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The Quantitative Analysis of Thiamin and Riboflavin and Their Respective Vitamers in Fermented Alcoholic Beverages

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ABSTRACT: This research aimed to develop a simple and effective method for analyzing thiamin (B_1), riboflavin (B_2) and their respective vitamers by high performance liquid chromatography (HPLC) in fermented alcoholic beverages. The method developed here employs a phosphate buffer/methanol gradient elution on a single reverse phase column, coupled with independent fluorescent detection regimes. It also employs a precolumn derivatization to convert thiamin to thiochrome via an alkaline potassium ferricyanide solution. The method described here allowed a spike recovery of better than 97%, with a typical linear detection range ($R^2 \geq 0.9997$) between ≤ 5 and ≥ 500 μg/L for all vitamers studied. Lager style beers were found to contain significantly ($p < 0.001$) less thiamin than other tested styles of beers (lager, 35.7 μg/L; ale, 88.3 μg/L; stout/porters, 104.4 μg/L; wheat beers, 130.7 μg/L), which may be due to the raw material and extensive processing that occurs for this style. There was no statistical difference ($p = 0.608$) between the riboflavin content of each beer style. Furthermore, wines and ciders contain less thiamin and riboflavin than beer, which is also likely to be due to the base materials used and the differences in processing steps to produce these beverages.

KEYWORDS: thiamin, riboflavin, beer, vitamers, precolumn derivatization, HPLC

INTRODUCTION

Thiamin (vitamin B_1) and riboflavin (vitamin B_2) are water-soluble vitamins found in a variety of foods and beverages.[1−13] These vitamins are found not only in their free forms but also as the phosphorylated vitamers, thiamin mono- and diphosphate (TMP and TDP), flavin mononucleotide or riboflavin 5′-phosphate (FMN) and flavin adenine dinucleotide (FAD). These biologically active forms are generally associated with enzyme complexes that are involved in a variety of metabolic processes in all biological cells. They can be liberated from dying or damaged cells and easily end up in the final food products. Since beer is made from a malted grain (barley), the likelihood that these vitamins will be present in the final product is high and numerous authors have reported their presence.[11,14−26] However these studies have been limited in that only relatively small sample numbers have been used and any potential differences in beer styles have not been investigated.

Traditionally thiamin and riboflavin analysis were performed by microbiological and fluorimetric methods (e.g., AOAC 960.46), however with the advancements of newer and faster technologies a shift has occurred toward chromatographic methods. The use of high performance liquid chromatography (HPLC), ultrahigh pressure liquid chromatography (UHPLC), and liquid chromatography mass spectrometry (LCMS) are favored by many researchers due to their high efficiency and accurate results.[27] Unlike microbial and basic spectrophotometric methods, HPLC-based methods have the ability to distinguish between the different vitamers of thiamin and riboflavin.

Modern HPLC systems utilize a variety of detectors that can enhance the potential detection limits, including UV/vis, refractive index (RI), mass spectrometry (MS) and fluorescence detectors. Fluorescence detection has commonly been employed in the analysis of thiamin and riboflavin because of its sensitivity and its relatively low cost.[28−30] Unlike riboflavin, thiamin does not naturally fluoresce and requires derivatization to the highly fluorescent compound thiochrome. This procedure has been well described in the past by a variety of authors for spectrophotometric assays,[31−35] capillary electrophoresis[36,37] and HPLC analysis.[16,38−47] Since the reaction of thiamin to thiochrome occurs in alkaline conditions, thiochrome derivatives cannot be directly introduced into a HPLC system and need to be neutralized preinjection[48] or the derivatization needs to occur postcolumn.[16] However postcolumn derivatization requires an additional pump that works in precise unison with the main HPLC pump.[16] Precolumn derivatization has traditionally been time-consuming in comparison to postcolumn derivatization; but it does not require an external pump and mixing coils to deliver the correct volumes of derivatization reagent and subsequent rousing and reaction. The aim of this investigation was to develop relatively simple methods for the quantitative analysis of thiamin, riboflavin and their respective vitamers in a variety of commercially available beer, wine and cider samples.

