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A study on retention "projection" as a supplementary means for compound identification by liquid chromatography–mass spectrometry capable of predicting retention with different gradients, flow rates, and instruments

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ABSTRACT

Using current data analysis techniques, even the most advanced LC–MS instrumentation can identify only a small fraction of compounds found in typical biological extracts. Augmenting MS information with HPLC retention information allows many more to be identified. In fact, our calculations indicate that a quadrupole MS is able to identify more compounds than an FTICR-MS when the quadrupole spectrum is augmented with retention information. Unfortunately, retention information is extremely difficult to harness for compound identification. Here, we demonstrate the first use of isocratic data measured on one LC–MS to "project" gradient retention on to *different* LC–MS systems. Using 35 chemically diverse solutes chosen to encompass the full range of reversed-phase alkylsilica interactions, and using experimental conditions typical of metabolomics experiments, gradient retention was projected from one instrument to another with only 1.2–2.6% error—enough accuracy to considerably improve compound identification. Besides accounting for nonlinear relationships of retention versus solvent composition as well as dead time versus solvent composition, accounting for the precise shape of the gradient profile (not just the dwell volume) improved projection accuracy on one instrument by up to 4 fold whereas flow rate non-idealities likely caused considerable error on the other instrument. Thus, these two factors must be taken into account to accurately project retention on diverse instrumentation.

1. Introduction

Some of the most demanding uses of HPLC involve the separation of complex biological extracts, in which more than 25,000 unique small molecules may be present in an extract from a single species [1,2]. In these separations, mass spectrometry (MS) is typically used as the detector following gradient elution liquid chromatographic separation because it offers high sensitivity, detection of a wide range of compounds without derivatization, and the ability to resolve large numbers of compounds. Such is the case in the rapidly expanding field of metabolomics, where LC–MS has evolved into the central technique [3–5].

While LC–MS is capable of detecting large numbers of features, even the best analyses are only capable of identifying a small fraction of all metabolites detected in a single run. For example, in one of the most advanced of these experiments reported to date [6], an LC-Fourier Transform Ion Cyclotron Resonance (FTICR) MS was used to assign elemental compositions (not structures) to 643 unique compounds in an *Arabidopsis thaliana* leaf extract. With current estimates of the size of an individual plant metabolome, that corresponds to only 3–13% of all metabolites [1,2].

We expect that a methodology for accurate HPLC retention prediction (here we use the term "prediction" in the broadest sense to describe any approach for the alignment of *expected* and *experimental* retention data) could enable experimental retention data to be used in combination with MS information to considerably improve assignment of chemical identities to unknown chromatographic features. In calculations discussed below, we test the degree to which this is true for metabolites in a simulated biological sample. But one may also look to the field of proteomics, where use of peptide retention in combination with MS data is rapidly becoming very important. In this case, *a priori* retention times of peptides are usually literally predicted based on their amino acid sequence and the known retention times of peptide training sets [7–9]. With this approach, correlations between predicted and experimental

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peptide retention times have been reported with R^2 values as high as 0.99. Yet this translates to a retention prediction accuracy of approximately ±1.8 min in a 60 min gradient (or ±3% of the gradient time, t_G). Clearly, there is still plenty of room for improvement. Even so, retention predictions based on analyte structure are increasingly being used as they significantly improve the quantity and quality of peptide identifications. Unfortunately, approaches to relate retention to chemical structure for small molecules such as metabolites are much less accurate and reliable because they encompass an extremely wide range of chemical diversity [10] compared to peptides.

Several other approaches have been reported for using retention information on small molecules, but none seem to have the necessary level of accuracy and/or generality to gain wide use. Compendia of retention times, relative retention times, or retention indices have by and large failed to be useful for analyte identification in liquid chromatography. This is largely because there are so many factors that control observed retention times – particularly in gradient elution, where even the make and model of the HPLC instrument strongly influences retention due to significant non-idealities in the gradient and flow rate profiles which they produce (see Section 4.3 for examples of such differences). Therefore, existing approaches to retention prediction that emphasize accuracy require a highly restrictive set of experimental conditions. One such approach requires that the entire system be standardized: the make/model of column, the column dimensions, the gradient program, the temperature, the mobile phases, the flow rate, *even the HPLC instrument itself*. This is precisely the type of system currently being developed in several labs [4,11,12]. Though such a system may provide an accurate means for using retention for identification, we believe that a more universal methodology must be developed in order for it to become widely useful and not become immediately obsolete as instrumentation is updated.

