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Natural products research constantly has to deal with problems of analysis. Therefore, orthogonal techniques are extremely valuable when analyzing complex mixtures that are often seen in fungal extracts. Analysis of results of fungal fermentation experiments can influence the design of further experimentation. Earlier results can then expedite the process of analysis. Quantitative nuclear magnetic resonance spectroscopy (qNMR) offers analysis of complex mixtures at early stages and with several benefits over some more common methods of quantitation, including ultraviolet absorption spectroscopy (UV) and mass spectrometry (MS).

Several experiments were conducted to construct a methodology for use in analysis of natural products samples. A broadly applicable method was sought for use in both pure and complex mixtures. An externally calibrated method was used to quantify the solvent peak inside of a single batch of DMSO-d_6, which was used repeatedly to quantify interesting analytes. Thereby, a method was constructed that did not require contamination with calibrant for quantification of analyte signals. The method was implemented to measure the biosynthetic yield of griseofulvin and dechlorogriseofulvin from three fungal isolates. One isolate, a *Xylaria* sp. coded MSX648662, was found to biosynthesize griseofulvin in the greatest yield, 149 ± 8 mg per fermentation, and was selected for further supply experiments.

INVESTIGATION INTO qNMR FOR USE IN NATURAL PRODUCTS RESEARCH

by

Wilson C. Brooks

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Approved by

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APPROVAL PAGE

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

INTRODUCTION AND INITIAL qNMR ENDEAVORS

Introduction

In natural products research, analysis of samples is a frequent problem. Different techniques of qualitative and quantitative analysis have differing benefits and pitfalls that make their usefulness in application varied. Therefore, it is important to learn about and assess new methods for analysis in order for a researcher to be able to select the best method for their specific purpose.

Nuclear magnetic resonance (NMR) spectroscopy has frequently been used as a qualitative method at the University of North Carolina at Greensboro[1-4] (UNCG) and in natural products research in general.[5] NMR is frequently used in qualitative structure elucidation procedures. It is useful due to the unique signal pattern that arises from each molecule. These patterns are then used to locate structural features of the corresponding molecules. Thereby, in combination with information other techniques, structural information from a purified compound can be pieced together until the molecule is completely elucidated.

In the natural products field of science, research has begun to find NMR useful for other respects. Software advances have allowed for qualitative and quantitative measurements of mixtures of compounds. More recent advances in computational software for NMR spectra manipulation have allowed for qualitative and quantitative analysis of very complex spectra in a holistic manner, including metabolite profiling[6, 7] and advanced

spectral summation such as ^{1}H iterative full spin analysis (HiFSA).[8] NMR measurements have been performed on proteins inside of living cells using techniques to speed up the acquisition process of multi-dimensional NMR.[9] Since the 1990s, NMR has been used for quantitative studies of compound mixtures.[5, 10] However, there has been some hesitation to accept NMR as a technique to quantitatively analyze purity in spite of numerous studies for its validation and comparison to existing quantitation techniques.

Purity assays of natural products continue to be an important way of determining the interference with bioactivity and other testing on samples. Knowing the precise composition of a sample that is being tested for bioactivity has shown multiple times to be extremely important. There have been many examples of minor contaminants that have been responsible for bioactivity that was initially accredited to the primary constituent of a natural product sample.[11, 12] Traditional quantitation methods – most commonly high-performance liquid chromatography (HPLC) or ultra high-performance liquid chromatography (UPLC or uHPLC) coupled with ultraviolet absorption detection (UV)[13] and/or mass spectrometry (MS) – are able to provide a direct signal to mass ratio of particular components of each sample. However, these methods have a downside in their detection probe; the detection probe responds differently to different molecules based on features of the structure. For example, UV absorbance depends on the degree of electronic mobility within the molecule. Generally, the UV probe will show increased response – i.e. absorbance – to an organic molecule that has a high degree of conjugation compared to a molecule with relatively little conjugation.[14] In UV spectroscopy, the signal to response translation constant is termed the extinction coefficient of the molecule. MS has a similar flaw that offers some compounds greater response based on their ionization efficiency.[15]

This difference in response makes quantifying compounds guess-work without well characterized standards for each compound for the creation of calibration curves.

In natural products, the isolation of compounds often results in isolates that have never been discovered. This limits the usefulness of UV as a quantitative measure against other components in the sample due to a distinct lack of available standards with which to calculate an extinction coefficient. In addition, the broad scope of natural product chemistry often results in the isolation of compounds that have little or no UV absorption compared to contaminants, which makes UV absorption nearly useless as a detection method of these compounds. Furthermore, literature shows that there are examples where HPLC with UV detection has overestimated the purity of natural product samples.[13]

However qNMR has a universal response to organic compounds; every compound that has a proton in the structure has the potential for measurement via ^{1}H NMR. Additionally, a signal of one nonexchangeable proton from one molecule should be completely proportional to its concentration. This bypasses some flaws in UV detection by giving the same signal to mass conversion for every constituent within a sample. Thereby, the strength – i.e. integration – of signals from different constituents are in direct proportion to their relative molar concentrations, assuming no exchangeability of the protons. In addition, NMR has the potential to be a very sensitive technique as well, thanks to the advances in recent instrumentation and software.[13] The comparative sensitivity of NMR is also aided by the relative immobility of the sample that allows for prolonged exposure when compared to UV detection. While UV detection typically relies on chromatography to differentiate constituents, NMR spectroscopy has an innate separation of signals in due to the nature of chemical shifts. Therefore, for natural products, purity

analysis via ^{1}H quantitative NMR (or qNMR) could be more analytical and useful than purity analysis via UV detection.[13] Furthermore, the orthogonality of qNMR makes it a convenient method for crosschecking analysis via other methods, such as in the case of verifying reference standards. Recent studies have also added validity to the accuracy of qNMR and deem it interchangeable with UV quantitation[16] and superior to the reproducibility of HPLC-MS, HPLC coupled evaporative light scattering detection (HPLC-ELSD) or thin layer chromatography (TLC).[17]

qNMR is a tempting technique for the quantitation of small molecules and its use is growing in natural products science.[18] The following studies have been conducted in light of the increasing excitement and use of qNMR in efforts to add the technique to the arsenal of methods available for UNCG researchers.

Signal Based qNMR Analyses

Initial methodology was constructed based on some common lab practices. An estimation of purity (P%) via UV absorption can be done by calculating a ratio (P_{signal}%) between the peak area of one constituent and the summation of all peaks in the chromatograph:

$$P\% \cong P_{signal}\% = \frac{I_A}{I_A + I_{C1} + I_{C2} + \ldots + I_{Cn}} \quad (1)$$

where I represents an integral of a particular peak, A represents the primary analyte, and Cn represents the n-th contaminant peak. Thereby, the purity of the analyte would be proportion of the signal response of all constituents.

The rationale for this technique of purity assessment is that generally as mass increases for each constituent, so should their cumulative integrated peak area. As long as each compound absorbs in a similar way, the result will be a fairly valid estimate of purity.

Isosilybin A

Isosilybin A was chosen as an initial model compound because of the familiarity of the Principal Investigator with its chemistry.[8, 19-23] Milk thistle compounds, including isosilybin A, have been studied for their hepatoprotective and chemopreventative properties.[24, 25] Differentiation of these compounds via NMR was has been well characterized previously and quantified by Napolitano and Pauli in 2013.[8] Their study used computer modeling to identify and report small differences in chemical shifts of the compounds and calculate the unambiguous composition of four closely related analogues. Their analysis showed the extreme potential of quantitation via NMR. However, a more simple methodology would be preferred.

A purified sample of isosilybin A (**1**) that was deemed to be 97.2 % pure by UPLC-UV analysis was analyzed by qNMR. Samples were prepared in DMSO-d_6 for qNMR analysis to 10 mM. Samples were analyzed at 25 °C using a JEOL ECA-500, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Eight scans were each acquired for 1.745 seconds using 16000 points per acquisition. The pulse angle used was 45° and the pulse duration was 6.6425 μs. Receiver gain was set to 50 for each run. Figure 1 shows a representative NMR spectrum of this sample.

