Characterization of Microcystin Products at Beagle Bioproducts, Inc.
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Harmful Algal Blooms (HABs) are a threat to people and the environment in part because toxins, especially microcystins in the American Midwest, can be produced by the resident cyanobacteria. Understanding this threat can be a challenge because reliable standards for monitoring toxins and for basic research purposes are not consistently available, if available at all. As a company poised to address this issue of availability, Beagle Bioproducts intends to be fully transparent about our products’ specifications, so that our customers can be confident not only in their purchases but more importantly in their conclusions. What follows is a description of how we determine the identity, purity, and quantity of our microcystin products, so the end user can have this level of confidence.

Identity

Initial identification of a microcystin is relatively straightforward. First, its absorbance spectrum is obtained, and has a maximum peak at 238 nm that corresponds to the signature Adda residue found within all microcystins. Specific congeners may yield additional unique signatures in the absorbance spectrum, like the peak also at 230 nm for microcystin-YR. Significantly, UV absorbance alone is not sufficient to verify the congener of microcystin; the toxin must be subjected to mass spectroscopic (MS) analysis to confirm it has the correct mass for a microcystin and to determine its congener identity. Also unique modifications, like desmethyl residues, will become apparent through the MS analysis. We are exploring the addition of nuclear magnetic resonance (NMR) analyses for our products, especially since there are natural isomers that typically are part of any microcystin preparation. While it would be nice to represent the isomer fractions for our products, at this time we must analyze whether the additional cost of doing NMR, which would be passed along to our customers, is worth it. We will write more about this in the future, and feel free to contact us at info@beaglebioproducts.com if you would like to offer an opinion on that point!

Purity

The end goal of a purity analysis is to simply quantify the amount of microcystin and compare that to the amount of total material; if the product is pure then both amounts will be the same. This requires the ability to separate the target compound from everything else, which for microcystins requires a reverse phase HPLC approach with a C18 column and photodiode array (PDA) detection. The column facilitates separation of microcystins from contaminating compounds, and the other factors dictating separation are properties of the contaminants themselves, and the properties of the mobile phase that delivers the compounds to the column. What is paramount is that the microcystin is resolved from all other impurities meaning that nothing co-elutes with the toxin, including other microcystin congeners. That is the hard part of purity analysis, which actually requires multiple analyses to be sure the microcystin has been completely resolved from the impurities.
To accomplish this resolution, Beagle employs at least two different HPLC approaches which differ in the composition of the mobile phase, usually with one based on acetonitrile and the other based on methanol. The affinities of different microcystin congeners for the column, as well as the affinities of impurities for the column, change with the different solvents in the mobile phases. For example, with one type of C18 column we use, microcystin LR and YR co-elute before microcystin RR when we use the acetonitrile-based mobile phase, but with the methanol-based mobile phase, microcystin RR elutes before YR, which elutes before and is resolved from LR. Therefore, if the purity of our product is the same when analyzed with these two mobile phases, then that is a good indication that there is no other co-eluting compound, because it is unlikely that an impurity has properties so similar to the microcystin that its retention time shifts the exact same way. Yet it is still possible that there are co-eluting compounds; in fact, it is technically impossible to be absolutely certain that there is nothing co-eluting using HPLC-PDA.

Common among the software of HPLC-PDA detectors, including Shimadzu’s LC Solutions software used at Beagle, is a feature called a peak purity analysis that assists in elucidation of co-eluting compounds “hidden” in what appears to be a peak from a single compound. Such an impurity might not reveal itself via a peak “shoulder.” If a peak is the consequence of an impurity that co-elutes at the same time as the compound of interest, and if that impurity has a different absorption spectrum than microcystin, then the part of the peak that also contains the impurity will have a different spectrum than the rest of the peak. To perform the analysis the software converts the spectrum at each time point to a vector and compares the cosine of the angle between vectors to a threshold value that is set by the noise of the chromatogram (less noise results in a more stringent, and therefore lower, threshold value). Similar spectra have similar directions when converted to vectors, so the angle between them will be near zero (or the cosine of the angle will be near one). If the angle between them is less than the noise threshold (or the cosine of the angle is greater) then the spectra are not different. A purity curve is created by plotting these values for each time point, so a simple examination of the purity curve will indicate the uniformity of the peak’s spectra. If the peak has a consistent spectrum then no co-eluting compounds were detected. Unfortunately, if the impurity has a similar absorption spectrum to a microcystin or if their retention times are very similar (making them less resolved) then this method will not be able to distinguish the impurities. As such, one should always view these automated purity analyses with some skepticism; they are a helpful tool but not a stand-alone decision maker.

