

Use of a Mixed Cationic-Reverse Phase Column for Analyzing Small Highly Polar Metabolic Markers in Biological Fluids for Multiclass LC-HRMS Method

Marco Roverso *, Iole Maria Di Gangi, Gabriella Favaro, Paolo Pastore[†] and Sara Bogialli[†]

Department of Chemical Sciences, University of Padua, Via Marzolo, 1, 35131 Padova, Italy; iole.digangi@gmail.com (I.M.D.G.); gabriella.favaro@unipd.it (G.F.); paolo.pastore@unipd.it (P.P.); sara.bogialli@unipd.it (S.B.)

* Correspondence: marco.roverso@unipd.it; Tel.: +39-049-8275181

Received: 17 September 2020; Accepted: 12 October 2020; Published: 14 October 2020



Abstract: The determination of small highly polar metabolites at low concentrations is challenging when reverse-phase (RP) chromatography is used for multiclass analysis. A mixed cationic-RP column coupled to high-resolution tandem MS (HR-MS/MS) was tested for highly polar compounds in biological fluids, i.e., trimethylamine N-oxide (TMAO) and the isobaric molecules beta-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (DAB). The efficient retention and separation of the above compounds were obtained with common and MS-friendly RP conditions, reaching high selectivity and sensitivity. The method was firstly assessed in plasma and urine, showing good linearity in the range 50–1000 µg/L and 500–10,000 µg/L for TMAO and both BMAA and DAB, respectively. Excellent precision (RDS < 3%) and good accuracies (71–85%) were observed except for BMAA in plasma, whose experimental conditions should be specifically optimized. Preliminary tests performed on compounds with biological relevance and a wider range of polarities proved the effectiveness of this chromatographic solution, allowing the simultaneous analysis of a larger panel of metabolites, from very small and polar compounds, like trimethylamine, to quite lipophilic molecules, such as corticosterone. The proposed LC-HRMS protocol is an excellent alternative to hydrophilic interaction liquid chromatography and ion-pairing RP chromatography, thus providing another friendly analytical tool for metabolomics.

Keywords: polar amino acids; mixed cationic-RP column; LC-HRMS; BMAA; TMAO

1. Introduction

The separation of small strongly polar compounds is challenging for reverse-phase (RP) chromatography. The weak interaction between the hydrophobic stationary phase and analytes usually leads to short retention times, matrix interferences and poor selectivity [1]. Derivatization steps and ion-pairing chromatography are possible solutions. However, derivatization procedures are usually time-consuming, and both reagents and secondary reactions may interfere with the analysis. Ion pairing is another useful trick but may result in low sensitivity with LC-MS. Hydrophilic interaction liquid chromatography (HILIC) is nowadays a quite widespread approach for the analysis of highly polar molecules such as amino acids, saccharides, nucleic acids, and phosphate-containing molecules [2]. In this case, very common drawbacks are peak distortion, large consumption of organic solvents, long equilibration times, and lack of solubility of some polar compounds in organic solvents. Mixed-mode liquid chromatography is an interesting alternative to the RP and HILIC stationary phases. These phases, firstly described in 1984 by Bischoff and McLaughlin [3], can be very effective for the simultaneous analysis of polar and non-polar compounds, due to the combined effects of different interaction mechanisms [4].

Beta-*N*-methylamino-*L*-alanine (BMAA) is a polar amino acid with a supposed neurotoxic activity. BMAA was described as a metabolite of several cyanobacterial strains, which can be bioaccumulated and biomagnified across the food chain [5]. Chronic exposure to BMAA has been associated with degenerative neurological conditions such as amyotrophic sclerosis, Parkinson's disease and dementia [6]. The risk assessment of BMAA is highly debated in the scientific literature since results are difficult to compare, because of the possible false-positives obtained by using different analytical techniques [7]. The main issue is the presence of several isomers of BMAA, such as 2,4-diaminobutyric acid (DAB), so that the chromatographic separation and the correct quantification in biological and food samples remain challenging [8,9]. Most of the existing analytical procedures are based on the derivatization of the analytes with 6-aminoquinolonyl-*N*-hydroxysuccinimide prior to RP chromatography, or HILIC and ion-pairing RP separation without derivatization [5,8–11]. Solid-phase extraction performed using strong cation exchange sorbents is also reported for improving the sensitivity of the method and for samples clean up [12]. Recent works take advantage of tandem MS to improve the selectivity of the method and avoid false positives [13].

Trimethylamine *N*-oxide (TMAO) is a highly polar metabolite whose production is controlled by gut microbiota and liver enzymes. The production of TMAO is strictly related to the dietary consumption of *L*-carnitine and lectin rich food. Recently, the alteration of TMAO concentration in plasma was positively associated with an increased risk for cardiovascular diseases, heart failure, obesity, impaired glucose tolerance, diabetes, and colorectal cancer [14,15]. The quantitative analysis of TMAO is usually performed with RP or HILIC chromatography coupled to electrospray (ESI) mass spectrometry [16–18]. BMAA and TMAO, such as other polar metabolites, are potentially useful biomarkers to be monitored in various biological fluids, but the experimental conditions conventionally used for RP mode can inhibit the simultaneous multiclass determination of very different compounds, which is a goal of the metabolomic approach.