EXPERIMENTAL PROCEDURES

Samples. Two hundred and four beer samples were selected from entries from the 2010 Australian International Beer Awards (AIBA) and were stored at 4 °C, in the dark, prior to analysis. Any green or clear bottles were covered in aluminum foil to avoid light-induced degradation of

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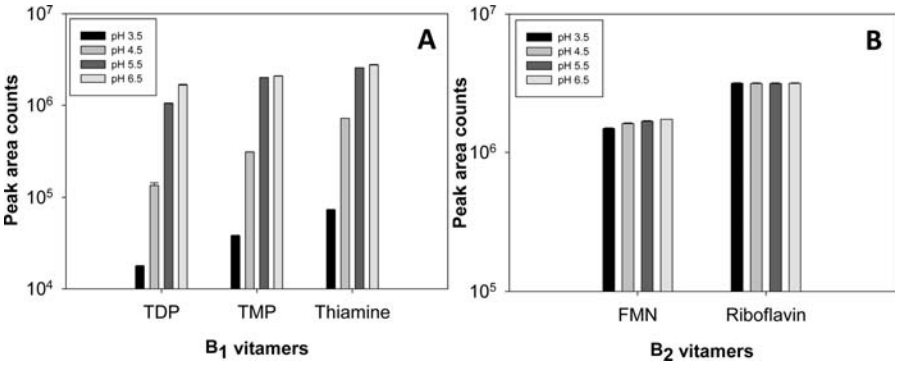

Figure 1. Effect of mobile phase pH on the fluorescent properties thiamin, riboflavin and their respective vitamers (injected concentration of each vitamer is 250 ng/mL). Error bars indicate the standard deviation of triplicate determinations of repeated experiments (i.e., $n = 6$).

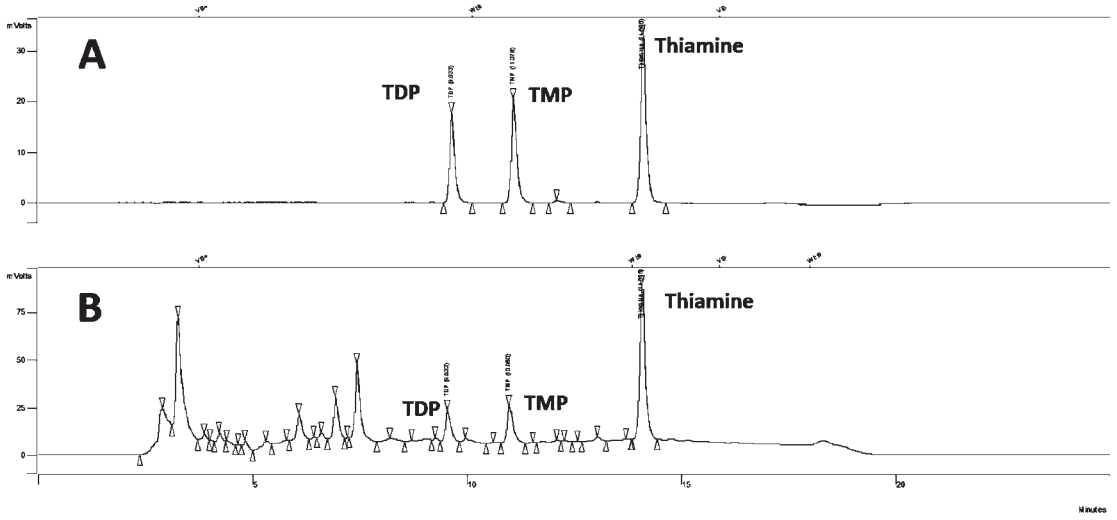

Figure 2. Chromatograms of TDP, TMP and thiamin in a standard (injected vitamer concentration is 250 ng/mL) (A) and beer sample (B).

riboflavin.[49,50] All beer samples were selected randomly from the following styles based on their entries into the AIBA: lagers, ales, wheat beers, stouts and porters. Furthermore, twenty-three commercially available cider, thirty-eight white wine, and fifty-one red wine samples were obtained and stored at 4 °C, in the dark, prior to analysis.

Reagents. Eighteen megohm water was purified using a Sartorius arium 611VF water purification system (Sartorius AG, Göttingen, Germany). Supragradient HPLC methanol (Scharlau, Barcelona, Spain) was obtained through Chem Supply (Adelaide, Australia). The 10 mM phosphate buffer (KH_2PO_4/K_2HPO_4) [filtered through 0.45 μm nylon filters (Phenomenex, Torrance, CA)] and thiamin and riboflavin vitamer solutions were prepared from commercially available products (Sigma-Aldrich, St. Louis, MO) that were at least 98% pure. Vitamin standards (50 mg/L of each vitamer) were prepared by dissolving 5 mg into 100 mL of 18 MΩ water and were stored at 4 °C in amber bottles. All working standards were freshly prepared by diluting with 10 mM phosphate buffer (pH 6.5) on the day of use. The derivatization reagent for the thiamin analysis was prepared by dissolving 1 g/100 mL potassium ferricyanide (Mallinckrodt, Giesheim, Germany) in 15 g/100 mL sodium hydroxide (Chem Supply, Adelaide, Australia) and was stored in amber bottles at 4 °C for up to one week.

