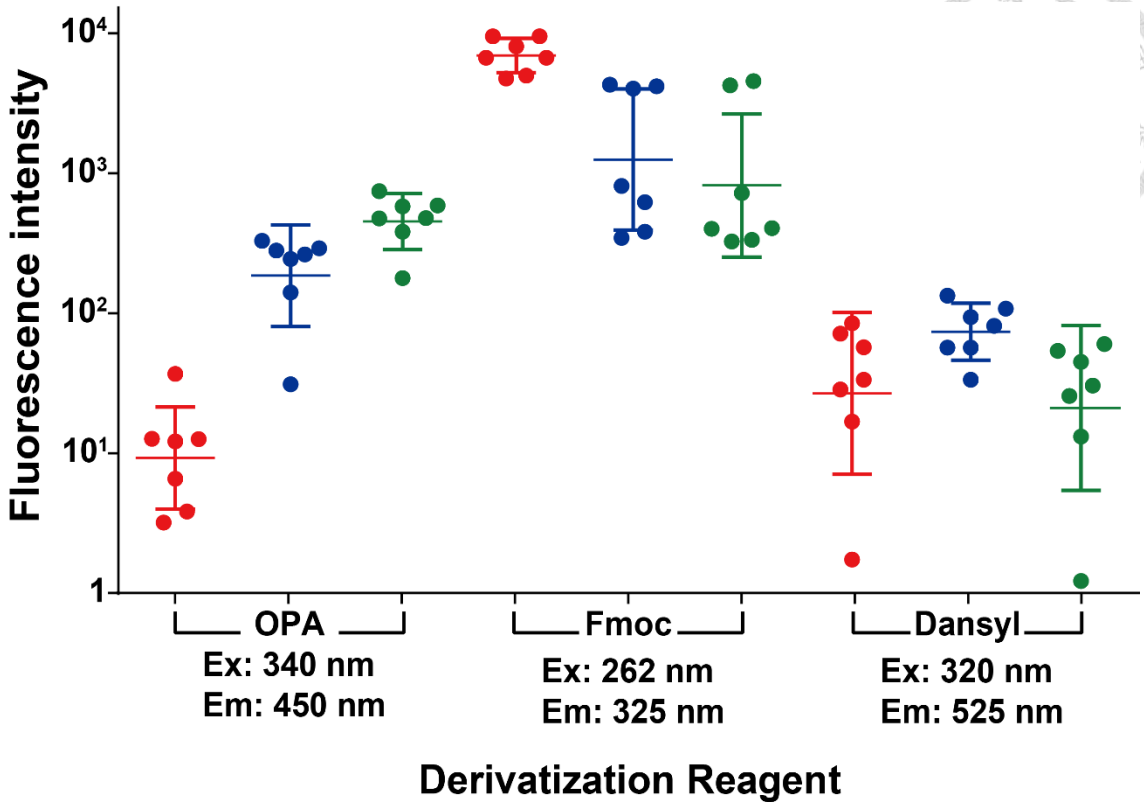

Figure 2-2 The effect of pH on the fluorescence performance of different derivatization products. Red color indicates pH 2.6 condition, blue color is pH 5 and green color is pH 8. The middle horizontal bar indicates mean fluorescence intensity of seven amino acids, while upper bar is mean plus two standard deviations and lower bar is mean minus two standard deviations.

For each reagent, we measured fluorescence under three different conditions with HPLC: 0.1% FA pH 2.6, AA 2 mM pH 5, ABC 2 mM pH 8. As shown in Figure 2-12 OPA has optimal fluorescence at pH 8 but very low fluorescence at pH 2.6. Fmoc has strongest fluorescence over the other reagents and optimal at pH 2.6. The fluorescence of OPA and Fmoc's products show greater pH dependence while Dansyl's products are only slightly influence by pH. In OPA method, the fluorescence intensity at pH 8 is nearly 100 times larger (two orders of magnitude) than pH 2.6. This huge difference may contribute to the

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protonation of the nitrogen atom on isoindole ring. After protonation, it lost aromatic character to further diminishing fluorescence performance. However, in Fmoc method, there is an opposite trend where acidic pH condition shows greater fluorescence intensity than basic pH condition. The possible reason of decreasing fluorescence intensity at basic condition may arise from deprotonation on fluorene ring. In Dansyl method, there is little difference in fluorescence intensity among three pH condition.

2.1.3 Limit of detection of Fmoc method

From the previous fluorescence result, Fmoc method displays highest intensity over OPA and Dansyl. In order to estimate the LOD and LOQ of Fmoc method, we prepared six serine amino acid standard solutions at different concentrations and measured in triplicate repeats. Figure 2-3 is the result of LOD and LOQ of Fmoc method in H_2O (contained 0.1% FA), pH 2.6. LOD and LOQ were defined as $3.3\times\frac{Sa}{b}$ for LOD, $10\times\frac{Sa}{b}$ for LOQ from ICH Q2(R1) document[91], where S_a and b symbolized the standard deviation of the y-intercept and the slop of regression line, respectively. As shown in Figure 2-3, all calibration curves showed good linearity (R^2= 0.9997). The LOD and LOQ of the Fmoc-Ser were $1.15*10^{-8}$ M and $3.48*10^{-8}$ M, respectively.

doi:10.6342/NTU201601534

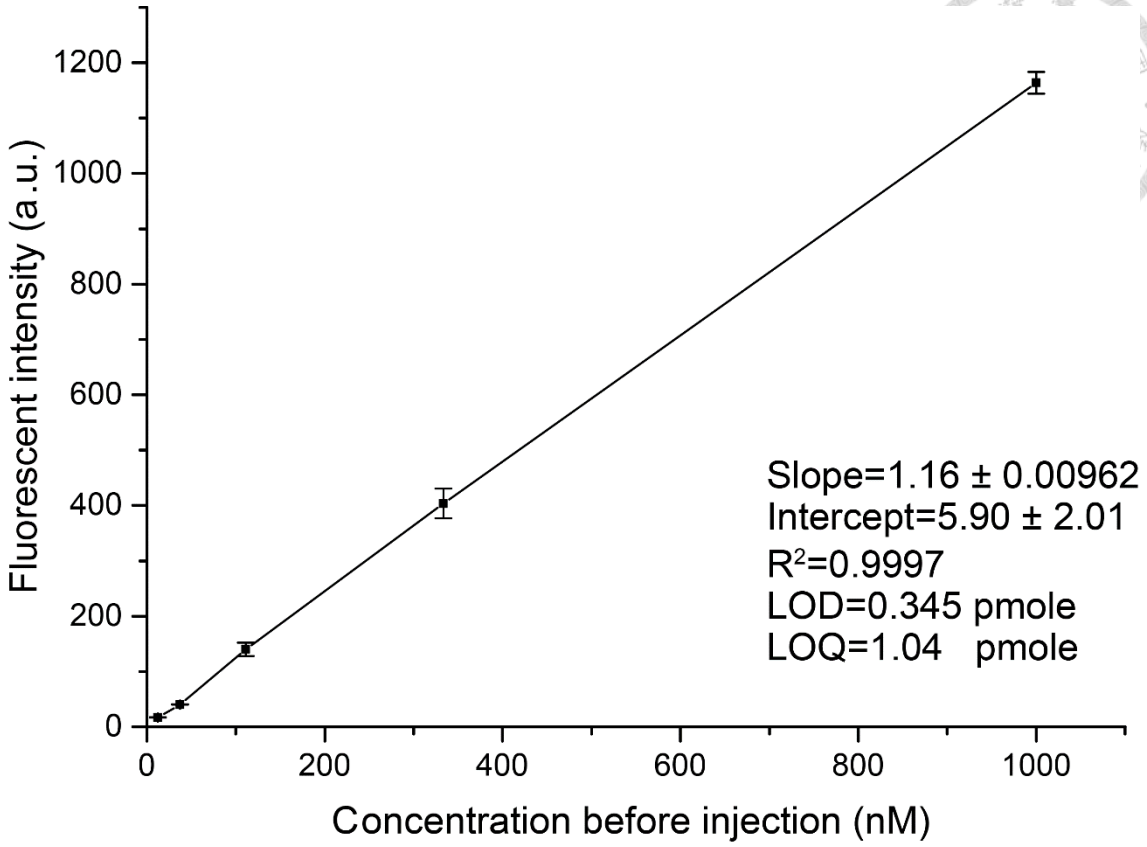

Figure 2-3. Results of LOD and LOQ of Fmoc method. (In the regression equation y=ax+b, x refers to the concentration (M), y indicates the fluorescence peak area, and R^2 is the correlation coefficient of the equation.)

2.2 Chromatographic performance

2.2.1 Retention time (hydrophobicity)

Strong hydrophilic compounds often have very short retention time on reverse phase columns, and the introduction of a hydrophobic group can increase their retention time and improve separation. There are three mobile phase conditions used to compare these reagents: (1) mobile phase (A) H_2O with 0.1% formic acid, mobile phase (B) 100% ACN. (2) mobile phase (A) 2 mM ammonium acetate pH 5 and mobile phase (B) 100% ACN. (3) mobile phase (A) 2 mM ammonium bicarbonate pH 8 and mobile phase (B) 100%

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ACN. Formic acid (FA), ammonium acetate (AA) and ammonium bicarbonate (ABC) is only added to mobile phase (A).

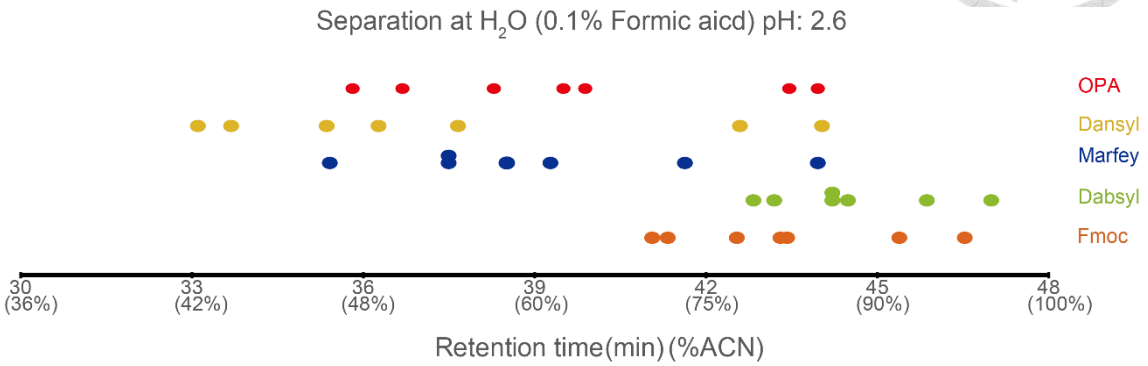

Figure 2-4. Chromatographic distribution of seven amino acids with different derivatization reagent (OPA, Marfey, Dansyl, Fmoc and Dabsyl) at H_2O, 0.1% formic acid, pH 2.6.

Figure 2-4 shows the retention time and hydrophobicity introduced by each derivatization reagent in an aqueous / organic (H_2O / ACN) mobile phase with 0.1% FA, pH 2.6. It is obvious that all five derivatization reagent enhance hydrophobicity significantly. The adducts elute out in a range of 40% ~ 100% ACN at linear gradient settings. Dabsyl and Fmoc exhibit the strongest hydrophobicity as their products elute out very late (at 75% ~ 100% ACN). Strong interaction of rigid tricyclic fused ring (Fmoc) and the longer molecular structure (Dabsyl) with C_{18} chain increases retention time.

2.2.2 Separation performance assay

In order to improve separation resolution, ionic buffer substances can be used in LC mobile phases. Ammonium acetate and ammonium bicarbonate are common volatile buffers used in LC-MS/MS experiments. They may improve good separation efficiency

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or enhance ESI ionization efficiency. 2 mM ammonium acetate or ammonium bicarbonate is sufficient to improve chromatographic separation and is compatible with mass analysis.

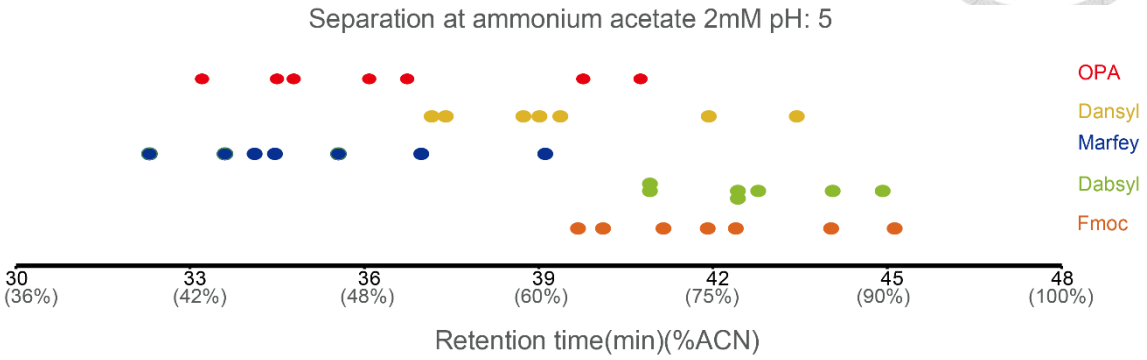

Figure 2-5. Chromatographic distribution of seven amino acids with five derivatization reagents (OPA, Marfey, Dansyl, Fmoc and Dabsyl) with 2 mM ammonium acetate pH 5 in H_2O/ACN gradient.

The experimental result in Figure 2-5 shows that 2 mM of ammonium acetate is sufficient to improve separation with OPA, Marfey and Fmoc but not Dabsyl and Dansyl. This condition has difficulty to separate Ser/Glu and Gly/Ala pairs in Dabsyl method.

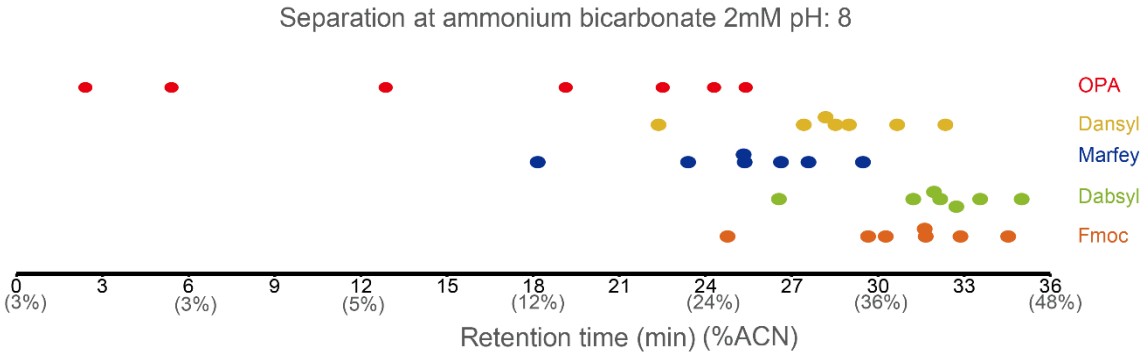

Figure 2-6. Chromatographic distribution of seven amino acids with different derivatization reagents (OPA, Marfey, Dansyl, Fmoc and Dabsyl) with 2 mM ammonium bicarbonate pH 8 in H_2O/ACN gradient.

