Application Note

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Analysis of Histamine in Wine Samples Using the Microplate Format

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Introduction

Bioactive amines are low molecular weight organic bases, formed by biological processes in all living organisms. One such bioactive amine is histamine. Wine, particularly red wine, is a beverage that contains significant amounts of histamine. Although there are many agents in red wine known to cause headaches (e.g. ethanol), histamine has been identified as the primary cause for intense headaches and migraines that some people experience after consuming red wine. Histamine is a bioactive amine that can be produced *in vivo* or ingested from dietary sources. Endogenous production of histamine is the result of enzymatic decarboxylation of the amino acid histidine, which is catalyzed by the enzyme L-histidine decarboxylase (Figure 1). Once formed, the compound is either stored or rapidly inactivated by the action of histamine-N-methyltransferase and diamine oxidase enzymes. Most tissue histamine is found in the metachromatic granules of mast cells of basophilic leukocytes. Other tissues containing histamine include the brain, where it acts as a neurotransmitter and the enterochromaffin cell of the stomach. In the course of type I allergy histamine is released from mast cells and basophils as a result of antigen binding to IgE on the surface of the cells, resulting in typical allergic reactions. In the case of ingestion of histamine, many of the same symptoms can appear without a true IgE allergic reaction taking place. This phenomenon is referred to as "food intolerance", with the term "food allergy" being used for true immunological reaction.

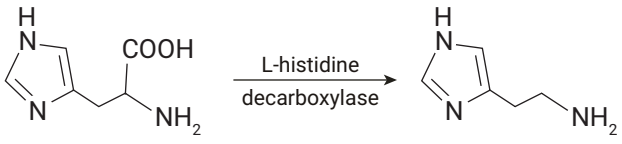

Figure 1. Formation of histamine by the enzymatic decarboxylation of the amino acid histidine.

Rash, diarrhea, nausea and vomiting, headache, and asthma clinically characterize food intolerances caused by elevated histamine levels. The extent of the reaction is directly related to the amount of histamine ingested and can be exacerbated by a lack of diamine oxidase. In addition to food intolerances, toxic reactions caused by very high histamine also exist. These toxic levels of histamine are usually the result of bacterial degradation of protein-rich food. This is particularly true with many fish species. Histamine content of many ichthyic species is the result of the degradation by bacteria on the fish skin. Post-mortem, these bacteria transform free histidine to histamine. High levels of histamine are therefore considered to be an early sign of decomposition.

There are a number of different analytical techniques for determining the levels of histamine in wine samples, mainly based on gas chromatography, liquid chromatography, HPLC and capillary electrophoresis. These methods often require several preparative steps to transform histamine into a detectable moiety. In addition, these methods are often time and reagent consuming, resulting in considerable expense in terms of instrumentation and reagents and precluding the measurement of large numbers of samples. The ELISA procedure described has the advantage of being easily performed on many samples using an Agilent BioTek ELx50 microplate strip washer and an Agilent BioTek ELx800 absorbance microplate reader.

The basis of the test is the antigen-antibody reaction. The wells are coated with specific antibodies against N-acyl-histamine. After sample preparation, histamine in samples and standards is derivatized into N-acylhistamine. Acylated histamine standards and samples in solution along with enzyme labeled N-acylhistamine (enzyme conjugate) are added respectively. Free and enzyme-labeled histamine compete for the antibody binding sites. Any unbound enzyme conjugate is then removed by a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colorless chromogen into a blue product. Addition of the stop reagent (sulfuric acid) causes a color change from blue to yellow. Measurement of color development is performed photometrically at 450 nm (optional reference wavelength ≥600 nm). The resulting absorbance values are inversely proportional to the histamine concentration of the sample (Figure 2).

