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# International interlaboratory study to normalize liquid chromatography-based mycotoxin retention times through implementation of a retention index system

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## ABSTRACT

Monitoring for mycotoxins in food or feed matrices is necessary to ensure the safety and security of global food systems. Due to a lack of standardized methods and individual laboratory priorities, most institutions have developed their own methods for mycotoxin determinations. Given the diversity of mycotoxin chemical structures and physicochemical properties, searching databases, and comparing data between institutions is complicated. We previously introduced incorporating a retention index (RI) system into liquid chromatography mass spectrometry (LC-MS) based mycotoxin determinations. To validate this concept, we designed an

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interlaboratory study where each participating laboratory was sent N-alkylpyridinium-3-sulfonates (NAPS) RI standards, and 36 mycotoxin standards for analysis using their pre-optimized LC-MS methods. Data from 44 analytical methods were submitted from 24 laboratories representing various manufacturer platforms, LC columns, and mobile phase compositions. Mycotoxin retention times ($t_R$) were converted to RI values based on their elution relative to the NAPS standards. Trichothecenes (deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol) showed $t_R$ consistency (± 20–50 RI units, 1–5 % median RI) regardless of mobile phase or type of chromatography column in this study. For the remaining mycotoxins tested, the RI values were strongly impacted by the mobile phase composition and column chemistry. The ability to predict $t_R$ was evaluated based on the median RI mycotoxin values and the NAPS $t_R$. These values were corrected using Tanimoto coefficients to investigate whether structurally similar compounds could be used as anchors to further improve accuracy. This study demonstrated the power of employing an RI system for mycotoxin determinations, further enhancing the confidence of identifications.

## 1. Introduction

Mycotoxins are fungal secondary metabolites that can cause harm to humans and/or animals. They are of interest to the agricultural sector due to their widespread presence in food and feed systems [33]. Liquid chromatography coupled to mass spectrometry (LC-MS) is a widely used and powerful technique for mycotoxin determinations. Laboratories commonly develop their own in-house analytical strategies for mycotoxin analysis using either a broad multi-mycotoxin screening method, or more targeted analyses for specific regulated mycotoxins. The development of these methods is also a reflection of the specific needs of individual labs, as mycotoxins are geographically distributed. As a result, the mycotoxin community has generated hundreds of unique methods, consisting of a large variety of instrument configurations, including manufacturers, mass analyzers, high performance liquid chromatography (HPLC) column chemistries, and mobile phase compositions [49]. The variety of mycotoxin chemical structures and physicochemical properties make MS analyzers ideal detectors for the determination of multiple classes of compounds simultaneously. Mycotoxin determination has been further complicated by the discovery of new and emerging masked or modified mycotoxins [8].

The accurate identification of mycotoxins by liquid chromatography tandem mass spectrometry (LC-MS/MS) has generally focused on the MS portion of the technique. Suspected mycotoxins can be annotated by comparing the experimental *m/z* value to a calculated exact mass value. Tandem MS (MS/MS) spectra can also be used to compare measured product ion patterns with available databases. Nielsen & Smedsgaard [36] previously introduced the concept of combining ultraviolet (UV) fingerprints with LC—HRMS/MS time of flight (ToF) data as a means of mycotoxin dereplication, and demonstrated its successful application to fungal extracts from prepared database entries [36]. However, some mycotoxins including fumonisins are not UV-active, further complicating detection. The authors reported sensitivity issues surrounding certain types of mobile phase additives affecting ionization, thus requiring method redevelopment [36].

When analytical standards are available, the retention time ($t_R$) of the standard can be compared to the putative mycotoxin within a sample as a mandatory criterion for identification. Together, the annotated MS/MS spectra, $t_R$ and comparison to authentic standards are needed for identification. The Environmental Protection Agency (EPA) recently led a collaborative multi-laboratory study (ENTACT) for non-target analysis (NTA), where participants were provided synthetic mixes of analytical standards (between 95 and 365 substances) along with spiked reference material extracts from various matrices, and CompTox database access [35,48,55,65]. The ENTACT study underscores the importance of inter-laboratory normalization and better diligence for NTA when participants gain unbridled access to over 4000 compounds in the database. Results were also reported to be highly variable between laboratories, owing to differences between analysis approaches, instrumentation, or NTA workflow. As demonstrated by the ENTACT study, wide access to analytical standards remains an important feature for correct compound identification by LC-MS. This is important as many mycotoxins are not commercially available as analytical standards, difficult to isolate, unstable, or too expensive for routine applications.

One strategy used in liquid chromatography that could help improve consistency in mycotoxin determinations between laboratories is the incorporation of retention index (RI) standards [43]. Retention time ($t_R$) is an extrinsic factor that is impacted by numerous factors, including instrument platform type, void volume, LC column (supplier, chemistry, length, particle size), mobile phase composition and modifiers, gradient program, column compartment temperature, mixing volumes, delay and flow rate [43]. Retention index systems for LC are modelled after the gas chromatography (GC) Kovats *n*-alkane index system. Kovats index values are consistent across GC platforms, where a compound's $t_R$ is directly proportional to the stationary phase interaction, as there is very little mobile phase contribution [27]. For LC, RI systems often use a set of reference standards that elute predictably over the course of an LC run, where the $t_R$ of the RI standards are compared to the compounds of interest in the same analysis to produce a relative RI value. However, unlike GC, LC-RI systems will have analyte interactions from both the mobile phase and the stationary phase, which complicates normalization.

The application of RI systems for LC platforms has previously been proposed to normalize for $t_R$ variability within the laboratory and across different platforms. Rigano *et al.* [44] provided an extensive summary of RI systems for LC-MS that have already been developed, each having their own set of advantages and limitations [44]. Homologous series of alkylphenones and 1-nitroalkanes have previously been reported for HPLC, but due to poor ionization they are not amenable to MS detections [9,22]. The use of dansyl derivatized amino acids has also been employed, however it necessitates that analyte also be derivatized, limiting its widespread application [20]. Aalizadeh et al., [1] and NORMAN developed a homologous series of cocamide diethanolamine standards that were used to convert the $t_R$ in a given analysis into a more universal RI [1]. Although these standards ionize well in positive ionization mode, they ionize less efficiently in negative ionization mode. Alternatively to RI standard incorporation into analysis, there has also been interest in using machine learning, quantitative structure-retention relationships modelling (QSRR), and artificial intelligence (AI) for $t_R$ prediction [10,11,14,17,54].

For this interlaboratory study, the N-alkylpyridinium-3-sulfonates (NAPS) RI system, NAPS-RM-RILC (Reference Material for Retention Indices in LC systems) was selected [41]. NAPS is a series of *n*-alkyl congeners (RI 100–2000 for $n = 0$ - 19) with fixed positive and negative charges that are electrospray ionization (ESI) stable in both positive and negative modes and are ultraviolet (UV) active (Fig. 1). The NAPS RI system was selected for this study because it is commercially available to the public, and is compatible with reversed phase chromatography, which is used by most labs analyzing mycotoxins with LC-MS. It was previously demonstrated that NAPS was effective for the determination of 96 mycotoxins and fungal secondary metabolites using multiple chromatographic methods as a proof of concept [43]. The study demonstrated that false positive identifications could be reduced by incorporating the NAPS RI system and applying a secondary correction

factor. The authors also developed a software package that allows for the conversion of $t_R$ to RI and vice versa from a RI value to a predicted $t_R$. In a related study, NAPS-generated RI data was also shown to be effective at normalizing retention data between instruments for metabolomics studies [50]. In that study, the authors investigated whether variances due to differences in volumes between lower and higher pressure HPLCs, pump type (binary or quaternary), varying flow rates, and column particle sizes could be normalized. Relatively low deviations were reported between instruments when using the NAPS RI system, provided the analytical conditions were similar [50].

Compound retention via LC is largely based on its physicochemical properties. Compounds with similar structures have similar mobile/stationary phase interactions, and therefore similar RI values. To consider including RI systems in spectral deconvolution and retention time prediction, structural similarity should be factored in as an additional normalization measure in order to refine the accuracy of comparing RI values obtained from different methods. One way to accomplish this $t_R$ correction is the use of an 'anchor compound' approach. An anchor compound is a substance that has a reported RI on a database for which an analyst has a matching accurate $t_R$ on their chosen chromatographic method. Appropriate anchors are then selected based on structural similarity. For the structural similarity comparison, the Tanimoto Structural Similarity Coefficient (TS) was evaluated, which computes a comparative ratio of structural fingerprints from known molecular structures. The TS calculation is an approach to describe the structural similarity between two molecular structures using structural information derived from SMILES data. Identical structures have a TS value of 1.0, and two structures that do not share any structural features would obtain a TS value of 0.0. Examining the difference between the experimental RI of the anchor compound and its RI published on a database allows for corrections to be applied to structurally similar compounds. The TS score has been tested for structural fingerprint similarity calculations [4], and has been used in bio- and chemo-informatics databases [24,32], natural product mining [45], virtual drug target screening [13], and for assessing the confidence of assigned structural and molecular formula identifications from computational *de novo* MS/MS data [15]. Wen *et al.* used TS to support quantitative structure-retention relationships models for RPLC method development to successfully predict $t_R$ of early eluting drug candidates for nearly 70 % of the compounds tested to within 0.5 mins [60].

In this study, we conducted an interlaboratory evaluation of NAPS RI normalization of mycotoxin standards and evaluated whether RI could be feasibly adopted across different LC-MS platforms and chromatography conditions. The NAPS standard mix, as well as 36 mycotoxins of agricultural relevance including aflatoxins, fumonisins, ochratoxins, zearalenone and trichothecenes were provided to 24 participating laboratories. Each laboratory analyzed the standards using their respective established mycotoxin LC-MS methods, with suggested parameters provided for mycotoxins not yet optimized in user methods. We also evaluated whether NAPS RI could be used in tandem with current LC-MS dereplication strategies to predict the $t_R$ of unknown compounds with the help of TS. The lessons learned from this study will help improve the reliability of compound identifications, introduce $t_R$-curated databases, and promote data sharing between laboratories.