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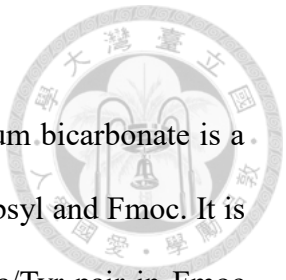

The experiment result in Figure 2-6 shows that 2 mM ammonium bicarbonate is a good buffer for OPA method but not suitable for Dansyl, Marfey, Dabsyl and Fmoc. It is difficult to separate Gly/Ala pair in Dansyl, Marfey, Dabsyl and Ala/Tyr pair in Fmoc method under this condition. Second, ammonium bicarbonate shortens the retention time of overall adduct of five derivatization reagents. All of the products elute out before 48% ACN. However, this phenomenon is not observed in ammonium acetate condition. This may be related to pH effect on silanol group of column. Because pKa value of silanol group is 6.5, residual silanol group deprotonate at pH 8. The carboxyl group on MPA molecule (OPA method) and amino acid also deprotonate at this condition. Charge repellence between deprotonating siloxide and carboxyl group become stronger. In OPA method, this repelling phenomenon is strongest due to two carboxylate group presence on structure. Glu-and Ser-adducts elute out before 6 minute.

The separation of OPA products at three mobile phase composition is different between different buffer pH (Figure 2-7).

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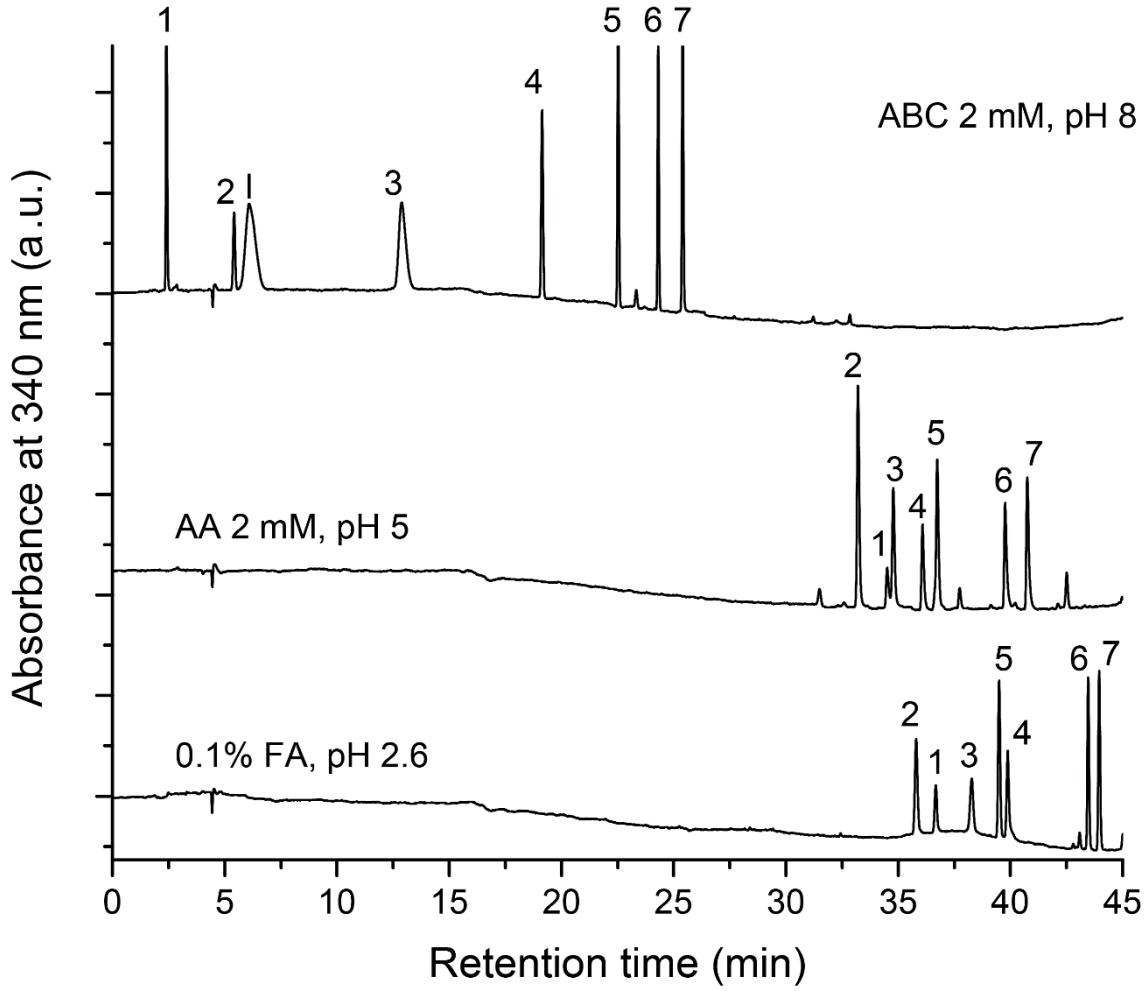

Figure 2-7. HPLC chromatogram of OPA products at different mobile phase composition. Number 1 denotes Glu, 2 denotes Ser, 3 denotes Gly, 4 denotes Ala, 5 denotes Tyr, 6 denotes Nva, 7 denotes Phe and I denote unidentified peak with *m/z*=446.

The separation of Marfey products at three mobile phase composition is different between different buffer pH (Figure 2-8).

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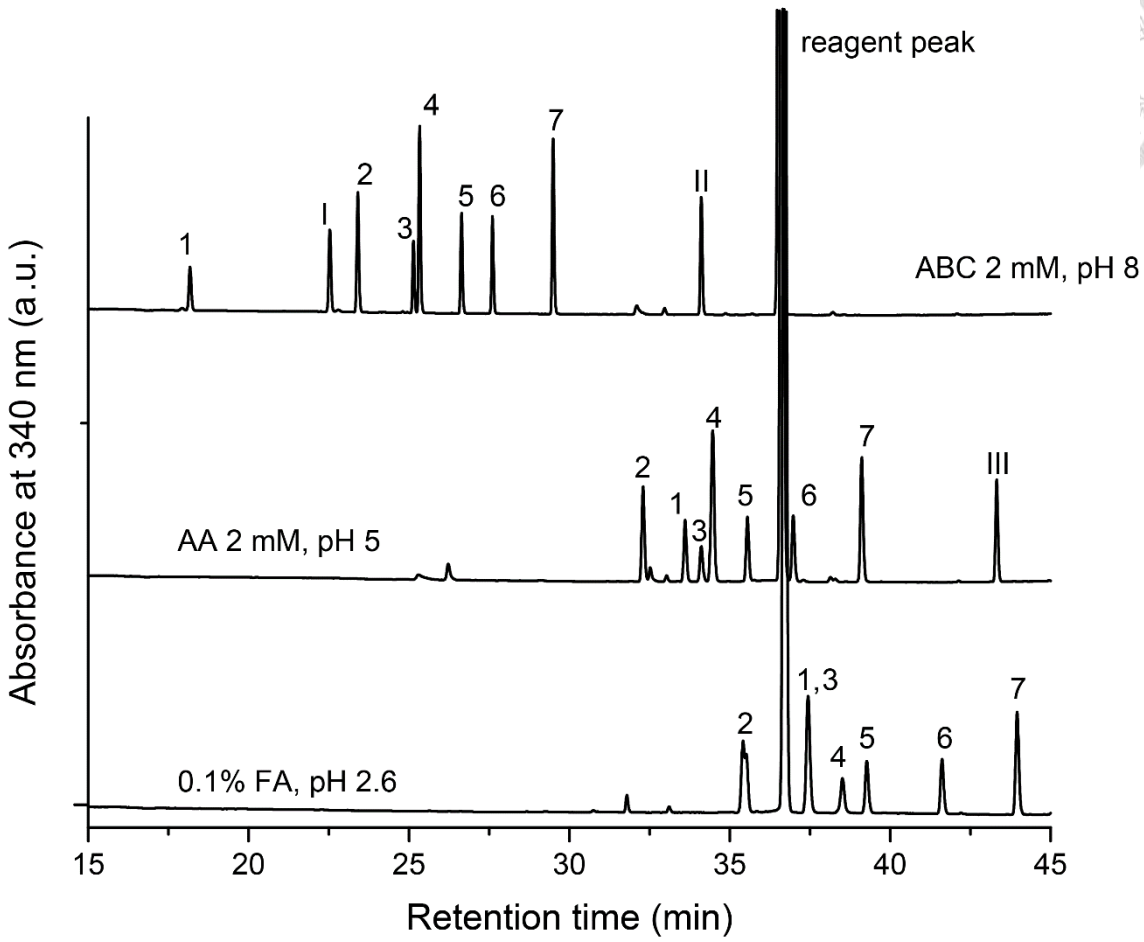

Figure 2-8. HPLC chromatogram of Marfey products at different mobile phase composition. Number 1 denotes Glu, 2 denotes Ser, 3 denotes Gly, 4 denotes Ala, 5 denotes Tyr, 6 denotes Nva, 7 denotes Phe and I denote unidentified peak with *m/z*=226, II denotes unidentified peak with *m/z*=237, III denotes unidentified peak with *m/z*=105.

The separation of Fmoc products at three mobile phase composition is different between different buffer pH (Figure 2-9).

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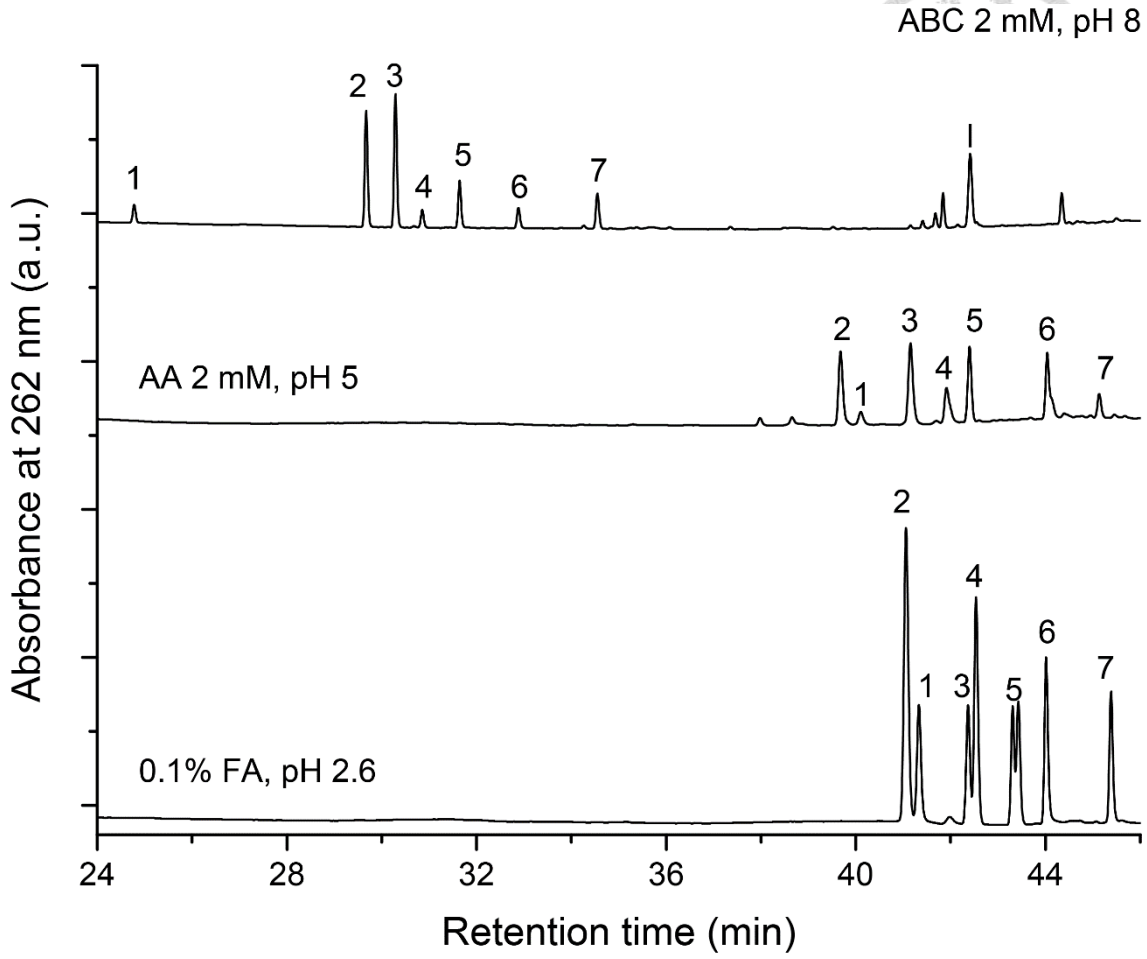

Figure 2-9. HPLC chromatogram of Fmoc products at different mobile phase composition. Number 1 denotes Glu, 2 denotes Ser, 3 denotes Gly, 4 denotes Ala, 5 denotes Tyr, 6 denotes Nva, 7 denotes Phe and I denote unidentified peak with *m/z*=166.

The separation of Dansyl products at three mobile phase composition is different between different buffer pH (Figure 2-10).

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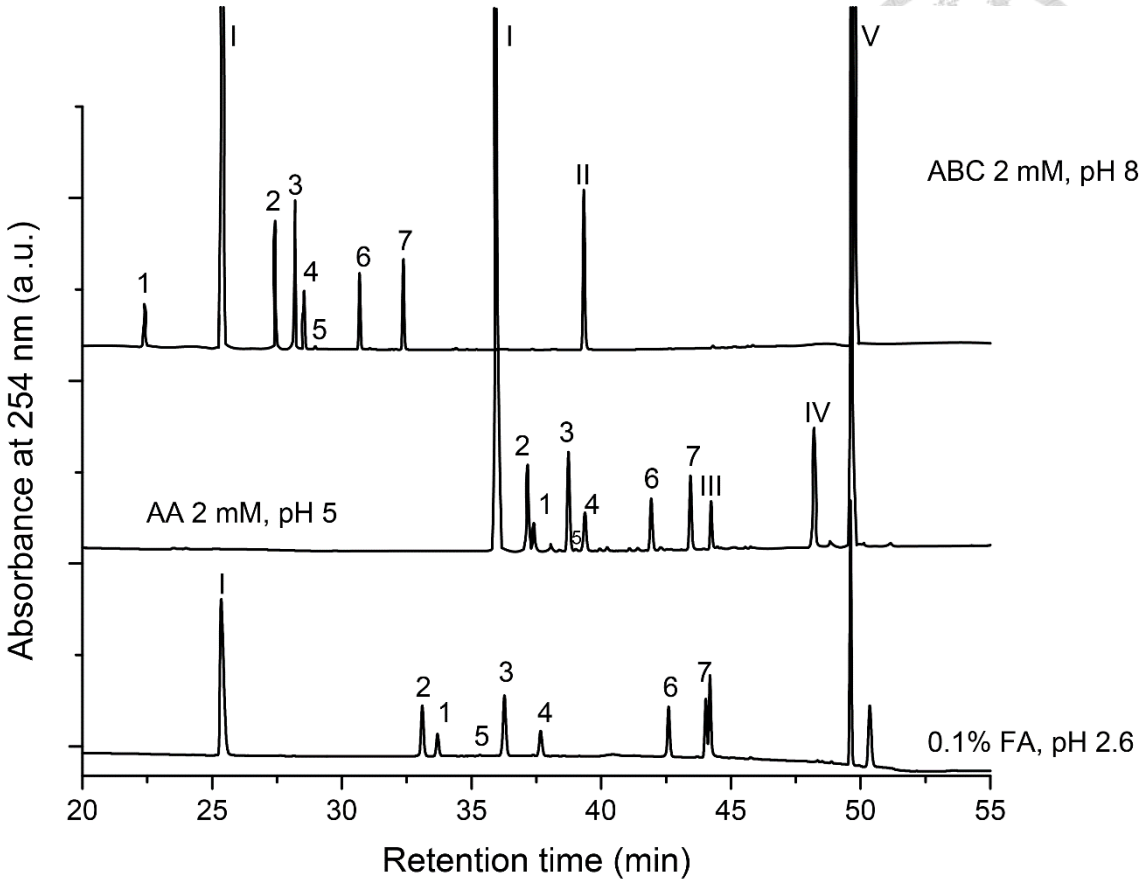

Figure 2-10. HPLC chromatogram of Dansyl products at different mobile phase composition. Number 1 denotes Glu, 2 denotes Ser, 3 denotes Gly, 4 denotes Ala, 5 denotes Tyr, 6 denotes Nva, 7 denotes Phe and I denote Dan-OH peak, II denotes unidentified peak with *m/z*=324, III denotes unidentified peak with *m/z*=376, IV denotes unidentified peak with *m/z*=245, V denotes Dan-heptylamine peak.