In this preliminary research work, a new separation approach, based on the use of a mixed cation-RP stationary phase, is evaluated to obtain the best retention and selectivity in the direct analysis of highly polar compounds such as TMAO, BMAA, and DAB. The analytical method is based on LC coupled to high-resolution tandem mass spectrometry (LC-HR-MS/MS) and applied to various biological matrices, such as plasma and urine, for clinical applications. Furthermore, the present study outlines future applications of this protocol to a wider panel of polar and non-polar analytes in the framework of metabolomic investigations.

2. Materials and Methods

2.1. Chemicals

Analytical grade BMAA, DAB, TMAO, deuterated trimethylamine *N*-oxide (D₉, 98%) (TMAO-d₉), trimethylamine, kynurenic acid, dopamine, homocysteine, carbidopa, picolinic acid, *L*-DOPA, 3-hydroxykynurenine, corticosterone and formic acid (FA) were purchased from Sigma-Aldrich Italy (Milan, Italy). LC-MS solvent grade acetonitrile was purchased from Carlo Erba Reagents (Milano, Italy). Ultrapure-grade water was produced by a Pure-Lab Option Q apparatus (Elga Lab Water, High Wycombe, UK). Standard stock solutions of the analytes under study were prepared in water/acetonitrile 50:50 at 1000 mg/L and stored at −20 °C until use. A mixed working standard solution was obtained by suitable dilutions in mobile phases. TMAO-d₉ was used as an internal standard (IS).

2.2. Instrumentation

The LC-MS/MS system was an Ultimate 3000 UHPLC coupled to a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

A Luna HILIC (100 mm × 2.0 mm i.d. × 3 µm, Phenomenex, Bologna, Italy) and an Acclaim Mixed-Mode WCX-1 (150 mm × 2.1 mm i.d. × 3 µm, Thermo Fisher Scientific, Waltham, MA, USA) were used as columns thermostated at 15 °C. The elution was performed in a gradient mode (0–6 min 0% B,

6–15 min 0% B to 100% B, 15–20 min 100% B, equilibration time 10 min) at a flow rate of 0.25 mL/min, using water (eluent A for Acclaim, B for HILIC) and acetonitrile (eluent B for Acclaim, A for HILIC) both 10 mM FA. This flow rate was a compromise between the chromatographic selectivity and the ESI-MS responses. The injection volume was 5 μ L. The MS conditions were the following: electrospray (ESI) ionization in positive mode, resolution 35,000 in MS and 17,500 in MS/MS (at m/z 200), AGC target 3×10^6 and 2×10^5 in MS and tandem MS, respectively; max injection time 200 ms, scan range 50–750 Da, isolation window 4.0 m/z , normalized collision energy 35 in HCD mode. The capillary voltage was 3.5 kV, the capillary temperature was 320 $^{\circ}$ C, auxiliary gas, and sheath gas was nitrogen at 40 and 20 a.u., respectively, while sweep gas was not used. Calibration was performed with PierceTM ESI Positive Ion Calibration Solution (Thermo Fisher Scientific, Waltham, MA, USA). The MS data were analyzed with the Xcalibur 4.0TM software (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Sample Preparation and Calibrations

A five-point external calibration curve was prepared from stock solutions of TMAO, BMAA and DAB. The concentrations of BMAA and DAB in the five solutions were 50, 100, 250, 750 and 1000 μ g/L, while TMAO was present at 5, 10, 25, 75 and 100 μ g/L. IS was added in all the calibration solutions to a final concentration of 250 μ g/L. The peak area ratio (A/A_{IS}) related to the selected fragment ions acquired in tandem MS mode for each compound was plotted against concentrations for calibration purposes. Linearity was assessed using the least-squares regression.

Mouse plasma and urine samples were collected as previously reported [19]. For the matrix-matched calibration, plasma samples were pooled from ten different animals and split into 100 μ L aliquots. Five aliquots were spiked with both BMAA and DAB at concentrations of 500, 1000, 2500, 7500 and 10,000 μ g/L and TMAO at concentrations of 50, 100, 250, 750 and 1000 μ g/L, respectively; one aliquot was treated as blank. The basal concentration of TMAO, which is a metabolite physiologically present in plasma and urine, was preliminarily estimated by the standard addition method. Plasma aliquots were extracted with 400 μ L of ice-cold acetonitrile added with 10 mM FA and 625 μ g/L of IS, vortexed and centrifuged at $14,000 \times g$ and 4 $^{\circ}$ C for 10 min. 100 μ L of the supernatant was further diluted 1:1 with water 10 mM FA and injected for the analysis. The same protocol was used for urine samples. The selected analytes were analyzed in their free form, i.e., not bound to proteins, which were removed during the sample preparation.