## 2. Materials and methods

### 2.1. Chemicals

Optima LC-MS grade water ($H_2O$), acetonitrile (ACN), methanol (MeOH) and formic acid (FA) (Fisher Scientific, NH, USA) were used for the LC pre-optimization of the study parameters. The NAPS-RM-RILC standard was purchased through the National Research Council of Canada (NRC) for initial study optimization [40]. Commercial mycotoxin analytical standard suppliers are provided in **Supplementary Table S1**.

### 2.2. Participating laboratories

Twenty-four laboratories agreed to participate in the study, including academic institutions ($n = 13$) and government agencies ($n = 11$ labs). Contributors were from Canada ($n = 10$), Austria ($n = 4$), Belgium ($n = 3$), the United States of America ($n = 3$), Italy ($n = 2$), Germany ($n = 1$), and the United Kingdom ($n = 1$). Each of the 24 participating laboratories were given a randomized code that was included with all communications, digital drive access, and file naming.

### 2.3. Pre-optimization of NAPS and mycotoxin mixture concentrations

Concentrations of the mycotoxin standard mixtures

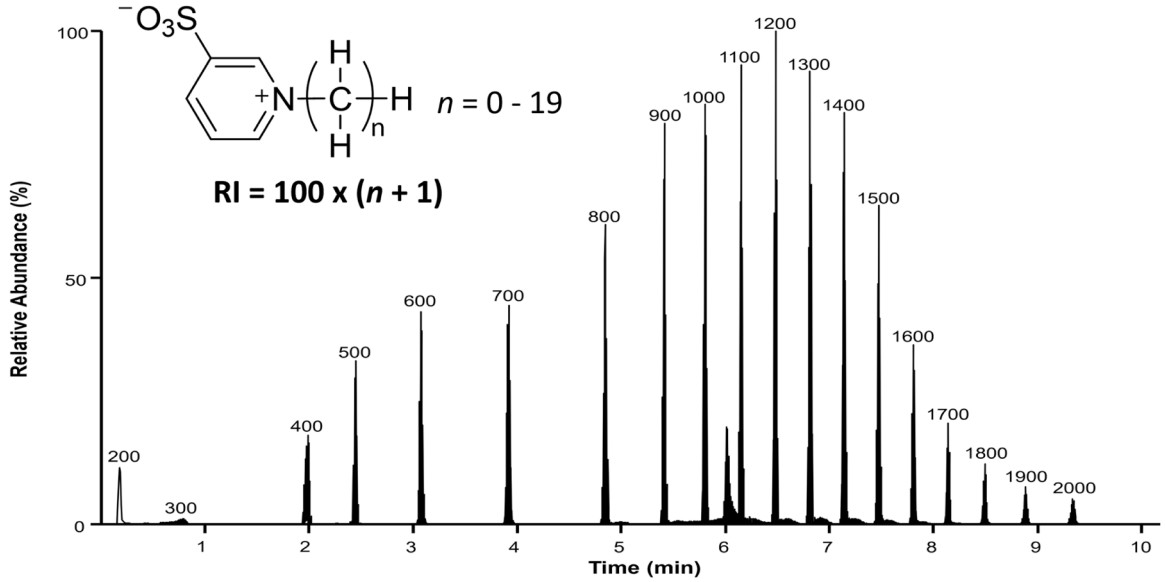



**Fig. 1.** Extracted ion chromatogram $[M + H]^+$ of N-alkylpyridinium-3-sulfonates (NAPS-RM-RILC) standards ($n = 0$–19, RI 100–2000) from NAPS standard optimization for the study (see methods) using the $m/z$ 160 product ion. The NAPS structure is shown top left, where RI is assigned to each compound as 100 times their alkyl chain length plus one ($n + 1$). RI 100 not shown due to its elution in the void volume.

(**Supplementary Table S1**) and the NAPS RM-RILC standards from the NRC [40] were designed to provide suitable sensitivity across a range of instrument platforms prior to dilution and shipment by the NRC [40]. The NAPS labelling convention was selected as the alkyl chain length ($n$ + 1) multiplied by 100 (NAPS-100–2000) per the notation used by the NRC. Optimal concentrations for testing were determined using a Thermo Vanquish HPLC coupled to a Thermo TSQ Altis triple quadrupole mass spectrometer using an Agilent RRHD Eclipse Plus C18 (2.1 × 100 mm, 1.8 μm particle size) column for separation, and a mobile phase composition of $H_2O$ + 0.1 % FA (A) and ACN + 0.1 % FA (B). For the NAPS standards, a suitable concentration was determined by assessing LC-MS sensitivity of NAPS 100–2000 using the associated product ion at 160 *m/z*. NAPS dilutions were evaluated in differing ratios of aqueous MeOH of 0, 25, 50, 75 and 100 % to ensure that the earlier (100–300) or the later eluting NAPS (1800–2000) remained consistent. For the mycotoxins, mixtures were similarly evaluated for sensitivity at different concentrations (from 0.5 μg/mL to 10 μg/mL) in 1:1 ACN:$H_2O$. A 1 μg/mL concentration was selected as a compromise between good instrument sensitivity and materials cost. Two mycotoxin mixtures were selected to minimize uncertainty in assigning retention times to isobaric compounds, such as 3-acetyldeoxynivalenol/15-acetyldeoxynivalenol (3-ADON/15-ADON) and Fumonisin $B_2$/Fumonisin $B_3$ (FB2/FB3) (Table 1). Both mycotoxin mixtures were evaluated for their short-term stability and compatibility for durations of 1 week, 2 weeks, and 1 month at −20 °C. All compounds showed excellent stability over the durations tested, (relative standard deviations of extracted peak areas < 5 %, data not shown).

### 2.4. RI package preparation - mycotoxin mixtures and NAPS solution preparation

For the 36 mycotoxins used in this study, two mixtures (Mycotoxin mix 1 and 2, Table 1, **Supplementary Table S1**) were subsequently prepared and dispensed in flame sealed argon purged glass ampoules at 1 μg/mL in 1:1 ACN:$H_2O$. Additional HPLC glass vials and inserts were provided to all participants. The NAPS solution was diluted 150-fold in 1:1 MeOH:$H_2O$ to provide concentrations of 0.3 μmol/L and 0.67 μmol/L for NAPS 100–300 and NAPS 400–2000, respectively for direct use by study participants. Mycotoxin mixes and the diluted NAPS solution were shipped in insulated containers with freezer packs.

### 2.5. Digital drive setup

Individual digital folders on Google Drive were prepared for each participant for downloading and uploading data, and access was provided following the shipment of the RI test package. Each digital folder contained a materials receipt form for upload on delivery to confirm materials were received in good condition. Laboratories were given general instructions to complete the study (**Supplementary, User docs RI Sample**), which included instructions on how to store and run the mycotoxin standard mixes and the NAPS mix, dilution recommendations, and example pre-optimized mycotoxin and NAPS MS/MS transitions and parameters for both low- and high-resolution instruments. A Microsoft Excel worksheet was included for participants to record method and instrumentation information (manufacturer platform, mobile phase compositions, column chemistries, column compartment temperature, flow rate, gradients, retention times, MS settings), and to report any deviations to the outlined analysis. Each LC-MS platform was assigned a letter (A, B, C, etc.), while each gradient or mobile phase combination received its own distinct number, (e.g. LC-MS Platform *A* + Gradient 1 = A1). This ensured that participants with different LC-MS platforms or labs utilizing different columns and gradients could easily report multiple analyses in the same document. Each laboratory was encouraged to use their own established methods for analyzing mycotoxins, and there was no limit on the number of methods or platforms tested. Though not mandatory, participants could include any additional mycotoxin standards not contained in mixes 1 and 2. Sample RI study documents are included in the Supplementary Information (Retention Index Sample, User Docs RI Sample). Individual laboratories were required to provide their instrument raw data files, as well as all retention times for the NAPS standard and mycotoxins for each method.

#### 2.5.1. RI conversion and data analysis

All reported $t_R$ were converted to RI values using the Retention Time Converter python script (https://github.com/sumarah-lab/Retention-Time-Converter). Briefly, this was accomplished by using an Akima cubic spline function to fit the $t_R$ of the NAPS standards against their defined RI values as previously described [3,43]. Data from each laboratory were individually verified from the uploaded instrument files where possible. Thermo Xcalibur software was used for .raw files, while Agilent .D files and Sciex .wiff files were subsequently converted into .mzml format and examined using MZmine 3.9.0 [46]. Waters and Bruker files could not be validated due to incompatibilities with vendor software, but affected laboratories were contacted individually for additional validation as required. Any reported $t_R$ that could not be validated was removed. All RIs from each laboratory are provided in the Supplementary (**RI reported by lab code.xlsx**). In some methods, the later eluting enniatins (ENNB & ENNB1) eluted after the NAPS- 2000