The separation of Dabsyl products at three mobile phase composition is different between different buffer pH (Figure 2-11).

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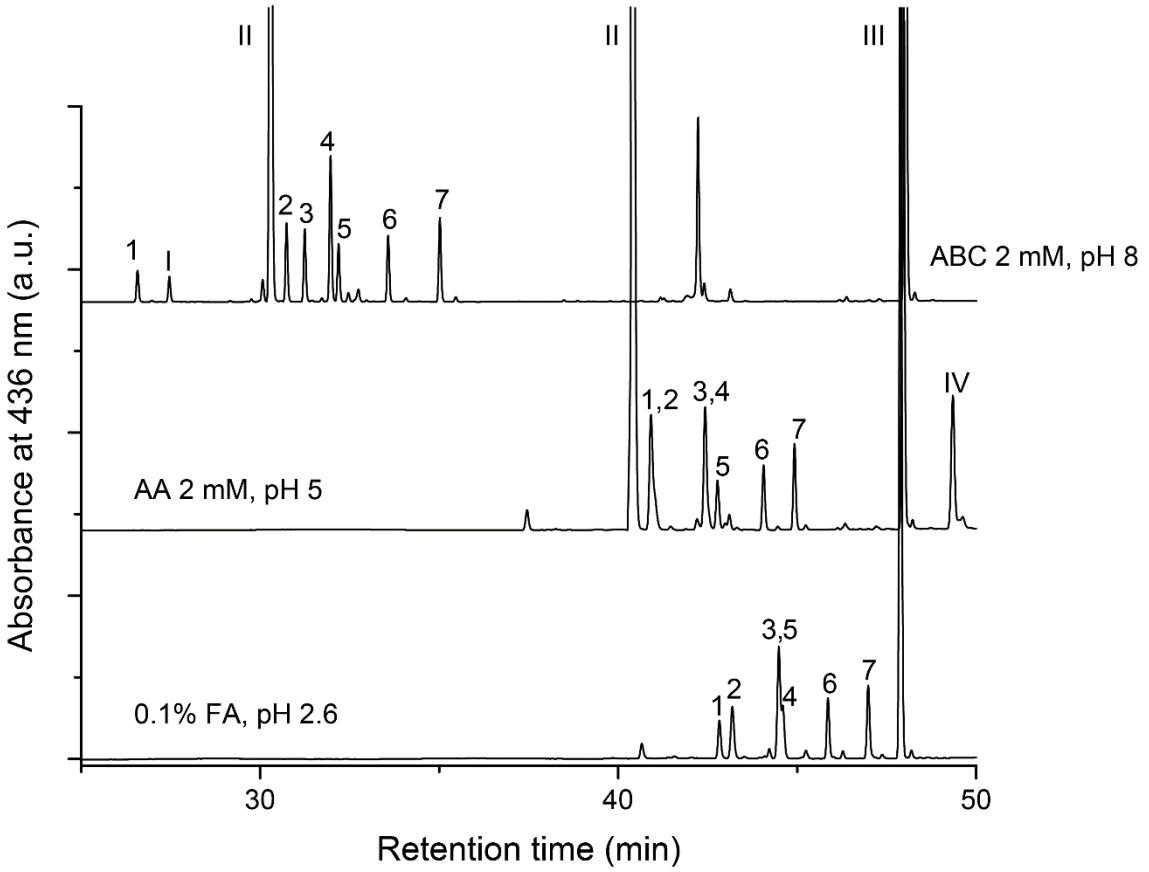

Figure 2-11. HPLC chromatogram of Dabsyl products at different mobile phase composition. Number 1 denotes Glu, 2 denotes Ser, 3 denotes Gly, 4 denotes Ala, 5 denotes Tyr, 6 denotes Nva, 7 denotes Phe and I denote unidentified peak with *m/z*=292, II denotes Dab-OH peak with, III denotes unidentified peak with *m/z*=334, IV denotes unidentified peak with *m/z*=378.

2.3 Ionization efficiency of different derivatization reagents

Introducing chargeable or easily ionizable moieties would help neutral compounds or poorly ionizing compounds increase ionization efficiency for MS detection. Typically, the introduction of basic chemical group, including primary, secondary, tertiary amine

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and ferrocene moieties, will significantly increase the signal response in the positive ESI mode.

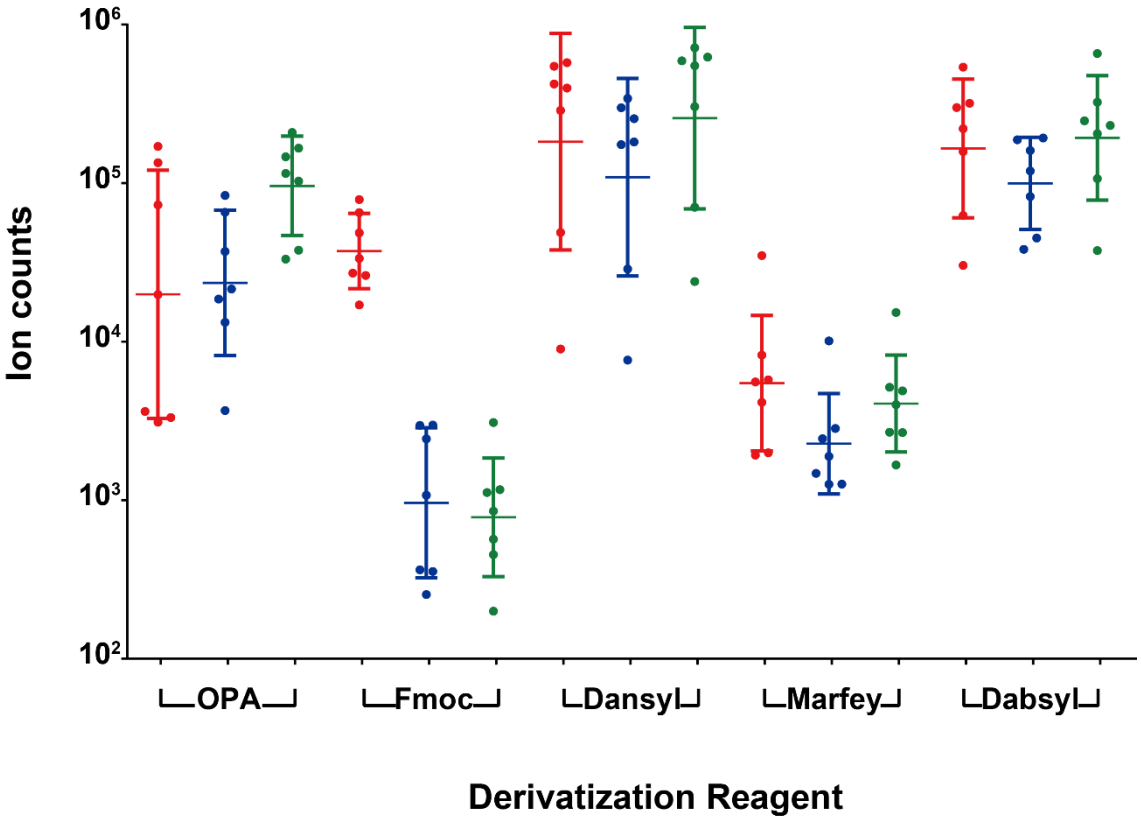

Figure 2-12. The ionization efficiency of seven amino acids with different derivatization reagents (OPA, Marfey, Dansyl, Fmoc and Dabsyl) at different pH value. Red color indicates pH 2.6 condition, blue color is pH 5 and green color is pH 8. The middle horizontal bar indicates mean of ion counts of seven amino acids of certain derivitization reagent, while upper bar is mean plus two standard deviations and lower bar is mean minus two standard deviations.).

In general, those from Dansyl, Dabsyl and OPA derivatives exhibit higher ionization efficiency than Fmoc and Marfey methods. This significant difference may arise from the tertiary amine group on Dansyl and Dabsyl structure. Tertiary amine group is prone to be

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charged in the positive-ion ESI mode. In OPA method, aromatic amine is also a basic chemical group, it contributes to ionization in the positive-ion ESI mode. On the other hand, Fmoc products show relatively high ion count in acidic condition with a much lower response when salt is presented at higher pH. Salt were known to deteriorate the detection of Marfey's derivatives[92]. Performing desalting before injection into HPLC or using pure aqueous/ organic mobile phase (H_2O/ ACN) may help increase low ionization efficiency.

2.4 Tandem mass spectroscopy

2.4.1 Fragmentation energy and fragmentation pattern

Reliable identification of metabolites cannot be solely determined by MS spectra. Further confirmation by MS/MS spectra can provide useful information for compound identification. In this study, we used CID to study the fragmentation properties of different derivatization reagents. The critical factor in MS/MS experiments is how collision energy affects the fragmentation pattern of derivatization products, which can be further used for the purpose for amine validation and MRM quantification.

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Characteristic ion \ Collision energy (eV)	2	4	6
m/z=179.09	+++	+++	+++
$[M+H]^+$	+	+	ND

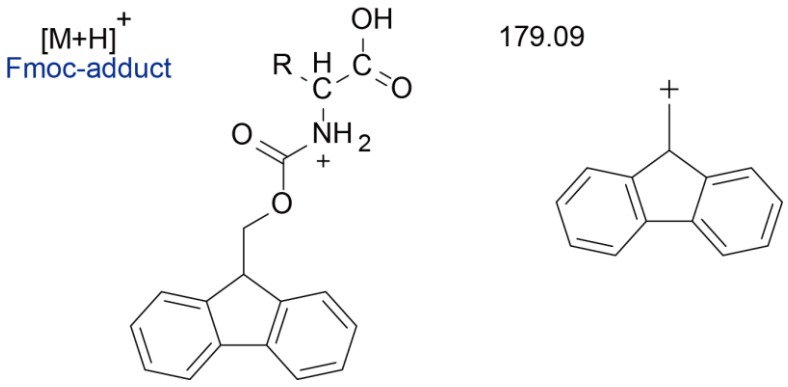

Figure 2-13. Summary of characteristic ion and fragmentation energy of Fmoc adduct. (ND: not detection, +, ++, +++ represents relative signal strength)

The Fmoc derivatives gave the characteristic cleavage at the carbamate bond by CID, and produced characteristic fragment ions at *m/z*=179, derived from the reagent moiety. This *m/z*=179 fragment was also reported by other paper and used in SRM[93] experiment. Its collision energy 2 eV is the lowest energy among the five derivatization methods.

We chose 2 eV as standard collision energy for Fmoc because it has a small abundance of precursor ion (Figure 2-14). Furthermore, the intensity of product ion at *m/z*=179 is very high.

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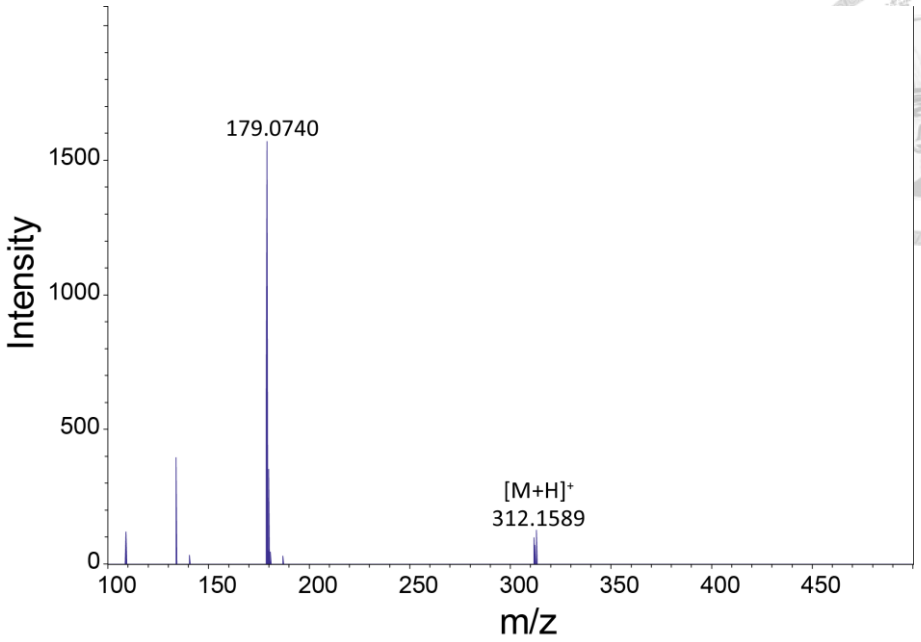

Figure 2-14. MS/MS spectrum of Fmoc-Ala adduct at collision energy of 2 eV.

Characteristic ion \ Collision energy (eV)	2	4	8	16	20
m/z=170.30, 171.10, 172.06	ND	+	++	+++	+
m/z=157.07	ND	ND	+	++	+++
$[M+H-CH_3]^+$	ND	ND	+	+	+
$[M+H]^+$	+++	+++	++	++	+

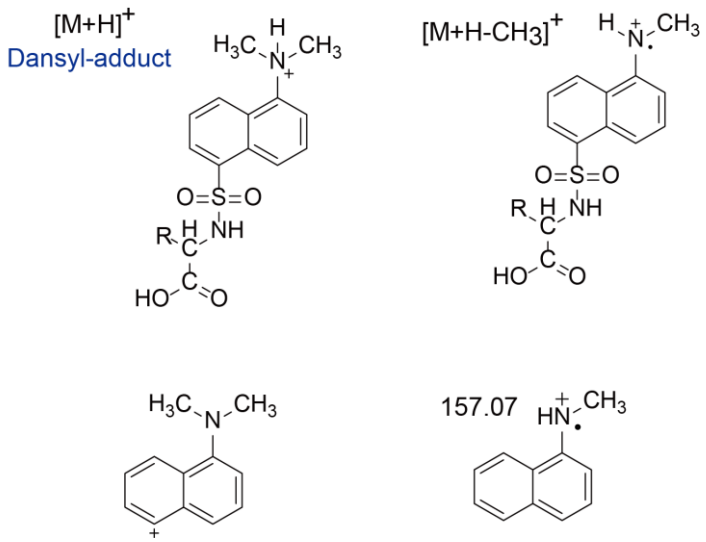

Figure 2-15. Summary of characteristic ion and fragmentation energy of Dansyl adduct.

doi:10.6342/NTU201601534

(ND: not detection, +, ++, +++ represents relative signal strength)

The fragmentation of Dansyl-derivatives gave the product ion at *m/z*=170, derived from protonated dimethylaminonaphtyl moiety (Figure 2-15). The other product ion had *m/z*=157 was formed after *m/z*=170 fragment lost a methyl group. The 172 Da and 157 Da fragments were also reported by other papers and used in SRM[94,95] or MRM[96] experiments. Howerer, the optimal fragmentation energy was 20 eV, which was much higher than Fmoc method.

