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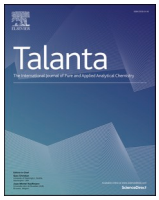

Identification and validation of small molecule analytes in mouse plasma by liquid chromatography–tandem mass spectrometry: A case study of misidentification of a short-chain fatty acid with a ketone body

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ABSTRACT

Recently, there has been growing interest in short-chain fatty acids (SCFA) and ketone bodies (KB) due to their potential use as biomarkers of health and disease. For instance, these diet-related metabolites can be used to monitor and reduce the risk of immune response, diabetes, or cardiovascular diseases. Given the interest in these metabolites, different targeted metabolomic methods based on UPLC-MS/MS have been developed in recent years to detect and quantify SCFA and KB. In this case study, we discovered that applying an existing validated, targeted UPLC-MS/MS method to mouse plasma, resulted in a fragment ion (194 *m/z*) being originally misidentified as acetic acid (a SCFA), when its original source was 3-hydroxybutyric acid (a KB). Therefore, we report a modified, optimized LC method that can separate both signals. In addition, the metabolite coverage was expanded in this method to detect up to eight SCFA: acetic, propanoic, butyric, isobutyric, 2-methylbutyric, valeric, isovaleric, and hexanoic acids, two KB: 3-hydroxybutyric, and acetoacetic acids, and one related metabolite: 3-hydroxy-3-methylbutyric acid. The optimization of this method increased the selectivity of the UPLC-MS/MS method towards the misidentified compound. These findings encourage the scientific community to increase efforts in validating the original precursor of small molecule fragments in targeted methods.

1. Introduction

Short-chain fatty acids (SCFA) are organic fatty acids characterized by having less than six carbons. These metabolites are produced by fermentation of dietary fiber, and serve as the principal energy source for colonocytes, contributing to about 10% of the daily energy requirement [1,2]. The remaining SCFA, which are not absorbed in the colon, travel to the liver where about 60% are removed from the portal circulation [3,4]. By contrast, ketone bodies (KB) are produced primarily in the liver through the oxidation of fatty acids. KB are used as an alternative energy source under nutrient deprivation and contribute about 5–20% to the total energy balance [5].

In recent years, there is increasing interest in determining the levels of SCFA and KB due to their anti-inflammatory effects [6,7], regulation of the immune system [8], and their role in gut-brain communication [9]. Both SCFA and KB have been described as potential biomarkers of health and disease [10,11]. For instance, diverse studies have found applications for these metabolites as biomarkers to monitor and reduce the risk of maladaptive immune response, diabetes, and cardiovascular diseases [12–16]. Similarly, the study of various neuropathologies has found altered SCFA levels [17–19]. Given the increasing interest of these compounds, suitable analytical methods have been developed, with

Abbreviations: 3NPH, 3-Nitrophenyl Hydrazine; AA, Acetic Acid; AcAA, Acetoacetic acid; ACN, Acetonitrile; A.U, Arbitrary Units; BA, Butyric Acid; EDC, N-(3-Dimethylaminopropyl)-N′-Ethylcarbodiimide; ESI, Electrospray Ionization; GC, Gas Chromatography; H_2O, Water; HA, Hexanoic Acid; HBA, 3-Hydroxybutyric Acid; HMB, 3-Hydroxy-3-Methylbutyric Acid; IBA, Isobutyric Acid; ISTD, Internal Standard; IVA, Isovaleric Acid; LC, Liquid Chromatography; MBA, 2-Methylbutyric Acid; MRM, Multi-Reaction Monitoring; MS, Mass Spectrometry; PA, Propanoic Acid; Qn, Quadrupole *n*; RP, Reverse Phase; RT, Retention Time; SCFA, Short-Chain Fatty Acids; UPLC, Ultra Performance Liquid Chromatography; VA, Valeric Acid.