**Table 1**
Mycotoxin mixtures 1 and 2 prepared at 1 μg/mL in 1:1 ACN:$H_2O$.

| Mycotoxin mixture 1 | | | Mycotoxin mixture 2 | | |
|---|---|---|---|---|---|
| **Aflatoxin $B_1$** | **AFB1** | **$C_{17}H_{12}O_6$** | **Ochratoxin A** | **OTA** | **$C_{20}H_{18}ClNO_6$** |
| Aflatoxin $B_2$ | AFB2 | $C_{17}H_{14}O_6$ | Fumonisin $B_3$ | FB3 | $C_{34}H_{59}NO_{14}$ |
| Aflatoxin $G_1$ | AFG1 | $C_{17}H_{12}O_7$ | Citrinin | CIT | $C_{13}H_{14}O_5$ |
| Aflatoxin $G_2$ | AFG2 | $C_{17}H_{14}O_7$ | Roquefortine C | ROC | $C_{22}H_{23}N_5O_2$ |
| Deoxynivalenol | DON | $C_{15}H_{20}O_6$ | Patulin | PAT | $C_7H_6O_4$ |
| T-2 toxin | T-2 | $C_{24}H_{34}O_9$ | α-Cyclopiazonic acid | CPA | $C_{20}H_{20}N_2O_3$ |
| HT-2 toxin | HT-2 | $C_{22}H_{32}O_8$ | Alternariol | AOH | $C_{14}H_{10}O_5$ |
| Zearalenone | ZEN | $C_{18}H_{22}O_5$ | Alternariol monomethyl ether | AME | $C_{15}H_{12}O_5$ |
| Fumonisin $B_1$ | FB1 | $C_{34}H_{59}NO_{15}$ | 15-Acetyldeoxynivalenol | 15-ADON | $C_{17}H_{22}O_7$ |
| Fumonisin $B_2$ | FB2 | $C_{34}H_{59}NO_{14}$ | Altenuene | ALT | $C_{15}H_{16}O_6$ |
| Sterigmatocystin | STE | $C_{18}H_{12}O_6$ | Penitrem A | PENTRA | $C_{37}H_{44}ClNO_6$ |
| Fusaric acid | FusAc | $C_{10}H_{13}NO_2$ | Ergocristine | ERGCRI | $C_{35}H_{39}N_5O_5$ |
| Diacetoxyscirpenol | DAS | $C_{19}H_{26}O_7$ | Ergocristinine | ERGCRIN | $C_{35}H_{39}N_5O_5$ |
| 3-Acetyldeoxynivalenol | 3-ADON | $C_{17}H_{22}O_7$ | Ergocryptine | ERGCRY | $C_{32}H_{41}N_5O_5$ |
| Enniatin B | ENNB | $C_3{}_3H_{57}N_3O_9$ | Ergocryptinine | ERGCRYN | $C_{32}H_{41}N_5O_5$ |
| Enniatin $B_1$ | ENNB1 | $C_{34}H_{59}N_3O_9$ | Mycophenolic acid | MPA | $C_{17}H_{20}O_6$ |
| NX toxin | NX | $C_{15}H_{22}O_5$ | Tenuazonic acid | TeA | $C_{10}H_{15}NO_3$ |
| | | | 3ANX toxin | ANX | $C_{17}H_{24}O_6$ |
| | | | Viridicatin | VIRI | $C_{15}H_{11}NO_2$ |

peak, which required a manual RI fit, resulting in RI values > 2000. Some participants also reported peak splitting, broad or tailing peaks (**Supplementary Table S3**) for some compounds where multiple RI values would be generated for each retention time reported. Upon validation of the instrument files, the predominant peak by extracted ion chromatogram was typically selected as the representative RI value for that compound. For major peak splitting (> 2 peaks) or major shifts ($t_R$ > 0.2 min), or where MS/MS fragmentation data were not included, the averaged RI value of the split peaks was reported. Any values with averaged RI values are denoted in the Supplementary Information as RI$. Boxplots and dotplots were prepared in R (v. 4.4.1) using the dplyr (v. 1.1.4) and ggplot2 (v. 3.5.1) packages [42,62,63]. RI values for each mycotoxin were visualized as scatterplots (**Supplementary Figures S4-S21**) compared to their median study value and plotted with 5 % median RI (± 100 RI units) and 10 % median RI (± 200 RI units). Regression coefficient analysis (caret (v. 6.0.94), glmnet (v. 4.1.8) and tidyverse (v. 2.0.0) packages) and random forest feature importance ranking (randomForest package v. 4.7.1.1) were calculated in R to investigate the significance ($p \leq 0.05$) of the meta information affecting the individual RI values [18,29,31,64]. Principal component analysis (PCA) was performed in R using FactoMineR, MetabolAnalyze, and factoextra packages [19,26,30]. Column information provided by participants was normalized using the Tanaka method via the ACD/Labs column selector tool, as well as the Synder method via the USP PQRI database [2,39,47,56], and is included in **Supplementary Table S18**. Method information for all 44 methods from the 24 participating laboratories (**Supplementary Tables S2-S11**) as well as converted RI values for all 36 mycotoxins is reported in the Supplementary Information (**RI reported by lab code.xlsx**).

#### 2.5.2. RI to retention time prediction and correction

Median mycotoxin RI values from the study were split by mobile phase composition, MeOH or ACN, and tested with the Akima cubic spline fit with the associated NAPS retention values for each method to first generate a 'predicted' retention time (**Supplementary Information- RI reported by lab code**). Tanimoto similarity coefficients were computed using SMILES notation with the RDKit python script (Open-source cheminformatics, accessed from: https://www.rdkit.org) for the 36 mycotoxins in the study (**Supplementary Figure S30**). Values ≥ 0.6 were considered for 'anchor' compounds and have been grouped by their structural similarity (**Supplementary Tables S20, S21, Supplementary Figures S30, S33**). Compound anchoring was accomplished using Eqs. (1) and (2):

$$Anchored\ RI = RIexp - \Delta\ RI \quad (1)$$

*Anchored RI* is the selected compound to anchor (corrected RI),

*RIexp* is the experimental RI value to be anchored (where TS ≥0.6 to chosen anchor), and

$$\Delta RI = (RI\ anchor - RI\ reported\ anchor) \quad (2)$$

*RI anchor* is the measured RI of the anchor compound,

*RI reported anchor* is the median study value (either MeOH or ACN) or database RI value of the anchor compound.

To evaluate the suitability of a compound for anchoring, the TS Coefficient (≥ 0.6) was used as a structure-guided cut-off value for anchor selection. This value was selected as a compromise for all mycotoxin classes in the mixtures to provide accurate structural information while also limiting potential false positives. A TS coefficient value of ≥ 0.5 has typically been used when evaluating structure activity relationships for screening large compound datasets [38,51]. Other applications utilizing more structurally related compound classes can use more stringent TS coefficients (≥ 0.8) to better suit their analyses.

## 3. Results and discussion

### 3.1. Results summary from all 44 methods

It is important to note that there are no correct or incorrect answers in this study. This study was designed to provide a better understanding of the challenges analysts face when developing and implementing multi-mycotoxin LC-MS determination methods, and to evaluate the feasibility of incorporating an RI system. The lessons learned from this study can be used to help improve the reliability of compound identifications, introduce $t_R$-curated databases, and promote data sharing between laboratories.

#### 3.1.1. LC method summary

Twenty-four laboratories across Europe (***n*** = 11) and North America (***n*** = 13) participated in the study. Each laboratory either used the method conditions provided or contributed with their own pre-optimized LC-MS method(s), which resulted in a total of 44 unique methods with paired RI information. Laboratories reported the use of six different LC manufacturers (Agilent, Thermo, Waters, Shimadzu, Sciex, and Bruker) (Fig. 2A, **Supplementary Tables S2a–2d**). Sample run times ranged between 9 and 55 min s, with a median time of 17 mins (**Supplementary Tables S2a – 2d, Supplementary Tables S3a – 3g, Supplementary Figure S2**). C18 was the most used column chemistry (~80 % of all methods), followed by pentafluorophenyl (PFP) columns (18 %) and biphenyl (BP) columns (2 %) (Fig. 2B). The majority (66 %) of the LC methods used $H_2O$-MeOH gradients (Fig. 2C, right) with the remaining 34 % using $H_2O$-ACN gradients. Mobile phase modifiers (Fig. 2C, left) included MS grade solutions of FA, (***n*** = 28 methods), acetic acid (HAc, ***n*** = 15 methods), ammonium formate (AF, ***n*** = 15 methods), ammonium acetate (AA, ***n*** = 7 methods), and aqueous ammonia solutions (***n*** = 2 methods), where some methods used more than one modifier.

### 3.2. RI value summary for all 44 reported methods

#### 3.2.1. NAPS relationship to retention time and retention index

The NAPS standard demonstrated excellent sensitivity by LC-MS for participating laboratories. The prepared NAPS standard mix (0.3 μM for NAPS 100–300, 0.67 μM for NAPS 400–2000) was detected across all 44 different methods, with many of the laboratories reporting additional dilution steps to better suit their instrumental sensitivity (**Supplementary Table S3**). As the length of the alkyl chain in the NAPS standards increased, their corresponding retention times also increased as expected due to the enhanced hydrophobic interactions with the stationary phase (**Supplementary Figure S3**). The linearity of the cubic spline fit for the NAPS standards in the study increased above NAPS−300 as previously reported by Renaud *et al.* [43]. Some users reported peak splitting, especially for the more polar NAPS 100, 200 and 300 standards, which has also been previously reported [43], with NAPS-100 and NAPS-200 typically eluting in the void volume. Peak splitting or deterioration can also be exacerbated if the NAPS solution is diluted in organic solvent such as methanol (> 50 %). Inclusion of the NAPS RI system may be difficult for smaller polar compounds, such as moniliformin, especially in rapid multi-toxin screening methods. Larger apolar compounds, including enniatins or other macrocyclic molecules, may also be difficult to fit to the NAPS RI system, as they may elute past NAPS−2000 on typical C18 reverse phase methods.