We chose 20 eV as standard collision energy for Dansyl because it has small abundance of precursor ion (Figure 2-16). Furthermore, the intensity of product ion at *m/z*=157 is very high.

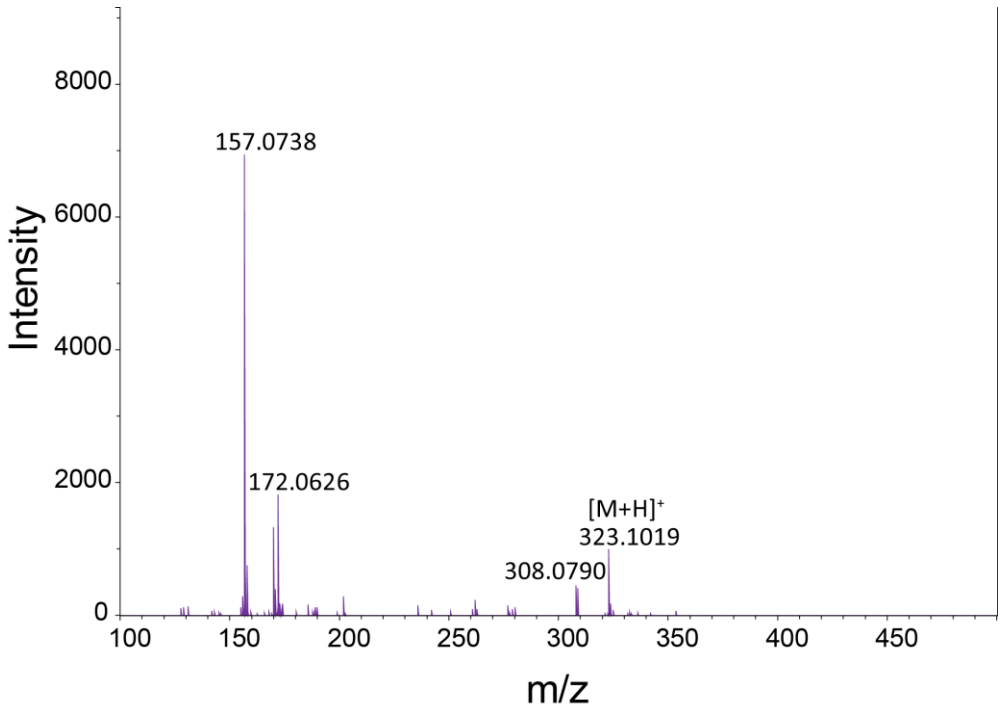

Figure 2-16. MS/MS spectrum of Dansyl-Ala adduct at collision energy of 20 eV.

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Collision energy (eV) / Characteristic ion	2	4	8	16	20	m/z
[M+H-MPA]+	ND	ND	+	++	+++	X-105.10
$[M+H]^+$	+++	+++	++	++	+	X

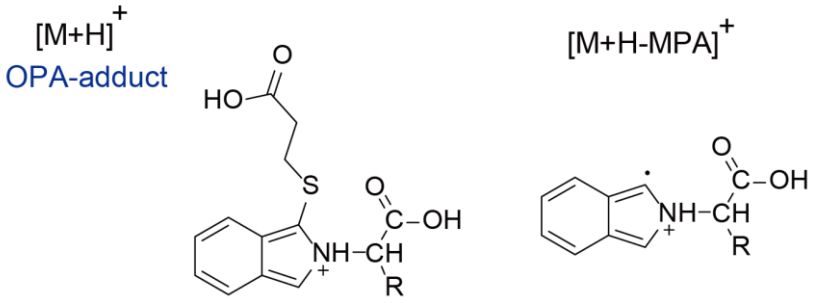

Figure 2-17. Summary of characteristic ion and fragmentation energy of OPA adduct. (ND: not detection, +, ++, +++ represents relative signal strength)

The fragmentation of OPA adducts generated the product ion in the form of $[M+H-MPA]^+$ fragment by CID, due to the loss of mercaptopropionic acid moiety. It did not produce a fixed *m/z* product ion like Dansyl and Fmoc method. On the other hand, it lost a fixed side chain moiety and resulted in a fixed loss of *m/z* relative to its native structure (for example, amino acid in our model). The optimal fragmentation energy was 20 eV similar to the Dansyl method.

We chose 20 eV as standard collision energy for OPA because it has small abundance of precursor ion (Figure 2-18). Furthermore, the intensity of product ion at $[M+H-OPA]^+$ is very high.

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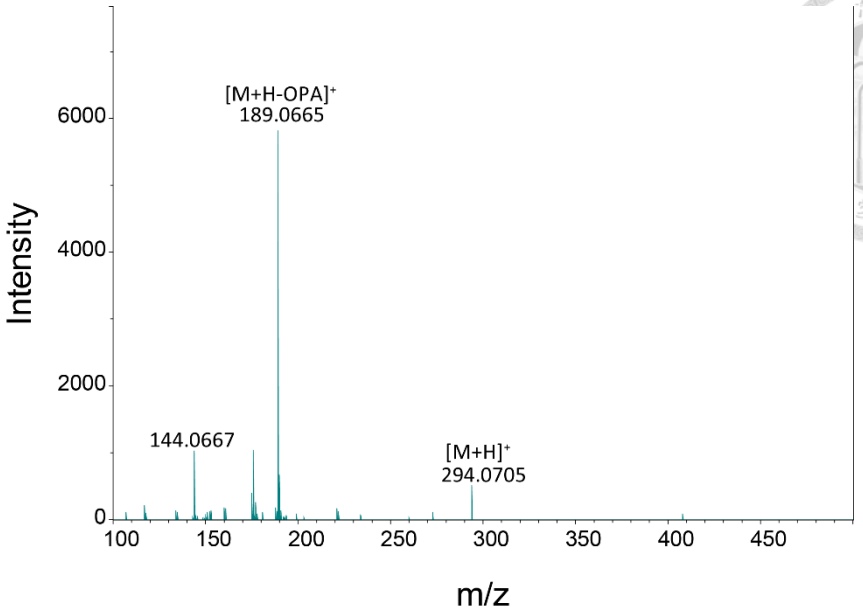

Figure 2-18. MS/MS spectrum of OPA-Ala adduct at collision energy of 20 eV.

Collision energy (eV) / Characteristic ion	2	8	16	20	25
m/z=305.10	ND	ND	+	+	+
m/z=224.11	ND	+	++	++	+++
m/z=134.08	ND	ND	+	++	++
m/z=120.08	ND	+	++	+++	+++
m/z=106.06	ND	ND	+	+	+
$[M+H]^+$	+++	+++	++	++	+

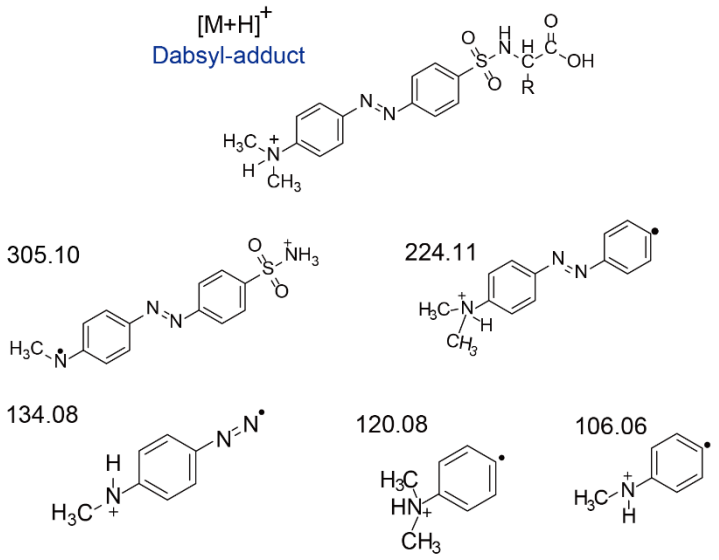

Figure 2-19. Summary of characteristic ion and fragmentation energy of Dabsyl adduct.

doi:10.6342/NTU201601534

(ND: not detection, +, ++, +++ represents relative signal strength)

The fragmentation of Dabsyl derivatives gave several major product ions at *m/z* values of 224, 134, and 122 by CID, derived from the protonated adduct. The 224 Da fragment was assigned to carbon-sulfoxide bond cleavage product; 134 Da fragment was assigned to aromatic sp^2-hybridized C-atom-azo group bond cleavage. The 120 Da fragment was assigned to the pronated dimethylamino phenyl. 305 Da and 106 Da species were minor product ions, and hence they are not suitable for the choice of SRM or MRM experiments. The 305 Da fragment may come from neutral loss of amino acid α-carbon side moiety and methyl group. The 106 Da fragment was assigned to the loss of methyl group from the 120 Da ion. The 224 Da fragment were also reported by other paper and used in SRM[97] or MRM[98] experiments. The optimal fragmentation energy was 25 eV for Dabsyl, which was the highest among the five methods.

We chose 25 eV as standard collision energy for Dabsyl because it has small abundance of precursor ion (Figure 2-20). Furthermore, the intensity of product ion at *m/z*=224 and 120 is very high.

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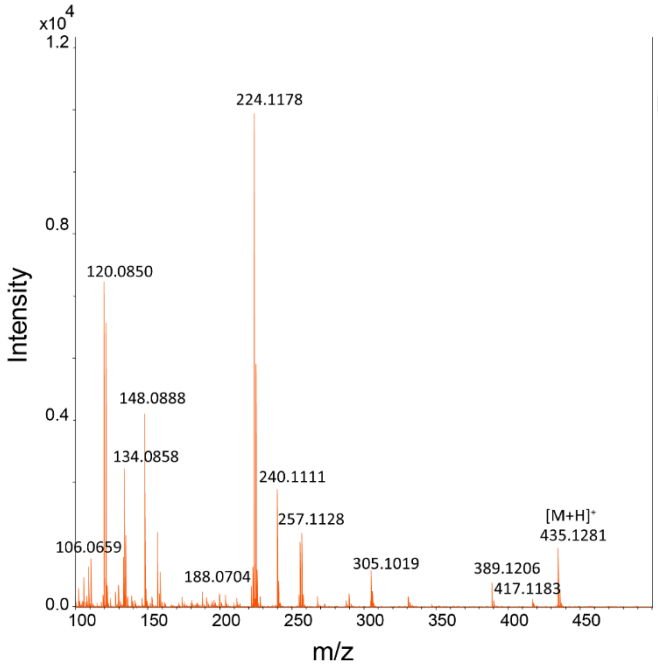

Figure 2-20. MS/MS spectrum of Dabsyl-Ala adduct at collision energy of 20 eV.

Marfey-adduct

Collision energy (eV) / Characteristic ion	2	4	6	8	10	m/z
$[M+H-NO_2]^+$	+	++	+++	+++	+++	X-45.02
$[M+H-NO_2-CONH_2]^+$	+	++	++	+++	+++	X-90.00
$[M+H-NO_2-CONH_2-OH]^+$	+	+	+	++	++	X-107.00
$[M+H]^+$	+++	++	++	+	ND	X

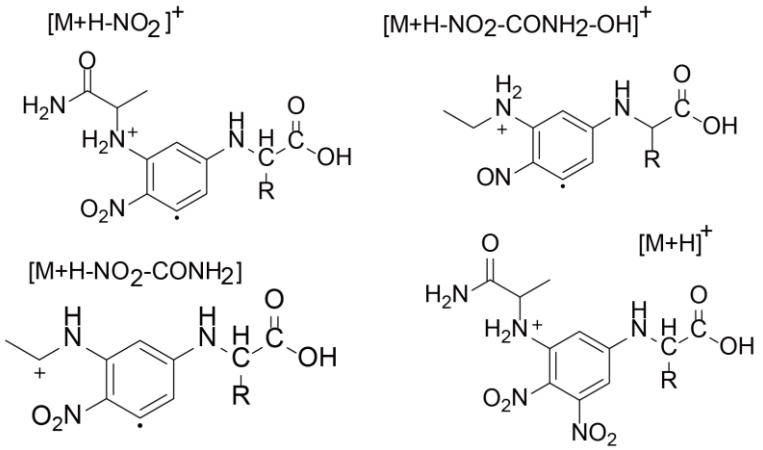

Figure 2-21. Summary of characteristic ion and fragmentation energy of Marfey adduct.

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(ND: not detection, +, ++, +++ represents relative signal strength)

The fragmentation of Marfey produces was similar to the OPA method, showing the loss of a fixed side chain moiety and resulting in a fixed loss of *m/z* value relative to its native structure. Nitro-aromatic compound had two routes[99] by which nitro group could be lost: (1) directly losing NO_2 group (2) losing OH radical first, followed by dissociation of the NO group. In Marfey's method, it was easy to lose the NO_2 group, generating $[M+H-NO_2]$ product ion. Furthermore, dissociation of the hydroxyl radical was also detected, which led to the generation of the $[M+H-NO_2-CONH_2-OH]$ product ion. $[M+H-NO_2-CONH_2]$ fragment was assigned to the loss of terminal amide (sequential neutral loss of NH_3 and CO group) and the nitro group.

We chose 6 eV as standard collision energy for Marfey because it has small abundance of precursor ion (Figure 2-22). Furthermore, the intensity of product ion $[M+H-NO_2]^+$ is very high.

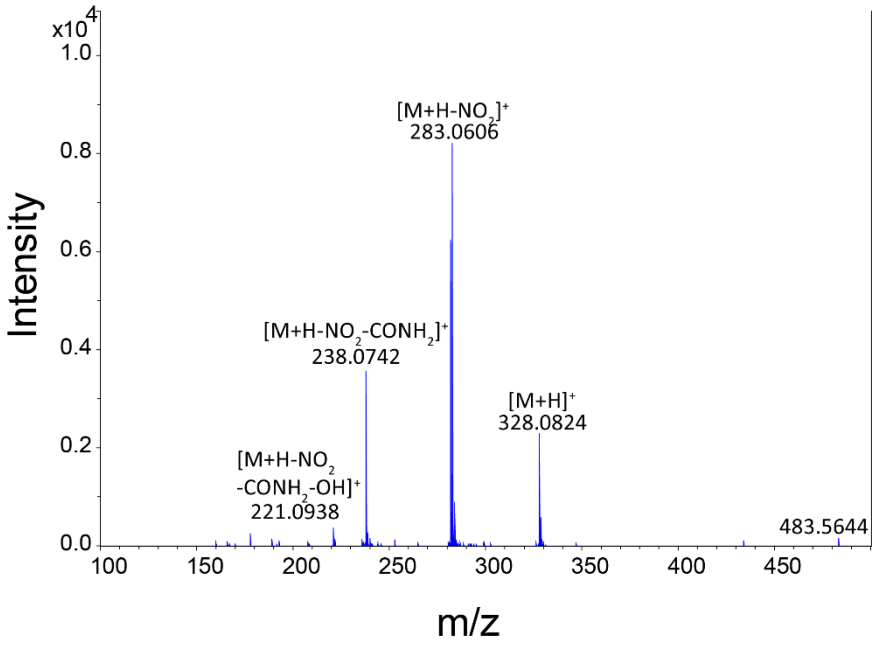

Figure 2-22. MS/MS spectrum of Marfey-Ala adduct at collision energy of 6 eV.