A somewhat more general approach than mere retention or retention relative to a fixed standard is to report retention times relative to two "bracketing" standard compounds. This retention metric, frequently termed a retention "index", is very widely used in gas chromatography (GC) because it is relatively insensitive to small differences in instrument-dependent variables (e.g. temperature and carrier gas flow rate non-idealities, column geometric factors, etc.), effectively accommodating major differences between makes and models of gas chromatographs [13]. However, in GC, retention indices measured from isothermal and programmed-temperature experiments are not the same. Moreover, different programmed-temperature conditions (i.e. different initial temperatures, program rates, flow rates, and column geometries) produce different retention indices [14]. Several methods have been reported that enable the transfer of retention indices measured under one temperature program to a different one with improved agreement [15–17]. Still, without rigorously reproducing the experimental conditions in which a set of programmed-temperature retention indices were originally measured, their inter-laboratory reproducibility is questionable [18,19]. Despite these limitations, retention indexing is routinely used in GC and continues to gain popularity at an increasing rate [20,21].

The success of retention indexing in GC has spurred many researchers to develop analogous systems for reversed-phase LC [22–24]. However, the accuracy of LC retention indexing systems are *fundamentally limited* because they assume that the sensitivity of retention to the volume fraction of organic modifier in the eluent, ϕ, is constant for all compounds. This is a *very* rough approximation. For example, Fig. 1 shows three retention (in terms of $\log k$) vs. ϕ relationships measured on a typical C_{18} column. The shapes of the curves are highly compound-dependent. Admittedly these are extreme examples chosen to illustrate the gravity of the problem. Therefore, the isocratic retention of a compound relative to two "bracketing" standards cannot be used to accurately predict its isocratic retention at a different solvent composition because the shape of the compound's $\log k$ vs. ϕ relationship may differ significantly from the $\log k$ vs. ϕ relationships of the standards. For example, a change in isocratic mobile phase composition from 10 to 30% methanol caused the retention index of aspirin to change from 302 to 8 [25]. In the same way, retention indices measured under one gradient program and flow rate cannot be used to predict retention under a different gradient or flow rate. In fact, when gradient and flow rate non-idealities produced by different makes and models of HPLC instruments are sufficiently large, the retention indices are not even transferable between different instruments run with the same instrument conditions. This is discussed more in the accompanying manuscript [26].

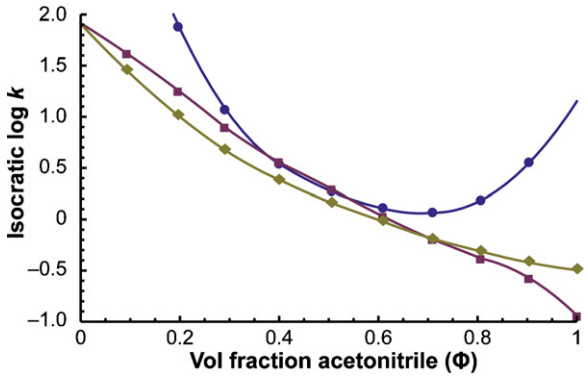

Fig. 1. Isocratic $\log k$ vs. ϕ for amitriptyline (●), indole (■), and acetophenone (◆) run on a Zorbax SB-C18 column (2.1 mm × 100 mm, 3.5 μm particles, 8 nm pore size).

Several peak-alignment, or "time warping", algorithms have also been reported to predict retention [27,28]. These systems "warp" the time scale of chromatograms to bring similar chromatographic *features* (i.e. groupings of analytes) into alignment in separate runs. While time warping algorithms have proven very useful for comparing features of nearly identical runs, they are not designed to be, nor would they be useful as a general tool for compound identification. First, they rely on the presence of similar chromatographic features in each run—they cannot compare chromatograms containing a significantly different set of features. Second, they do not provide any information about the chemical identity of the features. But even if you knew the chemical identity of a feature (perhaps by running a standard beforehand), a time warping algorithm could only reproduce its retention accurately under almost exactly the same experimental conditions in which the standard was run. Like retention indexing systems, time warping algorithms are fraught with assumptions about the relative behavior of neighboring peaks.

In contradistinction, in this work, we study the use of retention "projection" as a theoretically sound basis for gradient retention prediction. The term "projection" is used because gradient retention on one instrument is "projected" from isocratic measurements in a database (or library). We believe retention projection will overcome the major obstacles in the development of a reasonably accurate and reliable LC retention prediction system that accounts for all important factors controlling retention while still maintaining a useful degree of generality. Ideally, a retention projection system should not only be able to accurately predict retention (a) among different HPLC/UHPLC instruments and (b) with chemically diverse solutes, but it should also be accurate (c) under a range of gradient and flow rate conditions. This is particularly important in metabolomics, where no standard gradient or flow rate protocol has been agreed to and a wide range of flow rates are required to meet the demands of diverse experiments and instrumenta-