NMR spectra were analyzed using MestReNova Lite software. Careful phasing of the baseline was first applied to the spectrum to give an even baseline and regular peak shape. The chemical shifts were adjusted to the DMSO-d_6 peak at 2.50 ppm. Automatic peak

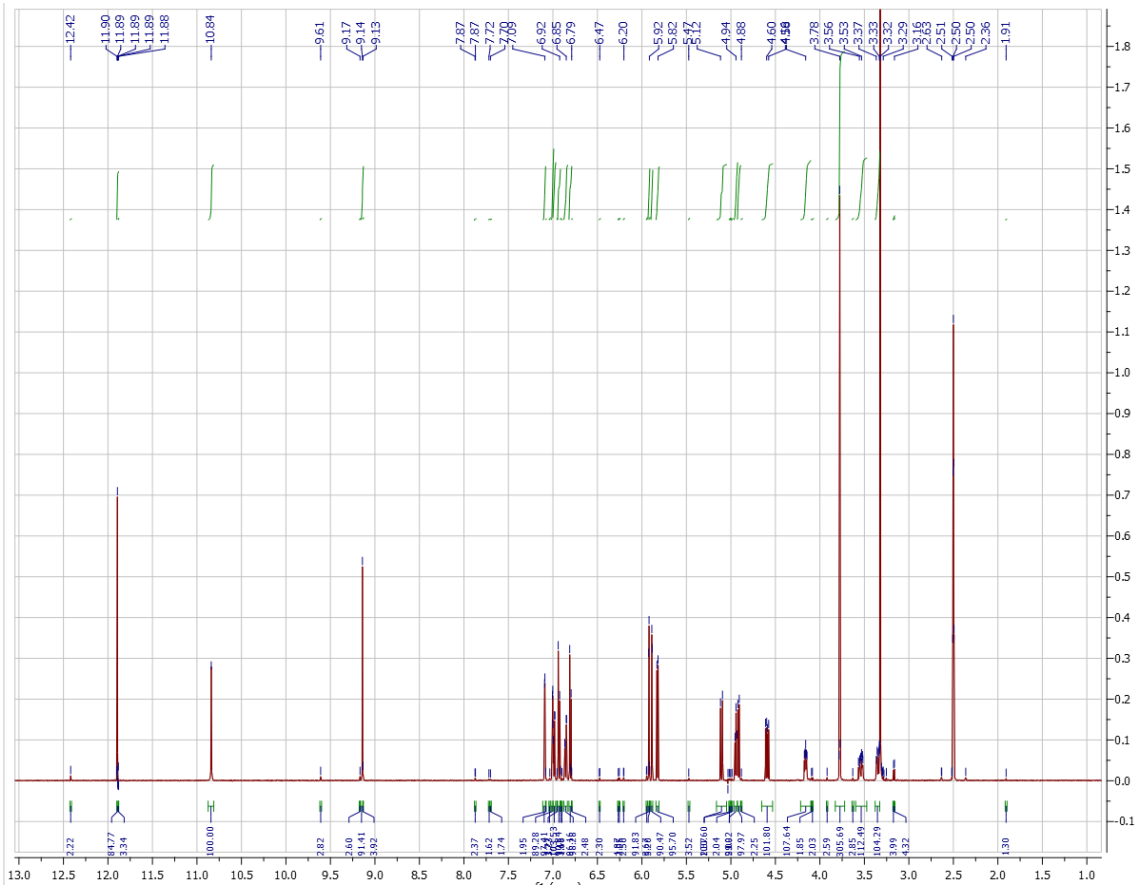


Figure 1. *S*pectrum of a 97% Pure Sample of Isosilybin A. – Baseline phase correction was applied first, followed by chemical shift correction; this was followed by peak area standardization. Numbers at the top are chemical shifts and numbers at the bottom are peak areas. Individual peaks are shown as vertical blue lines above each peak.

picking was used to select peaks that were 2.5 intensity above the baseline. Integration of peaks was carefully corrected to separate contaminant peaks as precisely as possible. Peaks were selected for inclusion into the qNMR analysis based on integration of the peak area. The integral area of the peak at 10.84 ppm was set to a peak area = 100. The peak at 10.84 ppm was chosen for normalization due to its isolation from other signals and regular peak shape. If integration of any peak yielded < 0.75 peak area, the peak was not included in the purity calculation, as this was deemed to be, in practicality, indistinguishable from baseline noise. The edited and corrected spectrum that was used to calculate purity is shown in Figure 1.

Peak areas were entered into a spreadsheet where they were sorted into contaminant and target analyte peaks (Appendix A). Target analyte peak areas were summed to be 2160.08 for 22 protons. This sum was then divided by the sum of all peak areas from the spectrum, 2241.95. This resulting quotient of 96.35 is termed the *purity of the sample* by ^{1}H peak area (P_{signal}%).

Defining purity by peak area is different than the traditional definition of purity which is defined based on the relative masses of the analyte and the contaminants. This definition uses only their integrated peak areas as the basis of purity. This assumption is valid in a general way. Generally, the hydrogen content of an organic compound increases as the mass of the compound increases. However, as conjugation and hydrogen deficiency differs, the variability of this assumption increases.

This assumption can be overcome in the cases of solutions where contaminants are known compounds with known ^{1}H spectra. Relative masses can be calculated using the peak integrations, which can then lead us to the traditional definition of purity. In the case where

the contaminants are unknown, this is potentially problematic due to the lack of mass information for each signal. The method proposed earlier in this report (Eq. 1) gives the opportunity to analyze samples with unknown contaminants by removing the necessity of mass information from the purity equation.

Removal of mass from the purity equation both hurts and helps the qNMR analysis. It helps compared to UV purity analysis due to the method's ability to pick up all contaminants that have a proton with great sensitivity and include them into the purity calculation. Purity by UV/Vis under-represents or fails to include many organic contaminants that don't or poorly absorb UV or visible light. Additionally, eliminating mass from purity saves time for those analyzing samples with constantly changing contaminants, such as natural products research. However, purity solely based on ^{1}H integration area, when compared to a method that includes mass in the calculation of purity, will over-represent those compounds with low hydrogen deficiency and under-represent those compounds with high hydrogen deficiency, due to their different hydrogen to carbon and hetero-atom ratios. Thereby, the elimination of mass from purity by qNMR recreates a problem found in UV/Vis purity analysis: some compounds will simply create more signal per mass unit than other compounds.

Ustilaginoidin F

Ustilaginoidin F (**2**) was isolated from filamentous fungi coded MSX51755. The structure was confirmed via NMR analysis. Several species of fungi are known to produce **2** and analogues.[26, 27] Compounds from this family of naptho-γ-pyrones have known cytotoxic properties[27, 28] and have recently been reported with HIV-1 integrase inhibition[29] and antitubercular activity.[30]

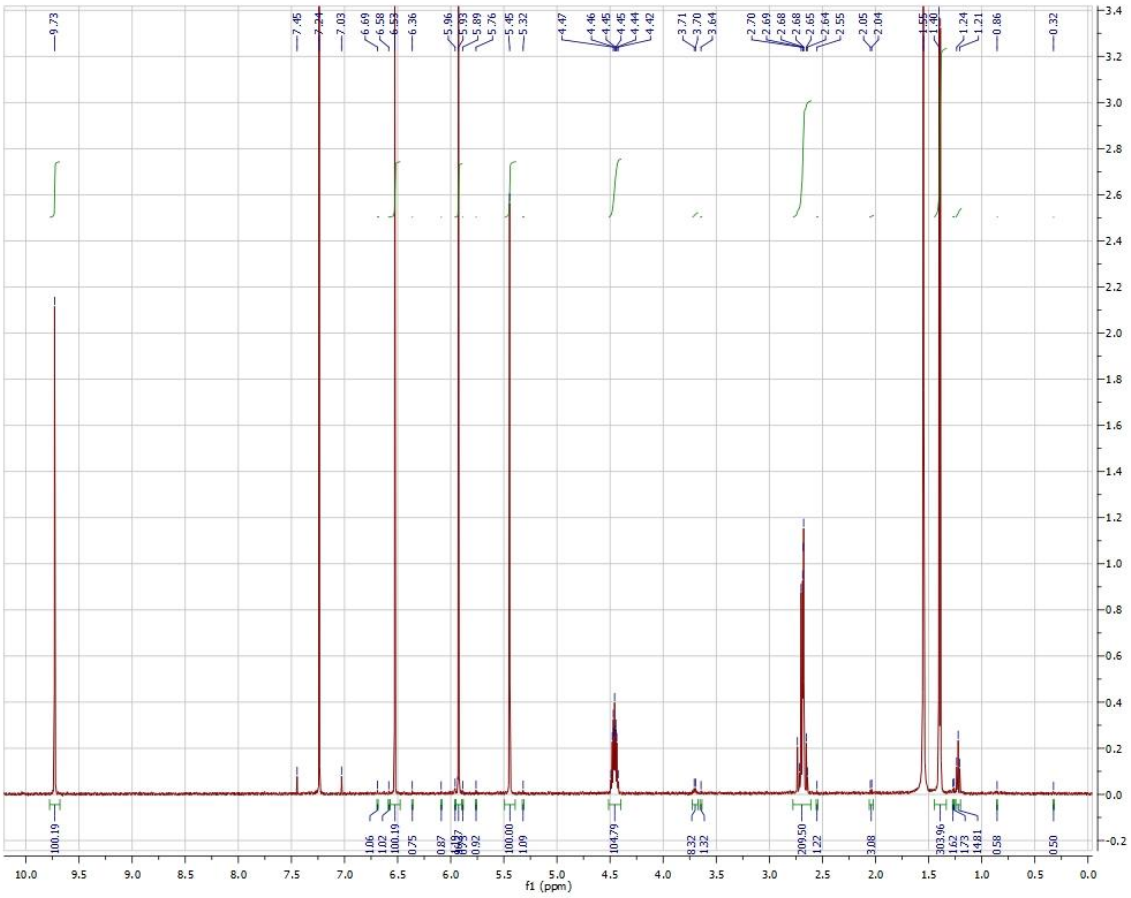


Figure 2. Corrected Spectrum of a Sample from MSX51755. – Baseline phase correction was applied first, followed by chemical shift correction; this was followed by peak area standardization. Numbers at the top are chemical shifts and numbers at the bottom are peak areas. Individual peaks are shown as vertical blue lines above each peak.

