

A Novel Derivatization Procedure for Chemicals with Carboxylic Acid and Its Application in LC-MS/MS Bioanalysis

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INTRODUCTION

Chemical derivatization has long been employed in LC–MS bioanalysis to address analytical challenges such as poor ionization efficiency, lack of diagnostic product ions, compound instability, limited selectivity, and unsatisfactory chromatographic performance. Despite these advantages, derivatization is often considered a last-resort strategy due to concerns related to incomplete reactions, formation of multiple derivative species, low recovery, and experimentally unfriendly conditions, including extreme temperatures, specialized containers, and non-standard solvents. Consequently, the routine use of derivatization in regulated bioanalysis has been limited. A derivatization procedure that is simple, fast, robust, and compatible with existing bioanalytical workflows would significantly expand its utility, particularly for endogenous biomarkers and structurally diverse carboxylic acid compounds.

METHOD

Sample Preparation

The compounds were extracted from 30 μ L of biological fluid with 120 μ L of methanol. After the sample's vortexed and centrifuged, 100 μ L of the supernatant was transferred into 2nd sample tube, before derivatization reaction being applied. 20 μ L of O-benzylhydroxylamine (O-BHA, 0.3 M) and 20 μ L of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC, 0.6 M) were sequentially added at ambient condition, and mixed well. After 20-30 min reaction, 50 μ L of stop solution was then added before transferred for sample analysis.

Liquid Chromatography:

HPLC System:

Analytical Column:

Mobile Phase A)

Mobile Phase B)

Gradient

Flow rate:

Injection Volume:

Mass Spectrometry

MS System:

Condition:

MRM Transition:

Derivatized Pivalic acid :

Derivatized DMB :

Derivatized lauric acid :

Derivatized MMA :

Derivatized Isoleucine :

Shimadzu Nexera L2

BEH C18 1.7 μ m ,50 $\times$ 2.1 mm

or HSS T3 1.8 μ m, 50 $\times$ 2.1 mm

0.1% formic acid in Water

0.1% formic acid in ACN

0.5 mL/min

5 μ L

Sciex API-4000 or API-6500 +

LC/(+)ESI-MS/MS (MRM)

208.2 $\longrightarrow$ 57.1

222.1 $\longrightarrow$ 71.1

306.1 $\longrightarrow$ 124.1

329.1 $\longrightarrow$ 124

237.2 $\longrightarrow$ 86

RESULTS

Couple carboxylic acid derivatization procedures have been summarized in two published literatures. One method was carried out under mild condition (Journal of Pharmaceutical and Biomedical Analysis 138 (2017) 43–53), but reaction efficiency was poor as well as lower sensitivity. The other method gave a better efficiency and sensitivity (Bioanalysis (2019) 11(08), 741–753).

In order to remove extra reaction chemicals and pyridine solvent, a liquid-liquid extraction (LLE) was applied in this method, and eventually there are a lot of interference in the LC-MS analysis, which limited the method application into other carboxylic acid compounds.

In our study, a completely new method was developed (as shown in Sample Preparation Section) , in which a simple protein crash instead of LLE was applied. The pyridine was replaced with acetonitrile/water, and the amount of reaction reagents was reduced by 5X. In this way, a high reaction efficiency has been achieved with low interference in LC-MS analysis. The whole experiment including both extraction and derivatization could be completed within one hour. This technology has been evaluated for different type of carboxylic acid compounds in plasma: pivalic acid (Figure 1) and 2,2-Dimethylbutyric Acid (DMB, short chain fatty acids), Lauric Acid (long chain fatty acid, Figure 2), Methylmalonic Acid and Succinic Acid (MMA and SA, dual carboxylic acids, Figure 3), isoleucine (Figure 4) and lysine (amino acid).

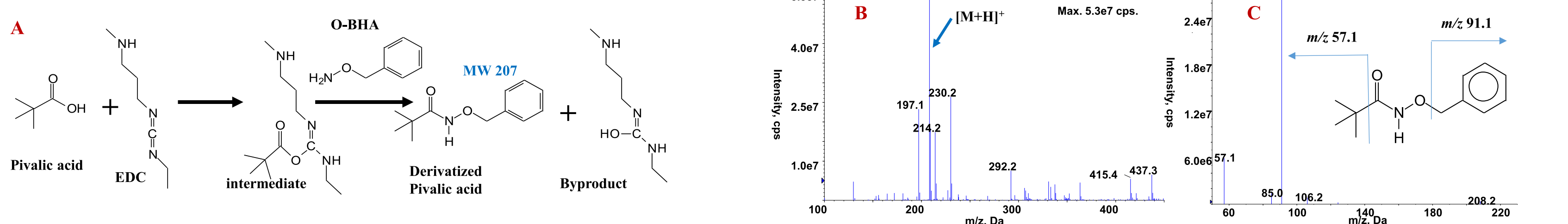


Figure 1, (A) Reaction scheme depicting the derivatization of pivalic acid with O-BHA using the crosslinker EDC, (B) MS derivatized pivalic acid molecular ion spectrum, and (C) MS product ion spectrum

