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What Experimental Factors Influence the Accuracy of Retention Projections in Gas Chromatography-Mass Spectrometry?

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Abstract

Programmed-temperature gas chromatographic (GC) retention information is difficult to share because it depends on so many experimental factors that vary among laboratories. Though linear retention indexing cannot properly account for experimental differences, retention times can be accurately calculated, or “projected”, from shared isothermal retention vs. temperature (*T*) relationships, but only if the temperature program and hold-up time vs. *T* profile produced by a GC is known with great precision. The effort required to measure these profiles were previously impractical, but we recently showed that they can be easily back-calculated from the programmed-temperature retention times of a set of 25 *n*-alkanes using open-source software at www.retentionprediction.org/gc. In a multi-lab study, the approach was shown to account for both intentional and unintentional differences in the temperature programs, flow rates, and inlet pressures produced by the GCs. Here, we tested 16 other experimental factors and found that only 5 could reduce accuracy in retention projections: injection history, exposure to very high levels of oxygen at high temperature, a very low transfer line temperature, an overloaded column, and a very short column (15 m). We find that the retention projection methodology acts as a hybrid of conventional retention projection and retention indexing, drawing on the advantages of both; it properly accounts for a wide range of experimental conditions while accommodating the effects of experimental factors not properly taken into account in the calculations. Finally, we developed a four-step protocol to efficiently troubleshoot a GC system after it is found to be yielding inaccurate retention projections.

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Keywords

Gas chromatography-mass spectrometry; retention prediction; retention projection; compound identification; instrument non-idealities; programmed-temperature retention from isothermal retention

1. Introduction

Despite continued advances in mass spectrometry, the identification of small molecules from complex mixtures remains a major bottleneck. This is especially true in fields like metabolomics where it is often the goal to identify as many compounds as possible, though samples could easily contain tens of thousands of compounds [1,2]. There, GC-MS is one of the primary analytical tools for identification of volatile and semi-volatile compounds because it offers two independent and complimentary dimensions of information to help identify compounds: mass spectra and GC retention. Though mass spectral information has found wide use for compound identification, GC retention remains underutilized despite its potential utility because it is so difficult to reproduce across laboratories.

GC retention times are essentially irreproducible because they depend on a host of experimental variables that differ among laboratories and instruments. While some of those variables are controllable (temperature program, flow rate, carrier gas, etc.), some are not (temperature calibration errors, inexact column dimensions, flow rate non-idealities, etc.). Therefore, even if standard experimental conditions were universally adopted (which is unlikely), GC retention times would still be irreproducible because of the uncontrollable differences between GC systems.

In order to accommodate some differences in experimental conditions, linear retention indexing was developed [3]. In linear retention indexing, a series of standards are spiked into the sample before it is run in a temperature program. Retention is then reported not as a time, but as an index describing the position a compound elutes between its two bracketing standards. The idea is that, since the standards are subjected to the same experimental conditions as the analytes, linear retention indexing can accommodate some changes in the experimental conditions.

While linear retention indices are certainly *less* sensitive to changes in experimental conditions than retention times, they are still strongly affected by them [4–6]. Thus, in order for retention indices to be at all reliable, they must be used under precisely the same experimental conditions as they were originally collected (or used under a narrow range of translated methods [7–9]). But even then, small, unintentional differences between GC systems are enough to cause significant differences between the retention indices measured on each system [11]. Some of the unintentional differences between GC instruments can be minimized by retention time locking (specifically, inlet pressure and column length), which requires a user to adjust their inlet pressure until the retention of a standard matches that measured on the original GC [7], but it offers no way to easily calibrate out unintentional errors in the temperature program.

Instead of building a shared database of retention indices, a more general approach is to build a database of isothermal retention factor (k) vs. T relationships (two are shown in Figure 1). Using these relationships, each compound's programmed-temperature retention time can be calculated using the following equation [12–17] (note that it holds true except in constant flow rate mode under moderate gas decompression [9]):

$$\int_0^{t_R} \frac{dt}{t_{M,T}(k_T+1)} = 1 \quad (1)$$

where t_R is the retention time of the compound, and $t_{M,T}$ and k_T are the hold-up time and retention factor at temperature T . The equation essentially treats a programmed-temperature run as a series of infinitesimally small isothermal steps that closely approximate the true temperature program. Eq. 1 can be accurately solved for t_R if the following three relationships are precisely known: (1) the isothermal k vs. T relationship for the compound (as in Figure 1), (2) the T vs. time relationship produced by the GC instrument (the temperature profile), and (3) the t_M vs. T relationship produced by the GC instrument (the hold-up time profile). However, when any of those relationships are more complicated than simple linear functions, eq. 1 usually has to be solved numerically. To solve it numerically, the temperature program is broken up into a series of very short isothermal steps and in each step, the fraction of the column traveled by the compound is calculated based on its k at the T of that step and the t_M at that T . Using the following summation equation, analogous to eq. 1, for numerical integration:

$$\sum_{i=1}^n \frac{\delta t}{t_{M,T}(k_T+1)} \geq 1 \quad (2)$$

where n is the smallest integer that makes the inequality true, t_R can be calculated from:

$$t_R = \sum_{i=1}^n \delta t \quad (3)$$

We call this approach, “retention projection” because temperature-programmed retention times are projected from isothermal retention data. The big advantage of retention projection is that it can theoretically calculate accurate retention times under virtually any temperature program, flow rate, inlet pressure, column length, inner diameter, and film thickness. In fact, there are only two fundamental limitations: the stationary phase and the carrier gas must be the same as those originally used to measure the isothermal k vs. T relationships.