Isolation yielded a significant mass of **2**, 440 mg., from a screener-scale fermentation of MSX51755. This yield and the potent cytotoxicity of the extract led for **2** to be further analyzed by qNMR.

The procedures followed for the data collection and analysis were similar to the previous signal-based analysis of isosilybin A. A ^{1}H NMR experiment for 8 scans using the 500 MHz JOEL NMR was conducted on two samples of purified compound **2** (Figure 2). Each scan was acquired for 1.745 seconds using 16000 points per acquisition. The pulse angle used was 45° and lasted 6.6425 μs and the receiver gain was set to 50. ^{1}H NMR spectra were analyzed and corrected using MestReNova software (MestReLab Research). Spectra were carefully phased to give regular peak shape and even baselines. Chemical shifts were corrected to the DMSO – D_6 peak at 2.50 ppm. Automatic peak picking was used to select peaks that were 2.5% above the baseline noise. Integration of peaks was carefully corrected to separate contaminant peaks as precisely as possible. Peaks were selected for inclusion into qNMR analysis based on integration of the peak area. A peak representing a single proton was chosen from each spectrum and set to have an integrated area of 100. Peaks were selected for this based on a lack of neighboring peaks and a lack of surrounding contaminant peaks. Peaks with integrated areas of less than 0.75 were deemed "insignificant" compared to the baseline and were not included in tabulation of contaminants. Integrations of all peaks were summed and the summed integrations of the primary compound were divided by the total for each significant peak. The resulting quotient was termed the purity via ^{1}H NMR peak area. The purity of the initial flash chromatographic fraction containing **2** was found to be 96.3% pure via ^{1}H NMR peak area.

Conclusion

Using a quotient of peak areas has validity as an estimate of purity. However, the more complex the sample and the further that the constituents deviate in chemical properties from one another, the less accurate of an estimate a signal quotient becomes. A traditional definition of purity is a mass percentage:

$$P\% = \frac{m_A}{m_A + m_{C1} + m_{C2} + \ldots + m_{Cn}} = \frac{m_A}{m_{samp}} \quad (2)$$

where m is a mass of a particular analyte or contaminant inside of a sample. Thereby m_A is the mass corresponding to the analyte, A, and m_{samp} is the mass corresponding to the entire sample comprised of an analyte, A, and *n* contaminants - C_1, C_2, …, C_n. The Purity (P%) using the above signal based estimate (P_{signal}%) then would be accurate if each constituent in the sample had the same ratio between total peak area and mass:

$$\frac{I_A}{m_A} = \frac{I_{C1}}{m_{C1}} = \frac{I_{C2}}{m_{C2}} = \cdots = \frac{I_{Cn}}{m_{Cn}} \quad (3)$$

This condition may be met if the mixture was comprised of close analogues. But most mixtures would be unlikely to meet this condition, due to the variability of natural product small molecules. Therefore, it was determined that a mass based purity analysis would be more consistent for the general purposes of natural products research.

qNMR Analysis of Isosilybin A

Several experiments were designed based on methods from Pauli[13] and Krunic[31] to measure isosilybin A purity based on single peaks. An individual nonexchangeable proton peak yields an integration that is proportional to its mass. Therefore, by comparing an

individual analyte peak to a peak from a calibrant with known concentration, their relative concentrations, and thereby the analyte's absolute concentration, can be determined.

External solvent calibration is used to calibrate the residual solvent signal inside of a duterated NMR solvent. In theory, the concentration of DMSO-D_5 inside of DMSO-D_6 can be determined for an entire batch of solvent. Thereby subsequent use of the calibrated DMSO-D_6 solvent can then be used to measure numerous analyte samples without contamination by the calibrant. To determine the consistency of a single batch of solvent, several ampules of DMSO-D_6 from the same box were measured for consistency of the residual solvent signal. The absolute integrals of the corresponding samples of solvent had an average peak area of 133±2 (Figure 3). When the result from sample 4 was removed from the group, the average peak area changed to 132.6±0.9. Interestingly, a q-test then showed that the result from sample 4 can be rejected as an outlier from the rest of the group with greater than 99% confidence. Misplacement of a DMSO-d_6 vial into a box from a separate batch of solvent could explain this erroneous result. The solvent peaks were found to be consistent and usable for quantitation since their deviations were less than 2%.

A calibration curve was designed to measure the linearity of the peak areas over a small range. Isosilybin A was made up in DMSO-D_6 at 1.0 mg/mL, 0.30 mg/mL, and 0.070 mg/mL and the peak areas corresponding to the features 4'' chelating hydroxy proton, the 2' aromatic proton, the methoxy were plotted against their corresponding concentration (Figure 4). The slopes of the regressions for OH-4'' (10.4 M^{-1}), H2' (10.1 M^{-1}), and OCH_3 (30.1 M^{-1}) were in excellent proportion to the number of protons that gave rise to the signals. Additionally, the lowest of the three R^2 values was 0.9989, for both the OCH_3 and H2'

features. The measurement of various protons then seemed very linear in nature and had good consistency across several types of signals.

The consistency of solvent peaks and the linearity of the measurements were consistent with reports that deemed single residues as sufficient for quantitation due to their proportionality to the molarity of the entire compound. For example, the protons on the methoxy residue from isosilybin A were in triple the molar concentration relative to the hydroxy and aromatic protons, and therefore met the expectation for triple the peak area.

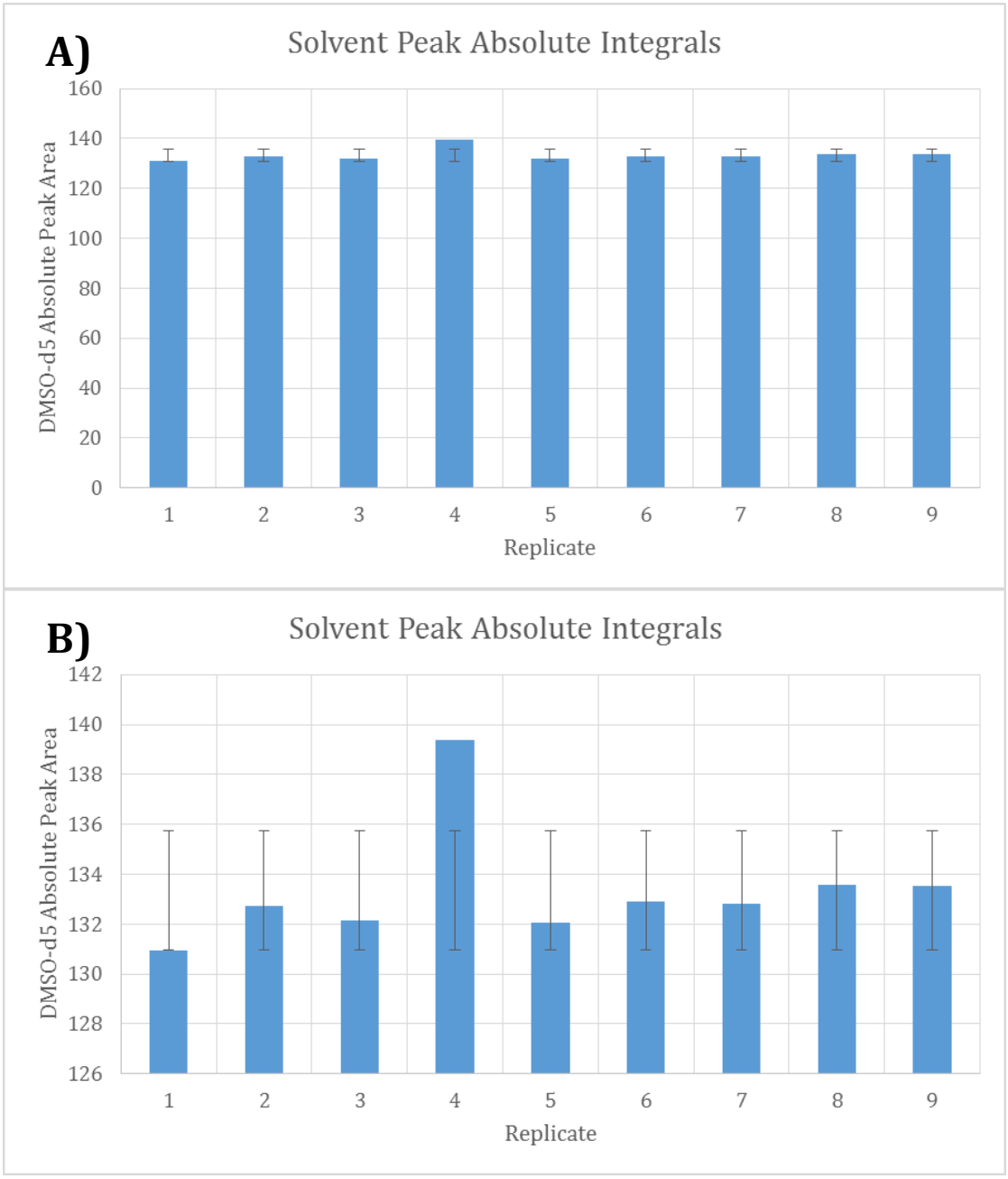


Figure 3. Absolute Integrals of DMSO-d_5 Solvent Peaks. Solvent peaks of 9 replicate samples were integrated and plotted. Replicate number 4 was shown with greater than 99% confidence via a q-test to not belong to the subset of the 8 samples. Error bars show the average and standard deviation for the combined set not including sample 4. A) Full height plot and B) zoomed in on the top region of the plot.