#### 3.2.2. Application of NAPS normalization to mycotoxin determination by LC-MS analysis

Submitted user methods reflect the analytical methodologies already optimized for each individual laboratory's needs, either for robust multi-mycotoxin determinations, or for application-driven analysis needs for specific classes of regulated compounds. As a result, not all of the 36 mycotoxins were reported in several of the datasets provided. Individual

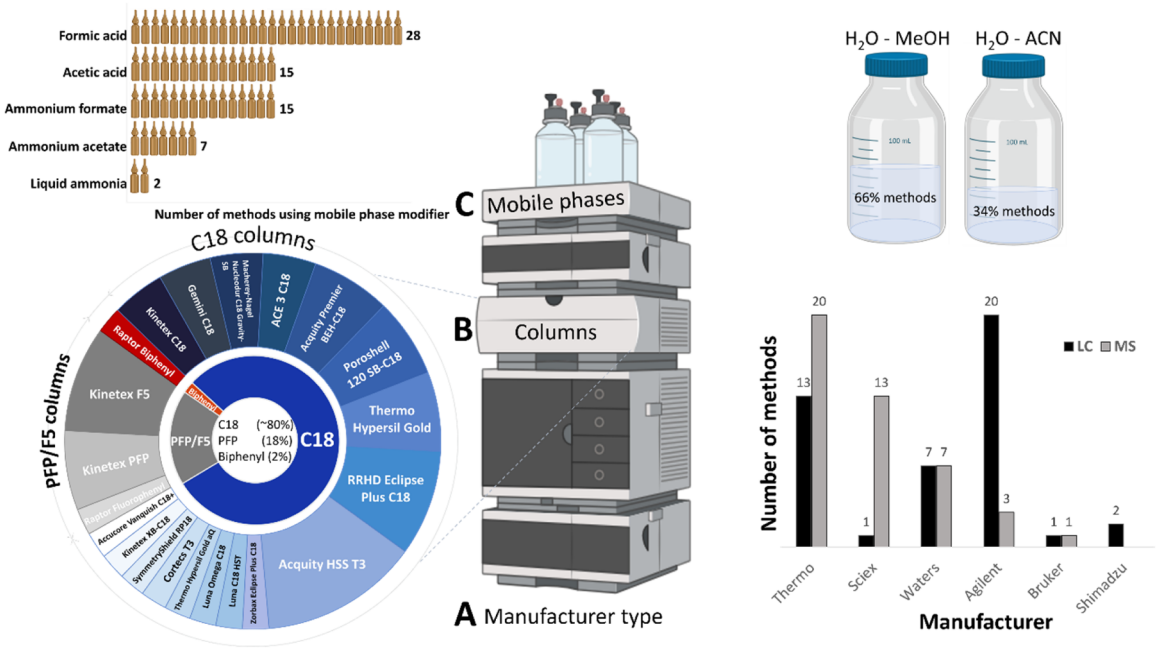



**Fig. 2.** Compilation infographic of diversity of platforms and LC conditions and parameters used in the study. (A) Platform information by manufacturer for both LC and MS instruments; (B) reported column use for the 44 methods (bottom left) with C18 formulations; (C) Mobile phase reported (%, top right) and mobile phase modifiers used in the 44 methods (top left). Figure created using images from BioRender.com.

RI plots of the 36 mycotoxins for all 44 methods are reported in the Supplementary Information (**Supplementary Figures S4-S21**).

Most participants reported the majority of the 36 mycotoxins targeted in this study (Table 2). For mycotoxins not currently established in their methods, parameters were included in the provided user documentation for MS pre-optimization (**Supplementary- User Docs RI Sample**). Detection issues following adding compounds to the method have been summarized in **Supplementary Tables S3a-g**. However, as the focus here was not to evaluate if all laboratories successfully modified or optimized their methods to include for each mycotoxin, discussion of detection centered around major trends in the data and user comments.

The mycotoxin with the smallest RI value was patulin (PAT), with a median study value of 473. Although there is a demonstrated need for PAT determination, it has traditionally been analyzed separately from other multi-mycotoxin analytical methods to circumvent its elution in the void volume [58]. Patulin had one of the lowest detection rates, with only 59 % of the total methods reporting it. Despite two outlier values (> ± 200 RI), 74 % of the reported PAT RI values were within 5 % (± 100 RI) of the median (Fig. 3**A, B, Supplementary Figure S18**). Similar detection issues were observed for the polar trichothecene, NX-toxin (NX, median RI 537), where only 45 % of the analytical methods detected it (Table 2). Several users noted LC-MS sensitivity issues for NX, which is structurally related to DON. Most of the NX detections (approximately 83 %) were achieved using high resolution instruments, likely because these instruments could confirm the presence of NX through additional ESI adducts. This approach bypassed the need for further method development, such as the need for tuning the compound for MS/MS transitions typically required for triple quadrupole instruments. While poor sensitivity of NX may have been a contributing factor, it is likely that the MS/MS parameters provided for participants did not align for all triple quadrupole MRM methods. For methods reporting NX, the NAPS RI system had similar agreement (Fig. 3**A, B, Supplementary Figure S17**), where approximately 95 % of the methods were within 5 % (± 100 RI) of the median RI.

### 3.3. *Factors affecting retention indices*

To explore the factors contributing to RI differences in the study, a PCA plot was produced using RI results from all 44 methods along with meta information from the methods including mobile phase composition, mobile phase modifiers, column chemistry, and universal column designations (Fig. 4).

Each point on the PCA plot represents an individual laboratory method, and represents all RI values included for the study. Data grouped closely together based on column chemistry (C18, PFP or BP) and also separated in the second dimension according to mobile phase selection (MeOH vs. ACN). The PCA highlighted that the biphenyl column tested could not be included with either set of C18 or PFP data. In terms of the mobile phase contribution to RI differences contributing predominantly to the second dimension, fumonisins, ROC and ergot alkaloids had higher measured RI values in MeOH compared to ACN. Conversely, the apolar enniatins had higher measured RI values in an ACN mobile phase compared to MeOH. The basis of using this RI is to describe the $t_R$ of any mycotoxin relative to the NAPS standards. As such, it is not surprising that differences in measured RI values would occur depending on whether a PFP or C18 stationary phase was used. Relative to C18, PFP columns show greater dipole selectivity and stronger retention of compounds based on π-π interactions. This was generally observed in this work, all but four of the tested mycotoxins had higher median RI values on a PFP column compared to a C18 column when methanol was the mobile phase (Table 2). When acetonitrile was the mobile phase, 22 mycotoxins had higher median RI values on PFP columns compared to C18 stationary phases as acetonitrile impedes π-π interactions [68]. This provided insight into which components from analytical methods impacted the RI of mycotoxins, which were further explored.

#### 3.3.1. *Mobile phase composition*

Six mycotoxins were reported for all 44 methods within 5 % (± 100 RI) of the RI study median for all 44 methods: 3-ADON, 15-ADON, 3ANX, DAS, DON, and HT-2. However, RI values reported for several mycotoxins including the ergopeptines and ergopeptinines (ERGCRI,

**Table 2**
Results summary for all 44 methods from 24 participating laboratories (left), and median RI values for C18 and PFP/F5 columns with MeOH and ACN mobile phases (right).

| All methods ($n = 44$) | | | | | | | C18 Columns ($n = 35$) | | PFP/F5 ($n = 8$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mycotoxin** | **RI (median)** | **RI (Min)** | **RI (Max)** | **Methods reporting** | **Methods* within 5 % ( ± 100) median RI** | **Methods* within 10 % ( ± 200) median RI** | MeOH ($n = 21$) | ACN ($n = 14$) | MeOH ($n = 7$) | ACN ($n = 1$) |
| PAT | 473 | 256 | 645 | 59 % | 74 % | 93 % | 473 | 474 | 535 | 442 |
| NX | 537 | 435 | 556 | 45 % | 95 % | 100 % | 547 | 502 | 519 | 482 |
| DON | 541 | 447 | 599 | 91 % | 100 % | 100 % | 556 | 502 | 528 | 481 |
| FusAc | 683 | 590 | 938 | 66 % | 90 % | 93 % | 699 | 601 | 668 | 601 |
| 15-ADON | 693 | 644 | 736 | 91 % | 100 % | 100 % | 699 | 658 | 717 | 644 |
| 3-ADON | 695 | 655 | 745 | 86 % | 100 % | 100 % | 698 | 664 | 728 | 655 |
| 3ANX | 702 | 647 | 755 | 77 % | 100 % | 100 % | 708 | 686 | 746 | 668 |
| AFG2 | 756 | 710 | 1800 | 100 % | 86 % | 95 % | 734 | 775 | 881 | 827 |
| AFG1 | 791 | 733 | 1747 | 100 % | 86 % | 98 % | 760 | 819 | 916 | 882 |
| ALT | 802 | 764 | 905 | 98 % | 98 % | 100 % | 802 | 781 | 893 | 803 |
| AFB2 | 807 | 756 | 1890 | 100 % | 82 % | 95 % | 795 | 818 | 950 | 871 |
| AFB1 | 837 | 780 | 1046 | 98 % | 84 % | 98 % | 818 | 861 | 981 | 928 |
| DAS | 838 | 795 | 920 | 95 % | 100 % | 100 % | 828 | 844 | 880 | 818 |
| TeA | 843 | 481 | 996 | 68 % | 70 % | 97 % | 849 | 851 | 798 | 830 |
| ERGCRY | 868 | 572 | 1790 | 95 % | 57 % | 76 % | 868 | 800 | 1243 | 994 |
| ERGCRI | 874 | 576 | 1789 | 95 % | 67 % | 76 % | 868 | 815 | 1261 | 1029 |
| ROC | 900 | 660 | 1676 | 100 % | 64 % | 86 % | 906 | 820 | 1071 | 927 |
| ERGCRYN | 914 | 617 | 1677 | 89 % | 44 % | 74 % | 933 | 807 | 1274 | 1029 |
| ERGCRIN | 918 | 616 | 1685 | 89 % | 54 % | 79 % | 949 | 852 | 1180 | 1060 |
| FB1 | 929 | 688 | 1576 | 98 % | 60 % | 91 % | 943 | 789 | 1088 | 856 |
| HT-2 | 932 | 892 | 994 | 89 % | 100 % | 100 % | 935 | 916 | 984 | 892 |
| AOH | 941 | 860 | 1274 | 100 % | 80 % | 82 % | 937 | 919 | 1218 | 1009 |
| CIT | 942 | 767 | 1930 | 98 % | 56 % | 88 % | 849 | 996 | 984 | 970 |
| FB3 | 995 | 768 | 1763 | 98 % | 56 % | 88 % | 1018 | 853 | 1198 | 930 |
| MPA | 1000 | 779 | 1283 | 98 % | 79 % | 93 % | 976 | 1012 | 1114 | 1027 |
| VIRI | 1030 | 995 | 1170 | 86 % | 95 % | 100 % | 1029 | 1030 | 1034 | 1015 |
| T-2 | 1053 | 911 | 1158 | 93 % | 93 % | 100 % | 1013 | 1096 | 1126 | 1073 |
| FB2 | 1070 | 826 | 1744 | 93 % | 51 % | 85 % | 1089 | 897 | 1261 | 980 |
| ZEN | 1123 | 1039 | 1413 | 98 % | 79 % | 86 % | 1097 | 1165 | 1333 | 1230 |
| OTA | 1149 | 842 | 1723 | 100 % | 66 % | 93 % | 1074 | 1161 | 1250 | 1189 |
| AME | 1161 | 1073 | 1555 | 100 % | 77 % | 80 % | 1152 | 1162 | 1482 | 1285 |
| STE | 1165 | 827 | 1962 | 95 % | 69 % | 88 % | 1141 | 1214 | 1397 | 1343 |
| CPA | 1277 | 846 | 1760 | 80 % | 54 % | 86 % | 1146 | 1336 | 1339 | 1392 |
| PENTRA | 1399 | 1226 | 1679 | 95 % | 55 % | 95 % | 1349 | 1526 | 1395 | 1492 |
| ENNB | 1641 | 1347 | 2114[x] | 95 % | 33 % | 71 % | 1469 | 1672 | 1919 | 1744 |
| ENNB1 | 1699 | 1405 | 2192[x] | 95 % | 40 % | 69 % | 1528 | 1734 | 1970 | 1829 |

