Short Report

# Identification of a frit-related sample carryover in newborn screening by tandem mass spectrometry

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## ARTICLE INFO

## ABSTRACT

The need for high-throughput analysis of multiple analytes for inborn errors of metabolism in newborn screening (NBS) has led to the introduction of tandem mass spectrometry (MS/MS) into the NBS laboratory. In a flow-injection analysis (FIA), the predominant MS/MS method utilized for NBS, samples are introduced directly into the mass spectrometer without chromatographic separation. When a high-throughput FIA-based MS/MS method is implemented on newer generations of mass spectrometers with increased sensitivity, the risk of carryover and contamination increases. In the present study, we report the carryover of ornithine identified during the implementation of the NeoBase™ 2 (PerkinElmer) non-derivatized kits on the Xevo-TQD platform (Waters Corporation) and describe the source of the carryover, which was traced to the stainless-steel frit-type inline filter. Furthermore, a possible compound-dependent interaction with the stainless-steel frit is suggested based on the structure of ornithine and its effect on separation techniques. Investigation and mitigation of carryover can be a time and resource consuming process, and to this end, our report on identification of a stainless-steel frit as the source of delayed elution and carryover of ornithine should be recognized as a rare, albeit possible source of carryover in FIA-MS/MS methods adopted for NST.

## 1. Introduction

In newborn screening (NBS), tandem mass spectrometry (MS/MS) has been rapidly adopted and expanded due to the need for a high-throughput analysis of multiple analytes for metabolic disorders. Because MS/MS is a multiplex method that measures several analytes simultaneously, >40 inborn errors of metabolism (IEMs) can be detected using a single dried blood spot (DBS) sample. Moreover, the MS/MS procedure is further simplified by introducing the extracted samples into the mass spectrometer (MS) by flow injection without chromatographic separation in a method called flow injection analysis by MS/MS (FIA-MS/MS) [1].

Due to its wide dynamic range and high throughput, MS/MS methods can be confounded by factors associated with the instrument hardware and/or the nature of samples. Sample carryover is a common issue in MS/MS methods, and sample carryover in NBS testing can be detrimental to the accuracy of NBS results by increasing false positive results and compromising the positive predictive value. In the present study, we report on carryover evaluated for all primary amino acids and acylcarnitines included in the 1st tier NBS, as identified during implementation of the NeoBase™ 2 (PerkinElmer Life and Analytical Sciences, Turku, Finland) non-derivatized kits on the Xevo-TQD platform (Waters Corporation, Milford, MA, USA) and describe the manner in which the source of the carryover was traced and eliminated. A special focus is given to ornithine, because the retention time difference of ornithine relative to other analytes in FIA not only prompted the investigation of

the carryover, but also suggested a compound-dependent factor contributing to the observed carryover.

## 2. Experimental

The FIA-MS/MS of metabolite profiles on DBS samples was performed using a NeoBase™ 2 Non-derivatized MS/MS kit (PerkinElmer) on an ACQUITY ultra performance liquid chromatography (UPLC) I-Class System configured with a flow through needle (FTN) Sample Manager (SM). An in-line filter (0.2 um pore size, stainless steel frit, Waters Corporation) was installed between the autosampler and MS/MS. The mobile phase consisted of 84:16 acetonitrile/water with 0.1 % formic acid. A 10 μL injection of the sample was analyzed. The initial flow rate was 0.100 mL/min; at 0.05 min, the flow was increased to 0.380 mL/min to push the sample plug to the MS/MS; at 0.20 min, the flow was decreased to 0.014 mL/min to increase the residence time of the peak in the MS/MS; at 1.00 min, flow was increased to 0.500 mL/min to help flush the sample completely from the system before the next injection was made, and between 1.25 and 1.60 min, flow was decreased to 0.100 mL/min. The total run time was 1.6 min. The UPLC was coupled to a Xevo™ TQD IVD MS/MS (Waters Corporation). The MS/MS was operated in positive ion mode using multiple reaction monitoring. The MS parameters, such as ion transitions, cone voltages, and collision energies, for each metabolite and its internal standards, are provided in Supplementary Table 1.