Unfortunately, retention projection is not accurate unless the temperature profile and the hold-up time profile actually produced by the GC are known with great precision. Small imperfections in the temperature calibration, column dimensions, or the flow rate/inlet pressure can cause large errors in projected retention times. Some researchers have successfully taken these imperfections into account by making meticulous measurements of them [13,18], but the amount of effort required is impractical for most users. Moreover,

these measurements would have to be re-made every time the experimental conditions are deliberately or inadvertently changed.

Alternatively, a new approach was recently reported to easily measure and account for such imperfections in the temperature and hold-up time profiles [19]. First, one spikes their sample with a set of 25 *n*-alkane samples and subjects it to temperature-programmed elution. Then the retention times of the *n*-alkanes are entered into online software at www.retentionprediction.org/gc. *The software uses the retention times of the standards to iteratively back-calculate the effective temperature program and hold-up time profiles that must have been produced in the run to give those retention times.* Finally, it uses the back-calculated profiles to project expected retention times for all the other compounds for which isothermal *k* vs. *T* relationships have been measured (currently, a database of only 97 compounds is available on the site, but we are in the process of expanding it).

In a multi-lab study [11] the retention projection methodology was shown to account for both intentional and unintentional differences between GC systems in different laboratories, enabling retention times to be calculated with the same level of accuracy regardless of the instrument, temperature program, or inlet pressure/flow rate used. Since the error was laboratory-independent, it was also possible to calculate the appropriate retention time tolerance windows for each projected retention time. This makes it possible to determine the probability of an incorrect peak assignment without needing to have a standard of the compound physically on hand. However, the retention time tolerance windows can only be trusted if one's system is in a "like new" state. Therefore, to ensure the integrity of the calculated tolerance windows, a simple system suitability check was developed [11]: After the software back-calculates the effective temperature and hold-up time profiles, it attempts to project the retention times of 12 chemically diverse test compounds. If error in the projected retention times of these 12 standards is under a certain threshold (which is calculated for a given run based on the temperature program, flow rate/inlet pressure, column dimensions, and film thickness used), the system is deemed suitable and subsequent retention time tolerance windows can be trusted.

The system suitability check has helped us to identify GC systems in an unsuitable state, but once they were identified, it was often unclear what caused them to fail the suitability check. This made us wonder, what factors *could* cause the system suitability check to fail (i.e. what could cause the accuracy of retention projections to worsen)? Or, more practically, if a system fails the system suitability check, what should one do to fix their GC system so that it passes the test? A similar question was explored in a report by Vezzani et. al. [13] where they calculated the sensitivity of conventional retention projections to small errors in a number of experimental factors including the ramp rate, inlet pressure, and column length. However, these were all factors that would affect the temperature and hold-up time profiles—the multi-lab study showed that the back-calculation methodology can take those types of errors into account. Once those major factors are out of the way, there remain a number of other experimental factors that one might expect could also affect the accuracy of retention projections.

In this work, we tested the impact of a broad range of the most common, practical experimental variables on the accuracy of the retention projection methodology to give a sense of what factors *can* cause the system suitability check to fail and what factors are irrelevant. We tested the accuracy with a poorly functioning GC oven (simulated by placing metal blocks in the oven), after multiple injections of a concentrated urine extract, with different makes/models of liners, poor manual injection technique, split and splitless injections, different inlet temperatures, straight and crooked column cuts, different lengths of column in the inlet, with and without column conditioning, after extended exposure to 6 ppm O₂ (approximately the level in industrial grade He) at high temperature, with different transfer line temperatures, different MS source temperatures, different injection amounts. We also tested the ability of the retention projection methodology to account for intentional differences in column length, inner diameter, and film thickness, and compared its accuracy to that of retention indexing. Based on all of these results, we developed a protocol to efficiently return a GC system to a state where it passes the system suitability test. The experiments also represent a test of the practical robustness of the retention projection methodology to a range of experimental conditions not specifically tested in the multi-lab study.

2. Experimental

2.1 Test Mixture

The test mixture was made up of 25 *n*-alkanes (C7-C26, C28, C30, C32, C34, and C36) and 12 chemically diverse test compounds (Figure 2), selected to represent each of the five types of interactions most common in GC (as represented by the Abraham descriptors) [20–24]. Four groups of three test compounds were selected—one group for each type of interaction except the gas-liquid partition coefficient since the test compounds in each group were selected to cover a range of gas-liquid partition coefficients. The hydrogen bond donors were (listed in order of increasing gas-liquid partition coefficient) phenol, resorcinol, and 1-naphthol; the hydrogen bond acceptors were *N,N*-dimethylisobutyramide, benzamide, and dextromethorphan; the compounds that interact by π and/or lone pair interactions were ethylbenzene, naphthalene, and anthracene; and the compounds that interact by dipole-dipole and dipole-induced dipole interactions were *N,N*-diethylacetamide, 4-nitroaniline, and caffeine [25,26]. (*N,N*-dimethylisobutyramide and *N,N*-diethylacetamide are listed in two different groups but they both interact by the same two mechanisms.) Even though a new DB-5MS phase does not have significant hydrogen bond donating capacity [27] we keep hydrogen bond acceptors in the test mixture because parts of the inlet or a dirty column may donate hydrogen bonds. All standard compounds were dissolved in ethyl acetate at 100 μ M concentration. All chemicals and solvents were purchased from Sigma-Aldrich® (St. Louis, MO), Alfa Aesar® (Ward Hill, MA), or TCI America (Portland, OR).