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2.5 Discussion

2.5.1 General description of five derivitization reagent at its optimal condition

Here we summarize the useful properties of each derivatization method under its optimal LC-MS/MS condition. We use a radar diagram to represent the following properties: (1) fluorescence intensity (2) LC separation efficiency (3) hydrophobicity (4) ionization efficiency (5) ease of fragmentation in CID (6) MS/MS fingerprint. In the MS/MS fingerprint category, a good response is defined by generating an intense and characteristic product ion, a medium response is defined by losing a fixed *m/z* value by neutral loss. A poor response is defined by random fragmentation. In the ease of fragmentation category, a good response is defined by the energy which is 1-9 eV range, a medium response is defined by the energy which is 10-19 eV range, a poor response is defined by the energy which is larger than 20 eV.

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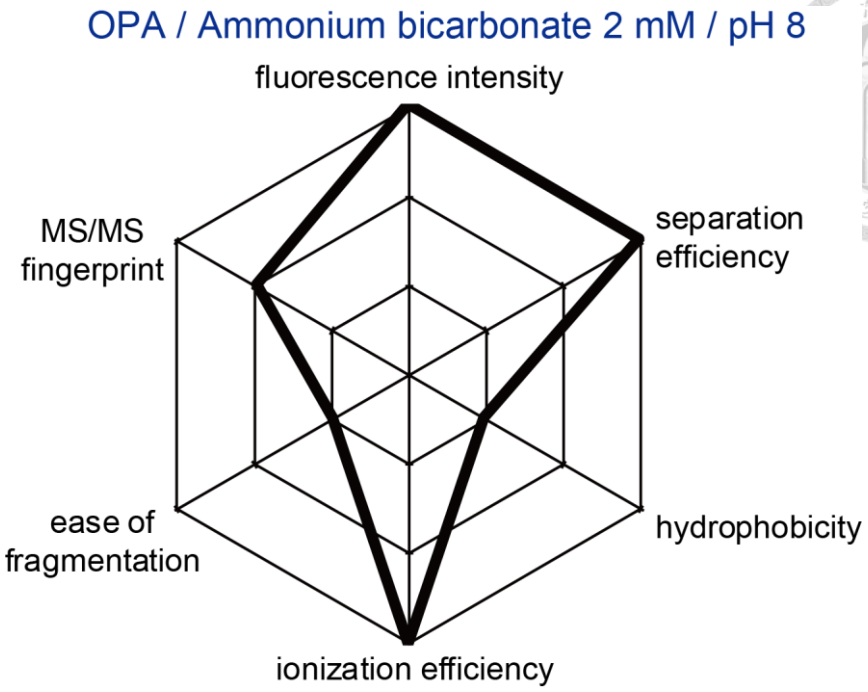

Figure 2-23. Radar chart of OPA method in its optimal condition (ammonium bicarbonate 2 mM, pH 8). The performance scale is given as none (center), poor, medium and good (perimeter). Bold black line indicates relative strength of the derivatization reagent compared to other reagents.

Under 2 mM ABC pH 8, the strength of OPA method showed strong fluorescence intensity, good separation and good ionization efficiency (Figure 2-23). The behavior of OPA's fluorescence is pH dependent, showing stronger fluorescence at basic condition. 2 mM of ammonium bicarbonate can improve the separation of OPA's derivatives as well as enhancing ionization efficiency. The weakness of OPA method is high fragmentation energy (20 eV) and poor hydrophobicity since its derivatives carry two carboxylate groups and. Its MS/MS fingerprint shows the loss of a fix mass instead of generating a characteristic ion.

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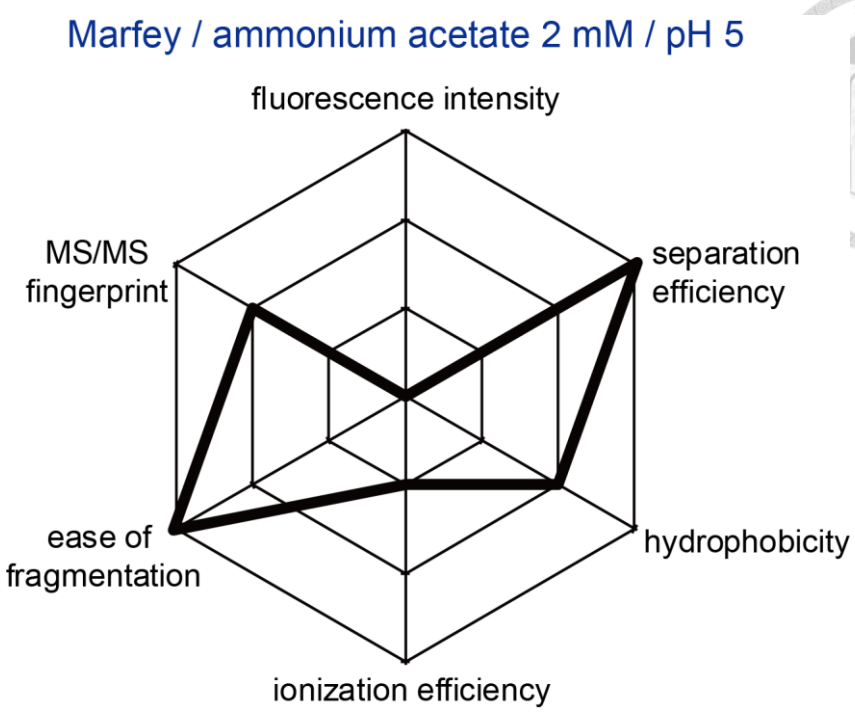

Figure 2-24. Radar chart of Marfey method in its optimal condition (0.1% FA, pH 2.6). The performance scale is given as none (center), poor, medium and good (perimeter). Bold black line indicates relative strength of the derivatization reagent compared to other reagents.

With 2 mM AA pH 5, the strength of Marfey method shows good separation and low fragmentation energy (Figure 2-24). 2 mM AA can improve the separation of Marfey's derivatives. Low fragmentation energy (6 eV) is also an advantage. However, the MS/MS fingerprint of Marfey derivatives involves losing a fix mass, instead of generating a characteristic ion. This MS/MS behavior is similar to OPA but different from Dansyl, Fmoc and Dabsyl methods. Marfey's derivatives lack fluorescence but it has strong UV/Vis absorbance. Medium hydrophobicity, absence of fluorescence and ease of interference by the presence of salt in mobile phase are three major weakness in Marfey's method.

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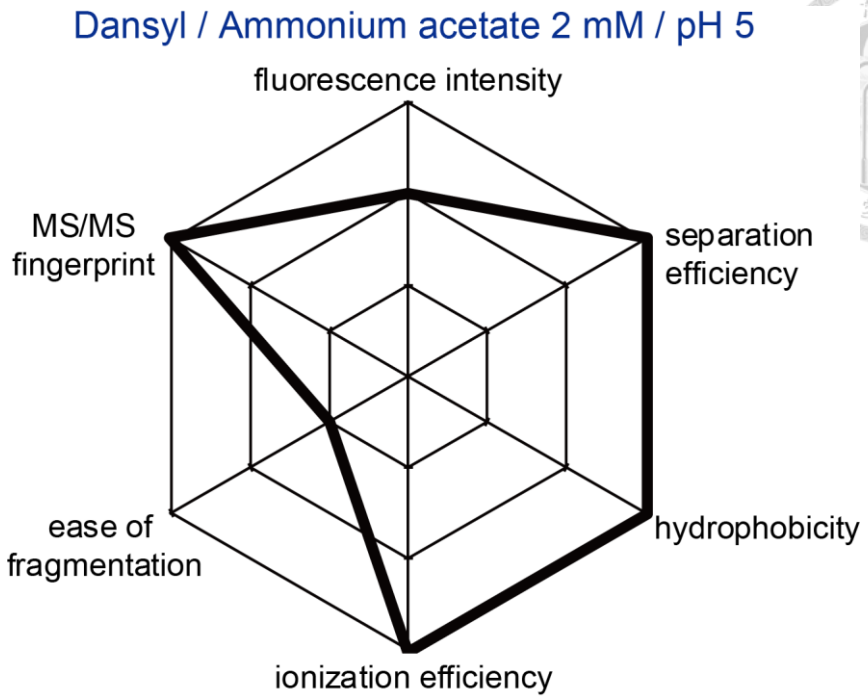

Figure 2-25. Radar chart of Dansyl method in its optimal condition (ammonium acetate 2 mM, pH 5). The performance scale is given as none (center), poor, medium and good (perimeter). Bold black line indicates relative strength of the derivatization reagent compared to other reagents.

With 2 mM AA, pH 5, the strengths of Dansyl method are good separation performance, large hydrophobicity, and good ionization efficiency (Figure 2-25). 2 mM of ammonium acetate is sufficient to separate Dansyl derivatives well. Naphthalene group provides high hydrophobicity, resulting in late retention time and its tertiary amine enhances the ionization efficiency. Although lower fluorescence intensity is its weakness (compared to OPA and Fmoc), its pH independence property can partially compensate this weakness. High fragmentation energy (20 eV) is also a drawback of Dansyl method and this should take into consideration for MS/MS experiments.

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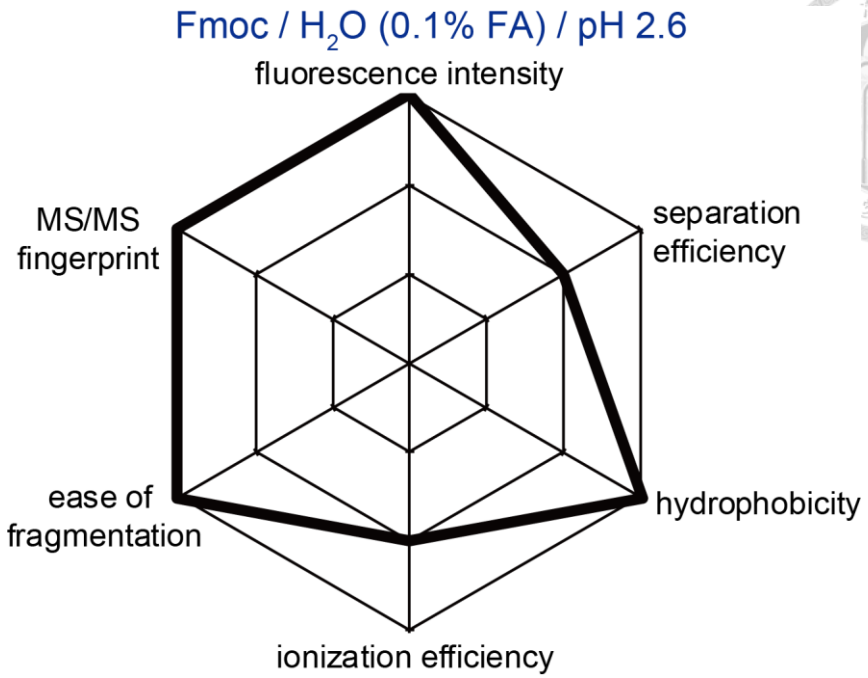

Figure 2-26. Radar chart of Fmoc method in its optimal condition (0.1% FA, pH 2.6). The performance scale is given as none (center), poor, medium and good (perimeter). Bold black line indicates relative strength of the derivatization reagent compared to other reagents.

With 0.1% FA, pH 2.6, the strength of Fmoc method shows high fluorescence intensity, high hydrophobicity, lowest fragmentation energy and a strong characteristic daughter ion (Figure 2-26). High fluorescence intensity and large hydrophobicity come from fluorene ring. The major weakness of Fmoc is medium separation and ionization efficiency. Water contained 0.1% formic acid may be the best aqueous mobile phase for Fmoc method.

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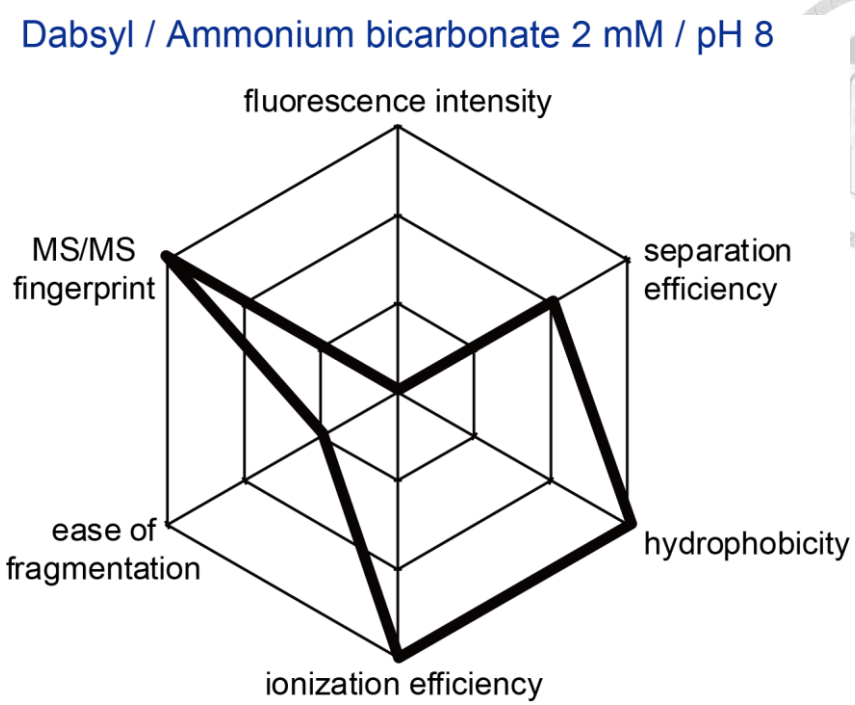

Figure 2-27. Radar chart of Dabsyl method in its optimal condition (ammonium bicarbonate 2 mM, pH 8). The performance scale is given as none (center), poor, medium and good (perimeter). Bold black line indicates relative strength of the derivatization reagent compared to other reagents.

With 2 mM ABC, pH 8, the strengths of Dabsyl are good separation performance, high hydrophobicity and high ionization efficiency (Figure 2-27). 2 mM of ammonium bicarbonate is sufficient to promote good separation. Its longer molecular structure interacts strongly with C_{18} carbon stationary phase and tertiary amine makes it prone to protonation under ESI. However, Dabsyl lacks fluorescence, the energy requirement for fragmentation is the highest (25 eV). Its MS/MS pattern is quite complex because of its larger molecular structure and many fragmentation points. Dabsyl appears to be less useful compared to OPA, Dansyl, Fmoc and Marfey.

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2.5.2 Practical considerations

UV/Vis absorbance detection:

UV/Vis absorbance is a more universal detection method than fluorescence, although the detection limit of absorbance is several orders of magnitude worse in general. The absorbance of Fmoc method is pH dependent, optimal at pH 2.6. On the other hand, the absorbance of OPA, Dansyl, Dabsyl and Marfey are less affected by pH and the intensities from high to low are Dansyl > Fmoc > Marfey > Dabsyl > OPA. Among these five reagents, only Marfey's reagent has the power to resolve enantiomers by forming diastereomers[37] (Figure 2-28). OPA can also be derivatized with chiral thiols like N-acetyl-L-cysteine to achieve enantiomer separation[100].