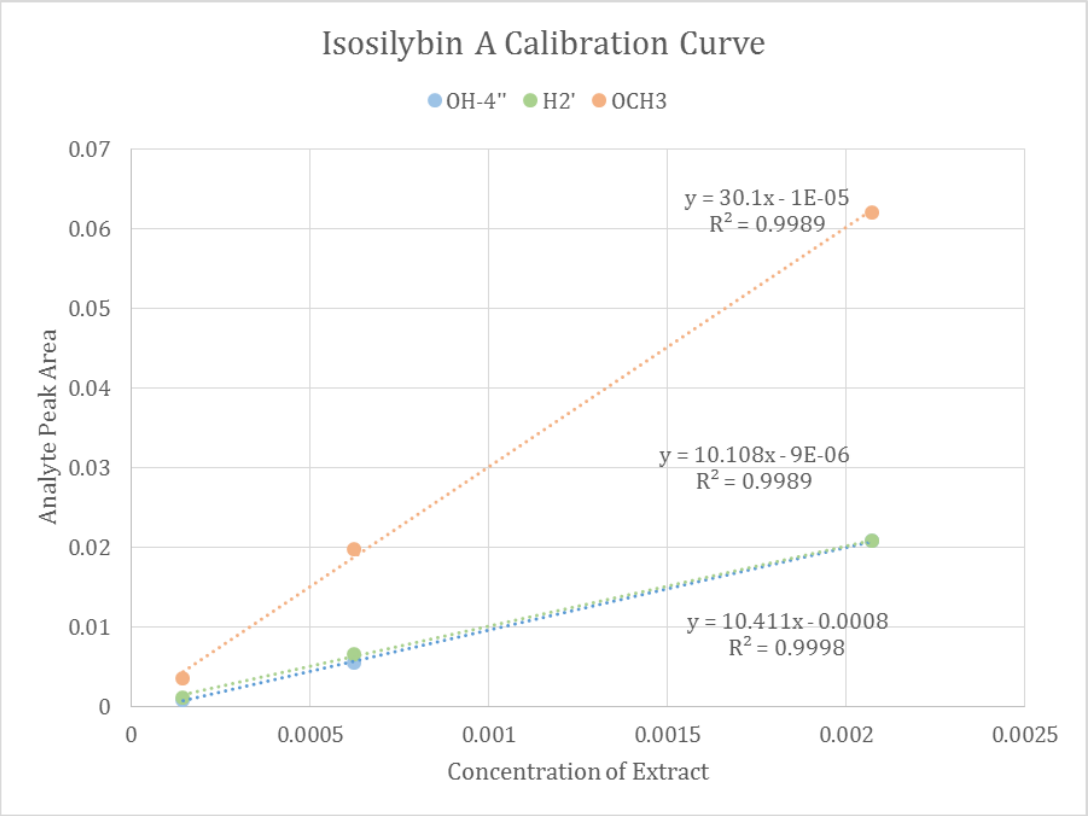


Figure 4. Calibration Curves with Regressions. Three different isosilybin A (**1**) peaks, OH-4'', H2', and OCH_3 were integrated at three different concentrations of sample. 1.0, 0.30, and 0.70 mg of sample per mL of solvent were used to span. Regressions were calculated for each peak showing that the peaks were linear. Since the methoxy peak represents three times the ^{1}H residues of the other two peaks, the signal increases proportionally, as shown by the slopes of the three lines.

CHAPTER II

EVALUATION OF FUNGAL ISOLATES FOR SECONDARY METABOLITE PRODUCTION USING qNMR

Introduction

In natural products research, pure compounds are often isolated in quantities of 1.0 mg or less. When isolates are in such small quantity, this amount is quickly consumed through biological testing and other experimentation. This stock of valuable compound then needs resupply through the fermentation of new batches of the fungal culture. In order to expedite future restocking, several organisms and complete media optimization studies may be tested to find the most productive organism and conditions for the resupply of the analyte of interest.[32-36]

The problem then falls to analysis of the samples. There are many ways that one could employ to analyze the various fermentations. Historically, analysis of resupply conditions re-isolated the compound of interest, so as to quantify the yield under new conditions.[33] However, this process can be time intensive, impeding further research on promising leads. Recent innovations in mass spectrometry, particularly ambient ionization techniques like LAESI,[37] DESI,[38] and MALDI,[39] or spatial mapping technique like droplet-LMJ-SSP[40] have drastically reduced the analysis time. These techniques can provide an *in situ* snapshot of the metabolite profile on a culture's surface well ahead of extraction or isolation processes. However, quantitative information is difficult to derive from this

information without further study due to lack of penetration of these techniques to analyze constituents embedded in the culture and media. LC-MS analysis of the raw extract is frequently used now to quantify individual mixture components,[41] and this requires the use of a standard reference sample of the analyte for determination of ionization efficiency or creation of a standard curve. This is often not possible if remaining pure sample after early experimentation is extremely low. Without calibration, LC-MS can only be used to give relative production of compounds of interest. While providing a useful picture of the relative biosynthesis, it would be useful to acquire quantitative information to plan for the productivity of further fermentations.

Quantitative NMR (qNMR) offers a method orthogonal to LC-MS. qNMR is a validated method[10] that can be used to quantify and analyze secondary metabolites upstream in the isolation and purification process. Aside from its non-destructive nature, qNMR offers several benefits over LC-MS. Quantitation does not necessitate a purified standard of the analyte to calculate a standard curve.[13] Additionally, NMR spectroscopy inherently contains some separation of constituent signals,[13] such that complex samples can be analyzed upstream of relatively pure samples in a way that is orthogonal to LC-MS or LC-UV quantitation. With these benefits, qNMR can be applied throughout the isolation process, providing quantitative measurements with which to compare differing culture conditions. Moreover, since NMR is frequently incorporated into natural products research schemes, [1, 2, 42-48] this process does not necessitate acquisition of new equipment or severe deviation in protocols. The end result is the selection of an efficient fungal strain and/or specific fermentation conditions and extrapolation of the quantitative information to a production scheme to resupply valuable compounds.

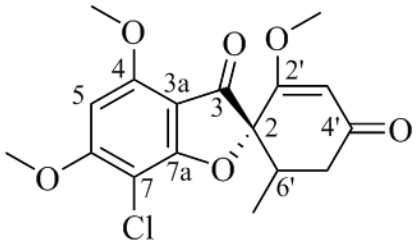
Griseofulvin (**1**) and dechloro-griseofulvin (**2**) (Figure 5, Appendices C, D) were observed in extracts of several fungi, which were coded MSX54665, MSX48662, and G536. Originally isolated from a filamentous fungus in 1939[49], compound **1** was one of the first antifungal compounds isolated from a natural product source and has been on the market for the treatment of several dermatological fungal infections in animals and humans.[50-52] The recent literature on **1** for activity against cancer and suppression of hepatitis C virus replication, in conjunction with the influx of patents for analogues of **1,** indicate the expanding interest in this class of compounds.[53-56]

In this study, these fungal isolates were evaluated for the production of secondary metabolites, **1** and **2**, to determine which would be most efficient for large scale production. A qNMR method was created and implemented to analyze extracts from these isolates in order to rank their biosynthetic potential. Additionally, two series of experiments were completed to investigate the robustness and repeatability of the qNMR method.