2.2 Measurements of Isothermal Retention vs. T Relationships

The isothermal retention factors and a detailed description of their measurement are available elsewhere [19]. Briefly, we measured isothermal retention factors for each of the 37 compounds in the test mixture at 20 °C intervals from 60 °C to 320 °C, using N₂ as the hold-up time marker. The isothermal retention factors, *k*, were then calculated from:

$$k = \frac{t_R - t_M}{t_M} \quad (4)$$

where t_M is the hold-up time and t_R is the retention time (measured from the apex of each peak). All isothermal measurements were made on an Agilent DB-5MS UI column (30 m long, 0.25 mm inner diameter, 0.25 μ m film thickness) using He carrier gas.

2.3 Instrumentation

The isothermal retention vs. T relationships were measured with a Hewlett Packard (HP, Palo Alto, CA) Model 5890 Series II GC equipped with an HP 5970 single quadrupole mass spectrometer. We used He carrier gas (99.999% pure), deactivated, straight quartz liners (2 mm inner diameter) containing deactivated quartz wool, an inlet temperature of 290 $^{\circ}$ C, and a transfer line temperature of 320 $^{\circ}$ C.

Most of the experiments below were performed with the same instrument, but the experiments with different MS source temperatures and different inner diameters were performed with a Thermo Scientific (Waltham, MA) Trace GC Ultra equipped with a Thermo TSQ Triple Quadrupole mass spectrometer.

Agilent Technologies (Santa Clara, CA) DB-5MS UI columns (30 m long, 0.25 mm inner diameter, 0.25 μ m film thickness) were used for all experiments unless specifically stated otherwise.

2.4 Test Conditions

Unless otherwise stated, the method used for each test was the following: temperature program, 60 $^{\circ}$ C for 5 min, then ramp at 26 $^{\circ}$ C/min to 320 $^{\circ}$ C, hold for 15 min; inlet pressure, 50 kPa; 1 μ L split injection with 1:10 split ratio; inlet temperature, 290 $^{\circ}$ C; MS transfer line temp, 320 $^{\circ}$ C; MS scan window, 57 to 271 m/z; scan rate, 2 Hz; He carrier gas.

2.5 Urine Sample

1 mL of cow urine was acidified to pH 2 with 1 M HCl. Then 3 mL of ethyl acetate was added and the mixture was vortexed for 30 s before being centrifuged for 15 min at 25,000 $\times g$. The ethyl acetate layer was recovered and allowed to evaporate under N_2 flow until the volume reduced by a factor of 10.

2.6 Software

The GC retention projection software was compiled for compliance with the Java 1.6 (Oracle, Redwood Shores, CA) runtime environment. It includes the Java OpenGL (JOGL) binding library version 2.0-rc11 (JogAmp, <http://jogamp.org>), the Unidata netCDF library version 4.2 (Unidata®, Boulder, CO), the Savitzky-Golay filter library version 1.2 by Marcin RŹenicki (<http://code.google.com/p/savitzky-golay-filter/>), the jmxML library [28], and the jmxReader library [29]. The source code may be downloaded from <http://www.retentionprediction.org/gc/development>.

3. Results/Discussion

3.1 What factors affect the accuracy of retention projections?

To test the accuracy of retention projections under each condition, we injected a mixture containing the 25 *n*-alkanes and a set of 12 chemically diverse test compounds in a single temperature program. The temperature program began with a 5 min isothermal hold at 60 °C, ramped to 320 °C at 26 °C/min, and finished with a 15 min isothermal hold at 320 °C (see Experimental section for other details). The retention times of the *n*-alkanes in each run were used to back-calculate the effective temperature and hold-up time profiles, which were then used to project retention times for the 12 test compounds as explained above. The projected retention times were then compared to their experimental retention times. In each case, we report the accuracy of retention projections as the root-mean-square (RMS) of the errors in retention projections among all 12 test compounds.

In order to calculate thresholds for a “passed” system suitability check, we first assumed that the major source of error affecting retention projections came from error in the isothermal retention factor measurements and that that error was a constant percentage of the isothermal retention factors (in this and previous work [11], we assumed $\pm 0.57\%$ error in k at one standard deviation). That error was then propagated to the programmed-temperature retention times that were projected for the 12 test compounds [11], giving an expected error for each one. The overall expected error, σ_{expected} , was then calculated from the root-mean-square of those 12 errors. The threshold for a “passed” system suitability check was set so that there would be only a 25% chance that a suitable system would have greater error, that is, the RMS error among retention projections for the 12 test compounds must be less than $1.15 \sigma_{\text{expected}}$ to pass. A less stringent threshold was also determined that we call “passed, but questionable”. This threshold was set where there would be only a 5% chance that a suitable system would have higher error, that is, the RMS error among retention projections for the 12 test compounds was less than $1.96 \sigma_{\text{expected}}$, but greater $1.15 \sigma_{\text{expected}}$.

For example, Table 1 shows measured retention times, projected retention times, the actual errors, and the expected errors at one standard deviation for each of the test compounds in the run. In this case, the system suitability check passed because the RMS of the error in retention projections among all 12 test compounds was ± 0.42 s, which is less than the threshold for a passed system suitability check (threshold for passed system suitability check = $1.15 \sigma_{\text{expected}} = 1.15 * \pm 0.58 \text{ s} = \pm 0.68 \text{ s}$).

With the experimental conditions used for each of the experiments below (except for the last three), an RMS error of less than ± 0.68 s constitutes a “passed” system suitability check and an RMS error between ± 0.68 s and ± 0.80 s constitutes a “passed, but questionable” system. The results of each experiment are summarized in Table 2.

3.1.1 Metal blocks in the GC oven

We previously showed [11] that *small, unintentional* differences in the temperature program (such as would be found among different GC instruments) did not affect the accuracy of retention projections. But what if unintentional errors in the temperature program are more extreme? To test this, we placed two Al blocks (dry bath heating blocks – approximately $9 \times$

8 × 5 cm) in the oven during the temperature-programmed run (Figure 3A) and measured the accuracy of retention projections.