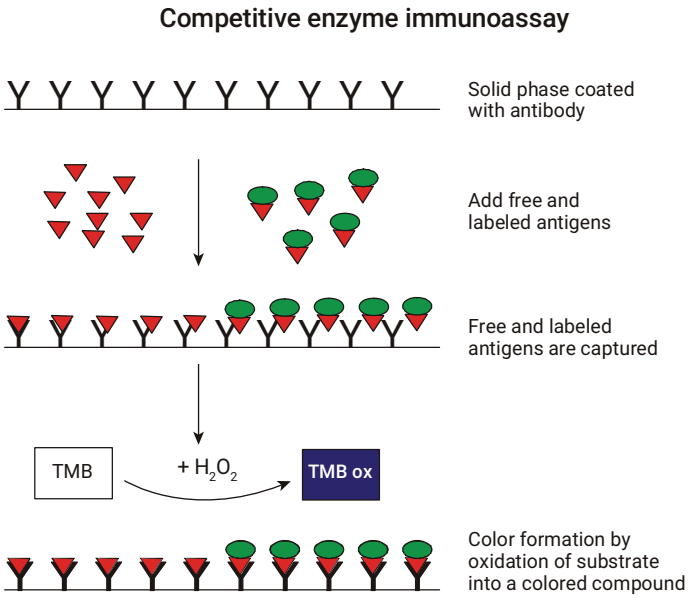

Figure 2. Competitive enzyme immunoassay. Free N-acyl-histamine and enzyme conjugated N-acyl-histamine compete for specific antibody binding sites. Because color development is directly related to the amount of captured conjugate while binding of free and conjugated N-acyl-histamine directly compete for binding, color development is indirectly proportional to histamine concentration of the sample.

Methods

Ridascreen Histamine (part number 1604) kits were purchased from R-Biopharm (Darmstadt, Germany). Each kit contained all of the necessary components, including an uncoated acylation plate, standards, reagents and a coated assay plate, to perform 48 determinations. The test procedure was performed according to the kit instructions. Briefly, samples and standards were first acylated by reacting 100 µL of sample or control with 25 µL of acylation reagent and 200 µL of acylation buffer supplied in the test kit. The reaction was allowed to incubate at room temperature for 15 minutes, after which the sample or standard was ready to assay. Using the antibody-coated plate supplied with the kit, 25 µL aliquots of samples and standards were pipetted into wells of the microplate. Each well also received 100 µL of antihistamine capture antibody and the mixture was allowed to incubate for 40 minutes at room temperature. After incubation, the microplate was washed using an ELx50 microplate strip washer 3 times with 250 µL of wash buffer.

After washing, 100 µL of conjugate was added and allowed to incubate for 20 minutes at room temperature. The plate was again washed with 3 cycles of 250 µL of wash buffer using the ELx50 washer followed by the addition of 100 µL of substrate/chromogen mixture. Color was allowed to develop for 15 minutes at room temperature in the dark. The reaction was stopped by the addition of 100 µL of acid stop solution and the absorbance of each well at 450 nm (630 nm reference) was determined using an ELx800 absorbance microplate reader. For all samples and standards, the ratio of the sample or standard to the mean of the zero standard (B/Bo) was calculated. The B/Bo calculation for the standards was then used to generate a standard curve.

Experiment 1

The first experiment was setup with three different sets of kit standards in duplicates. In addition to the standards provided by the kit, 6 replicates of the Negative Control, and 6 replicates of the Positive Control provided by the kit were also tested. Intra assay repeatability experiments involved multiple runs of standard curves using different kits. In each case standard curves were generated using a 4-parameter logistical fit to describe the data. For comparison, multiple experiments repeated the testing of the kit standards, as well as a number of white wine and white zinfandel samples were run in duplicate.

Results and discussion

These data demonstrate that the R-Biopharm kit, in conjunction with the ELx800 absorbance microplate reader and an ELx50 microplate strip washer, can be used to quantitate histamine in wine samples. As with any competitive ELISA reaction, increasing analyte concentration results in a decrease in absorbance (Figure 3). Repeated standard curves with different samples resulted in curves very similar in shape and parameters.

