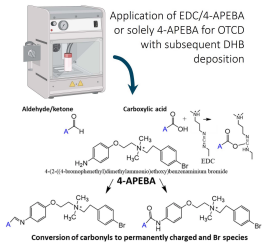
Jun 17, 2024 Version 2

A selective process for application of EDC and 4-APEBA for carbonyl containing metabolites by MALDI-MSI V.2



DOI
dx.doi.org/10.17504/protocols.io.ewov19d9ylr2/v2

Kevin J Zemaitis[1], Christopher R Anderton[1], Dusan Velickovic[1]

[1]Pacific Northwest National Laboratory

Kevin J Zemaitis
Pacific Northwest National Laboratory

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.ewov19d9ylr2/v2

Protocol Citation: Kevin J Zemaitis, Christopher R Anderton, Dusan Velickovic 2024. A selective process for application of EDC and 4-APEBA for carbonyl containing metabolites by MALDI-MSI. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.ewov19d9ylr2/v2 Version created by **Kevin J Zemaitis**

Manuscript citation:
Zemaitis, K. J.; Lin, V. S.; Ahkami, A. H.; Winkler, T. E.; Anderton, C. R.; Veličković, D. Expanded Coverage of Phytocompounds by Mass Spectrometry Imaging Using On-Tissue Chemical Derivatization by 4-APEBA. *Analytical Chemistry* **2023**, *95* (34), 12701-12709.
https://doi.org/10.1021/acs.analchem.3c01345

Veličković, D.; Zemaitis, K. J.; Bhattacharjee, A.; Anderton, C. R. Mass spectrometry imaging of natural carbonyl products directlyfrom agar-based microbial interactions using 4-APEBA derivatization. *mSystems* **2024**, *9* (1), e00803-00823.
https://doi.org/doi:10.1128/msystems.00803-23

Protocol status: Working
We use this protocol and it's working

Created: June 10, 2024

Last Modified: June 17, 2024

Protocol Integer ID: 101962

Funders Acknowledgement:
EMSL Intramural S&T Research Investment
Grant ID: 60091

Disclaimer

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to **protocols.io** is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with **protocols.io**, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

Abstract

Herein, we outline the protocol for application of 4-(2-((4-bromophenethyl)dimethylammonium)ethoxy)benzenaminium dibromide (4-APEBA) which we have previously demonstrated to be an effect reagent in tandem with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) to target carbonyl containing metabolites *in-situ* for matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI). Either with or without the selective application of EDC, MALDI-MSI is able to differentiate ketones and aldehydes from carboxylic acids lending more confidence to annotations. This has enabled metabolic pathway analyses with high-levels of coverage routinely enabling the detect of over a hundred metabolites containing carbonyls.

This reagent was previous introduced for LC-MS/MS purposes (see references), but generally through our on-tissue/on-target chemical derivatization (OTCD) protocol we find that (1) we have increased sensitivity and coverage of primary metabolites, (2) we have increased confidence within our annotations due to bromine isotopic patterns, (3) we do not delocalize metabolites within the process of OTCD, (4) we have multiplexed detection of non-derivatized metabolites and lipids in tandem with OTCD products, and (5) this is applicable to a broad variety of mammalian or plant tissues, and microbial cultures.

Safety warnings

Follow all environmental safety and health guidelines at your respective institutions prior to completing any additional work, and familiarize yourself with the chemicals and risks imposed by reading all SDS.

Considerations Prior to Starting Work

1 The points below are distilled from 100's of applications on various samples, and commonalities found to streamline the on-tissue/on-target chemical derivatization (OTCD) process and minimize batch effects. From experience, we suggest considering the following prior to starting work:

1.1 After initial methods validation, for completing small or large cohorts portion, weigh all derivatization (EDC/4-APEBA) reagents in 1-2 mL autosampler vials. Store under proper conditions per manufacturer guidelines within the freezer (-20°C) or fridge (2-4°C) for the duration of the project.

- While some manufacturers mention reagents can be stored as solutions, portion enough solid to reconstitute as 1-2 mL of solution within kits for each project.
- Dispose of all excess reagents after primary use.