Figure 3B shows the temperature and hold-up time profiles that were back-calculated with and without Al blocks in the oven. The blocks prevented the GC oven from keeping pace with the programmed ramp rate near the end of the run. Nevertheless, the accuracy of retention projections was unchanged: RMS error over all 12 compounds was essentially the same with and without blocks in the oven (± 0.43 s vs. ± 0.35 s).

For comparison, we also measured linear retention indices for each of the test compounds from the run *without* metal blocks and tried to use them to predict retention times in the run *with* metal blocks. The retention times predicted this way were only accurate to ± 2.6 s, which was over 6-fold less accurate than retention projections. The error was greatest for compounds eluting near the end of the temperature program, where deviation from the ideal temperature program was most pronounced. For example, retention indexing predicted the retention time of dextromethorphan (which eluted at 16.5 min) with an error of 5.5 s, but the projected retention time was only in error by 0.8 s because it properly accounted for the temperature deviation.

3.1.2 Liner make/model/geometry—A wide variety of GC inlet liners are available with different geometries and made of different materials. It would place a significant experimental restriction on the retention projection methodology if it was only accurate with a single make and model of liner, so we tried several different makes and models of liners to see if the accuracy was affected.

Table 2 shows the accuracy of retention projections with eight different liners. The liners were from five different companies, of three different inner diameters, two different lengths, two different geometries (straight or gooseneck), they were made from two different materials (quartz or glass), and they were packed with deactivated glass wool, deactivated quartz wool, or nothing. The accuracy of projections with each liner was indistinguishable, being between ± 0.38 s and ± 0.49 s. Of course, there are many more types of liners commercially available. While this result does not prove that all liners will allow for accurate retention projections, it suggests that at least with common liner makes, models, and geometries, there is no effect.

3.1.3 Injection history—As an example of the effect that prior injections of “dirty” samples can have on the accuracy of retention projections, we repeatedly injected a concentrated, underivatized cow urine extract (see Experimental section), and then tested retention projection accuracy. Seven injections caused a large drop in accuracy (± 1.3 s) that was enough to fail the system suitability check (Table 2). Though these were exaggerated conditions, they demonstrate that the history of injections on a particular column can strongly influence the accuracy of retention projections.

We were curious if this sort of change in selectivity was caused just by a change in the selectivity of the column or if it could also be from a change in the selectivity of the inlet. After all, the vaporized sample is only in contact with the inside surfaces of the inlet for a

short period of time during a split injection. This is certainly part of the reason that the liner make/model/dimensions did not significantly affect the accuracy of retention projections. To test whether a dirty liner alone can change the selectivity of the separation, we began with a system that easily passed the system suitability check (± 0.43 s error) and replaced the liner with a straight liner containing glass wool that was visibly brown from repeated injections of various biological samples. After switching the liner, the error increased about 4-fold, to ± 1.7 s, indicating that a dirty liner can strongly affect the accuracy of retention projections. We were also curious if a dirty inlet base seal could affect the accuracy, but that was evidently not the case. In another experiment, we replaced a clean base seal with one that was coated in the brown, charred remains of previous samples and the retention projection accuracy was unaffected (± 0.50 s).

3.1.4 Split and splitless injection—In a split injection, the vaporized sample is loaded onto the column in a relatively short period of time—any sample that does not make it into the column quickly is blown out of the inlet. On the other hand, in a splitless injection, the sample is loaded onto the column over a relatively long period of time, causing the retention times of early-eluting compounds to be somewhat later than in a split injection (later-eluting compounds are not affected nearly as much since they focus near the inlet of the column at the beginning of the temperature program when the column is coolest). This confounding factor is not taken into account by the retention projection methodology, so one might expect it to cause the accuracy of retention projections to suffer.

In order to compare the accuracy of split and splitless injections, we used a split ratio of 10:1 for the split injection, and for the splitless injection we diluted the test mixture by a factor of 10 in order to load the same amount of material onto the column. Under these conditions, we detected no difference in accuracy between split (± 0.42 s) and splitless (± 0.43 s) injections. However, the retention times of early eluting compounds were indeed later in the splitless injection. For example, the retention time of ethylbenzene (the earliest eluting test compound) changed from 6.160 min in the split injection to 6.210 min in the splitless injection, a difference of 3.0 s. Even so, the error in the retention projection for ethylbenzene remained small: 0.66 s for the split injection and 0.72 s for the splitless injection. This suggests that the retention projection methodology is able to accommodate the effect of a splitless injection to some extent without truly taking it into account.

Figure 4 shows the back-calculated temperature and hold-up time profiles from the split and splitless injections overlaid on one another. In the run with a splitless injection, the retention times of the early-eluting *n*-alkanes were slightly longer. To compensate, the back-calculated temperature profile averaged a little cooler at the beginning of the run and the back-calculated hold-up time profile became slightly longer. Of course, the splitless injection did not cause the *actual* temperature profile and hold-up time profiles to change. Rather, the effect of the splitless injection was “absorbed” into the back-calculated profiles, which in turn caused the projected retention time of ethylbenzene to also be later, putting it close to its actual retention time.

In this way, the retention projection methodology acts like a hybrid of linear retention indexing and conventional retention projection (“conventional” meaning where temperature

and hold-up time profiles are directly measured, not back-calculated as they were here). While linear retention indexing can accommodate small changes in experimental factors affecting retention, it cannot tolerate larger changes because it does not account for them in a theoretically sound way. On the other hand, while conventional retention projection *can* tolerate a wide range of values for many experimental factors, it cannot tolerate factors affecting retention that are not fully taken into account. Something like the effect of a splitless injection would be particularly difficult to take into account as it depends not only on the inlet geometry, but also injection solvent and possibly other factors as well. However, retention projection, as described in this work (i.e., where temperature and hold-up time profiles are back-calculated from measured retention times), has the advantages of both retention indexing and conventional retention projection: it properly accounts for a wide range of experimental conditions while being able to accommodate smaller factors affecting retention that are not taken into account by the model.