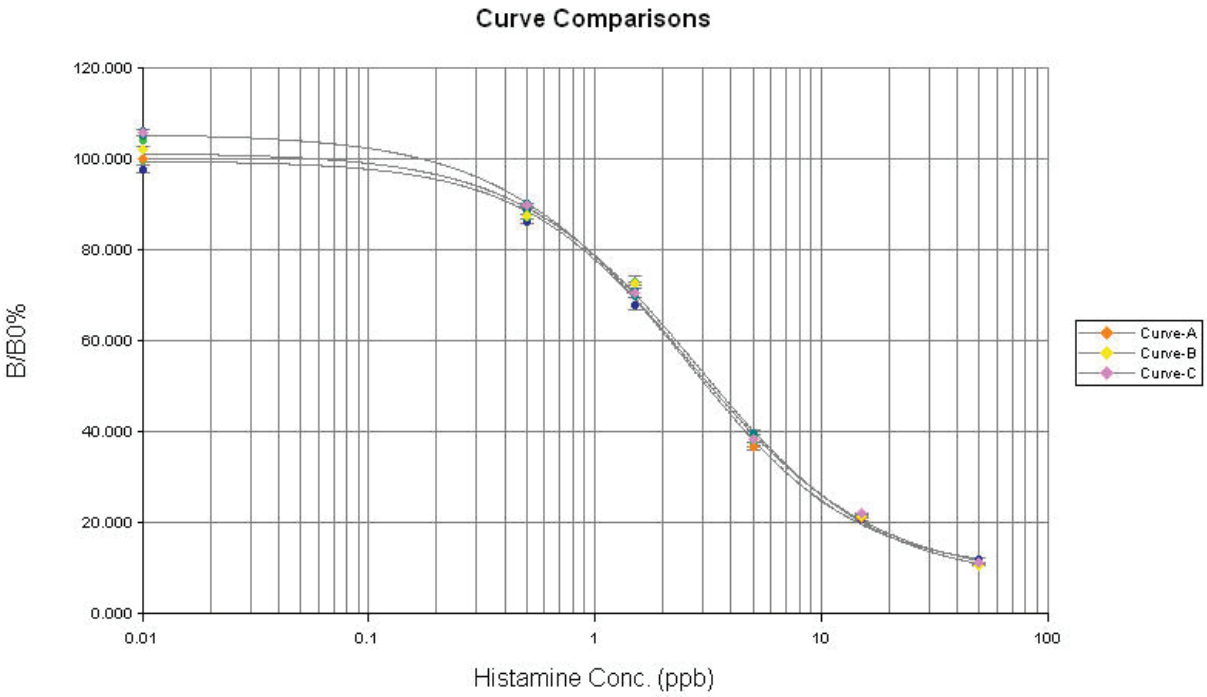

Figure 3. Typical standard curves. Three different standard curves were overlayed using Agilent BioTek Gen5 data analysis software and plotted on the same set of axis. Each data point represents the mean of duplicate determinations for the specific standard curve.

When individual samples at various known concentrations of histamine are compared, very good inter-assay repeatability is observed. Samples at 15, 5, and 1.5 ppb span most of the dynamic range of the standard curve. For each concentration, individual samples returned very similar calculated concentrations when measured on the same microplate (Figure 4). While similar samples, when measured at different times (intra-assay repeatability) showed an increase in variability, there was still very good agreement between different samples (Figure 5).

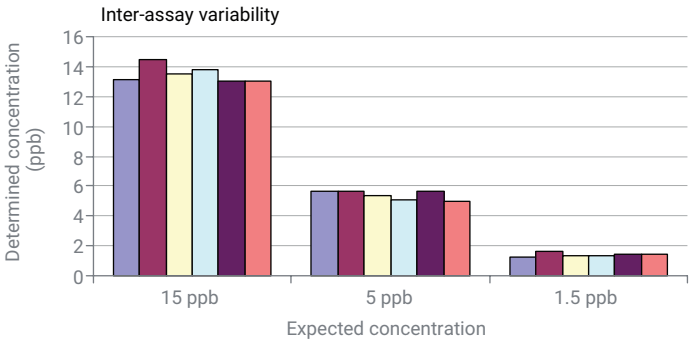

Figure 4. Inter-assay repeatability. Multiple replicates of samples at three different concentrations were assayed individually on the same microplate. The absorbance values for each sample were then used to interpolate a standard curve. The resultant calculated concentrations were exported to Microsoft Excel via the Power Export function of Agilent BioTek Gen5 and plotted.

The ELISA test kit is very specific towards acylated histamine. As demonstrated in Table 1, the antibody used only demonstrates a small amount of cross reactivity towards the closely related compounds N-methyl-histamine when assayed. Other compounds tested were found to be below the detection limits.

Table 1. Assay specificity for R-Biopharm ELISA**

Assay Specificity	
Compound	**Specificity**
N-Acyl-Histamine	100%
N-Methyl-Histamine	0.01%
5-Hydroxy-Indole-Acetic Acid	Below detection limits
Imidazole Acetic Acid	Below detection limits
L-Histadine	Below detection limits
N-Methyl Imidazole Acetic Acid	Below detection limits
Serotonin	Below detection limits