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those based on GC-MS and LC-MS the most widely used [20,21]. UPLC-MS/MS methods especially have recently increased in popularity due to fast sample preparation and shorter analytical run times [22–25]. Here we demonstrate how our attempts to implement and optimize an existing method from another lab and validated for fecal samples led to a surprise discovery of misidentification when applied to mouse plasma. In the following case study, the original method of Han *et* al. (2015) was optimized by ourselves in order to avoid the co-elution of an SCFA and a KB and avoid the subsequent misidentification and misquantification of both. Furthermore, we have expanded the coverage of the number of metabolites analyzed in a single analytical run albeit with longer run time. The optimized UPLC-MS/MS method was then used to evaluate the distribution of low abundant SCFA and KB in mouse plasma.

2. Materials and methods

2.1. Chemicals

All analytical standards acetic acid (AA), propanoic acid (PA), isobutyric acid (IBA), butyric acid (BA), 2-methyl-butyric acid (MBA), isovaleric acid (IVA), valeric acid (VA), hexanoic acid (HA), 3-hydroxybutyric acid (HBA), 3-hydroxy-3-methylbutyric acid (HMB), lithium acetoacetate (AcAA), as well as the reagents 3-nitrophenyl hydrazine (3NPH) hydrochloride, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) hydrochloride and pyridine were purchased from Sigma-Aldrich (Merck Group, Darmstadt, Germany) with purity ≥95.0%. All internal standards (ISTD) d3-acetic acid (d3-AA), d5-propanoic acid (d5-PA), d7-butyric acid (d7-BA), d9-valeric acid (d9-VA) and d11-hexanoic acid (d11-HA) were purchased from Supelco/Sigma-Aldrich (Merck Group, Darmstadt, Germany). Mouse plasma in sodium heparin (virus-free, non-sterile) was purchased from Rockland (Limerick, PA, USA).

2.2. Chemical derivatization and sample preparation

The final derivatization parameters, taken from Han *et* al. [23], were implemented in our lab and used to analyze pure standards dissolved in 50% (v/v) ACN:H_2O, and to evaluate the distribution of SCFA and KB in commercial mouse plasma. Briefly, 40 μL of matrix, either mouse plasma or mixed standard solution, was mixed in glass vials with 20 μL of 200 mM 3NPH, 20 μL of 120 mM EDC with 6% pyridine, and 10 μL of 50 μM ISTDs, and incubated at 40 °C for 30 min. Standard solutions were prepared fresh and kept at room temperature for the short time until derivatization. After incubation, 410 μL of 10% (v/v) ACN:H_2O was added, and the samples were centrifuged at 5500×*g* for 20 min at 20 °C. The supernatants were transferred to brown glass vials and directly subjected to UPLC-MS/MS analysis. Commercial mouse plasma was thawed and kept on ice until derivatization using the same method as above.

2.3. UPLC-MS/MS

An adapted method based on Han *et* al. [23] was used. The analyses were carried out in a UPLC system 1290 Infinity II (Agilent, Santa Clara, CA, USA) coupled to a TSQ Quantiva (ThermoFisher Scientific, Waltham, MA, USA). Briefly, 5 μL of sample was injected onto an RP C18 column ACQUITY BEH 1.7 μm 2.1 × 100mm (Waters Corp, Milford, MA, USA) at 40 °C. Gradient elution mode was used with a flow of 0.35 mL min^{-1} with the following mobile phases: H_2O + 0.1% (v/v) formic acid (Phase A) and ACN + 0.1% (v/v) formic acid (Phase B). Commercial standards were used to establish non-scheduled MRM methods. One precursor ion per compound was selected in Q1 to undergo fragmentation in Q2. Collision energies in Q2 were optimized for monitoring three transitions per compound in Q3, and isomers were monitored using the same precursor-fragment ion pairs. Q1/Q3 transitions were monitored using the following MS parameters: ESI in negative mode, spray voltage = static, negative ion voltage = 2500 V, sheath gas = 30 A U., sweep gas = 20 A U., aux gas = 0.8 A U., ion transfer tube temperature = 342 °C, vaporizer temperature = 300 °C, source fragmentation = 0, and the following scan parameters: scan rate = 500 Da/s, dwell time = 20 ms, Q1 resolution (FWHM) = 0.2, Q3 resolution (FWHM) = 0.7, CID gas = 1.5 mTorr. Raw data were processed and integrated into peak areas using Skyline (64-bit) 21.1.0.146. Fragment structures were obtained using an MS/MS fragment generator via an online-based toolbox for MS analysis (ms.epfl.ch).

