

Adding Analytical Metrics to the Production and Aging of Whiskey Using a Protein Sensor Assay

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Abstract

We have developed a protein-based sensor assay (PSA) that is both highly selective and broadly applicable for differentiating complex biological samples from one another. The PSA involves incubating samples with serum albumin protein, followed by analysis of bound molecules by mass spectrometry. Our previous work has shown that the assay greatly reduces the number of detected features while simultaneously improving the classification of complex biological samples. In this work, this protein based assay was applied to a set of whiskey samples produced by a craft distillery. The results of this analysis showed that whiskey samples aged for different lengths of time could be separated using unsupervised statistical analyses and a visible trajectory of the aging process was apparent. In a separate experiment designed to determine if subtle changes made during processing could be detected, partially aged whiskey was transferred to new barrels and subsequently tested. In this case, there were only subtle differences in taste between the samples, yet they were easily differentiated by the PSA. This analytical method shows great promise as a tool that can provide objective measures to food and beverage production which could improve quality and reduce cost.

Keywords: Bovine serum albumin; Protein sensor; Whiskey; Metabolomics; LC-MS; Flavor Profiling

Introduction

Differentiation of complex biological samples in a rapid and reliable manner is a major challenge for analytical chemists and biologists alike. Recently, the utility of a protein-based assay (PSA) for differentiating wine varieties, cellular stress, and urine was demonstrated [1,2]. The assay uses a highly abundant protein in mammalian blood, serum albumin (SA), as a sensor that works by binding a subset of the small molecules present in a sample. Analysis of the selectively bound molecules by liquid chromatography mass spectrometry (LC-MS) improves the ability to differentiate and categorize samples compared to direct analysis by LC-MS. The assay takes advantage of the biological role of SA as a transport protein in blood, picking up and delivering a cargo of fatty acids, drugs, metal ions, and other small molecules [3-8]. As an analytical tool, the PSA works by selective concentration; reducing sample complexity and time required for analysis [2]. This selective concentration results in enhanced differentiation of samples and clustering with respect to biological treatment. In this work, the utility of the PSA as an analytical tool for tracking the production and aging process of whiskey was evaluated.

The process of fermenting cereal grains into ethanol, and subsequently alcoholic beverages, has a long and storied tradition. It is thought that the first alcoholic beverage produced by fermentation came about in China, nearly 9,000 years ago [9]. The ancient Egyptians and later the Greeks refined the process and began to understand the benefits of consuming alcoholic beverages [10,11]. However, it was not until the 1500's when the distillation of fermented cereal grains began to grow in England, Ireland, and Scotland, and the production of whiskey was born [9]. Since then, whiskey has grown into a world market, with production in North America, Europe, and Asia [9]. As whiskey demand grew, so did production, and along with this came an increase in adulteration [9,12]. To amend this, countries put in place measures designed to standardize production and quality [9,12,13]. The rise of craft distilleries in the last decade is changing the landscape of the whiskey market and raising old questions [13-16]. From a business standpoint, a method for speeding up the aging process of whiskey has sparked a boom in approaches and businesses focused on maturing whiskey [17]. This has heightened the need for analytical methods that can provide metrics that allow distilleries to track the aging process and identify adulterated or suboptimal product.

The first reported use of a scientific assay for testing quality and consistency in the whiskey industry was in the early 1900s [18]. Before this there was a large fissure between distillers and scientists, with each wanting nothing to do with the other. In general the industry has been slow to adopt new methods and only in recent years has there been a move to apply analytical techniques such as mass spectrometry to aid production [19]. Methods for the analysis of whiskey to determine flavor profiles have largely been done using headspace or solid-phase microextraction (SPME) coupled to gas chromatography based mass spectrometry (GC-MS) [19-21]. These techniques are specific for volatile compounds. More comprehensive analyses based on LC-MS are now being developed [22]. The primary goal of the GC and LC-MS based analyses was to identify components common to specific whiskeys (for example Scotch) that could be used to authenticate origin and purity, as well as classify flavor profiles from different distilleries [19-23]. A limitation to widespread use of these techniques is the time and effort in sample preparation and analysis. Each sample can take as much as 90 minutes. A method that reduced sample preparation and acquisition time, while enhancing information pertinent to the analysis would be of significant value.