The carry-over evaluation was performed using quality controls (QCs) included in the NeoBase™ 2 kit. DBS QC samples were punched out into 3.2 mm diameter disks and extracted by the extraction working solution containing the internal standards for each analyte, based on the manufacturer's instructions. The high (H) and low (L) QC samples were injected in the following sequence H1-H2-H3-H4-L1-L2-L3-L4, and carryover (%) was calculated as follows [2]:

$$\text{Carryover}\,(\%) = \frac{L1 - \frac{(L3+L4)}{2}}{\frac{(H2+H3)}{2} - \frac{(L3+L4)}{2}} \times 100$$

The carryover evaluation was performed with the previous and the newly replaced stainless steel frit. The data were processed using the MassLynx™ Software V4.2 with NeoLynx™ or IonLynx Application Manager (Waters Corporation).

## 3. Results

During the initial validation, we observed that while the total ion chromatogram (TIC; the sum of all ion intensities) created a well-shaped peak (Fig. 1), the elution profile of the selected reaction monitoring chromatogram of ornithine showed a delayed appearance with major elution occurring between 1.0 and 1.25 min (Fig. 1). Moreover, an analogous late-eluting profile of selected reaction monitoring chromatogram was observed with the corresponding ornithine internal standard, $^2H_6$-Orn. The delayed elution of the ornithine peak in the final segment of the chromatogram indicated the scope for sample carryover. Additionally, when carryover was assessed with a low QC material, which was injected immediately following the high QC material, a significant percentage of carryover (>5%), as measured relative to the high QC, was present in multiple metabolites, including ornithine
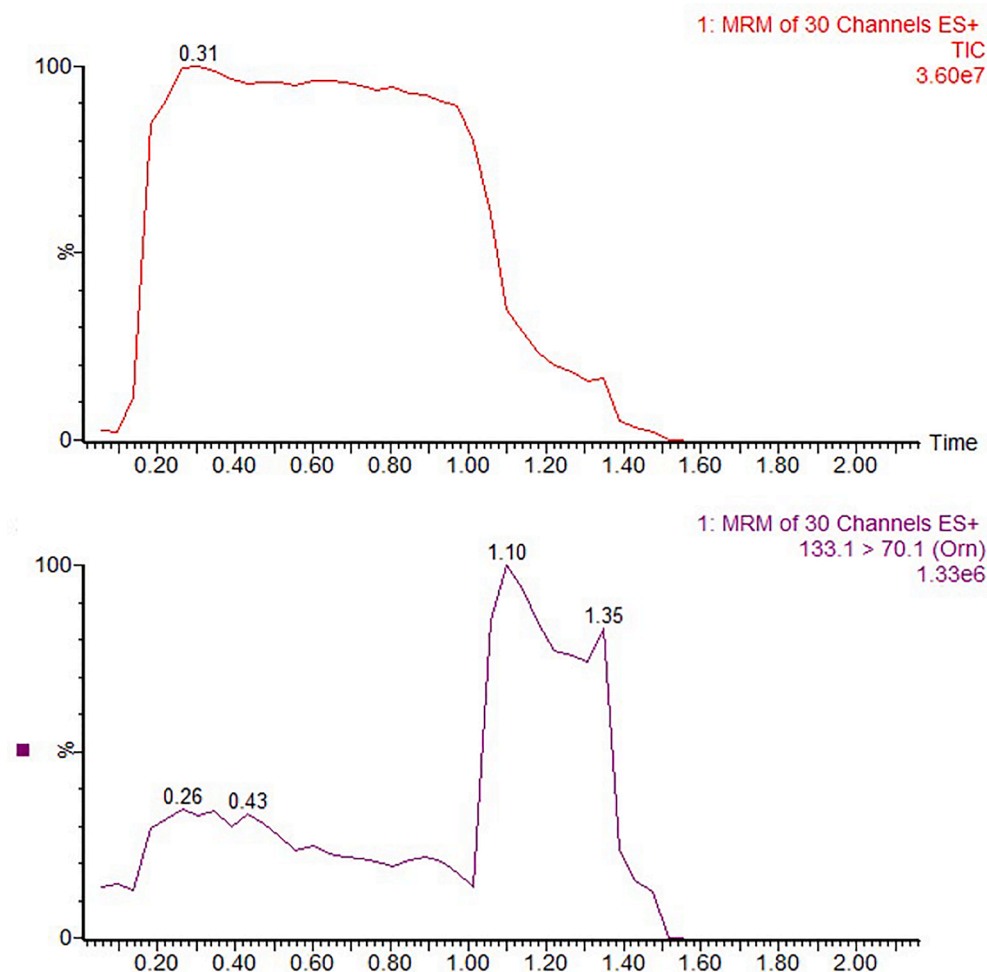


Fig. 1. Chromatogram of (A) total ion chromatogram and (B) selected reaction monitoring chromatogram of ornithine.