3.1.5 Manual injection technique—We tried manually injecting the sample two ways: a fast injection and a slow, uneven injection spread over the course of 5 seconds. Even with the long, uneven injection, we did not detect any change in the accuracy of retention projections (± 0.42 s vs. ± 0.44 s) despite the fact that the retention times of early-eluting compounds became later, much like they did with a splitless injection. As before, the effect of the slow manual injection would not have been properly taken into account, but it was accommodated by small adjustments to the back-calculated temperature and hold-up time profiles. This enabled the retention time of ethylbenzene, which was affected the most by the slow manual injection, to be projected with only 0.24 s error.

3.1.6 Inlet temperature—One might expect that at low inlet temperatures the accuracy of retention projections would suffer because it takes longer for some of the compounds to vaporize in the inlet, introducing a confounding factor not properly taken into account by the methodology. However, we tested a range of inlet temperatures between 200 °C and 300 °C and found that the accuracy was virtually unchanged, being between ± 0.42 s and ± 0.48 s. Even so, the retention times of the later-eluting compounds were affected. Hexatriacontane, the latest eluting compound in the test mixture, was affected the most. With an inlet temperature of 300 °C, its retention time was 27.700 min, but with an inlet temperature of 200 °C, its retention time was 27.789 min, 0.089 min (5.3 s) later. Such retention time differences caused there to be small differences in the temperature and hold-up time profiles back-calculated from each run, but the error in retention projections for the latest eluting test compound, dextromethorphan, remained low: With an inlet temperature of 300 °C, the error was 0.66 s and with an inlet temperature of 200 °C, the error was 0.48 s.

3.1.7 Crooked column cut—In our experience, when individuals found that the system suitability check failed, one of their first suggestions to fix the problem was to re-cut the column to make sure it was cut cleanly and squarely. We tested a clean cut (± 0.42 s) and an angled, rough cut (± 0.45 s) on the end of the column in the inlet, but it did not affect the accuracy of retention projections, nor did it affect the retention times.

3.1.8 Length of column in the inlet—We also tested whether the length of column pushed into the inlet affected the accuracy of retention projections. The user manual for the GC instrument recommended 0.2 cm (± 0.44 s), but we also tried 1.2 cm (± 0.39 s) and 2.2 cm (± 0.37 s), and found no significant effect.

3.1.9 Column conditioning—Laboratories have different protocols as to how long and in what way they condition their columns prior to use. The amount of column conditioning required to attain stable chromatographic selectivity likely varies between makes and models of columns, but the retention projection methodology currently requires use of the Agilent DB-5MS UI phase. Therefore, we tested whether conditioning made any difference to the accuracy of retention projections on that phase. We tested without any column conditioning (± 0.42 s) and with 3 hours of conditioning at 320 °C (± 0.45 s), but we found no effect on the accuracy of retention projections.

3.1.10 Column exposure to increased O₂ levels in the carrier gas—Laboratories also use a range of He purities for their carrier gas. For example, one laboratory we have worked with uses industrial grade He while others use only the highest purity He followed by additional purification steps. It is well known that exposure of GC stationary phases to O₂ at high temperature alters their selectivity [30–32], but we wondered just how sensitive the selectivity of DB-5MS UI columns are to O₂. If the system suitability check fails to pass soon after the installation of a new column, are impurities of O₂ in the carrier gas a likely cause?

To test how quickly oxygen impurities in the He carrier gas degraded the column to the point where it affected the accuracy of retention projections, we used ultra high purity (UPH) He spiked with 6.2 ppm O₂ (which is in the range typically found in industrial grade He) as the carrier gas in a GC column heated to 320 °C and periodically tested the accuracy of retention projections. After 72 hours of treatment, when we ended the experiment, the accuracy still had not changed at all (± 0.39 s). This suggests that the DB-5MS UI phase is relatively robust toward the amounts of O₂ in industrial grade He—if a system suitability check fails with a newly installed column, it is unlikely to be a result of O₂ impurities in the carrier gas. However, it must be mentioned that the DB-5MS UI phase cannot tolerate ambient levels of O₂ under heating. After just 24 hours exposure to laboratory air, the accuracy of retention projections became poor enough (>0.80 s) to fail the system suitability test.

3.1.11 Transfer line temperature—The transfer line is a heated tube housing the section of the GC column that passes from the GC oven to the MS source. Only a relatively short section of the GC column is in the transfer line (usually about 0.2 m), but this section is held at a different temperature than the rest of the column which could alter the selectivity of the separation. These changes would not be properly taken into account by the model used here to project retention; it assumes the entire column is held at the GC oven temperature. The general rule of thumb is to keep the temperature of the transfer line at or above the highest temperature in the temperature program. This minimizes the influence of the transfer line on the selectivity of the separation because its high temperature causes most compounds to be

unretained as they pass through it. However, we were curious how low the transfer line temperature could be before it caused significant error in projected retention times.

We tested the accuracy of retention projections with the transfer line temperature set as high as 320 °C, which was the same as the highest temperature in the temperature program, to as low as 160 °C. As the transfer line temperature decreased, the error in retention projections changed from ± 0.44 s at 320 °C to ± 0.66 s at 160 °C, a modest increase considering the large change in temperature. In fact, the system suitability check still passed at 160 °C. It appears that this is another situation in which the retention projection methodology was able to accommodate an experimental factor that it could not properly take into account. For example, the retention time of dextromethorphan, the latest eluting test compound, was affected the most by the drop in the transfer line temperature. Its retention time changed from 15.796 min at 320 °C to 15.871 min at 160 °C, a difference of 4.5 s. Nevertheless, at 160 °C its retention time was projected with an error of only 1.6 s. Of course, with a low enough transfer line temperature or a long enough transfer line, we expect the system suitability test would fail, but under normal circumstances we find that it is surprisingly insensitive.