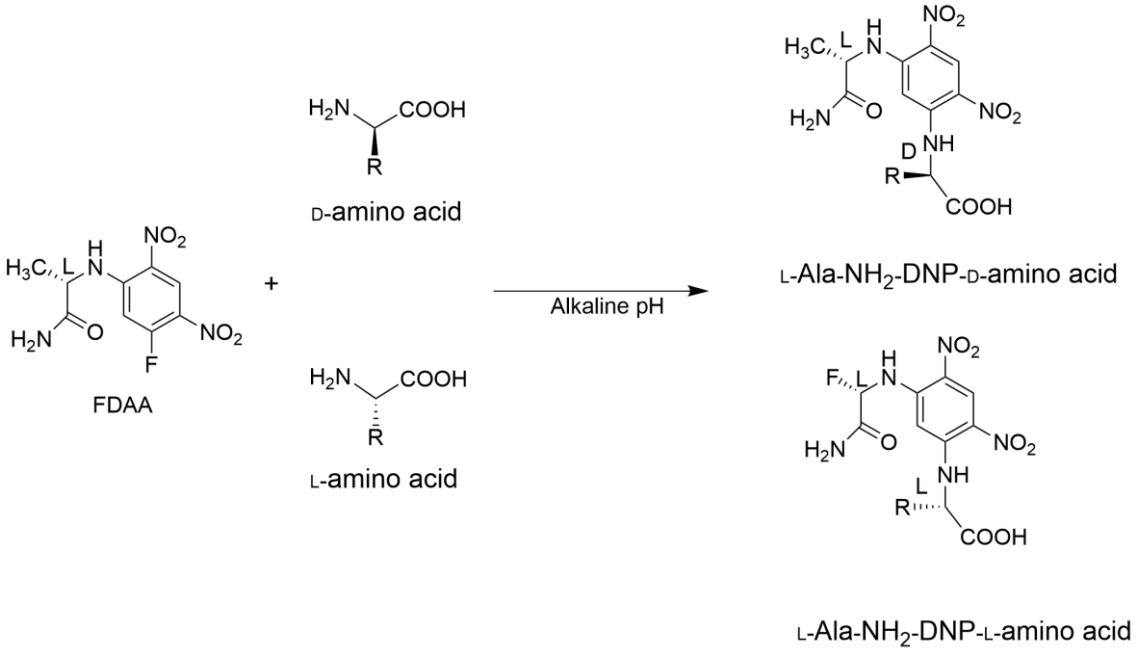

Figure 2-28. Reaction sequence for L-L- and L-D-diastereomers.

Fluorescence based detection:

If a very sensitive analysis is targeted, fluorescence detection is recommended over absorbance. Fluorescence properties of Fmoc and OPA methods are pH dependent with

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the acidic condition (0.1% FA) being optimal for Fmoc method, while the basic condition (2 mM ABC, pH 8) is suitable for OPA. The LOD and LOQ of Fmoc method at acidic condition (0.1% FA) are $1.15*10^{-8}$ M and $3.48*10^{-8}$ M, respectively. On the other hand, Dansyl method is less pH dependent, but also gave weak signals. Dansyl could be useful for neutral, weak acidic and basic LC eluent conditions. The order of fluorescence intensity is Fmoc > OPA > Dansyl.

Hydrophobicity detection:

It is difficulty to retain metabolites in RP-HPLC because metabolites are usually polar. Summarizing from the chromatograms, we rank five reagents as follows: Dabsyl > Fmoc > Dansyl ≈ Marfey ≈ OPA. This comes from the different of structures of different reagents. Fmoc contain three fused planar rings that enhance the interaction with C_{18} carbon stationary phase. The larger structure of Dabsyl also provides high hydrophobicity.

MS detection:

Ionization efficiency plays an important role in MS detection and we ranked them as follows: Dansyl ≈ Dabsyl > OPA ≈ Fmoc > Marfey. Because Dansyl and Dabsyl have tertiary amine, which improve ionization efficiency significantly. The aromatic nitrogen on isoindole ring also has good protonation ability, which improve ionization efficiency. Marfey has poor ionization efficiency relative to Dansyl, Dabsyl and OPA. The ionization of Fmoc depend on mobile phase composition and is optimal at 0.1% FA composition.

MS/MS detection:

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Generating a characteristic product ion can play a critical role in MS/MS experiments. Because it provides validation for amine-containing metabolites and the potential development in MRM application. Both Fmoc and Dansyl can generate a characteristic product ion but Fmoc has the lowest collision energy (2 eV). OPA and Marfey adduct ions both show neutral loss of a fixed *m/z* value. Marfey has the lower (6 eV) collision energy than OPA. Dabsyl has complex fragmentation products and therefore the worse MS/MS profile.

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Chapter 3 CONCLUSION

In this study, we compared five amine derivatization reagents for LC-MS/MS analysis and characterized their differences in the performance of UV/Vis absorbance, fluorescence, chromatographic separation, hydrophobicity, ionization efficiency and fragmentation energy and pattern. The best reagent overall in terms of detection sensitivity well is Fmoc, but it only works at acidic pH (2.6). Dansyl and OPA are also useful alternatives to Fmoc.

The general trend in absorbance is Dansyl > Fmoc > Marfey > Dabsyl > OPA.

The general trend in fluorescence is Fmoc > OPA > Dansyl. OPA is more fluorescent at basic condition (2 mM ABC, pH 8). The highest fluorescence intensity of Dansyl is under weak acidicly condition (2 mM AA, pH 5).

The general trend in hydrophobicity is Dabsyl > Fmoc > Dansyl ≈ Marfey ≈ OPA. This arises from the different structures of different reagents. Fmoc contains three fused planar rings that enhance the interaction with C_{18} carbon stationary phase. The larger structure of Dabsyl also provides high hydrophobicity.

The general performance in separation is 2 mM AA, pH 5 > water with 0.1% FA, pH 2.6 > 2 mM ABC, pH 8. Ammonium acetate buffer system can separate the products of OPA, Dansyl, Marfey and Fmoc efficiently but not Dabsyl. Water with 0.1% FA can separate OPA and Dansyl products efficiently but not Marfey, Dabsyl and Fmoc. Ammonium bicarbonate is the poorest among the three conditions, only working well for OPA.

The general trend in ionization efficiency is Dansyl ≈ Dabsyl > OPA ≈ Fmoc > Marfey. Dansyl and Dabsyl contain tertiary amines, which improves ionization efficiency significantly. The aromatic nitrogen on isoindole ring (OPA) also has good protonation

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ability, which improve ionization efficiency. Marfey has poor ionization efficiency relative to Dansyl, Dabsyl and OPA. The ionization of Fmoc depends on mobile phase composition, being optimal at strongly acidic conditions.

For SRM or MRM experiments, only Fmoc and Dansyl are suitable due to the generation of characteristic ion fragments.

Overall, we consider Dansyl to be the most versatile of all five reagents, useful in all detection methods and all elution conditions. It is also the current gold standard for metabolomics research for amine metabolites. Under acidic LC conditions, Fmoc may offer greater sensitivity than Dansyl. OPA is also a useful reagent and it may offer isotope labeling and enantioselective separation by choosing different thiols.

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Chapter 4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Equipment

The Agilent Technologies system consisted of a solvent delivery pump and with a built-in online degasser (The Agilent 1260 Infinity Quaternary Pump), an auto sampler (The Agilent 1260 Infinity Autosampler), a column oven (The Agilent 1260 Infinity Thermostatic Column Compartment), a diode array UV detector (The Agilent 1260 Infinity Diode Array Detector VL), a fluorescence detector (The Agilent 1260 Infinity Fluorescence Detector), we used two different C_{18} columns, one is Agilent, ZORBAX Eclipse Plus C18, 100×4.6mm, internal diameter 3.5μm; the other is YMC, Hydrosphere C_{18}, 250×4.6mm, internal diameter 5μm. The guard column is YMC HydrosphereC18 3PCS, 5 μm partical size gel, 23 × 4.0 mm. ESI-MS was conducted on a Bruker micrOTOF-QII high-resolution mass spectrometer (micrOTOF-QII, Bruker Daltonik GmbH, Bremen, Germany), that also coupled with the Agilent Technologies HPLC system.

4.1.2 Reagents

All reagents and solvents were reagent grade and were used without further purification unless otherwise specified. Commercial available reagents and solvents can be purchased from the following companies: 4-Dimethylaminoazobenzene-4'-sulfonyl chloride (Fluka), Fmoc-Cl (Fluka), o-phthalaldehyde (Sigma-Aldrich), 3-mercaptopropionic acid (Sigma-Aldrich), Dansyl-Cl (Sigma-Aldrich), heptylamine (Sigma-Aldrich), FDNP-Ala-NH_2 (Sigma-Aldrich), hexane (Macron), methanol (J.T. Baker), acetonitrile (J.T. Baker), acetone (Panreac), tetrahydrofuran (Macron), sodium

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bicarbonate (Acros), sodium carbonate (Acros), sodium tetraborate decahydrate (Acros), ammonium bicarbonate (Sigma-Aldrich), ammonium acetate (Bio Basic Inc), formic acid (Fluka).

4.1.3 Amino acid standards

The stock solution was composed of 7 amino acids (glutamic acid, serine, alanine, glycine, tyrosine, norvaline, phenylalanine) that had 25 mM for each amino acid in 0.1% perchloric acid. The working standard solution for MS/MS experiment was prepared by diluting the stock solutions to 2.5 mM for each amino acid with deionized water and also stored at -20°C. The working standard solution for LC-MS/MS experiment was prepared by diluting the stock solutions to 0.357 mM for each amino acid with deionized water and also stored at -20°C.

4.1.4 Derivatizing reagent

5 mg/mL OPA borate buffer solution was prepared freshly by dissolving 1.0 mg OPA with 20 μL methanol and mixing with 180 μL of 1 M borate buffer. OPA reagent, as the derivatizaing reagent, was also freshly prepared by mixing 200 μL OPA borate buffer solution with 0.84 μL of 3-mercapropionic acid. 1% Marfey's reagent solution was prepared by dissolving 3 mg of Marfey's reagent with 376 μL acetone and stored at -80°C. 7.5 mM Fmoc-Cl solution was prepared by dissolving 1.9 mg Fmoc-Cl with 1000 μL acetone and stored at -80°C. 50 mM Dansyl-Cl solution was prepared by dissolving 13.5 mg Dansyl-Cl with 1000 μL acetonitrile and stored at -80°C. 16 mM Dabsyl-Cl solution was prepared by dissolving 5.2 mg Dabsyl-Cl with 1000 μL acetonitrile and stored at -80°C.

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4.2 Methods

4.2.1 Derivatization protocol for five different reagents

Dabsyl: 20 μL of 0.5 M $NaHCO_3$ buffer (pH 9.0) was added into 20 μL of 17.5 mM amino acid working standard (2.5 mM for each amino acid) and derivatized by adding adequates volumes of 16 mM Dabsyl solution (16 equivalents). The mixture was incubated at room temperature for 1 hour. After incubation, the mixture was lyophilized overnight. The lyophilized powder was then dissolved with 200 μL of 75% ethanol solution and centrifuged at 14000 xg for 2 minutes. The supernatant was finally transferred to a new vial.

Dansyl: 20 μL of 0.5 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.4) was added into 20 μL of 17.5 mM amino acid working standard (2.5 mM for each amino acid) and derivatized by adding adequate volumes of 50 mM Dansyl solution (16 equivalents). The mixture was incubated at room temperature for 1 hour. After incubation, 11 μL of 0.5 M heptylamine was added to quench the reaction for 10 min at room temperature.

Fmoc: 20 μL of 0.5 M borate buffer (pH 8.0) was added into 20 μL of 17.5 mM amino acid working standard (2.5 mM for each amino acid) and derivatized by adding adequate volumes of 7.5 mM Fmoc-Cl solution (5 equivalents). The mixture was incubated at room temperature for 1 hour. After incubation, the mixture was extracted for 3 times with 0.5 mL hexane and the organic layer was discarded. The aqueous layer was collected and lyophilized overnight.

Marfey: 8 μL of 1 M $NaHCO_3$ (pH 8.17) and 31.5 μL of acetone were added into 20 μL of 17.5 mM amino acid working standard (2.5 mM for each amino acid) and derivatized by adding adequate volumes of 1% Marfey's solution (5 equivalents). The mixture was incubated at 40°C for 1 hour. After incubation, 6 μL of 2 M HCl was added to quench

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the reaction.

OPA: 20 μL of 1 M borate buffer (pH 10.7) was added into 20 μL of 17.5 mM amino acid working standard (2.5 mM for each amino acid) and derivatized by adding adequate volumes of 5 mg/mL OPA solution (16 equivalents). The mixture was incubated at room temperature for 1 minute.

4.2.2 LC-MS/MS experiment

The derivatization of 2.5 mM amino acid working solution (0.357 mM each) was conducted following same protocol as mentioned in section 4.2.1. The derivatives of each reagent were analyzed by a YMC Hydrosphere C_{18} column at 40°C. The fluorescence detector was set at an excitation wavelength of 340 nm and an emission waveltngth of 455 nm for OPA, excitation wavelength of 320 nm and an emission waveltngth of 525 nm for Dansyl, excitation wavelength of 262 nm and an emission waveltngth of 325 nm for Fmoc. The UV/Vis detector was set at 340 nm for both OPA and Marfey and 254 nm for Dansyl, 262 nm for Fmoc and 436 nm for Dabsyl. The chromatographic condition was shown below. The mobile phases A / B were: (1) water containing 0.1% FA, pH 2.6 / 100% ACN; (2) ammonium acetate 2 mM, pH 5 / 100% ACN; (3) ammonium bicarbonate 2 mM, pH 8 / 100% ACN. The LC pump was programmed with a flow rate of 1 mL min^{-1} with the following elution gradient:

Time	0	10	17	37	47	52	55	60
%ACN	3	3	10	50	100	100	3	3

Electrospray ionization (ESI) interface was used in positive mode and full MS^2 spectra were recorded (*m/z*=50 to 600). In most cases, the most abundant ions were $[M+H]^+$. ESI source parameters was: nebulizer gas (nitrogen) 0.3 bar, Drying gas

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(nitrogen) 4 L/min and drying gas temperature 180 °C. The optimal fragmentation energy acquired before was shown below.

Method	Fragmentation energy (eV)
Fmoc	2
Marfey	6
OPA	20
Dansyl	20
Dabsyl	25

4.2.3 MS/MS experiment

The derivatization of 17.5 mM amino acid working solution (2.5 mM each) was conducted following same protocol that mentioned in section 4.2.1. The derivatives of Dabsyl were analyzed by being directly injected into micrOTOF-QII and the fragmentation energy was as follows 2 eV, 4 eV, 6 eV, 8 eV, 10 eV, 12 eV, 14 eV, 16 eV, 18 eV, 20 eV, 25 eV, 30 eV, 40 eV, 50 eV. The derivatives of Dansyl, Marfey and Fmoc were separated by LC to prevent severe interference by each other. Mobile phase A consisted of 100% de-ionized water. Mobile phase B consisted of 100% ACN. Gradient program was as follows: 0-3 min, 2%; 3-10 min 2-50%; 10-15 min 50-100%; 15-18 min 100%; 18-20 min 100-2%; 20-23 min 2%. The eluent flow rate was 1.0 mL/min^{-1} and column was maintained at room temperature. 20 μL of the mixture was injected into HPLC system (overall volume was 400 μL). MS/MS fragmentation energy of Dansyl was tested as follows: 2 eV, 4 eV, 8 eV, 16 eV, 20 eV, 25 eV. MS/MS fragmentation energy of Fmoc was tested as follows: 2 eV, 4 eV, 6 eV. MS/MS fragmentation energy of Marfey was tested as follows: 2 eV, 4 eV, 6 eV, 8 eV, 10 eV, 15 eV. Because the derivatives of

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OPA eluted out quickly, the mobile phase mentioned above was not suitable for OPA. New mobile phase composition was choosing for OPA, which was suggested by Min-Han Shih. Mobile phase A consisted of 25 mM ammonium bicarbonate pH 8.15 with 0.5% THF. Mobile phase B consisted of 50% 25 mM ammonium bicarbonate pH 8.15, 35% methanol and 15% acetonitrile. Gradient program was the same with Dansyl experiment. The eluent flow rate was 1.0 mL/min^{-1} and column was maintained at room temperature. 20 μL of the mixture was injected into HPLC system (overall volume was set to 400 μL). MS/MS fragmentation energy of OPA was tested as follows: 2 eV, 4 eV, 8 eV, 16 eV, 20 eV, 25 eV.