1.2 Ensure all materials are present and instruments are working properly when you start the OTCD process.

- This OTCD reaction is a quick process, where incubation times beyond the time it takes to spray the reagents is not needed (nor is rehydrating the slides).
- It is best practice to be cognizant of timings, where induction of additional dry incubation periods before analyses can induce batch effects.
- Derivatized metabolites were found to be stable for several days.

1.3 Always drop-cast metabolite standards of interest onto the slides near the tissue/target sample (e.g., 1 µL of ~mM ketone, aldehyde, or carboxylic acid) for QA/QC.

- Make stock standard solutions and aliquot into vials for all samples within the analyzed cohort, ensure enough water of solutions is sufficient enough for adequate surface tension (e.g., ~50% MeOH).
- Mark all positions of dried droplets, and image the entire droplet. This ensures that chemical reactions were not problematic, and enables QA/QC metrics to be determined based on derivative standards intensities.
- When comparing in-tissue annotations it is best to exclude these ROIs, as it will effect maximum intensity color scales.

1.4 When switching between EDC and 4-APEBA, properly rinse the sprayer line and the syringe.

- Maintaining EDC/4-APEBA deposited ratios will ensure that applied matrix will be able to co-crystalize efficiently and there will not be larger matrix effects from OTCD (of the matrix itself). It is not recommended to combine EDC/4-APEBA into one single deposition, deposit individually in a two step process.

Instrumentation for OTCD

2 1. M5-Sprayer (HTX Technologies, Chapel Hill, NC) or equivalent

- Related HTX Sprayers also can be used (this was tested on a TM-Sprayer as well).

2. AZURA Pump P4.1S (Knauer GmbH, Berlin, Germany) or equivalent

- We utilize this pump with a 5 mL loop equipped for matrix deposition, usually within this process DHB is loaded and once EDC/4-APEBA is deposited lines can be cleaned the switched, and the process is for the third step of matrix deposition.
- The loop size can be smaller or the use of a secondary pump is not necessary if an external syringe pump is equipped.

3. External syringe pump

- We utilize the syringe pump for EDC/4-APEBA deposition due to low volumes of reagent we use initially to keep cost of OTCD at a minimum with a large loop on our Knauer pump.
- We use a 1 mL Hamilton gastight syringe (Model 1001 TLL, PTFE Luer Lock) connected to the secondary swage passthrough on the M5 Sprayer.

Materials for OTCD

3 All chemicals were used as received without any further purification, and synthesis of 4-APEBA was completed in house:

- 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride - CAS Number: 25952-53-8 - Sigma-Aldrich (>98.0%; St. Louis, MO) or Tokyo Chemical Industry Co. (>98.0%; Portland, OR)
- 4-(2-((4-bromophenethyl)dimethylammonium)ethoxy)benzenaminium dibromide (4-APEBA) - CAS Number: 1226984-28-6 was synthesized at PNNL according to protocols outlined elsewhere (***Note: several chemical suppliers do readily stock this reagent***)
- 2,5-dihydroxybenzoic acid (DHB) - CAS Number: 490-79-9 - Sigma-Aldrich (98%; St. Louis, MO)

Methanol (MeOH) - Fisher Chemical (HPLC grade; Fair Lawn, NJ)

- Water (H_2O) - Fisher Chemical (HPLC grade; Fair Lawn, NJ)

EDC and 4-APEBA Application

4 Both EDC and 4-APEBA are diluted within H_2O after the sample is mounted. No old reagents are kept or used within our process, and all reagents and matrix are applied using the HTX sprayer (reference below).

CITATION

Zemaitis KJ, Lin VS, Ahkami AH, Winkler TE, Anderton CR, Veličković D (2023). Expanded Coverage of Phytocompounds by Mass Spectrometry Imaging Using On-Tissue Chemical Derivatization by 4-APEBA..