While the peak purity analysis is performed to continue to assess the resolution of the microcystin, MS analysis is the method of choice to determine if there are co-eluting compounds. MS adds a second dimension to compound separation, if it is in line with an HPLC, by further separating the eluates based on their mass to charge ratio. Therefore if multiple compounds are in the same eluate, they can usually be detected because it is unlikely that compounds in the same eluate will have the same mass.

Presuming that differing mobile phases, peak purity analyses, and MS will resolve the microcystin from all the impurities, we can proceed to quantify the amounts of microcystin and the resolved impurities so that we can calculate the percent purity. Recall from the opening sentence of this section that this basic quantitation is actually the goal of the purity analysis. This is performed by
analyzing the absorption characteristics of the HPLC-separated compounds generated by the PDA. A major assumption is that all impurities can be detected by the PDA, meaning that they all have an absorption in the UV-Vis spectrum. If this is not the case, or the impurity is only weakly absorbing then the final purity quantitation will be overestimated. Unfortunately, there is no universal detector that can measure all imaginable impurities, but utilizing multiple detectors greatly reduces the probability that an impurity will remain unseen.

The second detector that is used in conjunction with the PDA is again an MS. With MS, a trace that monitors the total ion count can be generated and, when compared to the trace generated by the PDA, can bring to light any impurities that were not detected by the PDA. The MS can only be used for qualitative purposes in this manner because the identity of each impurity is not known; therefore, it is impossible to make a comparison to a standard or to know their individual ionization efficiencies. This same predicament also applies to data generated by the PDA, where the extinction coefficients for each impurity are not known, making it impossible to ascribe an actual mass amount to the impurities. That is why it is common to assume that all the extinction coefficients are the same.

Historically for microcystins, the single wavelength at 214 nm is used to visualize all compounds and to quantitate the purity by dividing the peak area of the microcystin by the total integrated area of the 214 peak to obtain percentage purity. With the advent of the PDA, an alternative method has become common that uses the wavelength of each compound’s maximum absorption ($\lambda_{\text{max}}$) instead of the single 214 nm wavelength. This “maxplot” is generated by plotting the maximum value of the entire absorption spectrum for each individual time point. Thus if a compound does not absorb at 214 nm or absorbs more strongly at a longer wavelength then it will not be under represented, but if an impurity does have a maximum absorbance at 214 nm then it will still be counted. Even this still assumes that the extinction coefficients at the $\lambda_{\text{max}}$ are the same, but there is no better way to compensate for different absorption characteristics without knowing more information about each impurity.

Once it has been confirmed that a certain HPLC method results in a resolved microcystin peak, the maxplot chromatogram is integrated using the default parameters of the LC Solutions software. The max plot monitors wavelengths between 210 and 400 nm because noise at the longer wavelengths in our detector can be more intense than the UV signal for minute impurities. Considering that the maxplot only records the highest absorbance, that steady noise can hide bonafide impurities. To analyze the entire UV-Vis range, a second maxplot chromatogram monitoring 400-800 nm of the same PDA data is also analyzed, but all impurities analyzed to date have had a stronger UV absorbance. Before the total integrated area value is calculated, peaks that also appear in an analysis of the solvent used to dissolve the sample are removed; therefore, impurities that are only found in the sample, not in post sample handling, are included in the purity analysis. The final purity percentage is calculated by dividing the area integrated under the resolved microcystin peak by the total integrated area, from a maxplot chromatogram, less the area for peaks that are due to the solvent. Due to the assumptions pointed out in this section, we aim for and report the purity as $\geq$ a percentage, typically $\geq 95\%$ for microcystins.
Quantity
The last specification is that the vial actually contains the quantity of microcystin that is reported and purchased. The concentration of microcystin (typically dissolved in MeOH) is derived from its absorbance at 238 nm and its extinction coefficient (ε). In the case of most microcystin congeners the exact ε is not known, so the ε for microcystin-LR, 39800 M⁻¹cm⁻¹, is used. Its concentration is also verified against an HPLC-based standard curve. We **strongly** encourage our customers, whether they've purchased their microcystin from us or another vendor, to make quantity verification by spec analysis the first step they do.

Quality Control
As a new provider of these products Beagle Bioproducts feels we shouldn’t expect our customers to just take our word that the products they get are what they purchased, and we offer upon request the raw data from analyses performed for a production lot. During this phase of establishing our credibility with our customers, we pay for third party verification of our results, and we also have a third party perform all of the MS analyses. Putting it plainly, we don’t have an MS in-house, but as described above we believe it is critical to truly ensuring the specifications of the product. The raw data from MS analyses will not be cut/pasted into our Certificates of Analysis, but we will indicate when the analyses have been done and we can make the data available upon request.

We welcome your input on our process described here ([info@beaglebioproducts.com](mailto:info@beaglebioproducts.com)), and hope you will agree that it offers the highest industry standard for product specifications presently available.