With the success of the PSA in previous work in mind, a series of experiments were undertaken to determine whether the PSA could be of use as an analytical tool in the production and aging of whiskey. To this end, experiments were set up to test the value of the PSA in tracking larger changes brought about through the aging of whiskey and if the approach was sensitive to the subtle changes brought about by alternative aging processes. Samples of whiskey were obtained from a local craft distillery and treated with the PSA before analysis by LC-MS. Results showed that the PSA was able to differentiate whiskey

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samples, both with respect to time and barrel type. Although more testing is needed, the work presented here could have implications for craft distillers and larger manufacturers alike, where the PSA could be used to give a metric to the quality control process in whiskey making. Additionally, the PSA can speed sample preparation, reduce data acquisition time and improve capabilities to differentiate samples.

Methods

Materials

Bovine serum albumin (BSA) (greater than 98% agarose gel electrophoresis pure) was purchased from Sigma-Aldrich (St. Louis, MO). Molecular weight cutoff spin filters and syringe filters were purchased from Pall (Port Washington, NY). The buffer agent 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and Sodium Chloride were purchased from VWR (Radnor, PA) at a purity of 99% or greater. All solvents were purchased as HPLC grade; water from Avantor (Center Valley, PA), methanol and acetonitrile from EMD Chemicals Inc. (Gibbstown, NJ). Formic acid (98% GR ACS) for use as an LC ion pairing agent was purchased from EMD Chemicals Inc. (Gibbstown, NJ). Wheat bourbon and rye bourbon whiskeys were supplied by Willie's Distillery Inc. (Ennis, MT). Samples of whiskey were taken from barrels, placed in Falcon tubes and stored at -80°C until analysis.

Preparation of BSA

BSA was prepared for use in the PSA exactly as previously described [1,2]. Briefly, a solution of BSA at 30 mg mL⁻¹ in 10 mM HEPES buffer (pH 7.0) was washed over a 3 kD molecular weight cutoff (MWCO) spin filter. The protein was washed three times with HEPES buffer to remove small molecules and contaminants remaining which would interfere with the assay. Following washing, BSA was re-suspended in 10 mM HEPES buffer (pH 7.0) at a concentration of roughly 30 mg mL⁻¹ and frozen at -20°C until analysis. Prior to analysis, the BSA stock was diluted to 0.1 mg mL⁻¹ with 250 mM NaCl, 10 mM HEPES (pH 7.0) buffer. Molecular weight cut-off (MWCO) spin filters were washed with deionized water prior to use to remove any preservatives as recommended by the manufacturer.

PSA treatment of whiskey samples

Prior to PSA treatment, whiskey samples were pre-filtered with a 10 kDa MWCO spin filter to remove proteins and particulates from the manufacturing process. A filtered whiskey aliquot of 100 µL was combined with 300 µL of H₂O and 100 µL of BSA at 0.1 mg/mL in HEPES buffer and allowed to equilibrate for 5 minutes. The solution was then washed over a 3 kDa MWCO spin filter and spun at 9500 × g for 5 minutes. The supernatant was removed and the filter was then washed with a solution of 10 mM HEPES buffer (pH 7.0, 250 mM NaCl) to remove nonspecific material. Following this, 70% MeOH was added to the top of the spin filters and centrifuged three times to remove metabolites bound to BSA. The supernatant containing metabolites which were bound was then dried using a speed-vac and stored at -80°C. To control for containments stemming from protein, solvent, and filters throughout the PSA process, experimental blanks were conducted.