2.4. Original gradient

First, the following gradient originally described by Han *et.* al [23] was used with Phase A and B: 15% B for 2 min, 15–55% B for 9 min, 100% B for 1 min, and finally 15% B for 2 min.

2.5. Optimized gradient

To achieve chromatographic resolution of two coeluting compounds (AA and HBA), a slower gradient was used as follows: 5% B for 5 min, 5–55% B in 12 min, 100% B for 1 min, and 2 min 5% B.

3. Results and discussion

3.1. Original method and misidentification of acetic acid

The method previously reported by Han *et* al. [23] was selected due to its reported superior sensitivity to SCFA among other UPLC-MS/MS approaches [24]. MRM methods were established for eight SCFA (AA, PA, BA, IBA, MBA, IVA, VA, and HA), two ketone bodies (HBA and AcAA), and one related metabolite (HMB). Five heavy-labeled ISTD were monitored and used for normalization purposes (Table S1). All the precursor-fragment ion pairs previously reported for SCFA were found (Table 1). Looking to expand the assay to enable some KB to be analyzed concurrently with the SCFA, we also monitored the precursor-fragment ion pairs for HBA using the same gradient. The precursor ion corresponding to the 3NPH derivative of HBA (HBA-3NPH) was detected with a nominal *m/z* value of 238, producing a fragment ion with the nominal *m/z* value of 194 (Fig. 1A), which is identical to the theoretical nominal mass of the 3NPH derivative of AA (AA-3NPH) (Fig. 1B). The HBA fragment 194 *m/z* was detected in Q1 when scanning a pure derivatized standard (Fig. 1A), indicating the occurrence of *in-source* fragmentation. Furthermore, the 194 *m/z* fragment ion was found even at low or no collision energy in Q2 during the collision energy optimization step (Fig. 1C), where the precursor ion with 238 *m/z* is filtered in Q1 to undergo fragmentation Q2 and the resulting fragment ions are scanned in Q3.

Both molecules also had identical retention times (RT) with the original gradient. Since both derivatives AA-3NPH and HBA-3NPH coeluted at a RT of 3.0 min, a large signal falsely attributed to AA-3NPH was always found when injecting pure derivatized HBA in the absence of AA (Fig. 2A), implying a false positive detection of AA and an undesired enrichment of AA signal in biological samples. To the best of our knowledge, this finding has not been reported in other studies which use different methods. For instance, Zeng *et* al. [24] optimized an UPLC-MS/MS method in positive mode for SCFA and KB in mouse plasma and feces using O-benzylhydroxylamine (OBHA) as a derivatization agent and reported coeluting AA- and HBA-OBHA derivatives. However, since the reported fragment masses of HBA-OBHA (194 *m/z)* and the protonated AA-OBHA (196 *m/z)* in positive mode are different, the authors did not report any problems with their identification or subsequent quantification. We briefly explored the possibility of positive mode ionization of AA-3NPH and HBA-3NPH, and found three common signals (174 *m/z*, 129 *m/z*, 80 *m/z*) for both compounds. However, none of the detected signals corresponded to the protonated molecular ion with theoretical *m/z* of 196 *m/z* and 240 *m/z* for AA-3NPH and

Table 1
Q1/Q3 transitions for SCFA and ketone bodies.