(Supplementary Table 2). The carryover estimated relative to the lower limit of quantitation (LLOQ) is also presented (Supplementary Table 2).

Sample carryover is a stubborn issue that can compromise the accuracy and precision of high performance liquid chromatagraphy (HPLC), liquid chromatography (LC)-MS, and LC-MS/MS assays [3]. Moreover, the investigation and minimization of carryover can be a time- and resource-intensive process, leading to reduced productivity and delays in the method development process. Carryover has many sources, but in general it is either column- or non-column-related. Non-column-related carryover occurs more frequently and normally results from inadequate setup or flush of the autosampler injection needle assembly, transfer tube, or injection valve [4]. Therefore, the initial investigation was directed at the autosampler. The autosampler was programmed for an injection volume of 10 μL, 50:50 water: methanol was used as needle wash solvent and the default (6 s post-injection) mode of needle wash was utilized. The ACQUITY UPLC I-Class SM FTN employed a FTN design where the needle is a part of the flow path, and the interior of the needle is flushed by the chromatographic mobile phase. Only the exterior of the needle is washed with the needle wash solvent, which enters at the bottom of the injection port. Hence, by design, the issue of sample carryover resulting from partial loop injection mode was ruled out [5]. As an initial step in troubleshooting the carryover issue, alternative needle wash solvents such as varying concentrations of water, acetonitrile, and methanol (90:10 water:methanol, 100 % methanol, 90:10 water:acetonitrile, 50:50 water:acetonitrile, and 100 % acetonitrile) were investigated to assess the impact of the wash-

solvent composition. No alternative needle wash solvents had a noticeable effect on the magnitude of carryover. The compression fitting on the injection valve and all the tube connections were checked because a similar occurrence of ornithine carryover resulted from an improper tubing connection at the electrospray ionization (ESI) source (N. H. Park, personal communication). However, the issue was not resolved.

To completely exclude the MS interface as a potential source of carryover or possible contamination, NeoBase 2 internal standards were directly injected into the MS followed by an injection of the mobile phase. The results showed no discernable carryover of $^2$H$_6$-Orn and ruled out the MS as a potential source of carryover or contamination.

Although the phenomenon of erroneous results was unquestionably serial in nature and most likely caused by residual analyte from a preceding sample analyzed earlier in the run, separate measures to investigate the nitrogen gas generator or its filters as possible sources of contamination were considered, and filters were replaced. However, this action did not resolve the carryover.