Mass spectrometry analysis of PSA treated whiskey samples

Analysis by LC-MS was done as described in detail previously [1,2]. Briefly, samples were re-suspended in 50% aqueous methanol (MeOH) and injected onto a Kinetex 1.7 µm C18, 150 mm x 2.1 mm column (Phenomenex, Torrance, CA) kept at 50°C. An Agilent 1290

UPLC (Santa Clara, CA) system with a flow rate of 600 µL min⁻¹ was used for LC separation. The solvent system consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile for solvents A and B, respectively. A solvent program of 2% B for 2 minutes, to 95% B over 10 minutes, with a hold at 95% B for 2 minutes, before finally returning to 2% B for 1 minute, for a total run time of 15 minutes was used. For MS acquisition, an Agilent 6538 Q-TOF Mass Spectrometer (Santa Clara, CA) was used, operating in positive ion mode, with a cone voltage of 3500 V and fragmentor voltage of 120 V. Drying gas (N₂) temperature was 350°C with a flow of 12 L min⁻¹ and the nebulizer was set to 60 psig. Spectra were collected at a rate of 2.5 Hz with a mass range of 50 to 1000 m/z.

LC-MS data analysis

A pipeline for analysis of LC-MS data from PSA treated samples was modified from previously described work from our lab [1,2,24]. Briefly, data was converted to mzML file format using Agilent Masshunter Software (Santa Clara, CA), and aligned using MZmine 2.14. Data files converted from Masshunter were imported into MZmine as raw data files. A crop filter was applied to each data file to remove the first minute of each run, where the LC stream was sent to waste. Features were extracted as centroid and lists of features with a minimum elution time span of 0.1 minutes and a minimum height of 5000 a.u. were generated. Peak lists were retention time normalized and then aligned into one list with a max tolerance of 0.02 m/z or 30.0 ppm to define the same peak across runs. This aligned peak list was then gap filled with the same tolerances to find missing peaks. This list was then exported as a .csv file for statistical analysis. An ANOVA was done using Excel and features which were found to be significant (fold >1.5, p value <0.05) between any two samples were retained for further statistical analysis. The resulting lists of significant features were analyzed using principal component analysis (PCA) and visualized using the R package rgl, while hierarchical clustering was done using XLStat [25-27]. Bubble plots were generated from significant features for each pairing of rye whiskey using the built in plot function in R [27].

Results and Discussion

The success of our prior work on the classification of wine, urine, and intracellular metabolites prompted us to try the PSA in the analysis of whiskey. As an initial test, a set of wheat based whiskey ranging in age from 0 to 9 months was treated with the PSA and analyzed by LC-MS. This test served two purposes, i) to develop a method for treating whiskey with the PSA and ii) determine the viability of the assay for further whiskey analysis. Samples needed to be pre-filtered with a 10 kDa MWCO spin filter and diluted with water to lower the ethanol content, before being incubated with BSA. The pre-filtering was necessary to remove particulates and large biomolecules to prevent clogging of the spin filter used in the PSA. Dilution of the pre-filtered whiskey was required to avoid denaturing the BSA upon mixing with the sample. This was accomplished by diluting 100 µL of pre-filtered whiskey 3-fold in water. Data containing details on the molecular weight, retention time, and intensity of each compound in the PSA treated samples was acquired using LC-MS. This information was imported into MZmine for retention time and feature alignment, and finally statistical analysis to compare the intensity of compounds between samples. To this end, an ANOVA was used to identify features that were significantly different between samples (fold change >1.5, p value <0.05).

Features found to be statistically significant in the ANOVA were used as input for principal component analysis (PCA) so that similarities and differences in samples could be visually assessed. PCA is an unsupervised method, meaning it provides an unbiased

analysis using all of the input data [28]. A 2-dimensional PCA plot of data from the aging test is shown in Figure 1. Technical replicates (same marker) were tightly clustered, while samples from each age group (different markers) were well separated. A logical trajectory beginning with the freshly distilled samples (squares) through to the most mature (pentagons) was observed. A dashed line has been added as a visual guide. The result of this analysis shows that the PSA can be used to track the aging process of whiskey. Importantly, the ethanol content of whiskey is high (60%) when it is first distilled and slowly drops as it ages, but did not appear to affect the PSA and its ability to differentiate samples. This means the method developed for treating whiskey with the PSA is robust enough to work across a wide range of ethanol concentrations, with the protein remaining intact and capable of binding molecules in a fashion such that it can be used to effectively differentiate time points/samples.