Compound	Precursor ion *m/z*	RT original gradient (min)	RT modified gradient (min)	Product ion *m/z*	Collision Energy (V)
AA-3NPH	194.046	3.0	8.6	152	−15
				150	−16
				137	−19
HBA-3NPH	238.083	3.0	9.1	194	−12
				152	−19
				137	−24
HMB-3NPH	252.075	N.A.	10.7	194	−14
				152	−20
				151	−18
PA-3NPH	208.121	4.5	11.0	165	−14
				152	−16
				137	−20
IBA-3NPH	222.163	5.8	12.8	Same for BA-3NPH	
BA-3NPH	222.163	6.0	13.1	179	−14
				152	−16
				137	−19
MBA-3NPH	236.129	7.2	14.6	Same for VA-3NPH	
IVA-3NPH	236.129	7.4	14.9	Same for VA-3NPH	
VA-3NPH	236.129	7.7	15.3	152	−17
				193	−14
				137	−21
HA-3NPH	250.167	9.4	17.3	152	−18
				207	−14
				137	−22
AcAA-$(3NPH)_2$	371.137	N.A.	18.2	218	−16
				150	−21
				178	−16

N.A.: Not applicable AA-3NPH: acetic acid derivative, HBA-3NPH: 3-hydroxybutyric acid derivative, HMB-3NPH: 3-hydroxy-3-methylbutyric acid derivative, PA-3NPH: propanoic acid derivative, IBA-3NPH: isobutyric acid derivative, BA-3NPH: butyric acid derivative, MBA-3NPH: 2-methylbutyric acid derivative, IVA-3NPH: isovaleric acid derivative, VA-3NPH: valeric acid derivative, HA-3NPH: hexanoic acid derivative, AcAA-$(3NPH)_2$: acetoacetic acid derivative.

HBA-3NPH, respectively (Figure S1), so we did not pursue this method further. Instead, we returned to Han's method. Since the MS signals for the HBA-3NPH fragment peak and the AA-3NPH precursor could not be isolated, the LC gradient was modified to avoid coelution.

3.2. Optimized gradient and expanded coverage

In order to separate the signals from AA-3NPH and HBA-3NPH, we instituted an improved gradient to achieve their chromatographic resolution. Although the use of this gradient delays the elution of all SCFA, it enables the complete resolution of AA with an RT = 8.6 min, and HBA with an RT = 9.0 min (Fig. 2B), without affecting the chromatographic separation of the isomeric SCFA (Fig. 2C). A clean chromatogram of pure derivatized HBA using the new gradient negates the possibility that contamination of the HBA standard with AA (Figure S2) is the cause of misidentification.

Furthermore, in order to expand the coverage of the method, the same reaction conditions were applied to derivatize HMB and AcAA. In our development, the precursor ion for AcAA was found to be the doubly-labeled 3NPH derivative with a nominal *m/z* value of 371 and not the singly-labeled 3NPH derivative with 236 *m/z* (Figure S3). The transitions for the molecular ions HMB-3NPH (252 *m/z*), and AcAA-3NPH (371 *m/z*) have not been reported previously, to the best of our knowledge. Importantly, the precursor ion of the 3NPH derivative of HMB (HMB-3NPH, *m/z*: 252) also produces a fragment ion with 194 *m/z*. However, AA-3NPH and HBM-3NPH elute at different RTs (Table 1), and therefore, there is no risk of a false-positive detection of AA due to fragmentation of HMB.