Due to the persistence of the issue, we considered that the issue could be related to the autosampler hardware. Therefore, the needle assembly, needle seal, sample loop, and injector valve were sequentially replaced. None of these substitutions resolved the issue. We also applied various rinse solvents to rinse ornithines off metals [6]: 0.5 % formic acid in isopropanol (IPA), 0.5 % formic acid in water/IPA (1:1 v/v), water, and methanol, but to no effect. After an increase in the backpressure (approximately 150 bar) was detected, we replaced the original frit ring
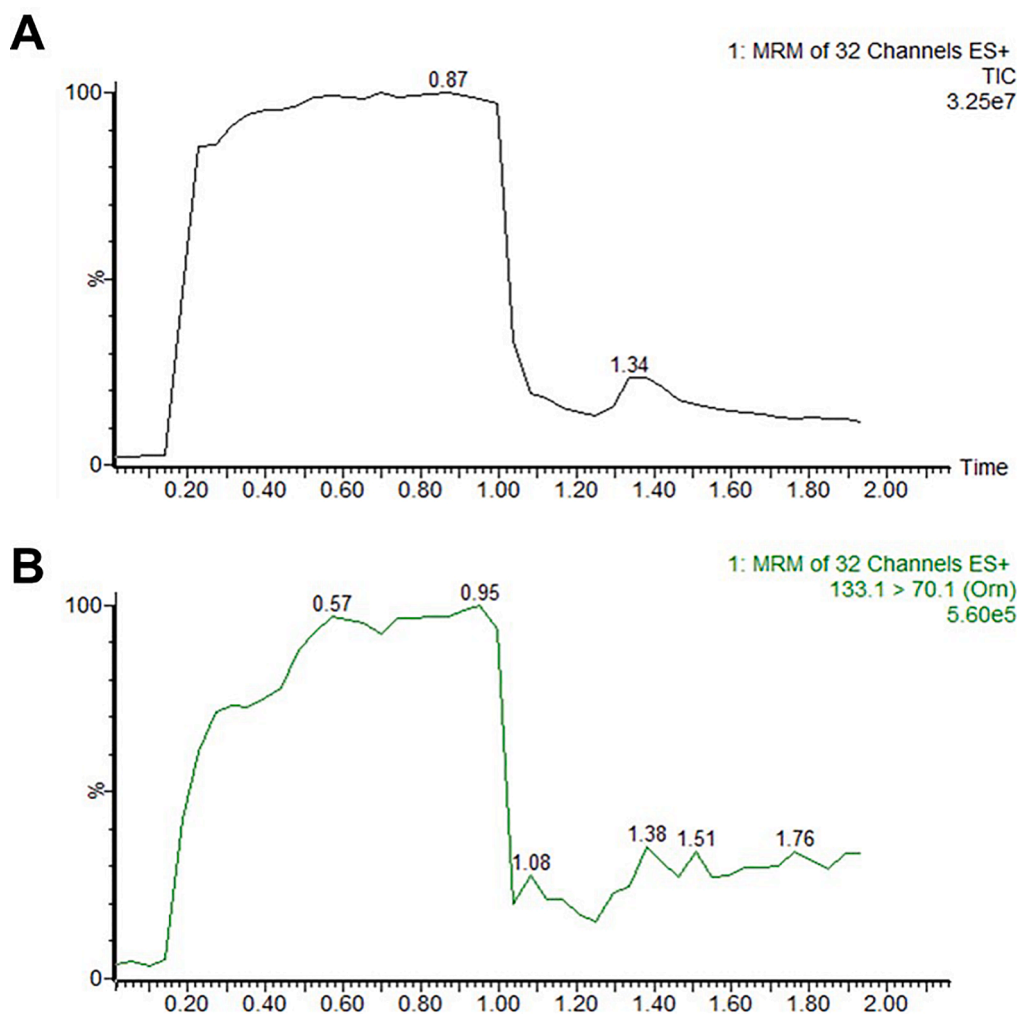


**Fig. 2.** Chromatogram of (A) total ion chromatogram and (B) selected reaction monitoring chromatogram of ornithine after the exchange of frit.

with another 0.2 um stainless steel frit, and the issue was remarkably resolved (Fig. 2). The comparison of percentage carryover before and after the change of frit is provided in Supplementary Table 2.

## 4. Discussion

In this study, we reported the delayed elution and carryover of ornithine, in addition to other multiple metabolites, during the implementation of NeoBase™ 2 non-derivatized kits on the Xevo-TQD platform, which was unwittingly alleviated after an exchange of frit. The resultant investigation of the source of carryover to the frit was unexpected because FIA-MS/MS approach operated without a separation column. Therefore, the investigation was directed at non-column-related sources of carryover, primarily focusing on the autosampler.

Carryover occurs when the analytes adhere to a surface that is shared during the analyses of subsequent samples or when residual analytes are stored in a space not washed by the mobile phase at constant pressure. For an LC-MS/MS assay, the surface or space may be the autosampler injection port, wall of the tubing leading to the ESI source, injection needle, space between the stator and rotor, frits, guard column, column or MS interfaces. Carryover issues can be one of the most vexing quandaries in LC-MS/MS, demanding considerable effort in tracking down the source of carryover. The various sources of sample carryover can be classified as column- or non-column-related. Column-related carryover occurs less frequently than non-column-related carryover and is much more challenging to mitigate. Additionally, column-related carryover can be associated with the separation column or the pre-column, including frit-type in-line filters.

Generally, column-related carryover in LC-MS/MS analysis is a compound-dependent issue and may be associated with the unique characteristics of the analyte. Of the metabolites that showed a significant % carryover (>5 %) (i.e., alanine, glutamine\lysine, ornithine, valine), ornithine was distinctive as it consistently displayed a late-eluting profile on the selected reaction monitoring chromatogram for both the analyte and its isotope-labeled internal standard. Ornithine contains a side chain with three methylene groups and an ammonium group attached to the end of the methylene groups, forming a δ-amino group. The amino group is in the protonated form under physiological conditions, which makes ornithine a basic amino acid with a isoelectric point (pI) of 9.8 and a molecular weight of 132.16. Ornithine exhibits polar properties in chromatography and makes it challenging to retain on reversed-phase HPLC columns [7].