PSA sensitivity to variations in the aging process was tested by analyzing partially matured samples transferred to different barrels for finishing. A rye based whiskey was distilled and placed into a 53 gallon barrel to develop. After two years, portions were transferred into two new 10 gallon barrels, originating from two different manufacturers. The original stock barrel and the two new barrels were then corked and left to age for an additional 4 months before sampling. The distillery conducted this as part of product testing aimed at developing new tastes and for assessing if barrel type changed the ageing process. Our goal was to assess whether the PSA could provide a metric for comparison. PCA was used to visualize the data from PSA treated samples. Once again, tight clustering of replicates was observed while the different samples were distinctly separated (Figure 2A). The samples transferred to the new barrels were separated from the stock barrel by PC1, which is composed of the features making the largest contribution to differences between samples. The samples placed in the new barrels were only separated from one another by PC2, which accounted for roughly one-third of the variation attributed to PC1 (60% in PC1 vs 20% in PC2). This indicates a similar, relatively large change between the new barrel samples and the stock, while a more subtle change differentiates the former. This subtle change is better understood when the data is displayed using agglomerative hierarchical clustering (AHC); a bottom up approach where each sample starts on its own, being merged as the tree is built [29]. Vertical lines indicate dissimilarity of samples in the tree. By AHC, the two new barrels had only a small amount of dissimilarity compared with the stock barrel, which was placed on a separate branch (Figure 2B). Taken together, these results show that the PSA is sensitive to both the small and large changes seen between whiskeys in different barrels, when aging time is kept constant.

The ability of the PSA to differentiate whiskey samples which have similar taste profiles, according to the distiller, prompted us to undertake a more thorough analysis of the features which were significantly different between the barrels based on ANOVA. Bubble plots displaying molecules with significant differences in pairwise analyses of whiskey barrels were generated to help understand the nature of the molecular species which are distinct in each barrel (Figure 3). For each plot, retention time and m/z for molecules are displayed on the x- and y-axis respectively; where the size of the circle indicates the fold change of a given feature and the hue signifies the p-value (larger, darker circles have a higher fold change and smaller, more significant p-value). The first two panels show the comparison of the stock barrel to each new barrel, while the third panel shows the comparison of the two new barrels. Most of the features shown in these plots appear to elute in the early to middle part of the chromatogram, which on a reverse phase column indicates they are more polar in nature. In comparing the stock

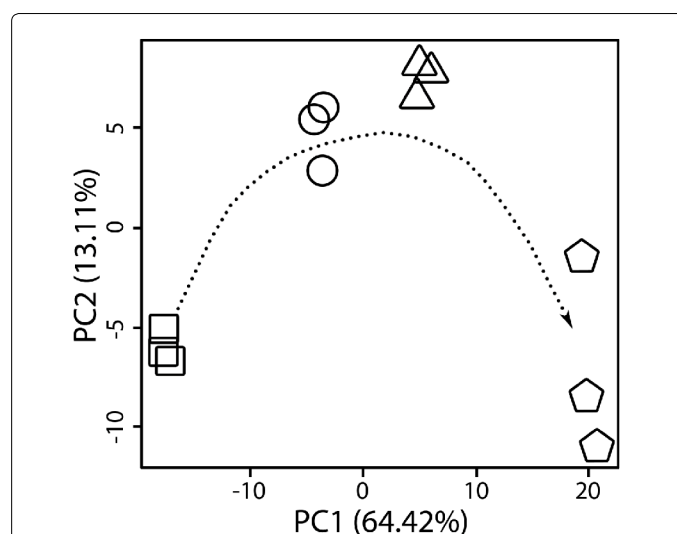


Figure 1: PCA plot of wheat bourbon whiskey samples treated with the PSA before LC-MS analysis. Samples represent four different stages of aging. Replicate samples from distillation (day 0, squares), day 174 (circles), day 180 (triangles), and day 240 (pentagons). A dashed line has been added as a visual aid showing the trajectory of ageing.