* Reporting methods, where %RI is calculated for the maximum NAPS length (2000).
[x] Extrapolation past NAPS-2000 necessary due to elution past NAPS-2000 standard. Mycotoxin acronyms are provided in Table 1.

ERGCRIN, ERGCRY, ERGCRYN), enniatins (ENNB, ENNB1), and fumonisins (FB1, FB2, FB3) were highly dependent on the mobile phases and column chemistries used (Fig. 3**A, B, Supplementary Figures S11-S15**).

NAPS standards have both a fixed positive quaternary imine and a fixed negative sulfonate moiety, which allows for both ionization stability and overall suitability for reversed phase chromatography [41]. Changes in mobile phase pH by different modifiers do not impact the NAPS $t_R$ due to the overall net neutral charges on the congeners [43,50]. However, several mycotoxins are strongly affected by changes in pH and mobile phase composition, resulting in a greater $t_R$ range relative to NAPS.

Several methods reported peak splitting and broadening for CIT, and only about half (56 %) of the total methods were within 5 % (± 100 RI) of the median RI value (942, Table 2 and Fig. 3**A, B, Supplementary Figure S9**). Citrinin ($pK_a = 3.5$) exists as both *p*- and *o*-quinone tautomers, and its retention is very sensitive to mobile phase pH [6,53,67]. The *o*- and *p*- quinone forms of CIT may have secondary interactions with the C18 functionalized silica or proprietary endcapping formulations resulting in retention shifts, peak splitting, or broadening.

Additional mycotoxins that are sensitive to pH are fumonisins (FB1, FB2, FB3), amino acid-containing mycotoxins such as enniatins (ENNB, ENNB1), PENTRA, ROC, and the ergopeptines and ergopeptinines (ERGCRI, ERGCRIN, ERGCRY, ERGCRYN). These analytes are more easily protonated by most typical acid-containing mobile phases, either FA or HAc, which would affect their interaction with the stationary phase. Alkaline buffered aprotic mobile phases are recommended to improve the separation of ERGCRI, ERGCRY, and their related ergopeptinines to maintain their stability and elution order on typical C18 columns [12]. Several method datasets included acidified buffering modifiers such as AA or AF to HAc/FA-containing mobile phases, likely contributing to the variable RI values between the 44 methods for these compounds (Fig. 3**A, B, Supplementary Figure S25 A).** Investigating metadata information significance on the main mycotoxin analytes (AFB1, FB1, OTA, ZEN, DON) using regression coefficient analysis and random forest feature importance ranking highlighted that mobile phase composition, especially $H_2O$-MeOH, was a significant contributor ($p < 0.05$) to RI values in the study (**Supplementary Tables S12-S17**), warranting further study. The PCA of C18 methods from **Figure S25 A** highlights that the acid modifier choice may play a role in RI variability, but its predominant separation still seemed to be due to mobile phase type. Both MeOH and ACN have differing solvent properties, which can influence solubility, stationary phase interactions and the analyte retention. However, the acid modifier selection, especially for acetic acid-containing mobile phases, may need to be investigated in future analyses.

Most of the methods (66 %) opted for protic (e.g. MeOH) organic mobile phases over aprotic organic mobile phases (e.g. ACN), which might be a cost-related consideration. Aprotic solvents like ACN typically have a greater elution strength for neutral compounds than MeOH, which results in a lower retention in ACN over MeOH. Penitrem A separation was strongly linked to the choice of mobile phase

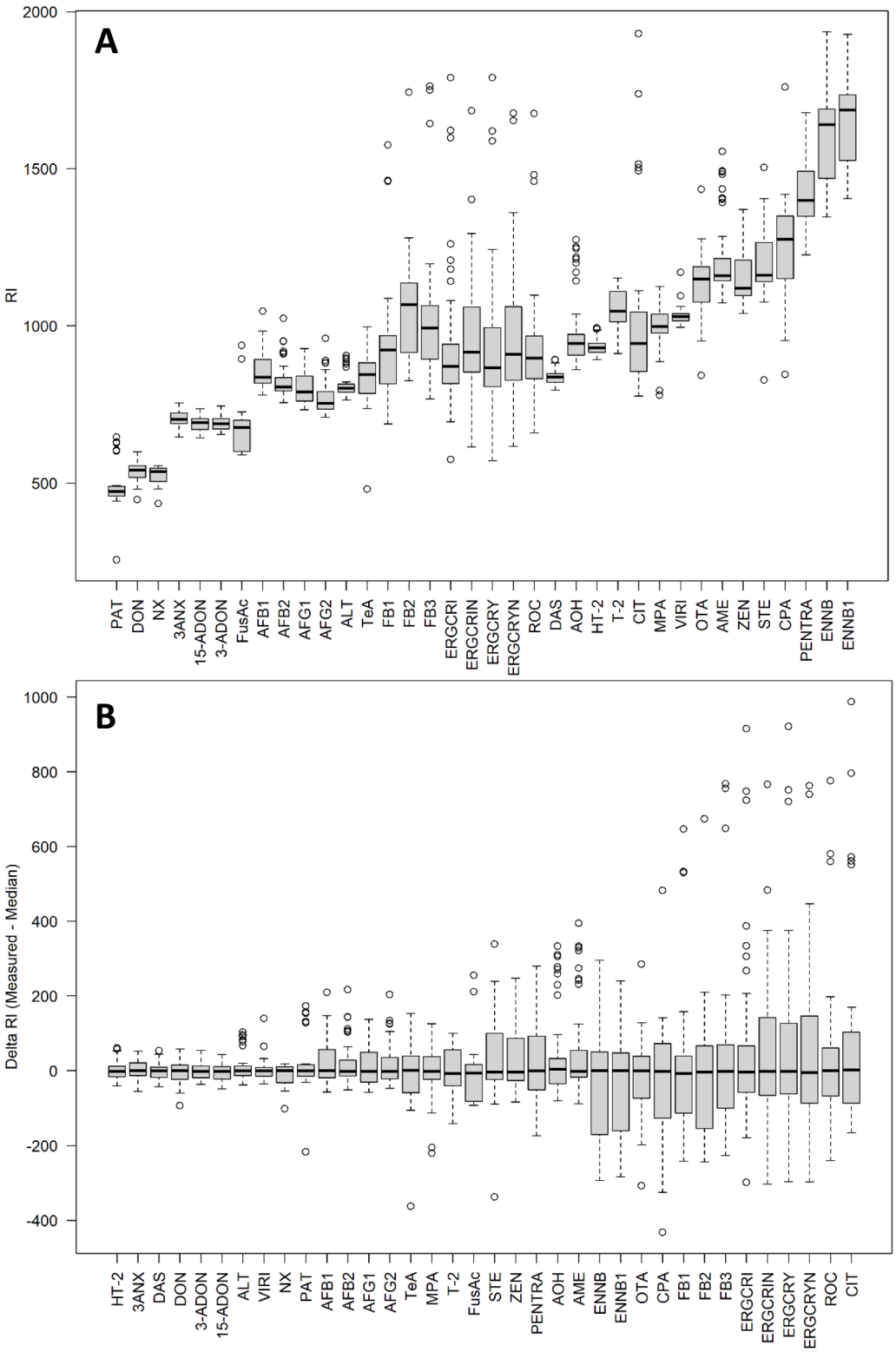



**Fig. 3.** (A) Boxplot of converted RI values by increasing RI for all 36 mycotoxins analyzed by 44 methods, and (B) converted RI values plotted as their difference from the median study values.

composition (**Supplementary Figure S27**).

In gas chromatography (GC), NIST databases contain separate Kovats RI values for apolar, and polar classes of GC-columns [37]. This allows a user to compare their data to reference values that best mimic their chromatographic method. Based on the RI results from the various methods reported here, the choice of a MeOH or ACN mobile phase has a significant role in the measured RI value. Therefore, analogous to the Kovats index, it would be advantageous to report the RI values of a given mycotoxin as either the $RI_{MeOH}$ or $RI_{ACN}$, allowing the analyst to choose the RI reference values obtained from methods that most closely resemble their approach.