** As reported in the kit insert.

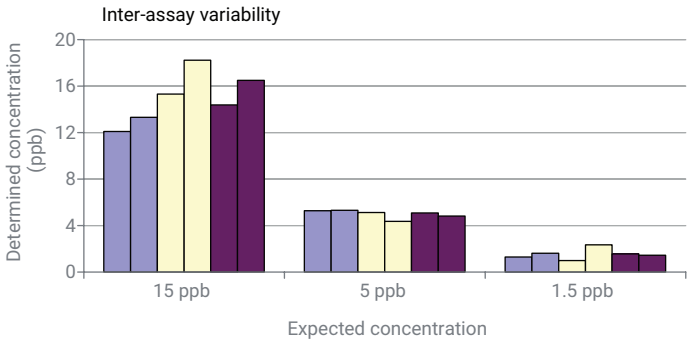

Figure 5. Intra-assay repeatability. Individual samples were run in duplicate on three different assay plates. The individual results were exported via the Power Export function of Agilent BioTek Gen5 to Microsoft Excel and plotted. Note that each color denotes the three different experimental plates.

When two different wines were assayed for histamine, very close agreement between separate samples was observed. The CVs for the absorbance values at 450 nm for either sample was less than 5% (Table 2). The low levels of histamine in these wine samples result in absorbance values that are located in portions of the standard curve that are relatively unchanging. This manifests in rather large changes in calculated concentrations with relatively small deviations in absorbance, leading to the higher CVs observed for sample concentrations.

Table 2. Wine sample measurements.

Sample	Raw 450	B/B0%	Mean	CV (%)	Conc.	X Dilution	Mean	CV (%)
SPL1	1.318	61.57			2.372	1186.0		
	1.387	64.80			2.107	1053.7		
	1.296	60.55			2.462	1230.8		
	1.307	61.06			2.417	1208.3		
	1.190	55.59			2.936	1468.0		
	1.284	59.99	60.16	4.13	2.512	1255.9	1251.9	8.99
	1.343	62.74			2.273	1136.7		
	1.250	58.40			2.658	1329.2		
	1.215	56.76			2.817	1408.6		
	1.273	59.47			2.558	1279.2		
	1.283	59.94			2.516	1258.0		
	1.307	61.06			2.417	1208.3		
SPL2	1.732	80.92			1.055	527.5		
	1.778	83.07			0.938	469.2		
	1.754	81.94			0.999	499.4		
	1.622	75.78			1.352	676.1		
	1.734	81.01			1.05	524.9		
	1.818	84.93	79.28	4.76	0.84	420.0	576.7	18.60
	1.794	83.81			0.899	449.3		
	1.647	76.95			1.282	641.1		
	1.582	73.91			1.468	733.9		
	1.660	77.55			1.246	623.2		
	1.600	74.75			1.415	707.6		
	1.642	76.71			1.296	648.0		

These data demonstrate that histamine can be quantitatively determined from wine samples using ELISA. Many of the other methods of determining histamine levels require extensive sample preparation, expensive equipment, and a great deal of technical expertise to perform. The ELISA method requires minimal sample preparation and a minimal amount of equipment, yet can be used for a large number of samples. In addition, ELISA can be used with a large number of different analytes without changing the basic procedure or instrumentation. Furthermore, the Agilent BioTek ELx800 absorbance microplate reader and Agilent BioTek ELx50 microplate strip washer are ideal cost effective tools to perform these types of assays.

Histamine is considered to be an allergen and a causative agent for headaches. While on average histamine in wine is 5.7 ppm and 3.4 ppm for red and white wine respectively, an extremely low histamine content is a desirable characteristic. The detection limit of the test kit is considered to be the lowest standard containing histamine (i.e. 0.5 ppb). Because wine samples are diluted 1:500, the lowest detectable limit in wine samples therefore would be 250 ppb. This is well below the typical ranges in wine, which are usually in the low ppm range. This large margin of sensitivity means that this assay can be used to monitor the formation of histamine by decarboxylation of histadine by lactic acid bacteria during fermentation.

The study of bioactive amines is of interest for a number of reasons. There is a certain amount of "risk" from the ingestion of exogenous histamine, particularly at high levels. Prevention of exposure to high levels in wine products is certainly of importance from a liability standpoint. In addition, there is a possible relationship between high amine content and the quality of the grape used, as well as the hygienic or sanitary conditions prevalent during the wine making process.[1] It has been reported as early as 1978 that routine checking of biogenic amine content of wine was a means to detect defective production procedures.[2] In addition, bioactive amine content of wines may be regulated in the future, such as those implemented for fish. Some countries have established limits for histamine in wines. Switzerland recommends 10 mg/L as a maximal level, Germany recommends 2 mg/L, while Belgium and France recommend 5 mg/L and 8 mg/mL, respectively.[3]

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