4.2.4 LOD and LOQ for Fmoc method

In order to estimate the LOD and LOQ for the Fmoc method, we chose serine as standard amino acid due to its fluorescence intensity was the highest among six amino acids. Standard amino acid solutions were prepared as six different concentrations in 0.1% perchloric acid solution from 10^{-5} M to 4.11×10^{-8} M and injected into HPLC after Fmoc derivatization, respectively. The fluorescence detector was set at an excitation wavelength of 262 nm and an emission wavelength of 325 nm. Six different concentration points were assayed in three repeats. Calibration curve was constructed by linear regression of the fluorescence peak area (y) versus the concentration before injection (x).

doi:10.6342/NTU201601534

REFERENCE

1 Nicholson, J. K. and Lindon, J. C. Systems biology: Metabonomics. *Nature* **455**, 1054-1056 (2008)

2 Goodacre, R. Metabolomics – the way forward. *Metabolomics* **1**, 1-2 (2005)

3 Nicholson, J. K., Lindon, J. C. and Holmes, E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **29** (1999)

4 Oliver, S. G. Yeast as a navigational aid in genome analysis. *Microbiology* **143**, 1483-1487 (1997)

5 Dettmer, K., Aronov, P. A. and Hammock, B. D. Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews* **26**, 51-78 (2007)

6 Nicholson, J. K. and Wilson, I. D. Opinion: Understanding 'Global' Systems Biology: Metabonomics and the Continuum of Metabolism. *Nature Reviews Drug Discovery* **2**, 668-676 (2003)

7 Harrigan, G. G. *et al.* Application of high-throughput Fourier-transform infrared spectroscopy in toxicology studies: contribution to a study on the development of an animal model for idiosyncratic toxicity. *Toxicology Letters* **146**, 197-205 (2004)

8 Fukushima, T., Usui, N., Santa, T. and Imai, K. Recent progress in derivatization methods for LC and CE analysis. *Journal of Pharmaceutical and Biomedical Analysis* **30**, 1655-1687 (2003)

9 Santa, T. *et al.* Synthesis of benzofurazan derivatization reagents for carboxylic acids in liquid chromatography/electrospray ionization–tandem mass spectrometry. *Biomedical Chromatography* **21**, 1207-1213 (2007)

doi:10.6342/NTU201601534

10 Santa, T. *et al.* Synthesis of 4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-N-methylhydrazino-2,1,3-benzoxadiazole (DAABD-MHz) as a derivatization reagent for aldehydes in liquid chromatography/electrospray ionization–tandem mass spectrometry. *Biomedical Chromatography* **22**, 115-118 (2008)

11 Matsuura, K. and Takashina, H. Effects of functional groups of acrylic acid derivatives as derivatization reagents for thiol compounds on molecular ion responses in electrospray ionization-mass spectrometry. *Journal of Mass Spectrometry* **33**, 1199-1208 (1998)

12 Higashi, T. and Shimada, K. Derivatization of neutral steroids to enhance their detection characteristics in liquid chromatography mass spectrometry. *Analytical and Bioanalytical Chemistry* **378**, 875-882 (2004)

13 Xu, F. *et al.* Quantification of fudosteine in human plasma by high-performance liquid chromatography-electrospray ionization mass spectrometry employing precolumn derivatization with 9-fluorenylmethyl chloroformate. *Journal of Mass Spectrometry* **41**, 685-692 (2006)

14 Ilisz, I., Berkecz, R. and Péter, A. Application of chiral derivatizing agents in the high-performance liquid chromatographic separation of amino acid enantiomers: A review. *Journal of Pharmaceutical and Biomedical Analysis* **47**, 1-15 (2008)

15 Gao, S., Zhang, Z. and Karnes, H. Sensitivity enhancement in liquid chromatography/atmospheric pressure ionization mass spectrometry using derivatization and mobile phase additives. *Journal of Chromatography B* **825**, 98-110 (2005)

16 Eggink, M. *et al.* Development of a selective ESI-MS derivatization reagent: synthesis and optimization for the analysis of aldehydes in biological mixtures. *Analytical Chemistry* **80**, 9042-9051 (2008)

doi:10.6342/NTU201601534

17 Liu, D. Q. and Hop, C. E. C. A. Strategies for characterization of drug metabolites using liquid chromatography–tandem mass spectrometry in conjunction with chemical derivatization and on-line H/D exchange approaches. *Journal of Pharmaceutical and Biomedical Analysis* **37**, 1-18 (2005)

18 Srinivas, N. R. Sensitivity enhancement in tandem liquid chromatographic mass spectrometric assays by summation of two transition ion pairs - perspectives. *Journal of Separation Science* **32**, 483-486 (2009)

19 Honda, A. *et al.* MPAI (Mass Probes Aided Ionization) Method for Total Analysis of Biomolecules by Mass Spectrometry. *Analytical Sciences* **23**, 11-15 (2007)

20 Tsukamoto, Y. *et al.* A further study on the combined use of internal standard and isotope-labeled derivatization reagent for expansion of linear dynamic ranges in liquid chromatography–electrospray mass spectrometry. *Biomedical Chromatography* **20**, 1049-1055 (2006)

21 Callejón, R. M., Troncoso, A. M. and Morales, M. L. Determination of amino acids in grape-derived products: A review. *Talanta* **81**, 1143-1152 (2010).

22 Anderegg, R. J. Derivatization in mass spectrometry: Strategies for controlling fragmentation. *Mass Spectrometry Reviews* **7**, 395-424 (1988)

23 Koller, M. and Eckert, H. Derivatization of peptides for their determination by chromatographic methods. *Analytica Chimica Acta* **352**, 31-59 (1997)

24 Roth, M. Fluorescence reaction for amino acids. *Analytical Chemistry* **43**, 880-882 (1971)

25 Simons, S. S. and Johnson, D. F. The structure of the fluorescent adduct formed in the reaction of o-phthalaldehyde and thiols with amines. *Journal of the American Chemical Society* **98**, 7098-7099 (1976)

26 Simons, S. S. and Johnson, D. F. Preparation of a stable, fluorescent 1-alkylthio-

doi:10.6342/NTU201601534

2-alkylisoindole. *Chemical Communications*, 374-374 (1977)

27 Jacobs, W. A., Leburg, M. W. and Madaj, E. J. Stability of o-phthalaldehyde-derived isoindoles. *Analytical Biochemistry* **156**, 334-340 (1986)

28 Kucera, P. and Umagat, H. Design of a post-column fluorescence derivatization system for use with microbore columns. *Journal of Chromatography A* **255**, 563-579 (1983)

29 Drescher, D. G. and Lee, K. S. Extraction of fixed, stained protein bands form gels for micro amino acid analysis using o-phthaldialdehyde. *Analytical Biochemistry* **84**, 559-569 (1978)

30 Benson, J. R. and Hare, P. E. O-phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 619-622 (1975)

31 Hou, Y. *et al.* Analysis of l-homoarginine in biological samples by HPLC involving precolumn derivatization with o-phthalaldehyde and N-acetyl-l-cysteine. *Amino Acids* **47**, 2005-2014 (2015)

32 Sanger, F. The free amino groups of insulin. *The Biochemical Journal* **39**, 507-515 (1945)

33 Zahn, V. H. and Meienhofer, J. Reaktionen von 1,5-difluor-2,4-dinitrobenzol mit insulin 2. Mitt. Versuche mit insulin. *Die Makromolekulare Chemie* **26**, 153-166 (1958)

34 Marfey, P. S., Nowak, H., Uziel, M. and Yphantis, D. A. Reaction of bovine pancreatic ribonuclease a with 1,5-difluoro-2,4-dinitrobenzene: i. Preparation of monomeric intramolecularly bridged derivatives. *Journal of Biological Chemistry* **240**, 3264-3269 (1965)

doi:10.6342/NTU201601534

35 Marfey, P. S., Uziel, M. and Little, J. Reaction of bovine pancreatic ribonuclease a with 1,5-difluoro-2,4-dinitrobenzene: ii. Structure of an intramolecularly bridged derivative. *Journal of Biological Chemistry* **240**, 3270-3275 (1965)

36 Marfey, P. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Research Communications* **49**, 591-596 (1984)

37 Bhushan, R. and Brückner, H. Marfey's reagent for chiral amino acid analysis: a review. *Amino Acids* **27**, 231-247 (2004)

38 Kochhar, S. and Christen, P. Amino acid analysis by high-performance liquid chromatography after derivatization with 1-fluoro-2,4-dinitrophenyl-5-l-alanine amide. *Analytical Biochemistry* **178**, 17-21 (1989)

39 Carpino, L. A. and Han, G. Y. 9-Fluorenylmethoxycarbonyl amino-protecting group. *The Journal of Organic Chemistry* **37**, 3404-3409 (1972)

40 Moye, H. A. and Boning, A. J. A Versatile Fluorogenic Labelling Reagent for Primary and Secondary Amines: 9-Fluorenylmethyl Chloroformate. *Analytical Letters* **12**, 25-35 (1979)

41 Kushnir, M. M., Urry, F. M., Frank, E. L., Roberts, W. L. and Shushan, B. Analysis of catecholamines in urine by positive-ion electrospray tandem mass spectrometry. *Clinical Chemistry* **48**, 323-331 (2002)

42 Chang, J. Y., Knecht, R. and Braun, D. G. Amino acid analysis in the picomole range by precolumn derivatization and high-performance liquid chromatography. *Methods In Enzymology* **91**, 41-48 (1983)

43 Tzeng, M.-C. A sensitive, rapid method for monitoring sodium dodecyl sulfate-polyacrylamide gel electrophoresis by chromophoric labeling. *Analytical Biochemistry* **128**, 412-414 (1983)

doi:10.6342/NTU201601534

44 Chang, J.-Y., Martin, P., Bernasconi, R. and Braun, D. G. High-sensitivity amino acid analysis: measurement of amino acid neurotransmitter in mouse brain. *FEBS Letters* **132**, 117-120 (1981)

45 Weber, G. Polarization of the fluorescence of macromolecules. II. Fluorescent conjugates of ovalbumin and bovine serum albumin. *The Biochemical Journal* **51**, 155-167 (1952)

46 Hartley, B. S. and Massey, V. The active centre of chymotrypsin. *Biochimica et Biophysica Acta* **21**, 58-70 (1956)

47 Guo, K. and Li, L. Differential 12C-/13C-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome. *Analytical Chemistry* **81** (2009)

48 Bergmann, F. and Pfleiderer, W. Nucleotides. Part XLII. The 2-dansylethoxycarbonyl(=2-{[5-(dimethylamino)naphthalen-1-yl]sulfonyl}ethoxy-carbonyl; dnseoc) group for protection of the 5?-hydroxy function in oligoribonucleotide synthesis. *Helvetica Chimica Acta* **77**, 481-501 (1994)

49 Edman, P. A method for the determination of amino acid sequence in peptides. *Archives of Biochemistry* **22**, 475-475 (1949)

50 Koop, D. R., Morgan, E. T., Tarr, G. E. and Coon, M. J. Purification and characterization of a unique isozyme of cytochrome P-450 from liver microsomes of ethanol-treated rabbits. *The Journal of Biological Chemistry* **257**, 8472-8480 (1982)

51 Bidlingmeyer, B. A., Cohen, S. A. and Tarvin, T. L. Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography* **336**, 93-104 (1984)

52 Heinrikson, R. L. and Meredith, S. C. Amino acid analysis by reverse-phase high-performance liquid chromatography: Precolumn derivatization with

doi:10.6342/NTU201601534

phenylisothiocyanate. *Analytical Biochemistry* **136**, 65-74 (1984)

53 Onisko, B. *et al.* Mass spectrometric detection of attomole amounts of the prion protein by nanoLC/MS/MS. *Journal of the American Society for Mass Spectrometry* **18** (2007)

54 De Montigny, P. *et al.* Naphthalene-2,3-dicarboxyaldehyde/cyanide ion: a rationally designed fluorogenic reagent for primary amines. *Analytical Chemistry* **59** (1987)

55 Matuszewski, B. K., Givens, R. S., Srinivasachar, K., Carlson, R. G. and Higuchi, T. N-substituted 1-cyanobenz[f]isoindole: evaluation of fluorescence efficiencies of a new fluorogenic label for primary amines and amino acids. *Analytical Chemistry* **59**, 1102-1105 (1987)

56 Shou, M., Smith, A. D., Shackman, J. G., Peris, J. and Kennedy, R. T. In vivo monitoring of amino acids by microdialysis sampling with on-line derivatization by naphthalene-2,3-dicarboxyaldehyde and rapid micellar electrokinetic capillary chromatography. *Journal of Neuroscience Methods* **138**, 189-197 (2004)

57 Huang, H.-M. and Lin, C.-H. Methanol plug assisted sweeping-micellar electrokinetic chromatography for the determination of dopamine in urine by violet light emitting diode-induced fluorescence detection. *Journal of Chromatography B* **816**, 113-119 (2005)

58 Sauvinet, V. *et al.* In vivo simultaneous monitoring of γ-aminobutyric acid, glutamate, andL-aspartate using brain microdialysis and capillary electrophoresis with laser-induced fluorescence detection: Analytical developments andin vitro/in vivo validations. *Electrophoresis* **24**, 3187-3196 (2003)

59 Quan, Z. and Liu, Y.-M. Capillary electrophoretic separation of glutamate enantiomers in neural samples. *Electrophoresis* **24**, 1092-1096 (2003)

doi:10.6342/NTU201601534

60 Beale, S. C., Hsieh, Y. Z., Wiesler, D. and Novotny, M. Application of 3-(2-furoyl)quinoline-2-carbaldehyde as a fluorogenic reagent for the analysis of primary amines by liquid chromatography with laser-induced fluorescence detection. *Journal of Chromatography* **499**, 579-587 (1990)

61 Pinto, D., Arriaga, E. A., Schoenherr, R. M., Chou, S. S.-H. and Dovichi, N. J. Kinetics and apparent activation energy of the reaction of the fluorogenic reagent 5-furoylquinoline-3-carboxaldehyde with ovalbumin. *Journal of Chromatography B* **793**, 107-114 (2003)

62 Stoyanov, A. V., Ahmadzadeh, H. and Krylov, S. N. Heterogeneity of protein labeling with a fluorogenic reagent, 3-(2-furoyl)quinoline-2-carboxaldehyde. *Journal of Chromatography B* **780**, 283-287 (2002)