Instrumentation. A Varian (Varian Inc., USA) LC system that consisted of a Prostar 230 solvent delivery system (flow rate 1 mL/min), a Prostar 410 autosampler (10 μL injection), a Prostar 500 column valve module (30 °C) and a Prostar 363 fluorescence detector (360/425 nm and 270/516 nm excitation/emission for thiamin and riboflavin vitamers respectively) was used. The column utilized was a Varian Pursuit C18 5 μm (250 mm × 4.6 mm) fitted with a Securityguard Cartridge C18 4 × 3.0 mm (Phenomenex, Torrance, CA).

Procedure. Analysis of both thiamin and riboflavin were conducted using gradient elution that consisted of a 10 mM phosphate buffer (KH_2PO_4/K_2HPO_4) at pH 6.5 (solvent A) and methanol (solvent B). The programmed elution of the mobile phases was as follows: 0−0.5 min 95:5 A:B; 0.5−10 min 65:35 A:B; 10−15 min 65:35 A:B; 15−16 min 95:5 A:B; at a total run time of 25 min. All standards and samples for thiamin analysis were converted to their respective thiochrome esters prior to injection by the use of an alkaline potassium ferricyanide solution. This was performed by adding 300 μL of 1% w/v potassium ferricyanide in a 15% w/v sodium hydroxide solution to 1 mL of standard/sample that had been previously degassed by filtration through a 0.45 μm RC filter (Phenomenex, Torrance, CA). The sample was vortexed for 15 s and neutralized with 600 μL of 1.33 M phosphoric acid solution. This solution was filtered through a 0.45 μm RC filter prior to injection. All samples for riboflavin analysis were degassed, filtered through a 0.45 μm RC filter and diluted 1:1 with a 10 mM phosphate buffer (KH_2PO_4/K_2HPO_4) at pH 6.5 prior to injection. The FAD content in this research was not investigated due to interferences from the samples hindering the accurate quantification of this riboflavin vitamer. All sample preparations and holdings were performed in triplicate, under subdued light and at 4 °C until analysis to minimize any potential losses.[29,51]

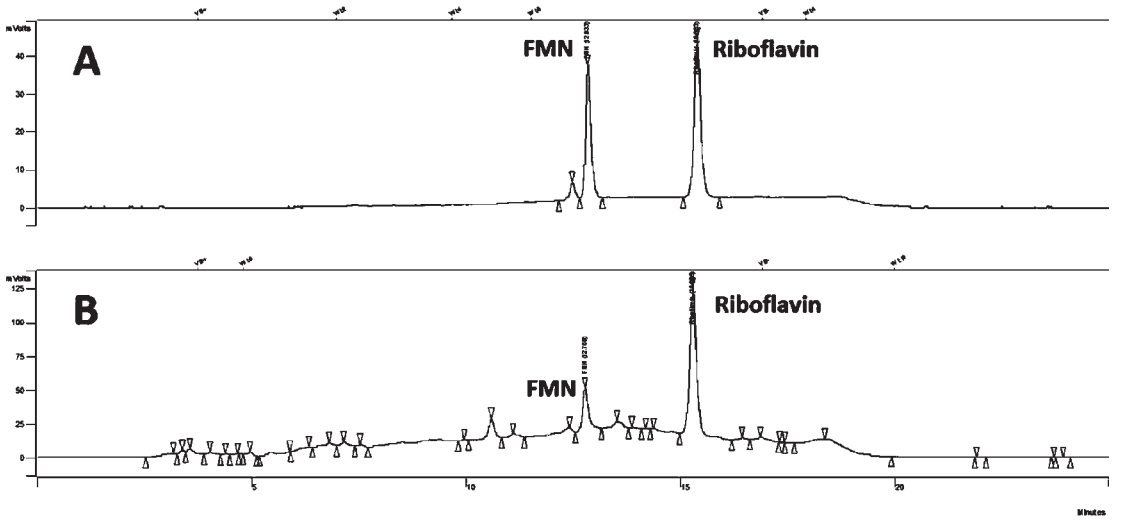

Figure 3. Chromatogram of riboflavin and FMN in a standard (injected vitamer concentration is 250 ng/mL) (A) and beer sample (B).