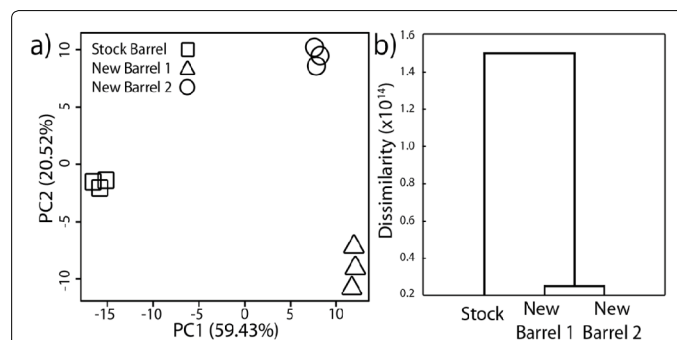


Figure 2: Influence of the barrel on whiskey maturation. A) PCA plot of rye bourbon whiskey samples treated with the PSA before LC-MS analysis. Samples are from the same batch of whiskey, which was aged in the stock barrel for 24 months, after which portions were transferred to new barrels of a different make. All three barrels were allowed to age for an additional 4 months. B) AHC of the three barrels of rye bourbon whiskey. The y-axis shows the dissimilarity, where length of the vertical lines are a measure of dissimilarity.

barrel with each of the new barrels, a majority of significantly different features were observed to increase in the new barrel samples (circles above the dashed line) (Figure 3A and 3B). This suggests that transfer to a new barrel introduced or enhanced the development of specific components. However, there are molecules that decrease in abundance as well (circles below the dashed line), suggesting the new barrels may in general speed removal of specific compounds.

Although the majority of the changes were between the stock and new barrel samples, differences were also detected between the two new barrel samples (Figure 3C). The features in this bubble plot, both above and below the dashed line, are compounds which are characteristic of each new barrel, highlighting the selective concentration property of the assay. Of the 80-100 features which are more intense in the comparison of the new barrels to the stock barrel, only a select few are retained when comparing the new barrels with one another. These select few are likely what give rise to subtle difference in flavor profile described by the distiller, and visualize in the PCA and AHC. The use of the PSA therefore adds a metric in the form of relative concentrations of specific

molecular features which can be used as a proxy for flavor profile. The rest of the features which showed significant change between the stock and new barrels (above the dashed line in Figure 3A and 3B) are the same compounds, and thus do not show up in the comparison of the two new barrels (panel 3C). Some of these compounds were only detected in samples from the new barrels, however, the majority were merely enhanced by exposure to the new barrels.

A plausible explanation for why introduction to a new barrel caused significant change is that the fresh surface for interaction facilitates changes in the whiskey. Contacting a fresh surface may allow

the whiskey in the new barrels to leech compounds from the wood and to have compounds depleted by absorbance of the wood. The number of features that were different between the two new barrels and the stock barrel was roughly 100. The number of different features between the two new barrels was just over 25. The small number of difference in the new barrels when compared to one another is consistent with the PCA, which shows them having almost no difference in PC1, with differentiation relying on PC2, which accounted for less of the total variance. It is also consistent with AHC which indicated that the two new barrels are highly similar, while there is significant dissimilarity between the stock barrel and the new barrels. The results of this analysis show that transfer to new barrels increases the rate of change, suggesting this as a method for speeding the process of aging. However, the nature of this change and if it is related to true ageing or represents a repeat of the initial process when first barreled will require further testing.