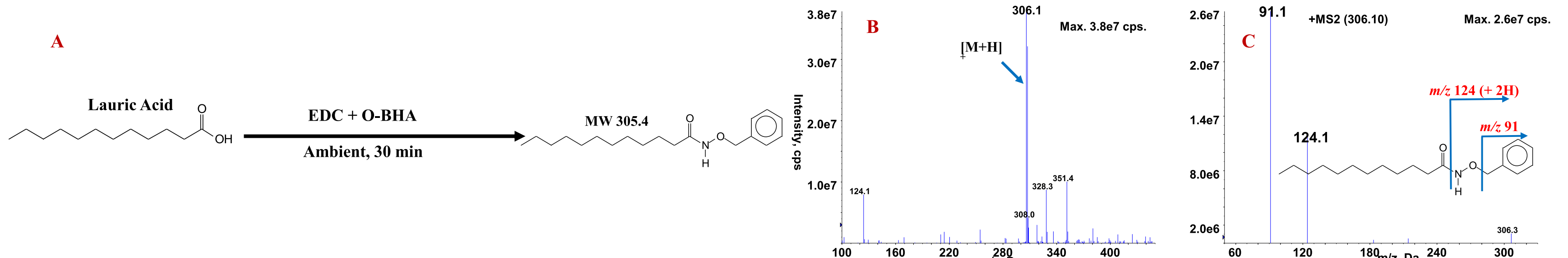


Figure 2, (A) Reaction scheme depicting the derivatization of lauric acid with O-BHA, (B) MS derivatized lauric acid molecular ion spectrum, and (C) MS product ion spectrum

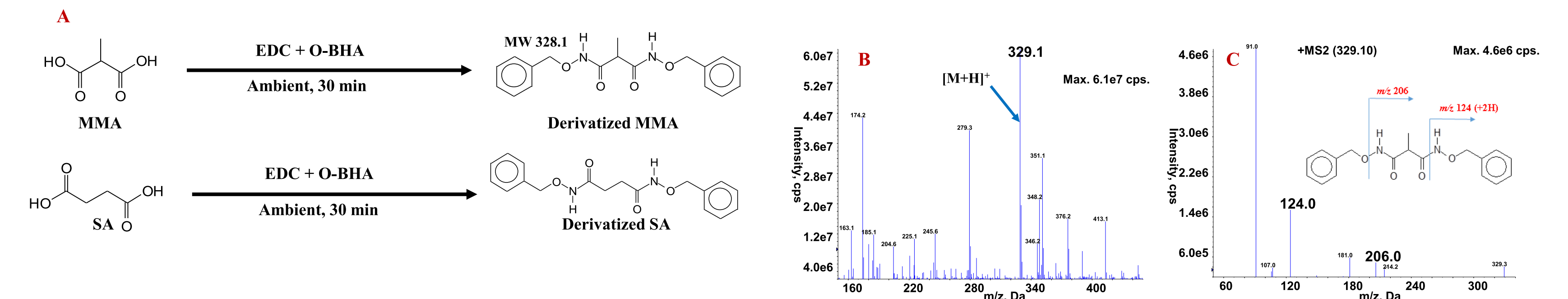


Figure 3, (A) Reaction scheme depicting the derivatization of Methylmalonic Acid (MMA) and Succinic Acid (SA) with O-BHA, (B) MS derivatized MMA or SA molecular ion spectrum, and (C) MS product ion spectrum

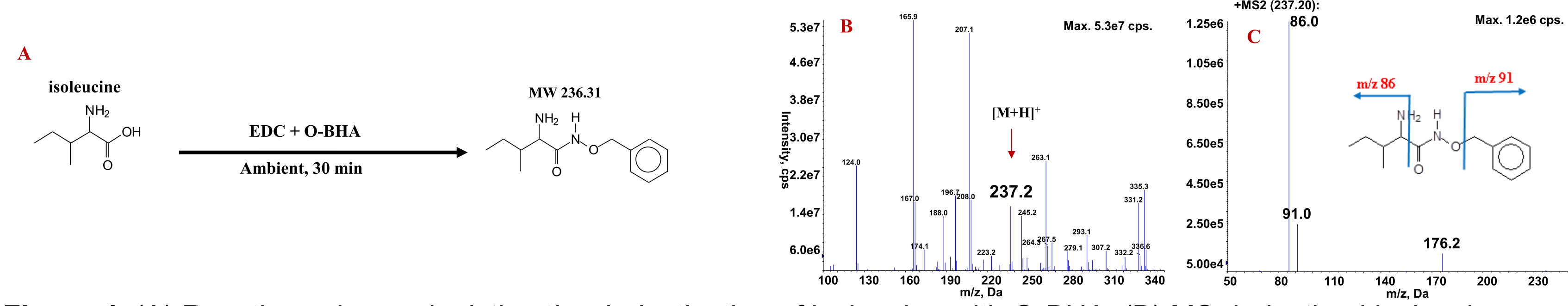


Figure 4, (A) Reaction scheme depicting the derivatization of isoleucine with O-BHA, (B) MS derivatized isoleucine molecular ion spectrum, and (C) MS product ion spectrum