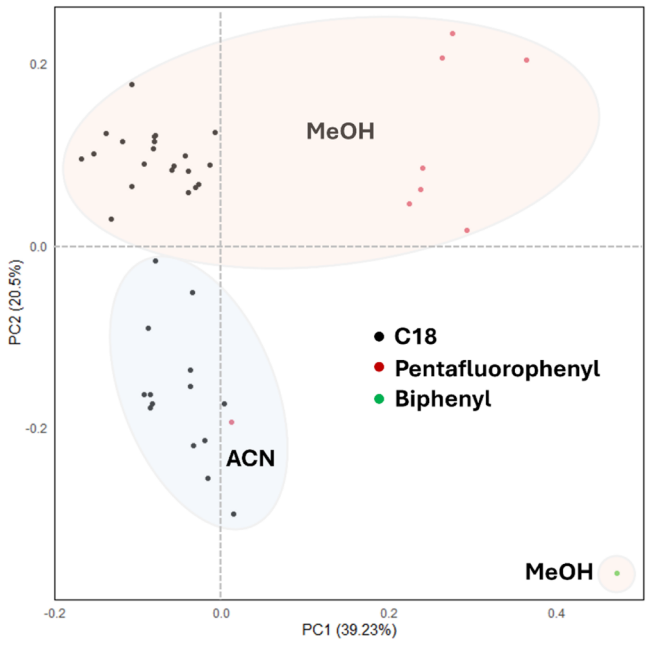



**Fig. 4.** PCA plot of mycotoxin RI values from all 44 methods in the study. Each point on the PCA represents an individual method, and has been coloured by column chemistry (C18, PFP or BP). Areas are shaded by mobile phase (orange, MeOH or blue, ACN).

### 3.3.2. Column chemistry

Most of the methods (~80 %) reported study results using C18 columns, and included differences in particle size, column endcapping and column core types (porous, poroshell/core shell) (Fig. 2**B, Supplementary Tables S2a – 2d**). The remaining 20 % of the methods used either PFP/F5 columns or biphenyl columns. To better compare between the columns used in the study, the columns were normalized by their hydrophobic retention, hydrophobicity, and steric selectivity using Tanaka and Snyder modelling (**Supplementary Table S18**) to determine whether column type affected RI. Unfortunately, it was unclear if the Tanaka and Snyder modelling constants, such as hydrophobic retention ($k_{PB}$) or hydrophobicity (H) had any direct impact on RI (**Supplementary Figure S25 B**).

The PCA analysis from Fig. 4 highlighted the contribution of column chemistry to RI values in the study. Further investigation of the significance values for mycotoxins (AFB1, FB1, OTA, ZEN, DON) from the metadata information similarly confirmed that using PFP/F5 column chemistry was a statistically significant contribution ($p < 0.05$) to the RI values in the study (**Supplementary Tables S12-S17**). While a number of mycotoxins had lower RI values on PFP, including DON, NX, FusAc, TeA, the RIs of some mycotoxins increased when PFP/F5 columns were selected over C18 columns. These increased differences were primarily observed for polar mycotoxins such as PAT, and mycotoxins containing aromatic groups, such as the dibenzopyrones, AOH and AME, (**Supplementary Figure S26**) and the ergopeptines and ergopeptinines. Differences in mycotoxin elution order were also more evident between PFP and C18 columns (**Supplementary Table S19**). Using C18 columns, ergopeptine eluted prior to ergopeptinine, but it was observed that the PFP/F5 column chemistry caused ergocryptinine to elute before ergocryptine in some instances. When methods running PFP/F5 columns were removed from the analysis, the RI differences (ΔRI, Measured RI – Median RI) for C18 columns decreased and improved overall compound identification confidence (Table 2, **Supplementary Figure S23 A, B**).

PFP/F5 columns were processed separately from the C18 columns, and had smaller RI differences (ΔRI) for the majority of the 36 mycotoxins (Table 2, **Supplementary Figure S24 A, B**). Methods utilizing PFP/F5 columns showed significant changes in NAPS RI values for ergopeptines, ergopeptinines and fumonisins (FB1, FB2, FB3). Even when comparing similar column chemistry, there were still large variations in RI values for these mycotoxins, with some ΔRI values of > 500 RI units (**Supplementary Figure S24 B**). RI differences were also observed within column chemistry types, such as the Phenomenex PFP Kinetex and Phenomenex Kinetex core-shell F5 columns. While these columns have the same chemistry, their core-shell formulations differ, affecting the analyte behaviour. West *et al.*, [61] previously reported that Phenomenex PFP (PFP-K) and Phenomenex F5 (PFP-K2) columns were highly dissimilar to each based on evaluation of ionic interactions for supercritical fluid chromatography [61]. Previous studies have shown that pentafluorophenyl columns have highly variable ion-exchange properties that have a strong affinity for basic compounds [7,61]. This ion exchange capability explains why some users reported peak splitting for some compounds in the study when PFP columns were used.

## 3.4. Retention time prediction and correction

### 3.4.1. Akima spline retention time prediction using NAPS retention time values

A recent multilaboratory collaborative study was designed by the US-Food and Drug Administration (FDA) and Canadian Food Inspection Agency (CFIA) to screen for pesticides by LC-MS. They still reported $t_R$ differences between laboratories despite highly controlled chromatographic parameters due to variations in instrument configurations [66]. In the current study, two different laboratories reported highly similar chromatographic conditions (gradients, mobile phases, and column chemistries), and their $t_R$ values were varied for many mycotoxins (**Supplementary Figure S29**). The barplot shows that their corresponding RI values are much less variable. If $t_R$ data between these two laboratories were instead normalized to RI, the overall average % difference would decrease from 7.8 % to 1.4 %, with 14 mycotoxins having less than 1 % difference. This increases the confidence of detections by decreasing the likelihood of reporting false positives, thereby introducing a more reliable method of direct comparability. This has been previously observed [50], where different void volumes introduced by uncontrollable variables, such as the HPLCs themselves (manufacturer differences, or HPLC vs. UHPLC), differing pump types (binary/quaternary), mixer type, or tubing differences (material, lengths, inner diameters) led to major variations in $t_R$ alone. This highlights a major challenge for sharing LC-MS data between laboratories, especially for regulated mycotoxins.

To investigate whether median RI values could be used to normalize retention time predictions, the NAPS retention times from each of the 44 methods were used to calculate a 'predicted' retention time for each mycotoxin based on the Akima spline fit of NAPS standards and the study median value (Fig. 5**A**).

Despite a few outlier RI values for both PAT and NX, the Akima cubic spline fit was reasonably accurate in predicting the $t_R$ based on NAPS $t_R$ values, with 20 and 13 methods being within 0.3 mins of the actual reported $t_R$, respectively (Fig. 5**A**). For emerging compounds of concern like NX where analytical standards are not yet commercially available, NAPS can be a crucial tool for determining its presence in a sample. On high resolution platforms, the *m/z* can be predicted, and its $t_R$ could also be putatively determined using NAPS, providing further confidence in detection. This is an especially useful strategy to overcome detection issues for compounds with low sensitivity, like NX. This strategy will also work for predicting $t_R$ in low resolution triple quadrupole instruments if MS/MS transitions are known. However, because *m/z* transitions and collision energies could vary across instrument platforms and *a priori* precursor and product ion knowledge are essential, this might be more difficult to accomplish in low resolution instruments.

Incorporating interaction contributions from the mobile phase and different column chemistries is necessary for RI prediction from molecular structure, and will be important towards the future goal of