The improved method was applied to determine the SCFA and KB

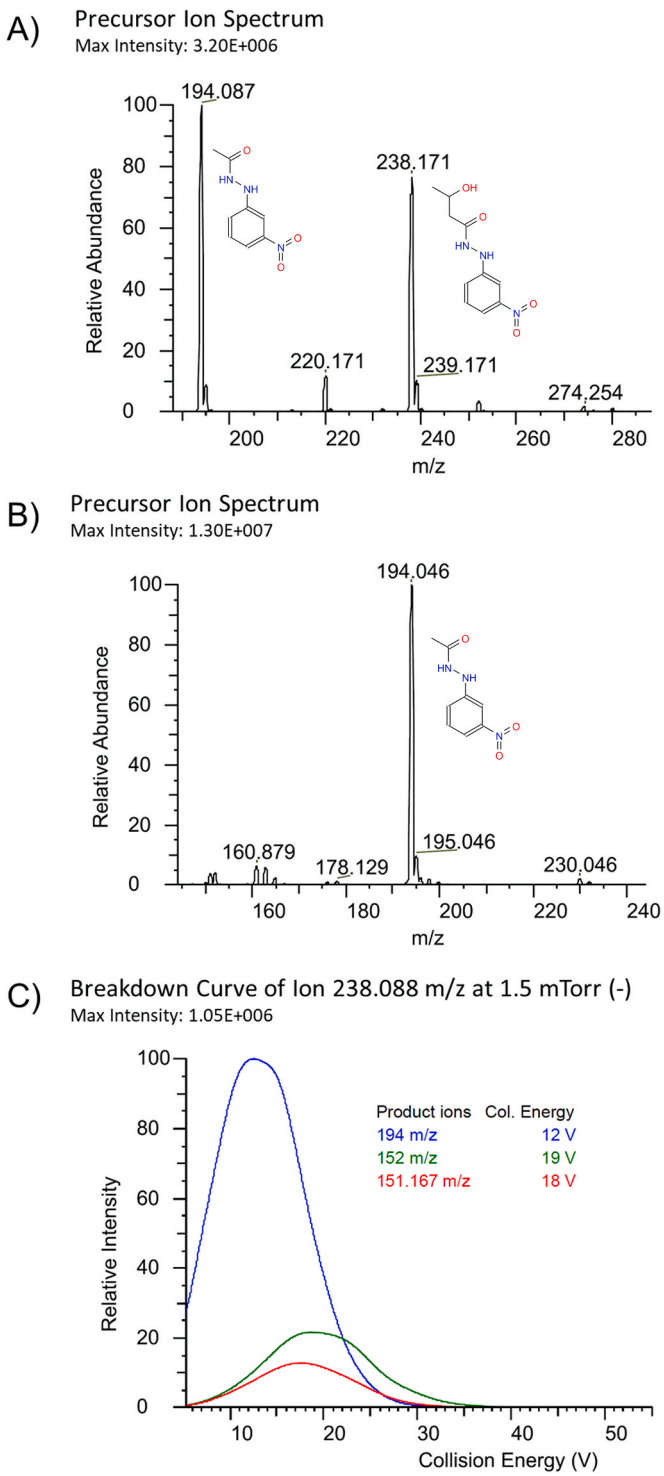

Fig. 1. Precursor ion spectrum for HBA-3NPH (A) and AA-3NPH (B). HBA-3NPH shows *in-source* fragmentation to the fragment ion with *m/z* values of 194.083. The nominal *m/z* of 194 corresponds to AA-3NPH as precursor ion. (C) Breakdown curve for HBA-3NPH showing the formation of 194 *m/z* fragment ion at low collision energy.

profile in commercial mouse plasma, showing abundances of these compounds in the μM range (Fig. 2D). The robustness of the method for mouse plasma was evaluated through linearity, precision, accuracy, and matrix effects, according to the European Medicines Agency (EMA) guidelines for method validation [26]. According to the EMA guidelines for method validation, when adapting a method with minimum changes