As a whole, the PSA performed well in the differentiation of whiskey and could be used as an analytical tool useful in tracking the aging process or investigating differences in flavor profile imparted by specific barrels. Aging is the key process that takes clear, bitter distillate and transforms it into the polished product ready for consumption and retail. The process requires skilled craftsman with years of experience to ensure a consistent and high quality product. Our analysis shows that the PSA can track the aging process and could be used in combination with a master distiller or as a stand-alone assay to provide metrics for the production process. The PSA was also sensitive to changes from a brief exposure to a new barrel which could be of interest to those seeking alternative strategies in the production process or as an analytical tool to assess aging methodology. Based on the retention times of the molecules in these experiments on a reverse phase column, many are semi-polar in nature. This work suggests that the PSA could be adapted as a straight-forward and sensitive test for counterfeit or adulterated product on a broad scale.

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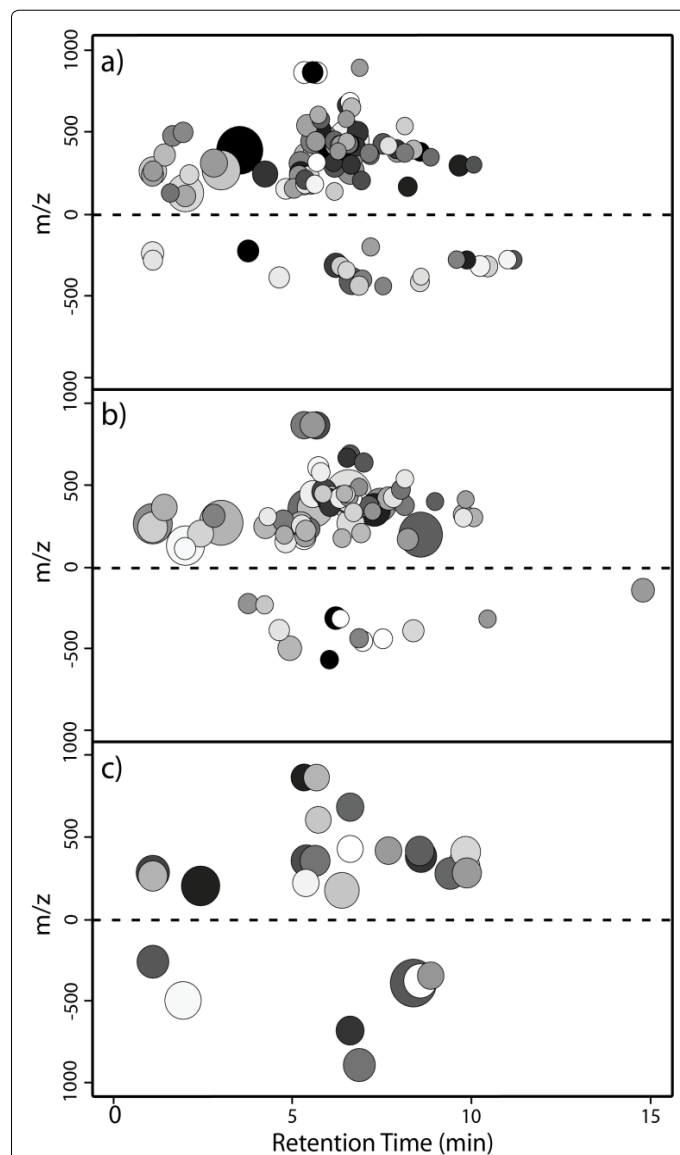


Figure 3: Bubble plots generated from the significant features (fold >1.5, p-value <0.05) between each pairing of rye based whiskey samples. For each plot, x-axis represents retention time of the given feature from reverse-phase chromatography, while the y-axis shows the m/z. Size of each bubble represents fold change, and hue represents the p-value, where a larger, darker, circle indicates a greater fold change and smaller p-value. A) Comparison of new barrel 1 and the stock barrel; below dashed line means more in stock barrel. B) Comparison of new barrel 2 and the stock barrel; above the dashed line means more in new barrel 2, below dashed line means more in stock barrel. C) Comparison of new barrel 1 and new barrel 2; above the dashed line means more in new barrel 1, below dashed line means more in new barrel 2.

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