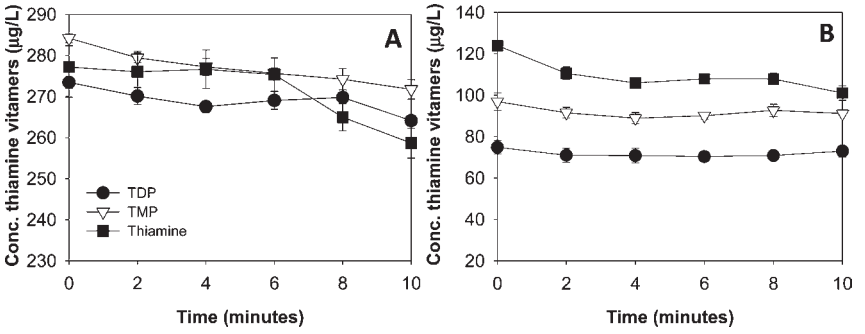

Figure 4. Effect of the derivatization time on the formation of thiochrome esters in a standard solution and a beer sample. (A) Thiochrome esters in standard solutions. (B) Thiochrome esters in beer samples. Results shown are the average and standard deviations of triplicate solutions and triplicate injections across duplicate experiments (i.e., $n = 18$).

Statistical Analysis. All statistical analysis was performed using an analysis of variance (ANOVA) and Dunn's all pairwise multiple comparison method utilizing commercially available Sigma Plot 11 software (Systat Software, Inc., USA).

RESULTS AND DISCUSSION

Optimization of HPLC analysis. The aims of this research were to develop a methodology which would allow the quantitative analysis of the vitamers of thiamin and riboflavin in fermented alcoholic beverages. This led to the use of a phosphate buffer at pH 6.5 as the mobile phase, which could be used for the analyses of both sets of vitamers through the same gradient elution method and allow for simple switching between the two methods (when run independently) with little to no down time. Moreover, this implies that either a multichannel fluorescence detector or two independent single-channel fluorescence detectors were employed such that both sets of vitamers could be simultaneously analyzed (not utilized in this research). A phosphate buffer was chosen over ion-pairing systems as this is a cheaper option and the phosphate buffer provided greater stability of the various phosphorylated vitamers, whereas ion-pairing systems such as tetrabutylammonia phosphate interfered with the fluorescence intensity of the thiochrome esters (data not shown). In order to optimize the mobile phase buffering system, 10 μL of various 250 μg/L standard solutions were injected into the HPLC while altering the pH of the mobile phase. The optimal fluorescent response for all thiamin and riboflavin vitamers was obtained with a mobile phase buffered at pH 6.5 (Figure 1).

Kawasaki et al.[29] described that pH 8 was ideal for achieving the greatest fluorescence for thiochrome derivatives; however this could not be used here as it would exceed the maximum operating conditions of the column employed (Varian Pursuit, C18), causing column degradation. In the present study a buffered system at pH 6.5 provided an acceptable fluorescent response and excellent reproducibility ($p < 0.05$) (Figure 1). A 10 mM phosphate buffer was chosen in the work because higher phosphate concentrations limited the methanol concentrations as the second eluent. Typical chromatograms for thiamin and riboflavin vitamers in standard solutions and a typical beer, using gradient elution, with the 10 mM phosphate buffer as the mobile phase are shown in Figures 2 and 3. A 25 mM buffer could be used; however a precipitate formed in the pump when it was applied over extended periods, limiting the long-term application of this method. An isocratic elution procedure, employing various phosphate and methanol concentrations, was attempted; however this resulted in the coelution of the vitamers of both