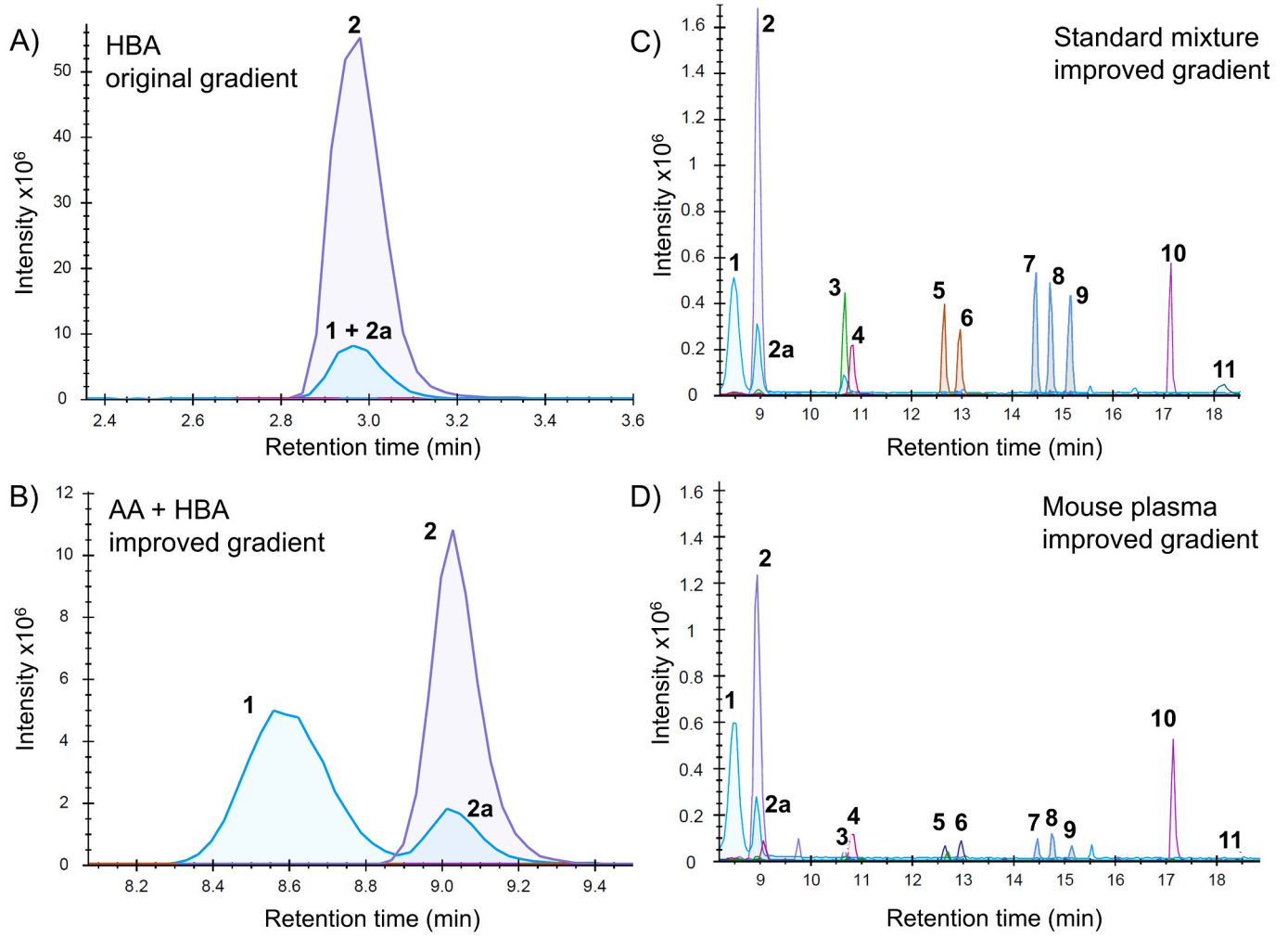

Fig. 2. A) Chromatogram of derivatized HBA standard (purple) measured with the original gradient (Han el at. 2015). A peak (blue) is clearly visible, which has the exact precursor-fragment ion transition (194 → 152) to be identified as AA (RT = 3.0 min) although it actually comes from early fragmentation of the HBA ion which produces a fragment ion also at 194 *m/z*. B) Chromatogram of an equimolar mixture (50 mM) of HBA (purple) and AA (blue) chromatographically resolved with an improved gradient (RT = 8.6 min for AA, RT = 9.0 min for HBA and HBA fragment with 194 *m/z*). C) Chromatogram of a mixture of SCFA and HBA (100 μM for AA and HBA, and 12.5 μM for the rest of SCFA) showing resolved AA and HBA as well as resolved isomeric SCFA. D) Chromatogram of a derivatized mouse plasma sample. 1: AA, 2: HBA, 2a: HBA fragment with 194 *m/z*, 3: HMB, 4: PA, 5: IBA, 6: BA, 7: MBA 8: IVA, 9: VA, 10: HA, 11: AcAA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