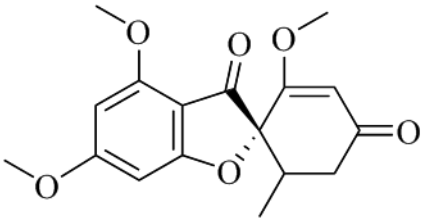
Experimental Section

Extraction

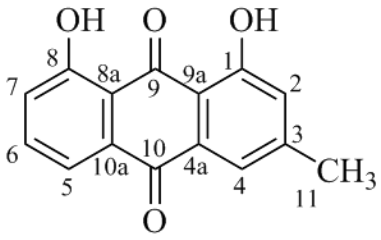
Separately, solid-substrate fermentations of the three fungi (Figure 6) were chopped with a spatula and shaken for 16 hr at 100 rpm with 500 mL MeOH/$CHCl_3$ in a 1:1 mixture. The supernatants were collected via vacuum filtration and solid substrates washed with 100 mL of 1:1 MeOH/$CHCl_3$. To the filtrates, 900 mL $CHCl_3$ and 1500 mL H_2O were added followed by 2 hr of stirring. The mixtures were transferred to separatory funnels, and the two layers were drawn off into independent flasks. The bottom layers were evaporated to dryness under vacuum and reconstituted in 300 mL of 1:1 MeOH/CH_3CN and 300 mL of hexanes. These solutions were transferred back to separatory funnels and shaken



Griseofulvin (**1**)



Dechlorogriseofulvin (**2**)



Chrysophanol (**3**)

Figure 5. Structures of compounds **1**-**3**.

MSX54665 G536 MSX48662

Figure 6. Solid Substrate (Rice) Fermentations of MSX54665, G536, and MSX48662.

vigorously. The MeOH/CH_3CN layers (i.e. organic extract) were evaporated to dryness under vacuum.

Sample Preparation

Organic extracts were reconstituted in DMSO-d_6 at 2.0 mg/mL for MSX 48662, 5.0 mg/mL for G536, and 10 mg/mL for MSX54655. The samples were weighed on a micro-analytical balance (XS105, Mettler Toledo), capable of giving readings of ± 0.01 mg. DMSO-d_6 99.9% (Lot #: PR-26893/10075DM1) was purchased from Cambridge Isotope Laboratories. Chrysophanol (**3**) (Figure 5) (99.3 % Lot No. 870622; Madaus, Germany) was used as a standard and was reconstituted using DMSO-d_6 at 0.50 mg/mL. All reconstituted fungal extracts were made in single stocks and then transferred in triplicate aliquots of 0.50 mL into standard 5 mm NMR tubes. Triplicate aliquots were used to achieve average quantities and to better avoid errors in quantitation.

Quantitative NMR

Quantitative NMR measurements were completed using a JEOL ECA-500, operating at 500 MHz for ^1H and 125 MHz for ^{13}C using parameters recommended in the literature.[13] For each set of 6 to 12 samples, auto-tuning was employed to optimize the probe. The autogain program routine was then run on the first sample in a dummy experiment to establish an optimal gain value. Each sample was then set to 90 % of the gain that was found optimal by the spectrometer on the first sample, in efforts to maximize signal while avoiding clipping. A 60 s relaxation delay was incorporated to ensure relaxation of most protons. Two dummy scans were applied to achieve steady state for each sample and was followed by 8 scans. A 90° pulse was applied to give maximum detector response. FIDs were acquired for 3 s. A large spectral width of 20 ppm was used, centered on 6.5 ppm to

prevent signal suppression from the decay that frequently occurs at the extremes of spectra.[5, 13] Sample temperature was maintained at 25 °C.

NMR files were processed using MestReNova software (Mestrelab Research, S.L.). Exponential apodization was applied using a value of 0.40 Hz, followed by phase correction. The baseline of each spectrum was corrected using the Whittaker Smoother routine included in the MestReNova software. The chemical shift was then adjusted to the DMSO-d_5 peak, which was set to 2.500 ppm. Analyte peaks used in quantitation were selected based on high intensity and relative isolation from neighboring peaks. Based on a close inspection of peak shape, peaks were selected if the majority of peak area was due to the analyte signal, rather than neighboring peaks. Peaks that didn't pass this scrutiny were also excluded from use for quantitation. Integration was then applied in a manner to exclude neighboring peaks. In the standard, and where possible in the analyte samples, integration was taken for a spectral width of 30 Hz. The solvent peak was integrated identically between the standard and analyte samples to ensure consistent integration and then was normalized to an arbitrary large value (i.e. 10,000.00). By setting the solvent peak to a large value, the relatively small analyte and standard integrals were comparable, so as to give a sense of variation of the concentrations in the samples. ^{13}C satelites were visible for the solvent peak but were not included in integration or purity calculations. Complete spectra and the assignment of peaks for **1** and **2** are included in the supplementary data and have been reported (Appendices C, D, E).[57]

Results and Discussion

Three separate fungal cultures were observed to biosynthesize griseofulvin (**1**) and dechlorogriseofulvin (**2**). The goal of this study was to determine which isolate produced

compounds **1** and **2** in greatest yield. The intensity of the analyte signals for MSX48662 were high enough to yield meaningful quantitative results at the initial concentration of 2.0 mg/mL. However, the organic extracts of MSX54665 and G536 made at 2.0 mg/mL had low signal intensity of **1** and **2** with S/N ratios ranging from 40-80 (Figure 7), which were not ideal for quantitation according to the literature.[5, 10, 13] In general, low S/N can hinder the ability to detect small impurities in the baseline surrounding analyte peaks. This has been seen to affect baseline correction and integration of analyte peaks, thereby introducing error into quantitation. Additionally, a high noise level can be seen to cause overcompensation of automatic baseline correction routines, which can skew integration downward. Minor impurities were likely to be hidden by noise if concentration was too low to allow their detection, due to the complexity of the spectral region of interest. To compensate for the low signal, the samples of MSX 54665 and G536 were concentrated to yield higher peak intensity, and then the NMR experiments were re-conducted to yield S/N ratio ranging from 120-240 for analyte peaks (Figure 8).

Purity Calculation

In order to calculate purity and yield from the various fungal cultures, amounts of **1** and **2** were calculated using the following formula:

$$m_x = \frac{S_x}{S_{cal}} \times \frac{N_{cal}}{N_x} \times \frac{M_x}{M_{cal}} \times m_{cal} \quad (1)$$

where x is the analyte being measured, cal is the calibrant being used, S integral of a particular analyte or calibrant peak, N is the number of protons represented by the peak being used for analysis, and M is the molar mass of the analyte or standard. In this case, m is

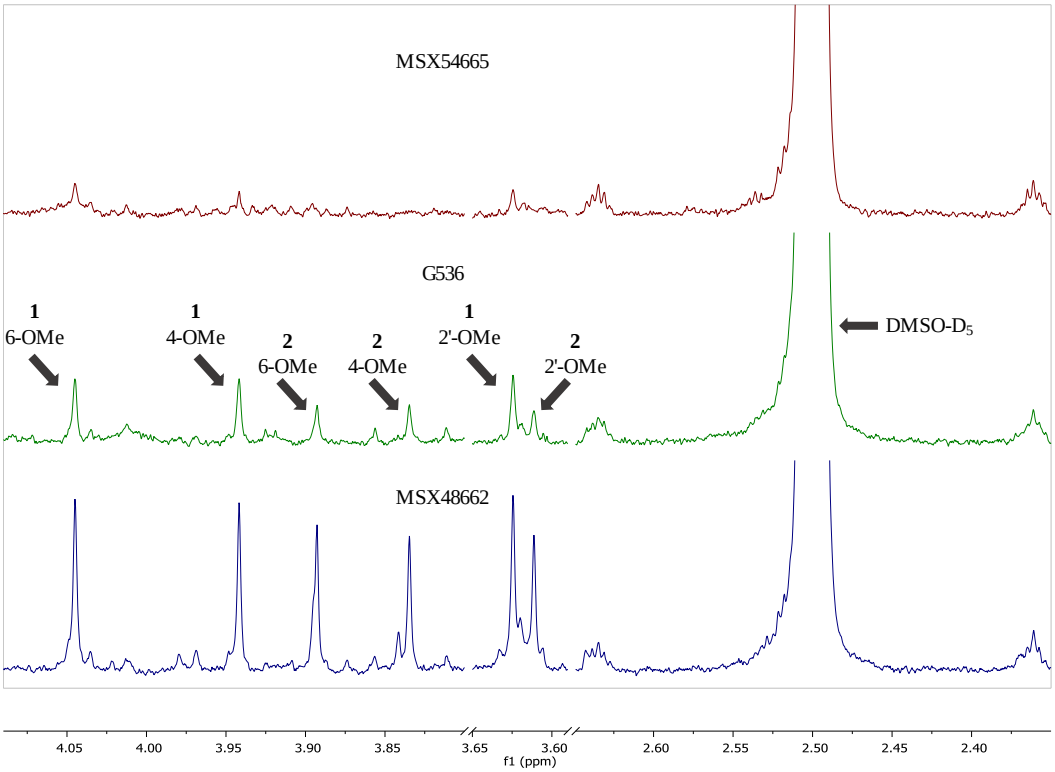


Figure 7. Selected Sections of the Spectra for MSX54665, G536 and MSX48662 at 2.0 mg/mL in DMSO-D_6. Given are the sections useful for quantitation of the methoxy groups. The DMSO-D_5 peak is also included for comparison.