3. Results and Discussion

The chromatographic separation of BMAA, DAB and TMAO is hindered by poor retention in RP mode, and HILIC conditions have been used to overcome these problems. Figure 1A shows the extracted ion chromatogram (EIC, mass accuracy 10 ppm) of $[M+H]^+$ precursor ions at m/z 119.0815, 76.0757 and 85.1322 for isobaric BMAA and DAB, TMAO and TMAO-d9, respectively, obtained from the analysis of a working standard solution at 2500 μ g/L in HILIC mode. Although the isobaric BMAA and DAB are greatly retained by this stationary phase ($rt = 7.03$ and 7.17 min, respectively), their separation is not effective. Conversely, TMAO and TMAO-d9 are not retained and are detectable at the column dead time. Although several attempts were made to increase the efficiency of the BMAA and DAB separation and the TMAO retention by varying both additive type and concentration (in particular, by changing FA concentration from 10 mM to 25 mM and by substituting FA with ammonium acetate, from 1 mM to 10 mM in both water and acetonitrile), no significant improvements were obtained. The effective separation of TMAO, and/or BMAA and DAB using HILIC chromatography are anyway reported in the literature, but the proposed methods are based on stationary phases that are quite different from the one herein described, e.g., ZIC-HILIC [8], ethylene-bridged hybrid (BEH) particles [12] or amide-based stationary phase [17].

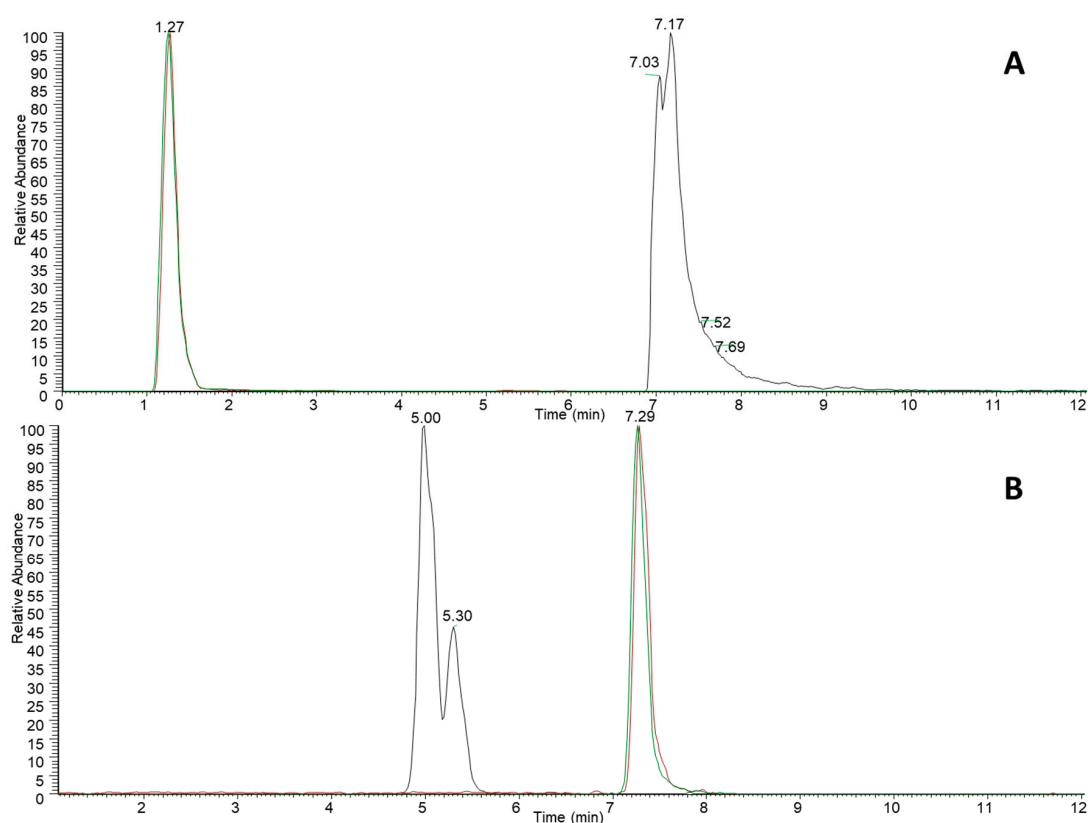


Figure 1. (A) Overlapped EIC chromatograms (accuracy 10 ppm) for BMAA ($[M+H]^+$, m/z 119.0815, black, $rt = 7.03$ min), DAB ($[M+H]^+$, m/z 119.0815, black, $rt = 7.17$ min), TMAO ($[M+H]^+$, m/z 76.0757.0815, red, $rt = 1.27$ min) and TMAO-d9 (IS) ($[M+H]^+$, m/z 85.1322, green, $rt = 1.27$ min) obtained with Luna HILIC. (B) Overlapped EIC chromatograms (accuracy 10 ppm) for BMAA ($[M+H]^+$, m/z 119.0815, black, $rt = 5.00$ min), DAB ($[M+H]^+$, m/z 119.0815, black, $rt = 5.30$ min), TMAO ($[M+H]^+$, m/z 76.0757.0815, red, $rt = 7.32$ min) and TMAO-d9 (IS) ($[M+H]^+$, m/z 85.1322, green, $rt = 7.29$ min) obtained with and Acclaim Mixed-Mode WCX-1. The concentration is 2500 $\mu\text{g/L}$ for each analyte.

Better results, in terms of peaks separation and retention, were obtained with a mixed cationic-RP column in reverse-phase conditions, as reported in Figure 1B. In this case, the isobars BMAA ($rt = 5.00$ min) and DAB ($rt = 5.30$ min) were retained and satisfactorily separated. Even for TMAO and TMAO-d9 ($rt = 7.29$ min) retention was considerably improved.

