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Residual Antibiotic Analyses-Ethanol Co-Products and Raw Material

It has become important for monitoring of antibiotic residues in a variety of materials. The impetus is based on an overall concern about release of antibiotics into the environment with subsequent development of antibiotic resistant bacteria. In addition, the various food and feed agencies have established acceptable levels in food and feed materials for consumption. As technology has advanced, the numbers and types and amounts of antibiotics have increased significantly, and there is a need to be able to identify and quantitate a number of combined and individual antibiotic materials in a wide range of matrices. In the ethanol industry, antibiotics are used to control bacterial growth that can interfere with ethanol fermentation. It is important for the industry to monitor if antibiotics are being concentrated into the final co-products, and if the antibiotics are being destroyed/inactivated by the fermentation process or processes involved in production of co-products such as syrup, thin stillage, wet distiller grains or dry distiller grains.

Over the years different types of antibiotic testing have been developed and utilized. The types of analyses can loosely be categorized into three types: microbial inhibition, ELISA, or instrument analyses. It is the intent of this narrative to review the different approaches and establish the strength and drawbacks of the various categories.

Historically, the original analyses have been based on microbial effects and this type of testing is still commonly used today. The basic procedure is to grow a population of bacteria on a surface that both provides a growth medium for the bacteria and a medium that the antibiotics can diffuse. The assumption is made that if an antibiotic is present; it will diffuse out from a central point and cause subsequent deterioration of the bacteria. Since the antibiotic should diffuse equally from a central point, a zone of inhibition is produced around a central point. The assumption is also made that the greater the concentration of the antibiotic, the wider the zone of inhibition. By measuring the diameter of the zone and comparing that zone to that produced by a known antibiotic at a known concentration, it is possible to quantitate how much antibiotic is present. The theory behind this type of test is scientifically based and has been supported by many studies and method validation efforts.

This procedure, however: does have a number of aspects that call the method into question. For one thing, the method measures the ability of a material to inhibit microbial growth, so it is more a measurement of the antimicrobial efficacy than the identification of a specific chemical. Associated with this is the assumption that the microbial population is affected by the antibiotic and that the diffusion of the antibiotic is unimpeded by factors such as depth of the agar, viscosity of the antibiotic, temperature, amount of extract, and the level nature of the agar material and well. In testing feeds, the manufacturers are adding a certain antibiotic at a certain level, making the final determination manageable. In ethanol and co-product production, there might be a single antibiotic used in the process, but the fermentation process may introduce other chemicals or by-products that potentially can inhibit microbial growth. The zone of inhibition that is formed can be caused by the antibiotics, a by-product, or a number of other factors such as metals or pH, but the final result is issued as a specific antibiotic being tested.

For example, if a producer introduces antibiotic "A" into the fermentation and then tests the final co-products for "A," any zone of inhibition will be assumed to be a result of antibiotic "A". It is possible that the fermentation process may destroy "A", but produce a metabolite or another chemical that is chemical "B" and chemical "B" can also inhibit microbial growth and produce a zone of inhibition. Since the test is looking for chemical "A," any zone of inhibition will be reported as antibiotic "A" but in fact, is due to "B" but the test does not show this.

Another potential drawback is the sensitivity of the method. In most cases, the levels of antibiotics added are in the grams/ton range (ppm) and many of the potential regulations may require sensitivity in the ppb (parts per billion) range. With the combination of a non-selective method and questionable sensitivity, the use of microbial methods may not be the best method in monitoring antibiotics at the residual level.

Another fairly recent method of analyses has been of test kits that use ELISA (enzyme linked immunoassay) where specific antibodies are produced against antigens. Again, the science is very sound and commonly used. The antibiotics are used to produce specific antibodies and these antibodies are attached to a variety of supports such as beads on plates or wells. If the antigen (antibiotic) is found in a sample, the two will combine as an antigen-antibody complex and then the complex is identified and quantitated. The most significant drawback to this method is the development of specific antibiotics. If the antibody being produced is not specific enough, it will combine with competing molecules and a false positive is reported or if the antigen does not bind to the antibody, a false negative can result.