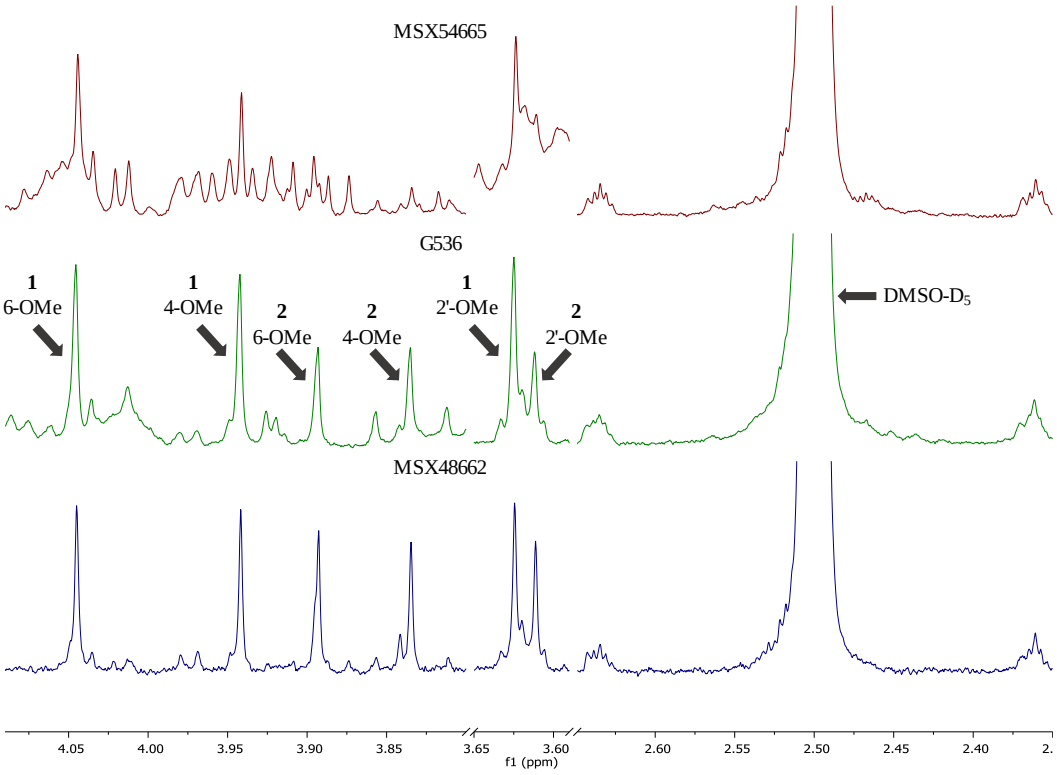


Figure 8. Selected Sections of the Spectra for MSX54665, G536 and MSX48662 at 10, 5.0, and 2.0 mg/mL, respectively, in DMSO-D_6. Given are the sections useful for quantitation of the methoxy groups. The DMSO-D_5 peak is also included for comparison. Peak height of spectra were normalized to the DMSO-D_5 peak.

the precise mass of the analyte or calibrant in the NMR tube. This value differs from the weighed sample amount, because it refers specifically to the analyte content and does not include the various "impurities" contained in the whole sample. In this project, **1** and **2** were analytes that were measured and **3** was used as a calibration standard. Simple analyte integrals were normalized to the solvent signal, so as to compensate for variation in integrals due to any dilution effect from contaminants (i.e. water), any signal variation between NMR tubes, or sample shimming in the magnet. As stated by Krunic and Orjala,[31] using one batch of DMSO-d_6 gave the best opportunity for proportional concentration of DMSO-d_5, which corresponds to the integrated area of the solvent peak.

Sample mass was then used to calculate purity (P_x) of the analyte in the sample using the following equation:

$$P_x = \frac{m_x}{m_{smp}} = \frac{m_x}{V_{smp} C_{smp}} \quad (2)$$

where smp is the sample that contains the analyte, V is the volume used in the NMR experiment and C is the concentration of sample inside the tube (Table 1). This concentration, C, refers to the weighed sample contained within the NMR tube and not just the analyte. This concentration was the anticipated concentration of sample from when they were prepared for NMR experimentation. This purity was then extrapolated to the extract to yield a calculated mass of analyte that was produced by the fungal strain using the equation:

$$m_{tot\,x} = m_{tot} \times P_x \quad (3)$$

where m_{tot} corresponds the mass of the organic extract, $m_{tot\,x}$ corresponds to the total content of the analyte in the extract (Table 2).

Result of Extract Comparison

The results indicated that MSX48662 yielded the highest total yield of **1** and **2** (149 ± 8 mg and 102±2 mg, respectively) (Table 2). The high mass of sample was indicative of a high rate of biosynthesis of the target compounds. Substantial degradation of **1** and **2** was not previously observed throughout research on this class of compounds and was therefore not attributed to the differences among isolates. This suggested that MSX48662 would be the best of the three candidate fungal cultures for the resupply of consumed stock of compounds **1** and **2**.

Difficulties of Analysis

During analysis, MSX54665 also showed a large population of signals between 3.8 and 4.1 ppm. These signals are similar in shape to methoxy signals that are seen for **1** and **2**. In addition to other contaminants, these signals may indicate a number of analogues that could be interesting from a research standpoint. Analogues would not necessarily be as evident when using LC-MS for quantification if deconvolution was used to analyze content. Furthermore, at the 10 mg/mL concentration of the organic extract of MSX54665, compound **2** was not readily identifiable from this large number of signals and was therefore not calculated (Table 1, Table 2, Figure 8).

Additionally, in the organic extracts of MSX48662 and G536, the 6-OMe signal of **1** and **2** had subtle shoulders that indicated peaks with little separation from the analyte peaks. These contaminant peaks wouldn't be differentiable without significant deconvolution procedures. Relatively, the 4-OMe peaks were less impacted by neighboring

Table 1. Purity of **1** and **2** in Fungal Extracts

	Compound **1**			Compound **2**		
Culture	Purity	SD	CV	Purity	SD	CV
MSX54665	0.0055	0.0003	5.8%	N/D	N/A	N/A
G536	0.0164	0.0005	3.3%	0.0095	0.0007	7.3%
MSX48662	0.076	0.004	5.0%	0.052	0.001	2.7%

Included are the results from one qNMR experiment. Each extract sample was weighed and aliquoted into triplicate NMR tubes and compared to a single triplicate set of standard. Purity represents a proportion of analyte in the extract; SD = Standard Deviation; CV = Coefficient of Variation.

Table 2. Mass of **1** and **2** in Fungal Extracts

	Compound **1**			Compound **2**		
Culture	Mass (mg)	SD (mg)	CV	Mass (mg)	SD (mg)	CV
MSX54665	5.4	0.3	5.8%	N/D	N/A	N/A
G536	4.6	0.1	3.3%	2.6	0.2	7.3%
MSX48662	149	8	5.0%	102	2	2.7%

Total mass of **1** and **2** in each organic extract are shown. Signals of 2 were not readily identifiable in the exract of MSX54665 and thereby mass of **2** was not calculated.

contaminants and were therefore used for quantitative measurement of **1** and **2** (Figure 8). These contaminants were relatively small compared to the analyte signals that were measured, and thereby their influence on the integration was assumed to be small, but may have affected the accuracy of the quantitative measurements i.e. overestimating the mass of each analyte. However, due to the complex nature of the organic extracts and the relativistic nature of this study, small error in the quantitative measurement would not be significant enough to change the results.

If extremely accurate quantitative information is required, such as in the analysis of reference standards, S/N can easily be improved by increasing the number of scans, enhancing the concentration, or with the use of a cryoprobe.[5] Moreover, minor impurities can be revealed if a higher magnetic field is used to increase spectral resolution, thereby separating closely shifted peaks.[5] An increase in resolution by using a higher magnetic field, or use of deconvolution methods could help to remove the influence of these contaminants or to elucidate the signals of **2** in the extract from MSX 54665.[8] In efforts to make the method generally applicable, however, standard instrumentation and a shorter timeframe was emphasized. Therefore, concentrations of the two extracts with low analyte signal were increased to give the desired S/N, and the method yielded meaningful comparative results in a reasonable timeframe.

Repeatability

Triplicate aliquots were made of a sample of **3** and MSX48662 at 0.50 mg/mL and 2.0 mg/mL, respectively. These aliquots were analyzed using the above method for content of **1**. The calculations were averaged to give purity and standard deviation. To determine the precision of the measurement, this process was completed twice more, each on separate

days to account for multi-day variability (Table 3). The average purity calculated from three days of the experiment was 7.4 ± 0.5 % (Table 3). The standard deviation was less on several of the individual days than for the average of all days. To estimate multi-day variability of the NMR spectrometer, a single set of standards and analytes from MSX48662 were run through the above qNMR analysis over three days (Table 4).

Previous literature has described the use of a solvent signal that is calibrated externally for quantification.[13] Using this method allows the sample to remain untainted from introduction of a calibrant into a valuable sample, but it requires careful weighing and pipetting. The smaller variability between days using the same sample and the higher variability between separate samples on separate days was taken to indicate that the majority of the error involved in the analysis occurs in the weighing and pipetting.