Greater sensitivity and selectivity can be achieved by acquiring data in high-resolution MS/MS mode, in particular for BMAA and DAB. The comparison between the MS/MS spectra of BMAA and DAB (Figure 2, panel A), shows the presence of two significantly different fragment ions for DAB and BMAA: signal at m/z 88.0399 was related to the loss of substituted amino moiety of BMAA, and signal a m/z 101.0714 due to the loss of the terminal NH_3 of DAB. Once selected the precursor ions, the EIC (mass accuracy: 10 ppm) related to the fragment ions at m/z 58.0880, m/z 68.1302, m/z 101.0714 and m/z 88.0399 for TMAO, TMAO-d9, DAB and BMAA, respectively, were acquired in parallel reaction monitoring mode (Figure 2, panel B), thus improving the MS sensitivity and the selectivity obtained by the chromatographic separation. The obtained separation is anyway necessary when lower selective detection systems, such as LC coupled to single-stage or low-resolution MS are used. In the last cases, the shared fragment ions can produce inaccuracies or false-positive results.

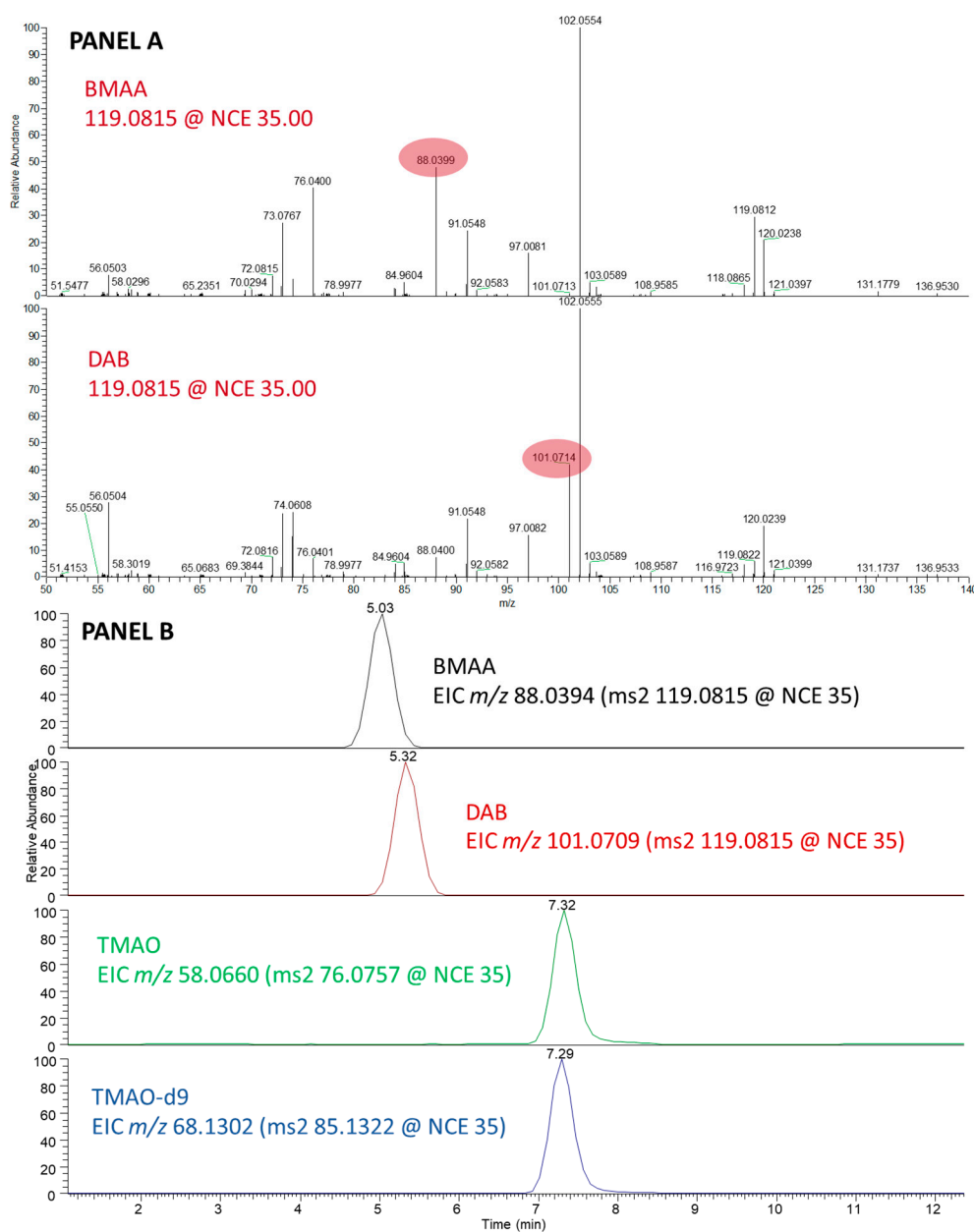


Figure 2. Cont.