Test kits are relatively cheap and easy to use, but the technology is not yet fully developed to make this a viable option, but hopefully in the future, more and more of these kits will become available. The second big drawback is that the kit would be specific for a single antibiotic and would not have the ability to screen samples for a large number of potential antibiotics.

It is because of the above method drawbacks that a number of instrumental methods have been developed. The advantages the instrumental methods have are that they can be selective for a certain chemical and also have the sensitivity to seek the low ppm or ppb levels. The drawbacks of the instrumental methods include the cost of analyses and the inability to measure microbial efficacy. It is not known if the chemical under investigation is active or an antimicrobial moiety, only the level of chemical is being determined. These instrumental methods can be further broken down into spectrophotometer methods or chromatography methods. The spectrophotometric methods are generally not considered to be common methods since a number of interferences can affect the final result. The spectrophotometric tests involve either the emission or adsorption of specific wavelengths of light, and again, the instrument is sensing light waves and not a specific chemical. Most of the instrument advancements have been in the chromatographic area, using either gas or liquid chromatography (GC or LC). As with many methods, there are advantages and drawbacks of both GC or LC methods and these will be discussed later. The chromatographic methods must be associated with a detector to measure what is leaving a column. The detector could be ultraviolet, flame injection, electron capture, refractive index or mass selective.

Overall, the industry is focusing on the mass selective detector since they are designed to identify chemicals by mass, making them very sensitive and very selective. The mass selective detector can be associated with either a GC or an LC (GC/MS or LC/MS). The major drawbacks of either GC/MS or LC/MS are the cost of instrumentation and the high level of operator expertise. These instruments need to be placed in fixed laboratory settings and operated by highly skilled analysts.
The base theory behind LC or GC is the use of a chromatographic column that separates the sample into components based upon how long they are retained in the column. Columns can be of a variety of types

based on their diameter, the polarity of the fixed phase and the length of the column, and a large number of other factors. The initial part of the procedure would be the extraction of the antibiotic from a sample, and it is hoped the extraction procedure can extract a wide range of suspected antibiotics; the extract should not produce hazardous wastes and not destroy or alter the antibiotics. Since residual levels (ppb) of antibiotics are needed, the extraction procedure must remove the chemical or chemicals of concern, but not extract other chemicals that are found in the matrix. The use of solid phase extraction can assist in this procedure, but any method must be investigated to ensure complete extraction of antibiotics without altering, destroying, or capturing all the antibiotics present.

The selection of GC or LC is based on the ability to volatilize the chemical. If it can be heated to form a gas, GC/MS can be used, and if the chemical can be solubilized into a carrier, use of liquid chromatography (LC/MS) is preferred. The advantage of the GC is the reduction of interferences because the chemical is in a gaseous state. For the LC method, it is important to obtain a solvent (mobile phase) that can carry the chemical or chemicals of interest through a column.

The existing science and instrumentation is leaning toward the LC/MS or LC/MS/MS methods because of the versatility of the method. Using LC/MS/MS instead of just LC/MS can also reduce the amount of sample preparation and increase the sensitivity and selectivity of the procedure. This is the direction Midwest Laboratories, Inc. is going. Midwest has purchased four LC/MS instruments. Two of the instruments are straight LC/MS while one is an LC/MS/MS and the fourth is an ion trap LC/MS. Midwest has validated an LC/MS method for Virginiamycin that is capable of easily reaching a 50 ppb detection level and another method for Erythromycin that is also validated at the 50 ppb level. It is the intent of the method development group to develop a screen that is capable of identifying and quantitating a considerable group of antibiotics at the ppb (50-100 ppb) level but that effort will take time, innovation, and money.