63 Chen, Z., Wu, J., Baker, G. B., Parent, M. and Dovichi, N. J. Application of capillary electrophoresis with laser-induced fluorescence detection to the determination of biogenic amines and amino acids in brain microdialysate and homogenate samples. *Journal of Chromatography A* **914**, 293-298 (2001)

64 Kraly, J. R. *et al.* Reproducible two-dimensional capillary electrophoresis analysis of Barrett's esophagus tissues. *Analytical Chemistry* **78**, 5977-5986 (2006)

65 Traut, R. R. *et al.* Methyl 4-mercaptobutyrimidate as a cleavable crosslinking reagent and its application to the Escherichia coli 30S ribosome. *Biochemistry* **12**, 3266-3273 (1973)

66 Jue, R., Lambert, J. M., Pierce, L. R. and Traut, R. R. Addition of sulfhydryl groups of Escherichia coli ribosomes by protein modification with 2-iminothiolane (methyl 4-mercaptobutyrimidate). *Biochemistry* **17**, 5399-5406 (1978)

67 Yang, W.-C., Mirzaei, H., Liu, X. and Regnier, F. E. Enhancement of amino acid

doi:10.6342/NTU201601534

detection and quantification by electrospray ionization mass spectrometry. *Analytical Chemistry* **78**, 4702-4708 (2006)

68 Münchbach, M., Quadroni, M., Miotto, G. and James, P. Quantitation and Facilitated de Novo Sequencing of Proteins by Isotopic N-Terminal Labeling of Peptides with a Fragmentation-Directing Moiety. *Analytical Chemistry* **72**, 4047-4057 (2000)

69 Yang, W.-C., Regnier, F. E., Sliva, D. and Adamec, J. Stable isotope-coded quaternization for comparative quantification of estrogen metabolites by high-performance liquid chromatography-electrospray ionization mass spectrometry. *Journal of chromatography. B* **870**, 233-240 (2008)

70 Weigele, M., De Bernardo, S., Leimgruber, W., Cleeland, R. and Grunberg, E. Fluorescent labeling of proteins. A new methodology. *Biochemical and Biophysical Research Communications* **54**, 899-906 (1973)

71 Castell, J. V., Cervera, M. and Marco, R. A convenient micromethod for the assay of primary amines and proteins with fluorescamine. A reexamination of the conditions of reaction. *Analytical Biochemistry* **99**, 379-391 (1979)

72 Tata, S. J. and Moir, G. F. J. Fluorescamine as a reagent for location of proteins after electrophoresis in starch gel or on paper. *Analytical Biochemistry* **70**, 495-498 (1976)

73 Miedel, M. C., Hulmes, J. D. and Pan, Y.-C. E. The use of fluorescamine as a detection reagent in protein microcharacterization. *Journal of Biochemical and Biophysical Methods* **18**, 37-52 (1989)

74 Felix, A. M. and Jimenez, M. H. Usage of fluorescamine as a spray reagent for thin-layer chromatography. *Journal of Chromatography A* **89**, 361-364 (1974)

75 Udenfriend, S. *et al.* Fluorescamine: a reagent for assay of amino acids, peptides,

doi:10.6342/NTU201601534

proteins, and primary amines in the picomole range. *Science (New York, N.Y.)* **178**, 871-872 (1972)

76 Tu, S.-I. and Grosso, L. Fluorescent labeling of proteins in sodium dodecyl sulfate complexes with fluorescamine. *Biochemical and Biophysical Research Communications* **72**, 9-14 (1976)

77 Sprinzl, M. and Faulhammer, H. G. Participation of X47-fluorescamine modified E. coli tRNAs in in vitro protein biosynthesis. *Nucleic Acids Research* **5**, 4837-4853 (1978)

78 Moore, S. and Stein, W. H. Photometric ninhydrin method for use in the chromatography of amino acids. *The Journal of Biological Chemistry* **176**, 367-388 (1948)

79 Moore, S. and Stein, W. H. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *The Journal of Biological Chemistry* **211**, 907-913 (1954)

80 Cavins, J. F. F., M. Automatic integration and computationof amino acid analyses. *Cereal Chem* (1968)

81 Dent, C. E. A study of the behaviour of some sixty amino-acids and other ninhydrin-reacting substances on phenol-;collidine' filter-paper chromatograms, with notes as to the occurrence of some of them in biological fluids. *The Biochemical Journal* **43**, 169-180 (1948)

82 Griffin, M. and Wilson, J. Detection of ε (γ-glutamyl) lysine. *Molecular and Cellular Biochemistry* **58**, 37-49 (1984)

83 Cashman, P. J., Beede, J. D. and Thornton, J. I. Ninhydrin: A Color Test for the Differentiation of Phenethylamines of Abuse. *Journal of the Forensic Science Society* **19**, 137-141 (1979)

doi:10.6342/NTU201601534

84 Ryan, E. A. and Kropinski, A. M. Separation of amino sugars and related compounds by two-dimensional thin-layer chromatography. *Journal of Chromatography A* **195**, 127-132 (1980)

85 Boppana, V. K. and Rhodes, G. R. High-performance liquid chromatographic determination of an arginine-containing octapeptide antagonist of vasopressin in human plasma by means of a selective post-column reaction with fluorescence detection. *Journal of Chromatography A* **507**, 79-84 (1990)

86 Wimalasena, R., Audus, K. L. and Stobaugh, J. F. Rapid optimization of the post-column fluorogenic ninhydrin reaction for the HPLC-based determination of bradykinin and related fragments. *Biomedical Chromatography* **17**, 165-171 (2003)

87 LaPorte, G. M. and Ramotowski, R. S. The effects of latent print processing on questioned documents produced by office machine systems utilizing inkjet technology and toner. *Journal of Forensic Sciences* **48**, 658-663 (2003)

88 Kyte, J. and Doolittle, R. F. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105-132 (1982)

89 Nakano, T., Takewaki, K., Yade, T. and Okamoto, Y. Dibenzofulvene, a 1,1-Diphenylethylene Analogue, Gives a π-Stacked Polymer by Anionic, Free-Radical, and Cationic Catalysts. *Journal of the American Chemical Society* **123** (2001)

90 Nakano, T. Synthesis, structure and function of π-stacked polymers. *Polymer Journal* **42**, 103-123 (2010)

91 International Conference On Harmonization (ICH) Of Technical Requirements for Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: Text and Methodology. ICH-Q2(R1), Geneva (1996).

doi:10.6342/NTU201601534

92 Jamindar, D. and Gutheil, W. G. A liquid chromatography–tandem mass spectrometry assay for Marfey's derivatives of l-Ala, d-Ala, and d-Ala-d-Ala: Application to the in vivo confirmation of alanine racemase as the target of cycloserine in Escherichia coli. *Analytical Biochemistry* **396**, 1-7 (2010)

93 Uutela, P., Ketola, R. A., Piepponen, P. and Kostiainen, R. Comparison of different amino acid derivatives and analysis of rat brain microdialysates by liquid chromatography tandem mass spectrometry. *Analytica Chimica Acta* **633**, 223-231 (2009)

94 Fox, S. D., Falk, R. T., Veenstra, T. D. and Issaq, H. J. Quantitation of free and total bisphenol A in human urine using liquid chromatography-tandem mass spectrometry. *Journal of Separation Science* **34**, 1268-1274 (2011)

95 Wu, M. *et al.* Liquid chromatography/mass spectrometry methods for measuring dipeptide abundance in non-small-cell lung cancer. *Rapid Communications in Mass Spectrometry* **27**, 2091-2098 (2013)

96 Luo, K., Gao, Q. and Hu, J. Derivatization method for sensitive determination of 3-hydroxybenzo[a]pyrene in human urine by liquid chromatography–electrospray tandem mass spectrometry. *Journal of Chromatography A* **1379**, 51-55 (2015)

97 Chen, Y.-H., Shih, L.-L., Liou, S.-E. and Chen, C.-C. Analysis of Dabsyl-Cl Derivated Amino Acids by High Performance Liquid Chromatography and Tandem Mass Spectrometry. *Food Science and Technology Research* **9**, 276-282 (2003)

98 Lacroix, C. and Saussereau, E. Fast liquid chromatography/tandem mass spectrometry determination of cannabinoids in micro volume blood samples after dabsyl derivatization. *Journal of Chromatography B* **905**, 85-95 (2012)

99 Holčapek, M., Volná, K. and Vaněrková, D. Effects of functional groups on the

doi:10.6342/NTU201601534

fragmentation of dyes in electrospray and atmospheric pressure chemical ionization mass spectra. *Dyes and Pigments* **75**, 156-165 (2007)

100 Bru¨ckner, H., Langer, M., Lupke, M., Westhauser, T. and Godel, H. Liquid chromatographic determination of amino acid enantiomers by derivatization with o-phthaldialdehyde and chiral thiols Applications with reference to food science. *Journal of Chromatography A* **697**, 229-245 (1995)

doi:10.6342/NTU201601534

Appendix

Working protocol for derivatizing amino acid

1.1.1 Derivatization procedure of OPA method

Prepare solutions:

2.5 mM (total amino acid concentration) pooled amino acid solution:

First prepare 25 mM of individual amino acid solution, mix 2.9 μL of each amino acid solution in enpendorf then add 980 μL of de-ionized water to 1000 μL

1 M borate buffer (pH 10.7):

Bought from Dr.MaischGmbH manufacturer

OPA solution (200 μL):

1 mg OPA+ 20 μL methanol+ 180 μL borate buffer+ 0.84 μL 3-MPA

50% ACN solution:

Mix equal volumes of acetonitrile and de-ionized water

Derivatization process:

(1) Mix 20 μL of 2.5 mM (total amino acid concentration) pooled amino acid solution with 20 μL of 1 M borate buffer (pH 9.0)

(2) Add 23.85 μL of 37.2 mM OPA solution.
[equivalent ratio of OPA to amino aicd is 16]

(3) Vortex the mixture and react for 90 s, then diluted with 156.15 μL 50% ACN/H_2O
[total volume 200 μL]

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1.1.2 Derivatization procedure of Fmoc method

Prepare solutions:

2.5 mM (total amino acid concentration) pooled amino acid solution:

First prepare 25 mM of individual amino acid solution, mix 2.9 μL of each amino acid solution in enpendorf then add 980 μL of de-ionized water to 1000 μL

0.5 M borate buffer (pH 8.0):

Dissolve 95.3 g of sodium tetraborate decahydrate into 500 mL of de-ionized water and sonicate for 30 minutes then titrate with HCl to pH 8.0

7.5 mM Fmoc solution:

Dissolved 1.9 mg of Fmoc-Cl into 1000 μL of acetone.

50% ACN solution:

Mix equal volumes of acetonitrile and de-ionized water

(1) Mix 20 μL of 2.5 mM (total amino acid concentration) pooled amino acid solution with 20 μL of 0.5 M borate buffer (pH 8.0)

(2) Add 33 μL of 7.5 mM Fmoc chloride (dissolved in acetone) to proceed the reaction [equivalent ratio of Fmoc to amino aicd is 5]

(3) Add 0.5 mL of hexane to extract excess Fmoc-Cl and Fmoc-OH. Remove hexane layer by pipet after 1 minute. The hexane layer is discarded. The remaining aqueous layer is extracted with two additional 1 mL portion of hexanes. Lyophilized the aqueous layer overnight

(4) Reconstituted with 50% ACN/H_2O

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1.1.3 Derivatization procedure of Dansyl method

Prepare solutions:

2.5 mM (total amino acid concentration) pooled amino acid solution:

First prepare 25 mM of individual amino acid solution, mix 2.9 μL of each amino acid solution in enpendorf then add 980 μL of de-ionized water to 1000 μL

0.5 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.4):

(1) 0.5 M $NaHCO_3$ solution: dissolved 4.2 g of sodium bicarbonate into 100 ml of de-ionized water

(2) 0.5 M Na_2CO_3 solution: dissolved 5.3 g of sodium carbonate into 100 ml of de-ionized water

(3) Mix two solutions with a ratio of 1:1 (v:v)

50 mM Dansyl solution:

Dissolved 13.5 mg of Dansyl-Cl into 1000 μL of acetonitrile

50% ACN solution:

Mix equal volumes of acetonitrile and de-ionized water

(1) Mix 20 μL of 2.5 mM (total amino acid concentration) pooled amino acid solution with 20 μL of 0.5 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.4)
(2) Add 16 μL of 50 mM Dansyl chloride (dissolved in acetone) to proceed the derivatization reaction and vortex the mixture for 5 seconds then incubate at room temperature for 60 minutes [equivalent ratio of Dansyl to amino aicd is 16]
(3) Add 1.6 μL of 0.5 M heptylamine solution to quench reaction, leave to react for 10 min at ambient temperature
(4) Diluted with 50% ACN/H_2O [total volume: 200 μL]

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1.1.4 Derivatization procedure of Marfey method

Prepare solutions:

2.5 mM (total amino acid concentration) pooled amino acid solution:

First prepare 25 mM of individual amino acid solution, mix 2.9 μL of each amino acid solution in eppendorf then add 980 μL of de-ionized water to 1000 μL

1 M $NaHCO_3$ (pH 8.17):

Dissolved 8.4 g of sodium bicarbonate into 100 mL of de-ionized water

1% Marfey solution:

Dissolved 3 mg of Marfey reagent into 375.5 μL of acetone

50% ACN solution:

Mix equal volumes of acetonitrile and de-ionized water

(1) Mix 20 μL of 2.5 mM (total amino acid concentration) pooled amino acid solution with 31.47 μL of acetone (enhance organic content to prevent Marfey reagent from forming colloid, ideally, water: organic = 7:10)
(2) Add 8.53 μL of 1% Marfey's reagent (dissolved in acetone) then vortex for 10 seconds [equivalent ratio of Marfey to amino aicd is 5]
(3) Add 8 μL of 1 M $NaHCO_3$ (pH 8.17) to activate the reaction, vortex the mixture 10 seconds and put the vial on BioShaker IQ (life science unlimited) at 40°C, 500 rpm for 1 hour
(4) Cool down at room temperature and add 6 μL of 2 M of HCl.
(5) Dilute with 156 μL of 50% ACN/H_2O [total volume: 200 μL]

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1.1.5 Derivatization procedure of Dabsyl method

Prepare solutions:

2.5 mM (total amino acid concentration) pooled amino acid solution:

First prepare 25 mM of individual amino acid solution, mix 2.9 μL of each amino acid solution in enpendorf then add 980 μL of de-ionized water to 1000 μL

0.5 M $NaHCO_3$ (pH 9.0):

Dissolved 10.5 g of sodium bicarbonate into 250 mL of de-ionized water then adjust by NaOH

16 mM Dabsyl solution:

Dissolved 5.2 mg of Dabsyl-Cl into 1000 μL of acetone

50% ACN solution:

Mix equal volumes of acetonitrile and de-ionized water

(1) Mix 20 μL of 2.5 mM (total amino acid concentration) pooled amino acid solution with 20 μL of 0.5 M $NaHCO_3$ (pH 9.0) buffer
(2) Add 50 μL of 16 mM dabsyl chloride (dissolved in acetone) to proceed the derivatization reaction and vortex the mixture for 5 seconds then incubate at room temperature for 60 minutes [equivalent ratio of Dabsyl to amino aicd is 16]
(3) After 60 minutes, lyophilize overnight
(4) Reconstitute the mixture with 200 μL of 75% ethanol and centrifuge for 2 minutes 14000 xg, and transfer to a new vial (it may loss 40 μL)
(5) Add adequate volumes of 50% ACN/H_2O (total volume: 200 μL)

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