LINK

https://doi.org/10.1021/acs.analchem.3c01345

4.1 The following method outlined within **Table 1** is used for both deposition of EDC and 4-AEPBA, separately.

	A	B
	Matrix	EDC/4-APEBA
	Solvent for EDC and 4-APEBA	100% H2O
	EDC Concentration (mg/mL)	6
	4-APEBA Concentration (mg/mL)	2
	Nozzle Temperature (°C)	37.5
	Number of Cycles	4
	Flow Rate (µL/min)	25
	Velocity (mm/min)	1200
	Track Spacing (mm)	3
	Pattern	CC
	Drying Period (sec)	2
	Nozzle Height (mm)	40
	N2 Flow Rate (L/min)	3
	Pressure (PSI)	10

	A	B
	EDC Coverage (µg/cm2)	~16.7
	4-APEBA Coverage (µg/cm2)	~5.6

Table 1: Critical method parameters for HTX Sprayers for the deposition of aqueous EDC and 4-APEBA.

4.2 After final deposition of 4-APEBA, an aqueous and organic wash of several syringe volumes are used to clean the HTX Sprayer lines and head according to laboratories best practice.

4.3 Note: 4-APEBA OTCD can be exploited for determining specific moieties on molecules (e.g., ketones/aldehydes vs caboxylic acids) as shown within *Veličković et al. 2024* referenced below.

- If EDC is not applied, caboxylates will not form an O-acylisourea intermediate, resulting in derivatization
  of only ketones and aldehydes
- If EDC and 4-APEBA are applied in sequentially, derivatization will occur for ketones, aldehydes, and carboxylic acids

CITATION

Veličković D, Zemaitis KJ, Bhattacharjee A, Anderton CR (2024). Mass spectrometry imaging of natural carbonyl products directly from agar-based microbial interactions using 4-APEBA derivatization..

LINK

https://doi.org/10.1128/msystems.00803-23

If following this protocol, it is highly recommended to complete EDC/4-APEBA and sole 4-APEBA deposition for the comparison.

DHB Application

5 Directly following the OTCD steps (either solely 4-APEBA or EDC/4-APEBA), matrix is applied. This deposition was previously optimized within the reference below. While DHB does contain moieties that can be derivatized, we found any derivatized DHB did not effect sensitivity. Additionally, these products provide additional lock masses that can be exploited for MS calibration.

CITATION

Veličković D, Zhang G, Bezbradica D, Bhattacharjee A, Paša-Tolić L, Sharma K, Alexandrov T, Anderton CR, KPMP Consortium (2020). Response Surface Methodology As a New Approach for Finding Optimal MALDI Matrix Spraying Parameters for Mass Spectrometry Imaging..
LINK
https://doi.org/10.1021/jasms.9b00074

5.1 The following method outlined within **Table 2** is used for both deposition of DHB.

	A	B
	Matrix	DHB
	Solvent	70% MeOH (*aq*)
	DHB Concentration (mg/mL)	40
	Nozzle Temperature (°C)	75
	Number of Cycles	12
	Flow Rate (µL/min)	50
	Velocity (mm/min)	1200
	Track Spacing (mm)	3
	Pattern	CC
	Drying Period (sec)	2
	Nozzle Height (mm)	40
	N2 Flow Rate (L/min)	2
	Pressure (PSI)	10
	DHB Coverage (µg/cm2)	~667

Table 2: Critical method parameters for HTX Sprayers for the deposition of DHB matrix.

.

5.2 After DHB matrix application, MALDI-MSI analyses can be performed.

MALDI-MSI Analyses

6 A variety of MALDI instruments have been used for the collection of 4-APEBA OTCD datasets.

Both a 7T scimaX FTICR MS and 12T solariX XR FTICR MS (Bruker Daltonics, Bremen, DE), as well as a timsTOF fleX MS (Bruker Daltonics).

Using at least one lock mass is recommended, ideally the $[4\text{-APEBA-}H_20]^+$ corresponding to $C_{18}H_{24}N_2OBr$ at *m/z* 363.1067, alternatively additional masses of $[DHB+4\text{-APEBA}]^+$ or other reaction byproducts and matrix clusters can be employed as well.