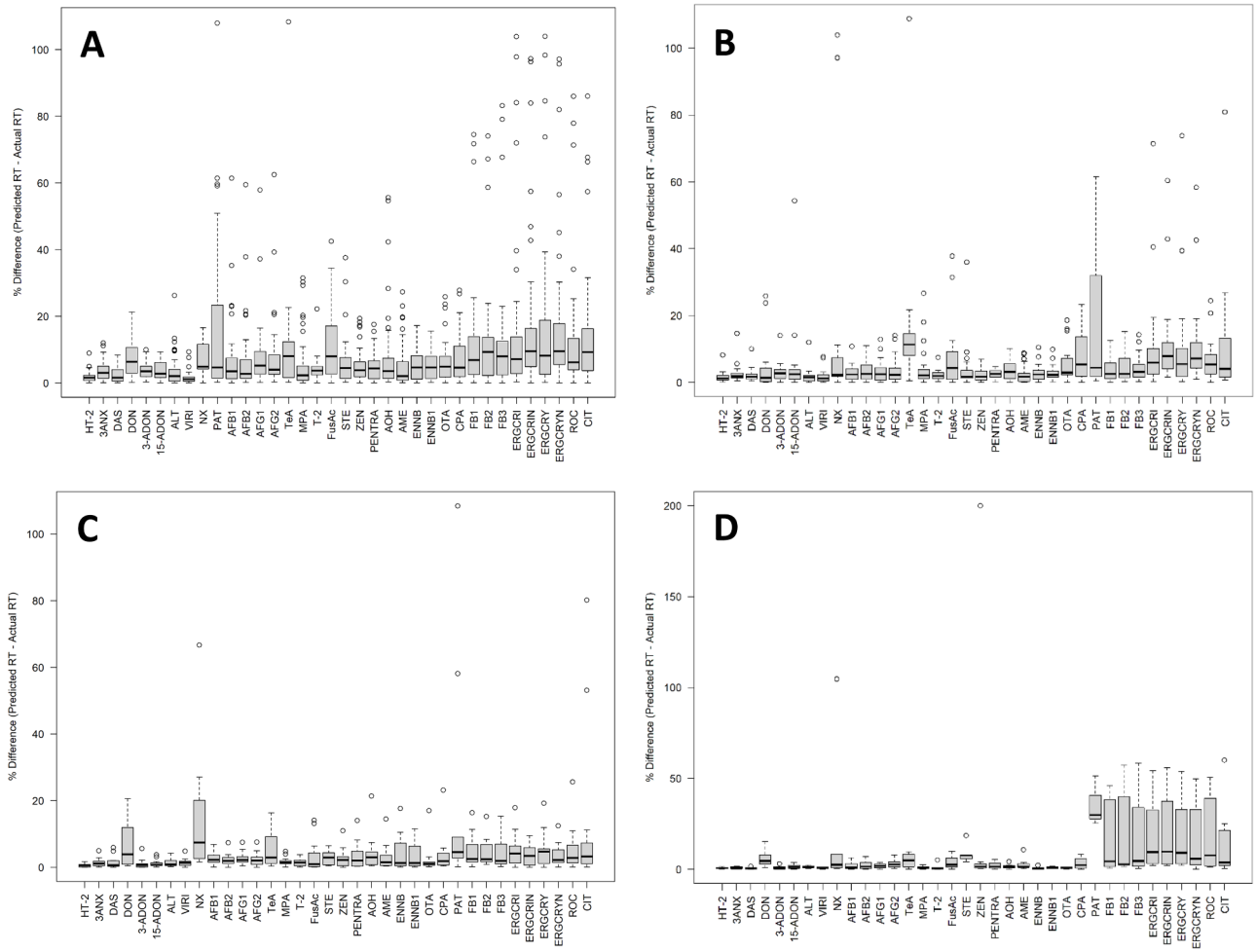



**Fig. 5-.** Percent differences for predicted mycotoxin retention times compared to the reported retention time by (A) Each of the 44 LC-MS methods, (B) Using C18 MeOH medians (21 methods), (C) Using C18 ACN medians (14 methods), (D) Using PFP MeOH medians (7 methods). Biphenyl and the single PFP ACN method are not included.

improved compound determination from retention data. Based on this, RI median values were split based on column chemistry (C18 vs. PFP/F5) and mobile phase composition (i.e. $H_2O$-MeOH or $H_2O$-ACN) to optimize the relationship between method performance and RI. Predicted $t_R$ were recalculated using the column and mobile phase-specific median values (Fig. 5**B–D**). For ERGCRI, 26 of 42 predicted $t_R$ values were within 10 % difference compared to the actual $t_R$ and had six outlier values when the 44 method study median was used (Fig. 5**A**). Using the C18 MeOH median for ERGCRI had 13 of 19 predicted $t_R$ values within 10 % difference with two outlier values (Fig. 5**B**). The C18 ACN median for ERGCRI had 10 of 13 predicted $t_R$ values within 10 % difference with only 1 outlier (Fig. 5**C**). When the PFP median was used for ERGCRI, four of seven predicted $t_R$ values were within 10 % difference and had no outliers. Using the optimized median values (Figs. 5**B–D**) removed many of the outlier values (Fig. 5**A**), and the data had smaller interquartile ranges, tighter clustering, and less skewed data. This approach supports separating RI by column type and mobile phase for a more robust $t_R$ prediction, but also facilitates the seamless integration of NAPS into pre-optimized mycotoxin methods (Table 2, **Supplementary- RI reported by lab code**).

### *3.4.2. Anchoring RI values to structurally similar compounds to normalize for unknown suspect screening using database searching*

To facilitate data sharing between laboratories, RI systems need to be accessible in currently available mycotoxin databases, with considerations of all critical intrinsic properties such as mobile phase composition and column chemistry. RI data could be filtered to include similar methods, or instead be anchored to structurally similar compounds to identify putative unknowns. A number of databases and open-source libraries exist for mycotoxin determination and prediction, including MycoCentral [21], MassBank [23], MycotoxinDB [25], METLIN Exposome [59], mzCloud [52], and the EPA CompTox dashboard, which filters results using TS coefficients. Connecting $t_R$ data to established databases could elevate the confidence level of putatively identified compounds during non-targeted analysis [65].

During routine sample screening or NTA, unknown compounds potentially related to mycotoxins or masked or modified mycotoxins for which no analytical standard is available, can be encountered. Using NAPS, RI values can be determined for all known and unknown peaks, and then compared to a database of RI values for confirmation. This approach can represent a valuable tool for differentiating structural isomers such as FB1 and FB6, which can be misidentified or misattributed in the literature. Similarly, FB2 and FB3 are structural isomers, so both compounds were provided to participants in separate vials. All reporting methods showed FB3 eluting before FB2, with an average ΔRI (FB2 – FB3) of 64.9.

We devised and tested two scenarios involving aflatoxins in participant datasets where normalizing RI could be useful at improving the confidence of putative candidates using RI in database format. For the first scenario, we investigated the feasibility of whether an analyst could elevate the confidence of their putative structurally related mycotoxin by ‘anchoring’ their computed RIs to a structurally related compound

with analytical standard(s) available. The RI value was then anchored to the database RI value entry for suspect compound identification (**Supplementary Figure S30**). Analysts could select RIs to compare by a reported median database value or be more selective towards methods reporting similar LC parameters, where the closest RI (ΔRI) value would be a putative candidate.

If unknown peaks are detected in complex matrices (ie., grain, milk, blood, plasma, urine etc.) by LC-MS, structural similarity to other known mycotoxins can be investigated by comparison to a matching accurate mass, or by performing additional MS/MS experiments to determine common product ions, MRM transitions, or neutral losses. For instance, aflatoxins share common neutral losses of carbon monoxide (CO), and can share the intermediate epoxide product ions or biosynthetic intermediates with other aflatoxins or other structurally related compounds like sterigmatocystin. If the unknown peaks are suspected to be putative aflatoxins (or structurally similar), the analyst can generate RI values for all unknown peaks. Then unknowns can be anchored to a structurally related aflatoxin standard (such as AFB1) to normalize RIs for direct database comparisons (**Supplementary Table S20**). To test the first scenario, we treated aflatoxins B2, G1, and G2 as suspected unknown aflatoxin candidates, but only AFB1 was available as a standard to analyze with the NAPS for comparison to open-source database entries. Based on the TS Coefficients for the aflatoxins (~0.9), AFB1 was selected as an anchor compound for the other three aflatoxin candidates (AFB2, AFG1 and AFG2, Fig. 6, **Supplementary Figure S31A, B, C**). Anchoring the putative "unknown" aflatoxin RI values to AFB1 resulted in a substantial decrease in ΔRI, especially for AFB2, where most methods were within ± 10 ΔRI units of the $H_2O$-MeOH C18-RI median. Instances where the ΔRI value increased for AFB2, AFG1, and AFG2 after anchoring were minimal, with AFB2 anchoring resulting in one overcorrection (3.6 RI units), AFG1 with four overcorrections (3.6 – 8.9 RI units), and AFG2 with five overcorrections (4.0 – 12.5 RI units). Once the aflatoxins were normalized, the RI values were compared to the median AFB2, AFG1, and AFG2 in the RI table (**Supplementary Figure S31**) to see if an assignment could be made, by the smallest difference to RI, without the need for an analytical standard. Aflatoxin $B_2$, AFG1, and AFG2 were correctly identified solely from their anchored RI values, except for three data points where AFG2 was misidentified as AFG1 (3/21 methods = 14 % misidentification, **Supplementary Figure S31**).

As the TS coefficient to AFB1 decreased for the other aflatoxins (AFB2 = 0.92, AFG1 = 0.81, AFG2 = 0.84), the anchored ΔRI to the median also increased slightly. Aflatoxin $B_1$, used as the anchor, has a different furocoumarin ring size compared to AFG1 and AFG2 that may result in slightly differing column retention. The difference in furocoumarin ring size could explain why overcorrections increased between AFG1 and AFG2 (Fig. 6).

RI median anchoring was repeated in the fumonisin series in ACN and MeOH mobile phases using FB1 as the anchor, (**Supplementary Figure S32**). Anchoring FB1 followed the same trend as the anchored aflatoxins. FB2 and FB3 had a higher TS coefficient to the FB1 anchor (FB2 = 0.99, FB3 = 0.96), and resulted in no overcorrections in the datasets. Although this scenario was based on a structural similarity relationship to known aflatoxins in the study, Tanimoto-guided anchoring could be applied to other putative unknown aflatoxins to suggest the degree of the structural similarity by RI difference.

While anchoring was efficient at aflatoxin normalization for database comparison for the NAPS RM-RILC RI system, it could be utilized with any RI system, provided users select the appropriate anchor compounds reflective of the structural similarity. The application of an anchored RI is limited to the 36 mycotoxins tested in the study, but it will improve as more mycotoxins are analyzed.