from one lab to another, a partial validation is sufficient to determine that the modified method is fit for use [26]. Therefore, we determined LLOQs, ULOQs, precision, and accuracy for each compound and evaluated the matrix effects in mouse plasma as described in the Supplementary material. The modified method showed acceptable linearity and reproducibility for all analytes in mouse plasma as matrix (Table S2). Moderate matrix effects in mouse plasma were observed only for HBA, AcAA, and AA when compared with solvent as matrix, and no matrix effects were observed for the rest of the compounds (Table S2).

The original developer of the method had used it to analyze fecal samples. We would emphasize that we have not tried their method on this matrix. The coelution of AA and HBA may not be of such importance in this sample type due to their relative ratios. Contrary to fecal samples, however, resolving AA-3NPH from HBA-3NPH becomes particularly important when analyzing plasma or serum samples because the concentration of circulating ketone bodies is much higher than circulating SCFA. In addition, the concentration of ketone bodies in mouse serum increases rapidly under fasting conditions, moving from the μM range to the mM range in 24 h [22]. Consequently, the magnitude of a misidentified signal may be large. Previous reports on concentrations of SCFA and KB show percentage ratios between HBA and AA (HBA/AA) in mouse feces of around 0.19% [24], in which case, the AA signal may not have a detectable enrichment. However, fecal contamination with urine is a potential source of HBA when analyzing feces. Diseased individuals in particular may present with high levels of HBA in their urine [27], thus leading to a risk of overestimating AA in fecal samples where HBA and AA cannot be properly resolved.

In the case of mouse plasma, an HBA/AA percentage ratio of 121% has been previously reported [24], and the mouse plasma used in the present study had a ratio of 34% (Table S3), highlighting the need for complete validation of both analytes. Before resolving the two compounds, HBA contributed around 20% of the detected signal attributed to AA in commercial mouse plasma (Table S4). With the proposed gradient, the selectivity of the method towards AA was significantly improved by preventing the undesired enrichment of AA signal due to the fragmentation of HBA.

4. Conclusions

We optimized and validated the quantification of SCFA and KB in mouse plasma by applying an existing UPLC-MS/MS method previously validated for human feces and based on the detection of 3NPH

derivatives of SCFA. An optimized gradient was established to avoid the misidentification of AA by the fragmentation of HBA. The proposed gradient allows the measurement and quantification of two chemically similar metabolite derivatives that jeopardize the selectivity of the method when coeluting. While in human feces the abundance of HBA is usually low, in mouse plasma the levels of HBA are high enough to interfere with the detection and quantification of AA. This may lead to misinterpretation of the results. This study demonstrates both the importance of exploring where fragment peaks are arising from, and why methods designed for one matrix may be inappropriate for other matrices. The proposed method is recommended for profiling SCFA and KB in plasma and serum samples, and any other biological matrix where KB are highly abundant. The findings of this case study intend to encourage the scientific community to improve the identification strategies of chemically similar analytes in targeted methods beyond the general method validation approaches.

Ethics statement

No ethics approval was required for this study as commercial mouse plasma was used. This plasma is for research use only and is not intended for therapeutic or diagnostic applications.

Author contributions

M.A.G.R.: Conceptualization, Investigation, Method development, Method validation, Formal analysis, Visualization, Writing-original draft, Writing – review & editing. A.F.O.: Investigation, Method development, Visualization, Writing-original draft, Writing – review & editing. U.B.: Investigation, Method development, Writing – review & editing. R.F.G.: Investigation, Method development, Writing – review & editing. J.A.K.: Conceptualization, Funding acquisition, Writing-original draft, Writing – review & editing, Supervision.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123298.

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