Since the majority are biomarker assays (except of DMB), surrogate matrix such as charcoal stripped plasma or methanol/water solvent were applied. Excellent linearity was obtained with correlation coefficient higher than 0.995. Parallelism assessment indicates that the slope difference between standard additional curve and surrogate matrix curve is below 20% (Tables from I-1 to IV-1) , and the difference of endogenous level measured with standard addition curve and measured with curve in surrogate matrix is also below 20% (Tables from I-2 to IV-2). The accuracy and precision of three level of QC (low, middle, and high) in regular plasma measured with the curve in surrogate matrix are all within 15% (Tables from I-3 to IV-3). The data implicated that the derivatization reaction is highly efficient and could be well reproduced. Since the experiment is simple, short time, and easy to be handled, it should be a general procedure for dealing with this type of the chemicals.

Table I-1. Parallelism between surrogate and normal HP curve evaluation of slope for pivalic acid

	Statistics Data		
	r	Slope	Intercept
Surrogate	0.9962	0.00417	0.0354
human plasma	0.9990	0.00457	1.0200
Difference (%)		-9.6	

Table I-2. Parallelism between surrogate and normal HP curve evaluation of Pivalic acid endogenous level

HP	Conc. in normal human plasma (ng/mL)		
Analyte	from Standard Addition	Calculated from Surrogate Matrix	Difference (%)
Pivalic acid	223	231	-3.4

Table I-3. QC of pivalic acid in HP recovery measured with surrogate curve

HP	QC concentration (ng/mL)			
	Control	QCL (15 ng/mL)	QCM (400 ng/mL)	QCH (800 ng/mL)
n	4	4	4	4
Mean	231	264	599	1158
RE (%)		7.22	-5.15	12.3

Table II-1. Parallelism between surrogate and normal RP curve evaluation of slope for lauric acid

	Statistics Data		
	r	Slope	Intercept
Surrogate	0.9956	923	23500
Rat plasma	0.9970	1070	176000
Difference (%)		-15.9	

Table II-2. Parallelism between surrogate and normal RP curve evaluation of lauric acid endogenous level

Analyte	Conc. in normal Rat plasma (ng/mL)		
	from Standard Addition	Calculated from Surrogate Matrix	Difference (%)
Lauric acid	164	175	-6.0

Table II-3. QC of lauric acid in RP recovery measured with surrogate curve

RP	QC concentration (ng/mL)			
	Control	QCL (60 ng/mL)	QCM (1600 ng/mL)	QCH (3200 ng/mL)
n	4	4	4	4
Mean	175	230	1963	3640
RE (%)		-2.23	10.6	7.9

CONCLUSION

A general, efficient, and user-friendly derivatization method has been developed for LC–MS bioanalysis of carboxylic acid–containing compounds in biological fluids. The simplicity, speed, and robustness of this approach support its broad applicability for both biomarker and small-molecule bioanalysis in regulated environments.

Table III-1. Parallelism between surrogate and normal DP curve evaluation of slope for MMA

MMA	Statistics Data		
	r	Slope	Intercept
Surrogate matrix	0.9991	0.00173	0.00208
Dog plasma	0.9974	0.00198	0.17800
Difference (%)		-14.5	

Table III-2. Parallelism between surrogate and normal DP curve evaluation of MMA endogenous level

Analyte	Conc. in normal dog plasma (ng/mL)		
	from Standard Addition	Calculated from Surrogate Matrix	Difference (%)
MMA	89.9	113	-20.4

Table III-3. QC of MMA in DP recovery measured with surrogate curve

MMA	QC concentration (ng/mL)			
	Control	QCL (30 ng/mL)	QCM (800 ng/mL)	QCH (1600 ng/mL)
n	4	4	4	4
Mean	113	145	990	1953
RE (%)		1.23	8.41	14.0

Table IV-1. Parallelism between surrogate and normal RP curve evaluation of slope for isoleucine

	Statistics Data		
	R	Slope	Intercept
Surrogate	0.9998	0.0251	0.00387
rat plasma	0.9971	0.0257	1.6500
Difference (%)		-2.4	

Table IV-2. Parallelism between surrogate and normal RP curve evaluation of isoleucine endogenous level

Analyte	Conc. in normal rat plasma (ng/mL)		
	from Standard Addition	Calculated from Surrogate Matrix	Difference (%)
isoleucine	64.2	54.4	18.0

Table IV-3. QC of isoleucine in RP recovery measured with surrogate curve

RP	QC concentration (ng/mL)			
	Control	QCL (3 μ g/mL)	QCM (80 μ g/mL)	QCH (160 μ g/mL)
n	4	4	4	4
Mean	54.4	61.3	149	230
RE (%)		6.71	10.5	7.28