While the FIA-MS/MS method for NST does not utilize a separation column, a frit type in-line filter is frequently installed to capture the paper fibers of the DBS and prevent clogging [8]. A clogged frit contributes to carryover, and retention time shifts may occur due to an increase in backpressure. Therefore, monitoring the backpressure during operations and replacing the frit ring if it is 10 to 20 % above the regular value are recommended [9,10]. However, the characteristic late-eluting profile of ornithine and its internal standard suggested a compound-dependent factor, which could have contributed to the carryover observed in our case, other than the physical clogging of the frit.

In many cases of LC analysis, including this study, the frits are made of stainless steel to make the columns resistant to pressure. Since the carryover and the late-eluting selected reaction monitoring chromatogram of ornithine could be resolved by changing the frit, we speculated, but did not further investigate, that the stainless-steel frit was responsible for the carryover and delayed elution of ornithine. Based on the structure of ornithine, and its effect on separation techniques, we suggest that the basic property of ornithine can be related to its high carryover effect, caused by an interaction with the stainless-steel frit. Column-related carryover involving one or several components of multicomponent samples that may be interacting with system materials, such as metals, has been reported [11]. For instance, ferrous metals in stainless-steel frits may bind to specific analytes, leading to carryover or selective irreversible sequestration, and the use of inert components

such as polyetheretherketone (PEEK) frits may reduce the carryover [5]. Though the hypothesis has not been verified experimentally in our study, the presence of high carryover effects (12.8 %) observed in lysine-glutamine isobars in the carryover study support this hypothesis because the molecular structure of lysine is similar to ornithine. Therefore, a common compound-dependent factor can be the reason for the frit-related carryover. Of note, arginine, which is also a basic amino acid, did not reveal significant carryover effect in this study (i.e., 2.7 % and 1.6 % before and after change of frit, respectively, as measured relative to the high QC). Although arginine is a basic amino acid, its structure is quite different from both ornithine and lysine, and this structural difference could be related to the insignificant carryover effect observed with arginine. Unfortunately, other charged amino acids, such as glutamate, histidine, and aspartate, which are expected to reveal a differentiated carryover effect, were not included in the carryover evaluation due to the limited kinds of analytes included in the QCs and/or NST method. By analyzing and comparing the carryover effect of these amino acids in future studies, the correlation between the polarity of amino acids and the stainless-steel frit-related carryover effects could be explored more clearly.

Carryover has always been an issue in LC methods. However, its significance and the risk of carryover increases with the continuous increase in sensitivity of new-generation LC-MS/MS instruments. Depending on the relative impact of carryover, we can choose to tolerate the bias, reject the sample, reject the batch, or raise the LLOQ. Regarding the clinical significance of the observed carryover in this study, while it is not possible to accurately estimate the clinical impact on 1st tier NBS testing, a hypothetical sample with concentrations of certain analytes (i. e., alanine, glutamine\lysine, ornithine, valine; analytes that had significant carryover) near the high QC material, will cause up to 13.6 % carryover if the following sample has values near the screening cut-offs (data not shown). Although clinical samples with analyte concentrations near the high QC will be rare, this will likely increase the number of samples classified as abnormal based on analyte cutoff values and may trigger unnecessary follow up and additional testing.

Therefore, it would be more prudent for the laboratory scientist to be motivated to investigate the origin and assess the impact of carryover when carryover is detected. This study suggests that stainless steel frit traced as a possible source of delayed elution and carryover of ornithine should be recognized as a rare, but possible, source of carryover in FIA-MS/MS methods used for NST. Therefore, to mitigate the carryover associated with stainless-steel frits, it is necessary to pay attention to the routine maintenance and replacement of in-line filters. This could be performed periodically or when backpressure increases above 10 % from baseline. In addition, routine in-process monitoring of carryover should be implemented, and finally, a careful analysis of selected reaction monitoring chromatograms can be used for early detection of analyte specific changes in elution and associated carryover.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2023.01.001.

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