A matching RI database comparison increases compound identification confidence, especially when paired with matching molecular formulae, elution order, and LC-MS/MS fragmentation or transition data from the literature or databases. RI can either support a putative identification, or trigger more in-depth natural product analyses such as compound isolation and structural characterization by NMR. Purchasing costly analytical standards or shifting towards compound isolation can then be justified for further confirmation and quantification. This is especially true for acquiring regulated compounds, as tight export control regulations can sometimes span across multiple countries and

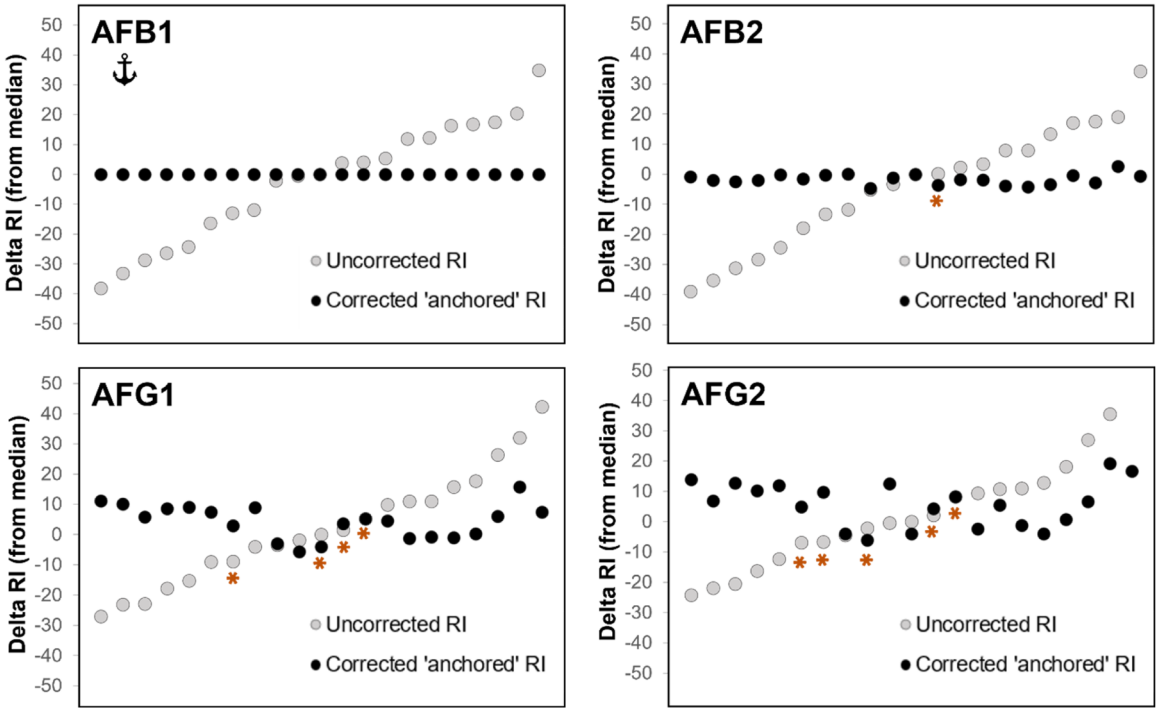



**Fig. 6.** Applying an RI correction to aflatoxins using AFB1 as an 'anchor' for AFB2, AFG1 and AFG2 in C18 $H_2O$-MeOH methods. Asterisks note where the delta RI values increased after anchoring. All other values were improved.

can be an extremely time-consuming process, especially for developing countries.

### 3.4.3. Predicting retention time using anchored retention index values of structurally similar compounds for known compounds with unknown retention times

Aflatoxin $M_1$ (AFM1) is the excreted hydroxylated metabolite of AFB1 and is commonly detected in milk and dairy products after cattle are fed aflatoxin contaminated feed. Due to climate change, aflatoxin-producing *Aspergilli* can become established in new geographic regions [5,16,34]. Thus, there is a need to add metabolites such as AFM1 to multi-mycotoxin analysis methods. However, AFM1, which was not included in this study, is an expensive standard to purchase, where 250 µg costs approximately 3000 CAD (EUR 2000) [A6428–250UG, Sigma Aldrich]. This additional cost can be prohibitive for some laboratories for method optimization and routine analysis. For the second scenario, it was investigated whether a laboratory could predict the $t_R$ of AFM1 for their method using only median RI database values and an anchor compound from the NAPS study.

Several participant laboratories submitted 'bonus' $t_R$ data (**Supplementary Table S21**) for AFM1 (median RI 741), which has a TS coefficient of 0.92 to AFB1 (**Supplementary Figure S33**). This allowed for a comparison of RI corrections and $t_R$ predictions between using a median RI value of AFM1 (RI 741), or anchoring to the structurally related AFB1 (TS 0.92). The use of the NAPS RI system to predict $t_R$ was evaluated prior to using only the NAPS $t_R$ values and the Akima cubic spline fit to the median value (Fig. 6). By anchoring the median RI C18-specific value to the AFB1 results, $t_R$ could be predicted within ±0.23 mins of the reported $t_R$ value (Table 3 and Fig. 7, **Supplementary Figure S34, Supplementary Table S22**). Using Tanimoto median anchoring not only assists $t_R$ prediction and putative compound identification but can also be employed for method development by pre-evaluating the separation of mycotoxins before using an analytical standard.

In this study, NAPS was evaluated for its interlaboratory variability for 36 commonly studied mycotoxin standards. A more extensive evaluation in real sample matrices is a natural continuation of this work. NAPS was previously applied to a *Penicillium roqueforti* extract against 96 mycotoxins to examine the use of RI in dereplication strategies and elimination of false positives [43]. Practical applications of the NAPS RI system could include longitudinal mycotoxin biomonitoring studies, or more NTA applications [28,57].

## 4. Conclusions

The current study highlights the feasibility of including the NAPS RI system within individually optimized methods for many of the agriculturally relevant mycotoxins. In this interlaboratory study, the NAPS RI system was evaluated to improve the reliability of mycotoxin determinations by LC-MS. Twenty-four laboratories across North America and Europe participated by submitting 44 datasets, representing a cross-section of mycotoxin analysis methods currently used worldwide.

The application of NAPS was demonstrated to be an effective tool for normalizing mycotoxin $t_R$. Some mycotoxins had variable RI values and were shown to be highly sensitive to the mobile phase selection, pH and column choice, thus highlighting a limitation that analysts should be aware of when attempting to normalize $t_R$ data using this RI system. RI values were further improved when TS coefficients were used to identify anchor compounds and correct for mycotoxin $t_R$.

This study provides a path forward for the mycotoxin community to better facilitate data sharing and comparison between laboratories. The application of an RI system will help improve the overall reliability of data generated for mycotoxins by improving the confidence of mycotoxin identifications reported in the literature. We believe that future work should focus on analyzing additional mycotoxins beyond the 36 tested in this study; validating matrices such as maize, wheat or peanuts; as well as working towards incorporating RI values into open access data repositories to help provide a data-driven strategy for identifying unknown mycotoxins. When combining RI with current LC-MS/MS dereplication, Tanimoto-anchored data can direct unknown compound identifications during non-target analysis or suspect screening beyond traditional targeted MS/MS analysis, thereby improving confidence, and facilitating ease of method development.

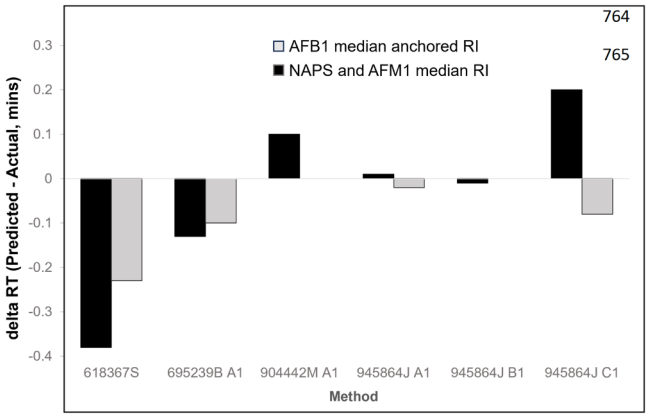



**Fig. 7.** Comparison of $t_R$ prediction methods, either using NAPS RI and median value only, or anchoring the median AFM1 to AFB1.

**Table 3**
Comparing between $t_R$ median prediction from NAPS (left) and Tanimoto-guided anchoring (middle) of AFM1 to AFB1 from the study.

| AFM1 ($n = 6$) | **Predicted $t_R$, median** | | **Predicted $t_R$, anchored** | | **Actual $t_R$** | |
|---|---|---|---|---|---|---|
| | RT (min) | RI (median) | RT (min) | RI | RT (min) | RI |
| 618367S A1 | 5.94 | 741 | 6.09 | 760 | 6.32 | 772 |
| 695239B A1 | 4.53 | 741 | 4.56 | 756 | 4.66 | 760 |
| 904442 M A1 | 7.83 | 741 | 7.73 | 733 | 7.73 | 732 |
| 945864 J A1 | 5.02 | 741 | 4.99 | 743 | 5.01 | 741 |
| 945864 J B1 | 4.86 | 741 | 4.87 | 742 | 4.87 | 742 |
| 945864 J C1 | 4.93 | 741 | 4.64 | 749 | 4.72 | 722 |

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## CRediT authorship contribution statement

**M.J. Kelman:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **J.B. Renaud:** Writing – review & editing, Validation, Methodology. **P. McCarron:** Writing – review & editing, Resources. **S. Hoogstra:** Writing – review & editing, Software. **W. Chow:** Writing – review & editing, Investigation. **J. Wang:** Writing – review & editing, Investigation. **E. Varga:** Writing – review & editing, Investigation. **A. Patriarca:** Writing – review & editing, Investigation. **A.Medina Vaya:** Writing – review & editing, Investigation. **L. Visintin:** Writing – review & editing, Investigation. **T. Nguyen:** Writing – review & editing, Investigation. **M. De Boevre:** Writing – review & editing, Investigation. **S. De Saeger:** Writing – review & editing, Investigation. **V. Karanghat:** Investigation. **D. Vuckovic:** Writing – review & editing, Investigation. **D.R. McMullin:** Writing – review & editing, Investigation. **C. Dall'Asta:** Writing – review & editing, Investigation. **K. Ayeni:** Writing – review & editing, Investigation. **B. Warth:** Writing – review & editing, Investigation. **M. Huang:** Writing – review & editing, Investigation. **S. Tittlemier:** Writing – review & editing, Investigation. **L. Mats:** Investigation. **R. Cao:** Writing – review & editing, Investigation. **M. Sulyok:** Writing – review & editing, Investigation.

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## 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.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.465732.

## Data availability

Data have been provided in the attached excel file. Raw data files can be provided on request, and with co-author permission

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# International interlaboratory study to normalize liquid chromatography-based mycotoxin retention times through implementation of a retention index system

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