Applicability

Use of this method gave clear indication that MSX48662 biosynthesized the largest amounts of **1** and **2** per fermentation. The analysis technique could be further used to analyze separate growth conditions as well, as it is well documented that different growth conditions produce varying metabolite profiles.[32] Additionally, because we have chosen to use a method that does not require any internal calibrant, the contamination of the analyte is not a concern. Thereby, this method also lends itself to use for partially purified and pure samples, as it is unnecessary to complete further purification steps to recover the measured material. In particular, this method is extremely useful for novel compounds for which no analytical standard is available. This method could also be used to verify purchased or isolated samples for use as reference standards in other quantitative and qualitative methods.

Table 3. Complete Repitition of Method on MSX48662 Extract

	Compound **1**			Compound **2**		
Repetition	Purity	SD	CV	Purity	SD	CV
1	0.076	0.004	5.0%	0.052	0.001	2.7%
2	0.078	0.006	7.6%	0.055	0.005	8.2%
3	0.069	0.002	3.5%	0.048	0.002	4.4%
Tot.	0.074	0.005	7.1%	0.051	0.004	7.5%

Results are shown for MSX48662. Each repetition represents a complete set of qNMR triplicates. Each repetition was made from aliquots of a separate weighing of MSX48662 and **3**. Each repetition was completed on a separate day to include multi-day variation.

Table 4. Muliti-day Measurements of Single MSX48662 Sample

	Compound **1**			Compound **2**		
Day	Purity	SD	CV	Purity	SD	CV
1	0.076	0.004	5.0%	0.052	0.001	2.7%
2	0.076	0.002	2.0%	0.054	0.002	3.3%
3	0.081	0.001	1.5%	0.0561	0.0007	1.3%
Tot.	0.077	0.003	4.0%	0.054	0.002	4.0%

Results are shown for MSX48662. One set of triplicate samples was measured over 3 days. The triplicate set corresponds to a single weighing of MSX48662 that was separated into 3 aliquots. The results imply that variation due to the day-to-day drift of the NMR spectrometer is small compared to other factors.

Various techniques and instrumentation were observed to further assist in the quantitation of various analytes and could be useful for analysis of complex mixtures. In the case of heavy contamination of the analyte signals, use of flash chromatography to give simpler metabolite profiles could yield less contamination without greatly increasing the preparation. With a stronger magnet, the peak resolution was subsequently increased to allow heightened accuracy and confidence in measurements in any particular analyte. Modern cryoprobes heighten the sensitivity to low abundant samples by improving noise reduction,[31] and their use consequently increased the S/N ratio. Reduced volume NMR tubes have effectively allowed for measurement of reduced mass of sample by increasing the signal of the analyte.[31] Additionally, as stated before, the number of scans can be increased to easily increase the S/N ratio at the expense of time, with diminishing returns.

Conclusion

In natural products research, the world's supply of a promising compound can be rapidly consumed in follow-up bioassay studies. Analysis of the experiments designed to efficiently resupply these compounds can be challenging without a well characterized reference sample. NMR has the ability to quantitate any proton, even in the absence of a reference standard. Thereby, nearly any secondary metabolite can be measured using this method. In this study, MSX48662 yielded the most of the target compounds and was therefore the best of the three fungal cultures to supply griseofulvin (**1**) and dechlorogriseofulvin (**2**) for further experimentation. The use of qNMR afforded an orthogonal method to LC-MS that enabled quantitation of **1** and **2** within crude extracts, which allows for the time efficient start of further fermentations.

CHAPTER III

CONCLUSION

An initial qNMR experimental design was based off of a rudimentary LC-UV signal-based method of quantitation. Total signal from a specific analyte would be divided by the total sum of signal from all compounds. This resulted in several experiments that measured the content of isosilybin A and ustilaginoidin F content of two samples. The results were "purities" with relationship to the proportion of signal that each analyte was responsible for. However, problems in this method stemmed from the inconsistent ^{1}H to mass ratio of organic compounds that causes a purity calculation to deviate from an analyte mass to total mass ratio for a sample, which is the accepted definition. The application of a signal based method would be limited to a subset of compounds whose masses correlate well to their resulting ^{1}H signals and thus further qNMR methodology more closely followed the mass-based definition of purity.

The subsequent experiments sought to analyze the consistency of the residual solvent signals and the linearity of the signal with varying concentration. Several experiments were conducted using samples dissolved in 99% DMSO-d_6 to conclude that the peak area of the residual DMSO-d_5 peak was very consistent such that outliers were readily identifiable. Three samples of increasing concentrations of isosilybin A were measured using established quantitative parameters.[13, 31] These samples were found to be linear with R^2 values greater than 0.9988 and intercepts of less than 0.001 mg per 100 mg of extract.

Additionally, slopes of the linear regressions were proportionate to the multiplicities of the ^{1}H residues responsible for the signals. These experiments gave confidence in the performance of ^{1}H NMR signal integration as a quantitative measure of analyte content.

A qNMR analysis was then performed on dried organic extracts of three filamentous fungi, G536, MSX54665 and MSX48662. qNMR was performed according to established collection and interpretation parameters and was used to gauge the biosynthetic potential of the three fungal isolates for griseofulvin, a valued secondary metabolite and an analogue, dechlorogriseofulvin. MSX48662 yielded the highest quantity of griseofulvin at 149±8 mg. Therefore MSX48662 would be the likely choice for the supply of griseofulvin for further experimentation.

External calibration was used to yield a single method that would be applicable to a wide variety of samples. An external calibration prevents the sample from contamination with the calibrant. Thus, the method is suited for use with the entire range of compound purities. Use of the JEOL 500 MHz NMR, 99% DMSO-d_6, and 5mm NMR tubes, all standard NMR equipment, also allow for routine use of the qNMR methodology.

Quantitative ^{1}H NMR offers a valuable and precise tool for analysis of natural product samples. qNMR removes many of the faults of LC-UV or LC-MS with natural products such as: imprecision in relative concentration determination due to variation among extinction coefficients or ionization efficiency; the lack of detection of organic compounds without conjugation or other capacity for electronic excitation. This makes qNMR extremely useful for samples in which many analytes are being measured; just one set of calibrant standards are needed in order to quantify all analytes. Additionally, qNMR is an increasingly popular technique[18] that can be utilized for absolute quantification of

analytes. The software tools to utilize qNMR in studying metabolite profiles or precise spectral modeling are becoming increasingly popular as well and offer a range of complexity to spectral interpretation.[7, 8] Its orthogonality allows qNMR to be used in addition to other techniques and has been touted as the newest standard in purity determination by its proponents.[17] Natural products research schemes would do well to learn from or adopt these increasingly popular analytical techniques.

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APPENDIX A

TABULATED PEAKS OF ISOSILYBIN A ANALYSIS

Isosilybin-A	84-1				
Peak shift (ppm)	Area	No. of protons	Class	% of signal	
3.339	104.29	1	primary	0.046517541	
3.532	112.49	1		0.050175071	
3.776	305.69	3		0.136350052	
4.167	107.64	1		0.048011775	
4.596	101.80	1		0.045406900	
4.912	97.97	1		0.043698566	
4.957	99.92	1		0.044568345	
5.105	100.60	1		0.044871652	
5.836	95.70	1		0.042686055	
5.884	90.47	1		0.040353264	
5.919	91.83	1		0.040959879	
6.803	96.16	1		0.042891233	
6.854	98.28	1		0.043836838	
6.933	90.84	1		0.040518299	
6.986	103.53	1		0.046178550	
7.003	89.28	1		0.039822476	
7.092	97.41	1		0.043448783	
9.159	91.41	1		0.040772542	
10.857	100.00	1		0.044604028	
11.900	84.77	1		0.037810834	
Total	**2160.08**	**22**		**0.963482682**	**0.963482682**
1.91	1.30		Impurity	0.000579852	
3.16	4.32			0.001926894	
3.17	3.39			0.001512077	
3.63	2.85			0.001271215	
3.92	2.59			0.001155244	
4.08	2.03			0.000905462	
4.09	1.85			0.000825175	
4.88	2.25			0.001003591	

4.99	2.04			0.000909922	
5.00	2.57			0.001146324	
5.02	2.00			0.000892081	
5.47	3.52			0.001570062	
5.91	5.2			0.002319409	
5.94	3.67			0.001636968	
6.20	2.50			0.001115101	
6.25	2.05			0.000914383	
6.27	1.87			0.000834095	
6.47	2.30			0.001025893	
6.79	2.48			0.001106180	
6.90	1.66			0.000740427	
6.92	3.15			0.001405027	
6.95	1.77			0.000789491	
7.04	3.23			0.001440710	
7.08	1.95			0.000869779	
7.70	1.74			0.000776110	
7.72	1.62			0.000722585	
7.87	2.37			0.001057115	
9.13	3.92			0.001748478	
9.17	2.60			0.001159705	
9.61	2.82			0.001257834	
11.88	3.34			0.001489775	
12.42	2.22			0.000990209	
Grand total	**2241.95**				
Peaks <.75 peak area not included.					
2.5	590.53		Solvent	DMSO – D_6	
3.33	926.12			H2O	

Impurity intensities are heat mapped. Green are impurities with smaller peak areas. Yellow and orange are impurities with moderate peak areas. Red are impurities with substantial peak areas.