3.1.12 MS source temperature—One might initially dismiss the effect that mass spectral parameters could have on chromatographic selectivity, but a small section of the column is held inside the MS at a different temperature than either the GC oven or the transfer line. Additionally, at very low MS source temperatures, peaks become strongly tailed due to their condensation on the inner surfaces of the ion volume. We tested the accuracy of retention projections at several MS source temperatures down to 100 °C (Table 2). Though peaks were strongly tailed at 100 °C, the accuracy of retention projections was unaffected (± 0.52 s).

3.1.13 Injection amount—In all prior experiments, we injected 10 pmol of each test compound. One would expect that when the amount injected is too large, the accuracy of retention projections will decrease as the column becomes mass overloaded. Indeed, when we gradually increased the amount injected, the accuracy grew noticeably worse at 50 pmol and above (Table 2). At 100 pmol and above, the system suitability check failed (Table 2). On the other hand, when we lowered the concentration by a factor of ten to 1 pmol, the accuracy was virtually unaffected (Table 2).

Therefore, care must be taken to avoid a situation in which the column is mass overloaded by one or more components of the sample. This is especially important because the problem would not be detected by the system suitability check. If some components of a sample were at a high enough concentration to overload the column, the system suitability check may still pass (especially if it is run separately from the sample, not spiked into it), but the calculated retention time tolerance windows would be incorrect. However, if there was any question as to whether a sample component was overloading the column, one could dilute a sample and run it again. If the retention times of the peaks change noticeably, it was probably overloading the column.

3.1.14 Different column lengths—Just as the retention projection methodology can account for differences in the temperature program, flow rate, and inlet pressure, it should theoretically also be able to account for differences in column length, inner diameter, and film thickness. To test how well it can account for differences in column length, we measured the accuracy of retention projections with columns of four different lengths between 30 m and 15 m (Table 2). While retention projections were accurate to ± 0.44 s with the 30 m column, they were accurate to ± 0.66 s with the 15 m column.

Interpretation of this data is slightly more complicated since the threshold for a passed system suitability check changes as a function of column length. As columns get shorter, the thresholds should become a little smaller as uncertainty in the projected retention times decreases. However, the actual error follows the opposite trend, increasing to a small extent with shorter columns. We are not sure specifically what caused the error to increase with shorter columns, but we plan to explore this in future work. Regardless, the system suitability check still passed at each column length, though in the case of the 15 m column it fell into the “passed, but questionable” region with an accuracy of ± 0.66 s (the “passed” threshold was ± 0.64 s and the “passed, but questionable” threshold was ± 0.76 s).

The retention projection methodology accounted for differences in column length considerably better than linear retention indexing. When retention indices were measured on the 30 m column and used to predict retention times with other column lengths, they were only accurate to ± 4.9 s (25 m), ± 5.8 s (20 m), and ± 9.4 s (15 m). Even on the 25 m column (only 5 m difference in column length), retention indices were over 10-fold less accurate than retention projections because they could not properly account for the difference in column length. Retention indices were the least accurate on the 15 m column, where they were 14-fold less accurate than retention projections.

3.1.15 Different column inner diameter—To test whether the methodology can properly account for differences in column inner diameter, we measured the accuracy of retention projections on a column of larger inner diameter (0.32 mm). In this case, the thresholds for a “passed” and a “passed, but questionable” system suitability check were ± 0.81 s and ± 0.97 s. The accuracy of retention projections on this column was ± 0.84 s, putting it into the “passed, but questionable” category.

We suspected that the slightly lower accuracy of retention projections on the 0.32 mm column was a result of the fact that the specified inner diameter is only approximate. A difference between the *actual* inner diameter and the *nominal* inner diameter would have caused error in the projected retention times because they would have been calculated with an imprecise phase ratio. To test whether we could correct for the assumed difference in the inner diameter of the column, we projected retention times assuming a range of inner diameters to find the one that gives the lowest error. Figure 5A shows the retention projection error as a function of the inner diameter that was entered into the software. Projected retention times were almost twice as accurate (± 0.41 s) if an inner diameter of 0.36 mm was assumed instead of the nominal value of 0.32 mm. In that case, the retention projection error was well under the threshold necessary to pass the system suitability check. This suggests that the retention projection methodology works as well as theory predicts

with a different inner diameter, but the difference from the nominal inner diameter must be taken into account. We suspect that this only needs to be done once for each inner diameter, as they are likely to be consistent from column to column. We plan to test more columns with a wider range of inner diameters in the future.

We then compared the accuracy of retention projections on the 0.32 mm ID column to that of retention indexing. When retention indices measured on a 0.25 mm ID column were used to predict retention times on a 0.32 mm ID column they were only accurate to ± 14.5 s, which is 35-fold worse than the accuracy of retention projections (after correcting for error in the column inner diameter).

3.1.16 Different film thickness—We also tested how well the methodology can account for differences in film thickness by attempting to project retention times on a column of 1 μm film thickness. In this case, the thresholds for a “passed” and a “passed, but questionable” system suitability check were ± 0.76 s and ± 0.90 s. The accuracy of retention projections on this column was ± 1.2 s, putting it into the “failed” category.