Data Analyses

7 4-APEBA-based OTCD MSI data is annotatable using METASPACE (**https://metaspace2020.eu/**) with the chemical modifier of ($+C_{18}H_{22}N_2Br$, +345.0966 Da).

Filtering using the adducts tool searching for $[M]^+$ adducts with the chemical modifier ($+C_{18}H_{22}N_2Br$) and starting with FDR of 10% is a good starting point.

It is vital to use the diagnostics tool, and also search through the full profile data in a different visualization package to ensure proper annotation of Br isotopic pattern.

You may find at >20% FDR there can be annotations which are true positives, but were annotated with low FDR due to isotopic complexity.

Additionally, [M+H]+ or [M+Na]+ or [M+K]+ of biogenic amines and other primary metabolites or phospholipids are commonly detected as well. Note, the carboxyl ester of the acyl chains within lipids are protected functional groups that are not able to be derivatized.

Protocol references

For description of original reagent development please see these seminal papers on LC-MS/MS:
Eggink M, Wijtmans M, Kretschmer A, Kool J, Lingeman H, de Esch IJP, Niessen WMA, Irth H. Targeted LC–MS derivatization for aldehydes and carboxylic acids with a new derivatization agent 4-APEBA. *Analytical and Bioanalytical Chemistry.* 2010, 397(2), 665-675.
https://doi.org/10.1007/s00216-010-3575-1
Kretschmer A, Giera M, Wijtmans M, de Vries L, Lingeman H, Irth H, Niessen WMA. Derivatization of carboxylic acids with 4-APEBA for detection by positive-ion LC-ESI–MS(/MS) applied for the analysis of prostanoids and NSAID in urine. *Journal of Chromatography B.* 2011, 879(17), 1393-1401.
https://doi.org/10.1016/j.jchromb.2010.11.028.

For description of protocol development for OTCD for MALDI-MSI please see these papers:
Zemaitis KJ, Lin VS, Ahkami AH, Winkler TE, Anderton CR, Veličković D. Expanded Coverage of Phytocompounds by Mass Spectrometry Imaging Using On-Tissue Chemical Derivatization by 4-APEBA. *Analytical Chemistry.* 2023, 95(34), 12701-12709.
https://doi.org/10.1021/acs.analchem.3c01345
Veličković D, Zemaitis KJ, Bhattacharjee A, Anderton CR. Mass spectrometry imaging of natural carbonyl products directlyfrom agar-based microbial interactions using 4-APEBA derivatization. *mSystems.* 2024, 9(1), e00803-00823.
https://doi.org/doi:10.1128/msystems.00803-23

For additional information on DHB spraying optimization please see this paper:
Veličković D, Zhang G, Bezbradica D, Bhattacharjee A, Paša-Tolic L, Sharma K, Alexandrov T, Anderton CR, KPMP Consortium. Response Surface Methodology As a New Approach for Finding Optimal MALDI Matrix Spraying Parameters for Mass Spectrometry Imaging. *Journal of the American Society for Mass Spectrometry*. 2020, 31(3), 508-516.
https://doi.org/10.1021/jasms.9b00074

Citations

Step 4

Zemaitis KJ, Lin VS, Ahkami AH, Winkler TE, Anderton CR, Veličković D. Expanded Coverage of Phytocompounds by Mass Spectrometry Imaging Using On-Tissue Chemical Derivatization by 4-APEBA.
https://doi.org/10.1021/acs.analchem.3c01345

Step 4.3

Veličković D, Zemaitis KJ, Bhattacharjee A, Anderton CR. Mass spectrometry imaging of natural carbonyl products directly from agar-based microbial interactions using 4-APEBA derivatization.
https://doi.org/10.1128/msystems.00803-23

Step 5

Veličković D, Zhang G, Bezbradica D, Bhattacharjee A, Paša-Tolić L, Sharma K, Alexandrov T, Anderton CR, KPMP Consortium. Response Surface Methodology As a New Approach for Finding Optimal MALDI Matrix Spraying Parameters for Mass Spectrometry Imaging.
https://doi.org/10.1021/jasms.9b00074