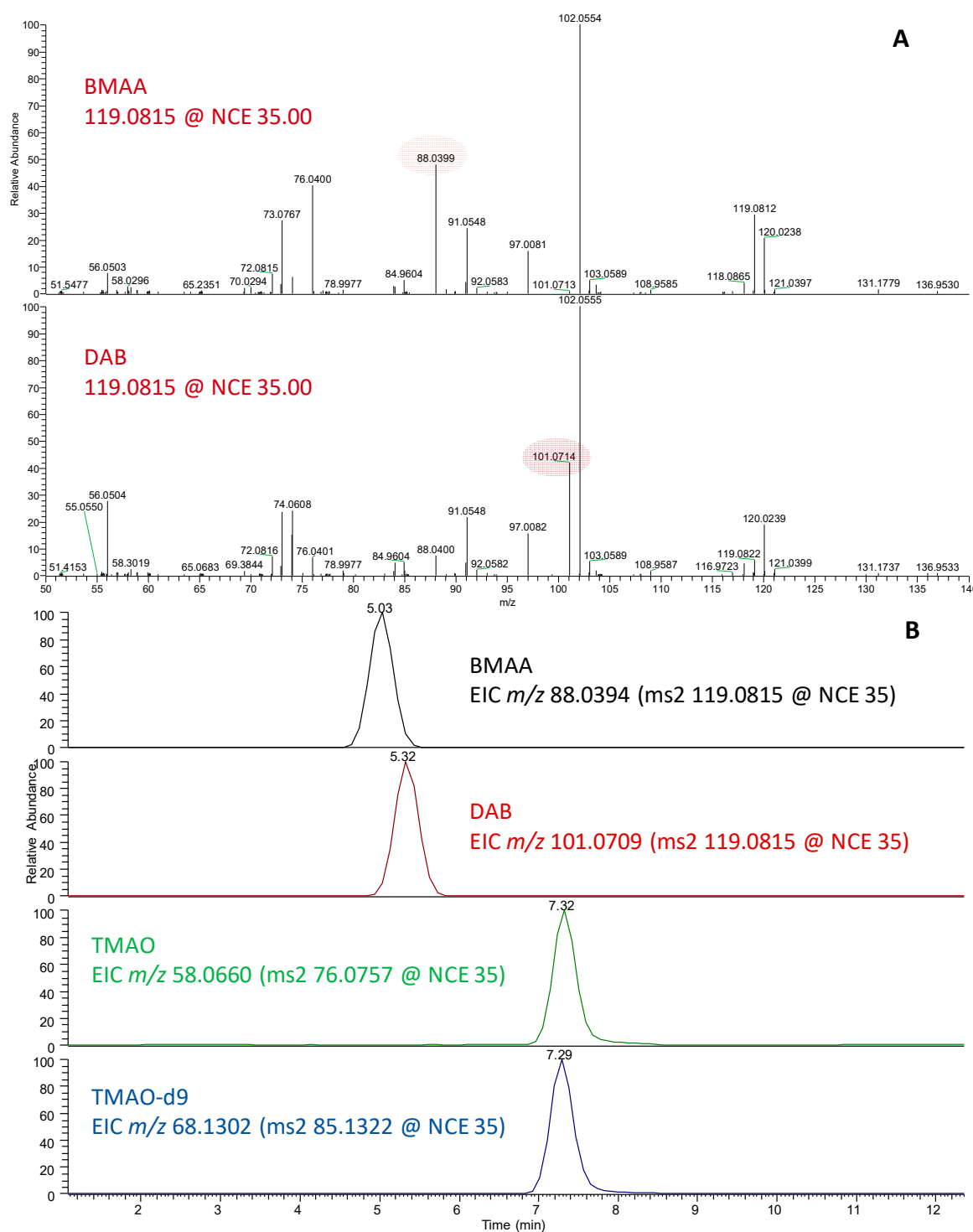


Figure 2. (A) Comparison between the MS/MS spectra of BMAA and DAB, both obtained in HCD mode with a normalized collision energy (NCE) of 35. (B) EIC MS/MS chromatograms (accuracy: 10 ppm) obtained with Acclaim Mixed-Mode WCX-1: BMAA: EIC for m/z 88.0394 (parent ion: m/z 119.0815, NCE:35); DAB: EIC for m/z 101.0709 (parent ion: m/z 119.0815, NCE:35); TMAO: EIC for m/z 58.0660 (parent ion: m/z 85.1322, NCE:35); TMAO-d9 (IS): EIC for m/z 68.1302 (parent ion: m/z 85.1322, NCE:35). The concentration is 2500 $\mu\text{g/L}$ for each analyte.

The method linearity, precision and accuracy, the last intended as the combined contribution of matrix effects and recoveries, were evaluated in plasma and urine to perform a preliminary evaluation

of the performances. Matrix-matched calibration curves were performed in triplicate and were obtained by spiking blank samples at the same nominal concentrations of the external calibration curves. A very good linearity ($R^2 > 0.99$) was observed for all the selected analytes in water, plasma and urine matrices. Precision, evaluated from the standard deviations of the regression slopes, was excellent and showed relative standard deviations (RSD%) $< 3\%$ for all the matrices taken into consideration. Matrix effects and recoveries were evaluated from the percent slope ratio of the matrix-matched and external standard calibration curves. Values within the range 70–110% are generally acceptable, as strong matrix effects and poor recoveries can be excluded. Considering the excellent precision of the method, it is possible to assume good recoveries and a limited matrix effect for BMAA and DAB in urine, as the values obtained for the percent slope ratio were 71% and 85%, respectively. Results obtained for plasma are acceptable for DAB (73%) and quite good for TMAO (81%). Such method performances were already proved to be reliable for the quantification of TMAO in mouse plasma (RSD of the IS $< 15\%$ for $N = 77$ samples) [19]. Nevertheless, the combined matrix effect, in terms of ESI signals variation and recovery, was not acceptable in plasma for BMAA, as values are lower than 50%. In this last case, probably due to the interfering compounds co-extracted from this complex matrix, it will be necessary to increase the dilution of the sample before analysis or modify the extraction procedure or the chromatographic conditions. The choice of a suitable IS could anyway improve its accuracy. LODs, assessed from the lowest point of the matrix-matched calibration curve, and corresponding to an S/N value of 3, were estimated to be 10 $\mu\text{g/L}$ for BMAA and DAB and 2 $\mu\text{g/L}$ for TMAO.