We again suspected that the lower accuracy of retention projections on the 1 μm film thickness column was a result of the fact that the specified film thickness is only nominal. To test whether we could correct for the assumed difference in the film thickness, we projected retention times assuming a range of film thicknesses to find the one that gave the lowest error. Figure 5B shows the retention projection error as a function of the assumed film thickness. Projected retention times were over twice as accurate (± 0.53 s) if a film thickness of 0.9 μm was assumed instead of the nominal value of 1.0 μm . That level of error was well below the threshold necessary to pass the system suitability check, suggesting that the retention projection methodology works as well as theory predicts when the film thickness is changed, but that the difference from the nominal film thicknesses must be taken into account. We expect the difference from nominal film thickness only needs to be measured once and then it can be applied to other columns of the same film thickness.

We then compared the accuracy of retention projections on the nominally 1 μm film thickness column to that of retention indexing. When retention indices measured on a column of 0.25 μm film thickness were used to predict retention times on a column of 1 μm film thickness, they were only accurate to ± 15.7 s, 30-fold worse than the accuracy of retention projections (after they were corrected for error in the film thickness).

3.2 Protocol to troubleshoot a GC system after a failed system suitability check

Based on these and previous experiments [11,19], we developed a protocol to efficiently restore a system to a state where it passes the system suitability check. After each of the following steps, one should re-run the system suitability check to test whether the problem has been fixed:

Step 1: Make sure experimental conditions conform to the limits of the methodology—Currently, there are six requirements: 1) the stationary phase must be Agilent DB-5MS UI, 2) the carrier gas must be He, 3) the lowest temperature in the temperature program may not be less than 60 °C and the highest may not exceed 320 °C, 4)

the data collection rate should be fast enough so as not to introduce significant error into the measured retention times (we find that two or more scans per second is enough under most conditions), 5) the transfer line temperature must be greater than or equal to the highest temperature in the temperature program, and 6) the column length must be >15 m (though once we fully understand the cause of the reduced accuracy with short column lengths, this requirement may be unnecessary).

Step 2: Check the amount of the test mixture injected—The amount of each component of the test mixture should be less than or equal to 10 pmol (e.g., 1 μ L injection of the test mixture with each component at 100 μ M at a split ratio of 10:1).

Step 3: Replace/clean the liner—The make/model/geometry of the liner did not matter among the ones we tested, but it had to be clean.

Step 4: Clip or replace the column—If the above steps fail to make the system suitability check pass, the column itself must be damaged, probably due to oxygen exposure or injection history. One can first attempt to restore the column by clipping off a short length of the column on the inlet side, but if that fails to fix the system, the entire column should be replaced.

4. Conclusions

Previous work showed that the accuracy of retention projections are on the level expected by theory regardless of the temperature program, flow rate/inlet pressure, or the GC instrument used. Here, we tested 16 other experimental factors and found that only 5 could be responsible for reduced accuracy in retention projections (i.e., a failed system suitability check): injection history, exposure to air at high temperature (though the level of oxygen in industrial grade He did not show an acute effect), a very low transfer line temperature, an overloaded column, and use of a very short column (<15 m). This dramatically reduces the number of experimental factors that need to be considered when a GC system fails the system suitability check. Based on this information, we developed a four step protocol to efficiently troubleshoot a failing system.

Even though some of the experimental factors we tested (splitless injection, manual injection speed, inlet temperature, and transfer line temperature) significantly affected the retention times of some of the test compounds and were not properly taken into account by the methodology, they showed very little or no effect on the accuracy of retention projections. We found that the effects of those experimental factors were “absorbed” into the back-calculated temperature and hold-up time profiles, shifting the projected retention times in the right direction without truly accounting for the cause of the retention time shifts. In this way, we find that the retention projection methodology acts as a hybrid of conventional retention projection and retention indexing, drawing on the advantages of both; it properly accounts for a wide range of experimental conditions while accommodating the effects of experimental factors not properly taken into account in the calculations.

In addition, the methodology was shown to properly account for differences in film thickness, inner diameter, and to some extent, column length, as well as would be expected by theory. Under these conditions, retention projections were 10- to 30-fold more accurate than retention indices. However, when using a column of an inner diameter other than 0.25 mm or a film thickness other than 0.25 μm , we found it improved the accuracy of retention projections to indirectly measure and account for what we presume to be small differences from the nominal film thickness and inner diameter. We are in the process of building a much larger database of isothermal retention vs. T relationships to enhance the practical value of the methodology.

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Highlights

- The retention projection methodology proved to be uniquely robust
- It acts as a hybrid of “conventional” retention projection and retention indexing
- Of 16 experimental factors tested, only 5 affected the accuracy of projections
- It accounted for different inner diameters, film thickness, and column lengths
- A short protocol was developed to fix a GC system yielding poor accuracy

Figure 1. Isothermal retention vs. T relationships of two different compounds, 1-naphthol and n -tetradecane.

Figure 2.

The 12 chemically diverse test compounds used to measure the accuracy of retention projections.

Figure 3.

A) Photograph of the GC oven containing two Al blocks (circled). B) Comparison of back-calculated profiles with and without the blocks in the oven. When the blocks were placed into the oven, the temperature profile actually produced by the GC was highly distorted, but the accuracy of retention projections was unaffected.

Figure 4.

Back-calculated temperature profiles (top) and hold-up time profiles (bottom) from a split injection (10:1 ratio) and a splitless injection. When the splitless injection lengthened the retention times of the early-eluting *n*-alkanes, the back-calculation algorithm compensated by slightly altering the temperature and hold-up time profiles.

Figure 5.

A) Accuracy of retention projections on a column of nominally 0.32 mm ID as a function of the ID that is used in the retention projection calculations. Retention projections were most accurate when an inner of 0.36 mm ID was assumed instead of 0.32 mm. **B)** Accuracy of retention projections on a column of nominally 1.0 μm film thickness as a function of the film thickness that was used in the retention projection calculations. Retention projections were most accurate when a film thickness of 0.9 μm was assumed instead of 1.0 μm .