APPENDIX B

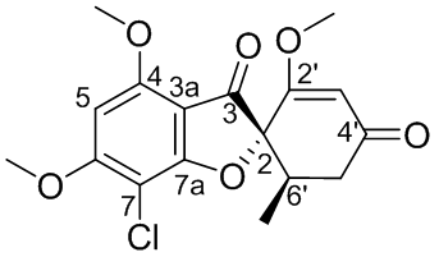
TABULATED PEAKS FROM USTILAGINOIDIN F ANALYSIS

Ustilaginoidin F		01037-147-2			
peak shift (ppm)	area	no. of protons	class	% of signal	
9.73	100.19	1	primary	0.095007349	
6.53	100.19	1		0.095007349	
5.93	96.77	1		0.09176426	
5.42	100.00	1		0.094827177	
4.45	104.79	1		0.099369399	
2.65	209.50	2		0.198662937	
1.4	303.96	3		0.288236689	
total	**1015.40**	**10**		**0.96287516**	**0.96287516**
				0	
1.22	14.81		Impurity	0.014043905	
1.26	1.73			0.00164051	
1.27	1.62			0.0015362	
2.04	3.08			0.002920677	
2.55	1.22			0.001156892	
3.64	1.32			0.001251719	
3.7	8.32			0.007889621	
5.32	1.09			0.001033616	
5.76	0.92			0.00087241	
5.96	1.19			0.001128443	
6.09	1.02			0.000967237	
6.36	0.75			0.000711204	
6.58	1.02			0.000967237	
6.69	1.06			0.001005168	
grand total	**1054.55**				
peaks <.75 peak area not included.					
7.24			Solvent	CDCl3	
1.55				H2O	

Impurity intensities are heat mapped. Green are impurities with smaller peak areas. Yellow and orange are impurities with moderate peak areas. Red are impurities with substantial peak areas.

APPENDIX C

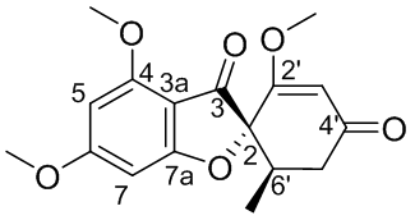
GRISEOFULVIN NMR DATA



Position	δC	Type	δH ppm, mult. (J in Hz)	HMBC
2	90.1	C		
3	191.2	C		
3a	104	C		
4	157.6	C		
5	91.3	CH	6.50, s	7, 3a, 4, 6, 3
6	164.5	C		
7	95.2	C		
7a	168.6	C		
2‘	170.2	C		
3‘	104.6	CH	5.60, s	2, 2‘, 4‘, 5‘,
4‘	195.5	C		
5‘	39.8	CH2	2.35, dd (16.7, 4.8)	2, 4‘, 6‘
			2.67, dd (16.7, 13.3)	
6‘	35.5	CH	2.80, dqd (13.3, 6.7, 4.8)	2, 3
6‘-Me	13.8	CH3	0.80, d (6.7)	6’, 5’, 2
4-OMe	56.6	CH3	3.94, s	4
6-OMe	57.6	CH3	4.05, s	6
2‘-OMe	57	CH3	3.63, s	2’

APPENDIX D

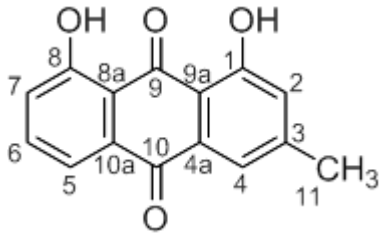
DECHLOROGRISEOFULVIN NMR DATA



Position	δC	Type	δH ppm, mult. (J in Hz)	HMBC
2	89.2	C		
3	191.3	C		
3a	103.2	C		
4	158.6	C		
5	93.4	CH	6.23, d (1.76)	3, 3a, 4, 6, 7
6	170.2	C		
7	89.1	CH	6.48, d (1.76)	3a, 5, 6, 7a
7a	175.4	C		
2‘	170.9	C		
3‘	104.4	CH	5.58, s	2, 2‘, 4‘, 5‘,
4‘	195.8	C		
5‘	39.8	CH2	α 2.33, dd (15.3, 3.4) β 2.68, dd (15.3, 13.2)	2, 3‘,4‘, 6‘
6‘	35.6	CH	2.71, m	2, 3, 4‘, 5‘
6‘-Me	13.9	CH3	0.81, d (6.3)	6‘, 5‘, 7
4-OMe	56	CH3	3.84, s	4
6-OMe	56.5	CH3	3.90, s	6
2‘-OMe	56.9	CH3	3.61, s	2‘

APPENDIX E

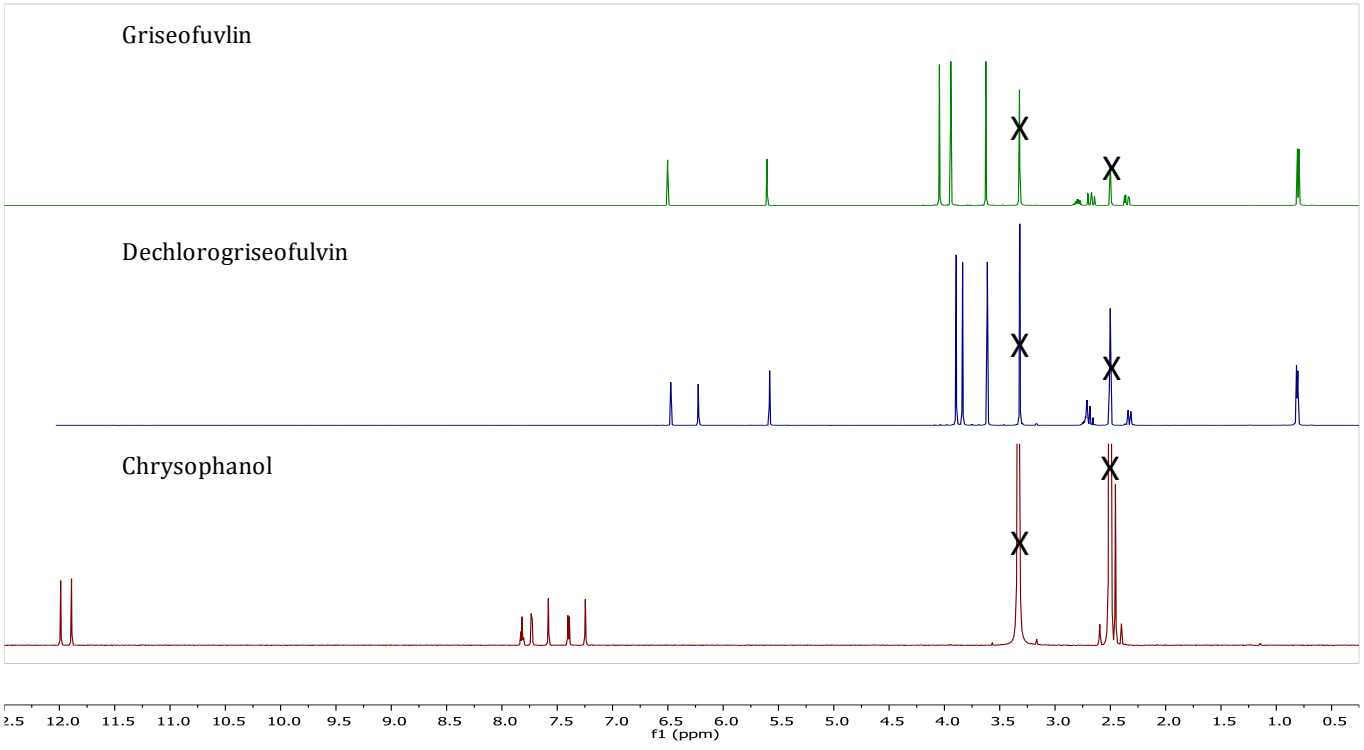
CHRYSOPHANOL NMR DATA



Pos.	δH ppm, mult. (J, Hz)
1	
2	7.24, d (1.7)
3	
4	7.58, d (1.7)
4a	
5	7.73, dd (1.1, 7.5)
6	7.82, dd (7.5, 8.3)
7	7.40, dd (1.1, 8.3)
8	
8a	
9	
9a	
10	
10a	
11	2.45, s
1-OH	11.9, s
8-OH	12.0, s

APPENDIX F

^{1}H NMR SPECTRA



^{1}H spectra for griseofulvin (**1**), dechlorogriseofulvin (**2**), and chrysophanol (**3**)