Preliminary quantitative data in plasma and urine samples were obtained only for TMAO, which was present as an average ($n = 5$) basal concentration of $121 \pm 8 \mu\text{g/L}$ in plasma and $193 \pm 9 \mu\text{g/L}$ in urine, respectively. BMAA and DAB were always lower than LODs in both plasma and urine samples, but the collection of specimens suspected to be positively correlated to BMAA has to be specifically planned, and it is beyond the focus of this work.

Further tests are in progress in our laboratory using the chromatographic method herein reported in order to expand the panel of analytes potentially quantifiable using this mixed cationic-RP column. The novel set of analytes included: other isomers of BMAA, such as beta-amino-*N*-methylalanine and *N*-(2-aminoethyl) glycine; levodopa, carbidopa and dopamine, which are compounds involved in the Parkinson's disease, and may be useful for the assessment of the possible adverse effects of BMAA; trimethylamine, short-chain fatty acids, e.g., butyric, isobutyric, valeric, isovaleric, hexanoic and acetic acid are key compounds, together with TMAO, linked to the gut microbiota, whose alteration was recently associated with the development of type 2 diabetes and obesity [20]; metabolites such as picolinic and nicotinic acid, tryptophan, kynurenic acid, 3-hydroxykinurenine were selected as representative of other specific metabolic pathways, e.g., the tryptophan metabolism. Corticosterone, an important intermediate for the synthesis of glucocorticoid hormones in humans, was selected in order to evaluate the chromatographic interactions of the mixed-RP stationary phase with lipophilic substances. Preliminary results regarding the chromatographic separation of some of the selected analytes obtained by the Acclaim Mixed-Mode WCX-1 column are reported in Figure 3. As shown, this column was suitable for retaining compounds with a wide range of polarities, from trimethylamine to corticosterone.