Table 1

Measured and Projected Retention Times for the 12 Test Compounds and the Expected Error Calculated for Each at One Standard Deviation

Test compound	Measured retention time (min)	Projected retention time (min)	Error	Expected error (1 SD)
ethylbenzene	6.160	6.1738	-0.0138	±0.0150
naphthalene	10.491	10.504	-0.0130	±0.0089
anthracene	14.259	14.263	-0.0040	±0.0087
N,N-diethylacetamide	8.372	8.3652	0.0068	±0.0100
4-nitroaniline	13.142	13.144	-0.0020	±0.0083
caffeine	14.328	14.333	-0.0050	±0.0083
phenol	8.069	8.0698	-0.0008	±0.0110
resorcinol	11.006	11.010	-0.0040	±0.0075
1-naphthol	12.630	12.628	0.0020	±0.0082
N,N-dimethylisobutyramide	8.022	8.0198	0.0022	±0.0110
benzamide	11.465	11.469	-0.0040	±0.0080
dextromethorphan	15.796	15.786	0.0100	±0.0093
RMS (min):			±0.0070	±0.0097
RMS (sec):			±0.42	±0.58

Table 2

Accuracy of Retention Projections Under a Range of Experimental Conditions

Experimental Condition	Accuracy of Retention Projections ^d
3.1.1 Metal blocks in the GC oven	
Without blocks in oven	± 0.43 s
With two Al blocks in oven	± 0.35 s
3.1.2 Liner make/model/geometry	
Agilent, 2 mm ID, 78.5 mm long, straight splitless, quartz	± 0.49 s
Agilent, 4 mm ID, 78.5 mm long, gooseneck, glass	± 0.44 s
SGE, 4 mm ID, 78.5 mm long, straight, glass	± 0.38 s
Restek Siltek, 2 mm ID, 78.5 mm long, straight splitless, quartz	± 0.45 s
Restek Sky, 4 mm ID, 78.5 mm long, straight, glass, packed w/deactivated glass wool	± 0.40 s
Supelco, 4 mm ID, 78.5 mm long, straight, glass, packed w/deactivated glass wool	± 0.40 s
Thermo, 5 mm ID, 105 mm long, straight FocusLiner, packed w/deactivated quartz wool	± 0.42 s
Thermo, 5 mm ID, 105 mm long, straight, packed w/deactivated quartz wool	± 0.43 s
3.1.3 Injection history	
Before injections	± 0.51 s
After 7 injections of the urine sample	± 1.3 s
Clean liner	± 0.43 s
Dirty liner	± 1.7 s
Clean base seal	± 0.47 s
Dirty base seal	± 0.50 s
3.1.4 Split or splitless injection	
Split (10:1)	± 0.42 s
Splitless	± 0.43 s
3.1.5 Manual injection speed	
Fast (~0.5 sec)	± 0.42 s
Slow (5 sec)	± 0.44 s
3.1.6 Inlet temperature	
300 °C	± 0.44 s
290 °C	± 0.42 s
280 °C	± 0.44 s
270 °C	± 0.43 s
250 °C	± 0.48 s
230 °C	± 0.45 s
200 °C	± 0.44 s
3.1.7 Column cut	
Straight	± 0.42 s
Angled	± 0.45 s
3.1.8 Length of column in inlet	
0.2 cm	± 0.44 s
1.2 cm	± 0.39 s

Experimental Condition	Accuracy of Retention Projections ^d
2.2 cm	± 0.37 s
3.1.9 Column conditioning	
None	± 0.42 s
3 hrs at 320°C	± 0.45 s
3.1.10 Exposure to O₂ levels in industrial grade He	
Before	± 0.42 s
After 72 hours exposure to 6.2 ppm O ₂ at 320°C	± 0.39 s
3.1.11 Transfer line temperature	
320 °C	± 0.44 s
280 °C	± 0.53 s
240 °C	± 0.57 s
200 °C	± 0.63 s
160 °C	± 0.66 s
3.1.12 MS source temperature	
300 °C	± 0.52 s
220 °C	± 0.50 s
180 °C	± 0.49 s
100 °C	± 0.52 s
3.1.13 Injection amount	
1 pmol	± 0.59 s
10 pmol	± 0.50 s
20 pmol	± 0.49 s
30 pmol	± 0.50 s
50 pmol	± 0.64 s
100 pmol	± 1.1 s
750 pmol	± 5.5 s
3.1.14 Column length	
30 m	± 0.44 s
25 m	± 0.45 s (LRI: ± 4.9 s ^b)
20 m	± 0.55 s (LRI: ± 5.8 s ^b)
15 m	± 0.66 s (LRI: ± 9.4 s ^b)
3.1.15 Column inner diameter	
0.25 mm	± 0.44 s
0.32 mm	± 0.41 s ^c (LRI: ± 14.5 s ^b)
3.1.16 Film thickness	
0.25 μ m	± 0.44 s
1 μ m	± 0.53 s ^d (LRI: ± 15.7 s ^b)

^a Accuracy of retention projections is given as the root-mean-square error (experimental minus predicted retention time) among all 12 test compounds. **Green** indicates the accuracy is within the threshold for a “passed” system suitability check, **orange** indicates “passed, but questionable”, and **red** indicates “failed”.

^b Accuracy of retention times calculated from linear retention indices that were measured under the standard 26 °C/min ramp with a 30 m column of 0.25 mm ID and 0.25 µm film thickness.

^c After correction for the difference from the nominal inner diameter

^d After correction for the difference from the nominal film thickness