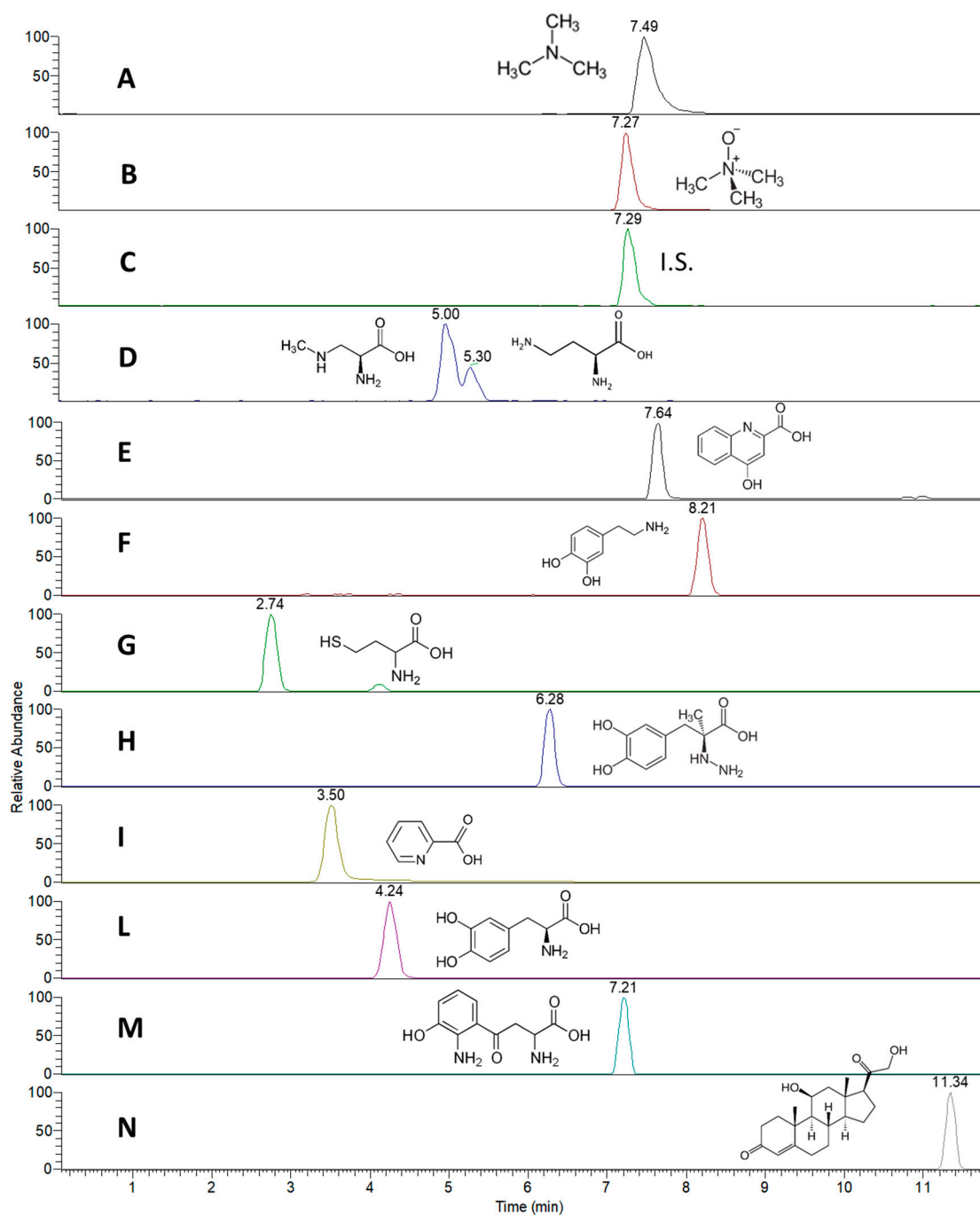


Figure 3. EIC chromatograms obtained with an Acclaim Mixed-Mode WCX-1 in ESI(+)-HRMS. (A) Trimethylamine, $[M+H]^+$, EIC at m/z 60.0808. (B) TMAO, $[M+H]^+$, EIC at m/z 76.0757. (C) TMAO-d9 (IS), $[M+H]^+$, EIC at m/z 85.1322. (D) BMAA (left peak) and DAB (right peak), $[M+H]^+$, EIC at m/z 119.0815. (E) Kynurenic acid, $[M+H]^+$, EIC at m/z 190.0499. (F) Dopamine, $[M+H]^+$, EIC at m/z 154.0863. (G) Homocysteine, $[M+H]^+$, EIC at m/z 136.0427. (H) Carbidopa, $[M+H]^+$, EIC at m/z 227.1026. (I) Picolinic acid, $[M+H]^+$, EIC at m/z 124.0393. (L) L-DOPA, $[M+H]^+$, EIC at m/z 198.0761. (M) 3-hydroxykynurenine, $[M+H]^+$, EIC at m/z 225.0870. (N) Corticosterone, $[M+H]^+$, EIC at m/z 347.2217. Mass accuracy: 10 ppm. The concentration is 2500 $\mu\text{g/L}$ for each analyte.

4. Conclusions

In this work, the effective chromatographic retention of selected highly polar metabolites was carried out by using a mixed cationic-RP column, simultaneously obtaining an efficient separation of

the isobaric BMAA and DAB without derivatization and ion pairing. The selectivity of the method was increased by HR tandem MS, avoiding the contribution of the partial co-eluted peaks. A preliminary evaluation of the method performances showed good linearity, acceptable recoveries and matrix effects for all the analytes in urine, and DAB and TMAO in plasma. The full validation of the method, including the assessment of the LOD, LOQ, repeatability and reproducibility, is in progress. Further evaluation of the column retention and selectivity started on a larger panel of analytes with different chemical properties and related to the metabolism of tryptophan, the short-chain fatty acids, and other isomers of BMAA and molecules related to the Parkinson disease. The versatility of this alternative chromatographic method is of particular interest in the field of metabolomics, where it is essential to analyze simultaneously various classes of molecules with very different chemical properties, in terms of polarity and molecular weight.

Author Contributions: Conceptualization, M.R., S.B. and I.M.D.G.; methodology, M.R., S.B. and I.M.D.G.; validation, M.R. and I.M.D.G.; formal analysis, M.R. and I.M.D.G.; investigation, M.R., S.B. and I.M.D.G.; resources, S.B. and P.P.; data curation, M.R., G.F. and I.M.D.G.; writing—original draft preparation, M.R., S.B. and G.F.; writing—review and editing, M.R., S.B. and P.P.; visualization, S.B. and P.P.; supervision, S.B.; funding acquisition, S.B. and P.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors are grateful to the research group of Gian Paolo Fadini (University of Padua) for collaboration with the TMAO project.

Conflicts of Interest: The authors declare they have no conflicts of interest.

References

1. Rojo, D.; Barbas, C.; Ruperez, F.J. LC-MS metabolomics of polar compounds. *Bioanalysis* **2012**, *4*, 1235–1243. [[CrossRef](#)] [[PubMed](#)]
2. Jandera, P. Stationary and mobile phases in hydrophilic interaction chromatography: A review. *Anal. Chim. Acta* **2011**, *692*, 1–25. [[CrossRef](#)] [[PubMed](#)]
3. Bischoff, R.; McLaughlin, L.W. Nucleic acid resolution by mixed-mode chromatography. *J. Chromatogr. A* **1984**, *296*, 329–337. [[CrossRef](#)]
4. Lemasson, E.; Richer, Y.; Bertin, S.; Hennig, P.; West, C. Characterization of retention mechanisms in mixed-mode HPLC with a bimodal reversed-phase/cation-exchange stationary phase. *Chromatographia* **2018**, *81*, 387–399. [[CrossRef](#)]
5. Manolidi, K.; Triantis, T.M.; Kaloudis, T.; Hiskia, A. Neurotoxin BMAA and its isomeric amino acids in cyanobacteria and cyanobacteria-based food supplements. *J. Hazard. Mater.* **2019**, *365*, 346–365. [[CrossRef](#)] [[PubMed](#)]
6. Kurland, L.T. Amyotrophic lateral sclerosis and parkinson's disease complex on Guam linked to an environmental neurotoxin. *Trends Neurosci.* **1988**, *11*, 51–54. [[CrossRef](#)]
7. Faassen, E.J. Presence of the neurotoxin BMAA in aquatic ecosystems: What do we really know? *Toxins* **2014**, *6*, 1109–1138. [[CrossRef](#)] [[PubMed](#)]
8. Faassen, E.J.; Antoniou, M.G.; Beekman-Lukassen, W.; Blahova, L.; Chernova, E.; Christophoridis, C.; Combes, A.; Edwards, C.; Fastner, J.; Harmsen, J.; et al. A collaborative evaluation of LC-MS/MS based methods for BMAA analysis: Soluble bound BMAA found to be an important fraction. *Mar. Drugs* **2016**, *14*, 45. [[CrossRef](#)] [[PubMed](#)]
9. Banack, S.A.; Downing, T.G.; Spácil, Z.; Purdie, E.L.; Metcalf, J.S.; Downing, S.; Esterhuizen, M.; Codd, G.A.; Cox, P.A. Distinguishing the cyanobacterial neurotoxin β -N-methylamino-L-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB). *Toxicon* **2010**, *56*, 868–879. [[CrossRef](#)]
10. Jiang, L.; Aigret, B.; De Borggraeve, W.M.; Spacil, Z.; Ilag, L.L. Selective LC-MS/MS method for the identification of BMAA from its isomers in biological samples. *Anal. Bioanal. Chem.* **2012**, *403*, 1719–1730. [[CrossRef](#)] [[PubMed](#)]

11. Bhandari, D.; Bowman, B.A.; Patel, A.B.; Chambers, D.M.; De Jesús, V.R.; Blount, B.C. UPLC-ESI-MS/MS method for the quantitative measurement of aliphatic diamines, trimethylamine *N*-oxide, and β -methylamino-L-alanine in human urine. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2018**, *1083*, 86–92. [[CrossRef](#)] [[PubMed](#)]
12. Yan, B.; Liu, Z.; Huang, R.; Xu, Y.; Liu, D.; Lin, T.F.; Cui, F. Optimization of the determination method for dissolved cyanobacterial toxin BMAA in natural water. *Anal. Chem.* **2017**, *89*, 10991–10998. [[CrossRef](#)] [[PubMed](#)]
13. Roy-Lachapelle, A.; Sollicec, M.; Sauvé, S. Determination of BMAA and three alkaloid cyanotoxins in lake water using dansyl chloride derivatization and high-resolution mass spectrometry. *Anal. Bioanal. Chem.* **2015**, *407*, 5487–5501. [[CrossRef](#)] [[PubMed](#)]
14. Janeiro, M.H.; Ramírez, M.J.; Milagro, F.I.; Martínez, J.A.; Solas, M. Implication of trimethylamine *n*-oxide (TMAO) in disease: Potential biomarker or new therapeutic target. *Nutrients* **2018**, *10*, 1398. [[CrossRef](#)] [[PubMed](#)]
15. Subramaniam, S.; Fletcher, C. Trimethylamine *N*-oxide: Breathe new life. *Br. J. Pharmacol.* **2018**, *175*, 1344–1353. [[CrossRef](#)] [[PubMed](#)]
16. Zhao, X.; Zeisel, S.H.; Zhang, S. Rapid LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine *N*-oxide, and creatinine in human plasma and urine. *Electrophoresis* **2015**, *36*, 2207–2214. [[CrossRef](#)] [[PubMed](#)]
17. Liu, J.; Zhao, M.; Zhou, J.; Liu, C.; Zheng, L.; Yin, Y. Simultaneous targeted analysis of trimethylamine-*N*-oxide, choline, betaine, and carnitine by high performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2016**, *1035*, 42–48. [[CrossRef](#)] [[PubMed](#)]
18. Wang, Z.; Levison, B.S.; Hazen, J.E.; Donahue, L.; Li, X.M.; Hazen, S.L. Measurement of trimethylamine-*N*-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry. *Anal. Biochem.* **2014**, *455*, 35–40. [[CrossRef](#)] [[PubMed](#)]
19. Scattolini, V.; Dalla Costa, F.; Cignarella, A.; Vettore, M.; Di Gangi, I.M.; Bogialli, S.; Avogaro, A.; Fadini, G.P. Interplay between gut microbiota and p66Shc affects obesity-associated insulin resistance. *FASEB* **2018**, *32*, 4004–4015.
20. Sanna, S.; van Zuydam, N.R.; Mahajan, A.; Kurilshikov, A.; Vich Vila, A.; Vösa, U.; Mujagic, Z.; Masclee, A.A.M.; Jonkers, D.M.A.E.; Oosting, M.; et al. Causal relationships among the gut microbiome, short-chain fatty acids and metabolic diseases. *Nat. Genet.* **2019**, *51*, 600–605. [[CrossRef](#)] [[PubMed](#)]

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).