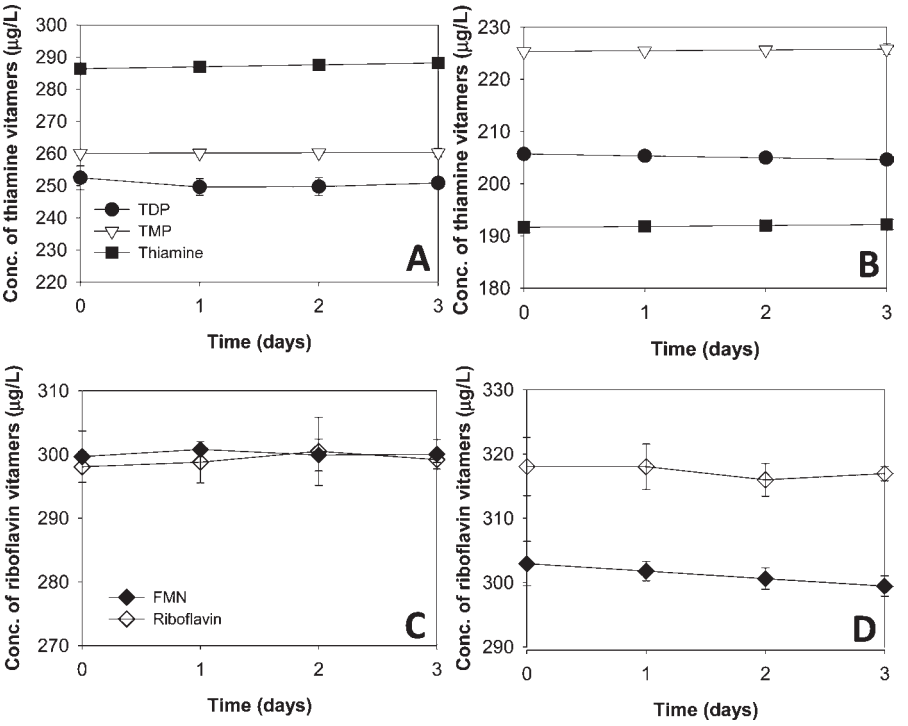

Figure 5. Stability of thiochrome and riboflavin vitamers after sample preparation. (A) Thiochrome vitamers in standard solutions. (B) Thiochrome vitamers in beer samples. (C) Riboflavin vitamers in standard solutions. (D) Riboflavin vitamers in beer samples. Results shown are the average and standard deviations of triplicate solutions and triplicate injection across duplicate experiments (i.e., $n = 18$).

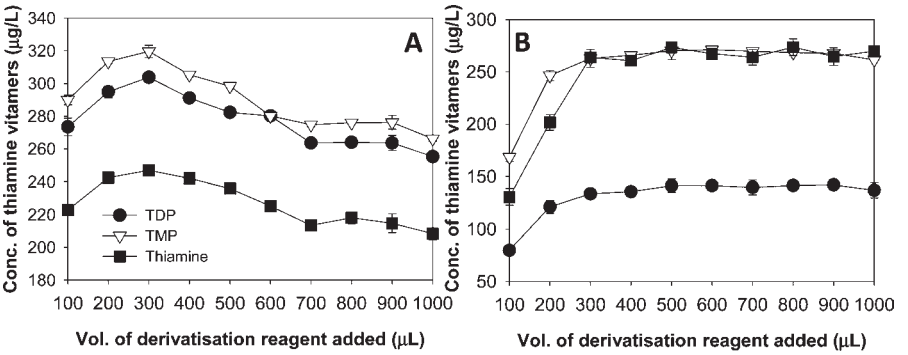

Figure 6. Effect of the derivatization volume on the formation of thiochrome esters in a standard solution and a beer sample. (A) Thiochrome esters in standard solutions. (B) Thiochrome esters in spiked beer samples. Results shown are the average and standard deviations of triplicate solutions and triplicate injections across duplicate experiments (i.e., $n = 18$). Spike recoveries of TDP, TMP and thiamin at 300 μL addition were 98.5, 99.6 and 102.1% respectively.

thiamin and riboflavin in standard solutions; while interfering compounds from actual samples were also observed.

Optimization of Thiamin Derivatization. A series of experiments were conducted to determine the ideal derivatization reaction time required to convert all thiamin and thiamin ester to their respective thiochrome forms. Both a standard solution and a commercially available pale ale sample (1 mL) were derivatized with 300 μL of a 1% w/v potassium ferricyanide in 15% w/v sodium hydroxide solution and vortexed for 15 s, and the solutions were allowed to sit at room temperature for a 10 min period with subsamples taken every two minutes and neutralized with a 1.33 M phosphoric acid solution.

The derivatization of thiamin or any of its vitamers to their respective thiochrome form appears to be instantaneous (Figure 4). Extending the contact time of the derivatization solution with the sample causes a decrease in the measured thiochrome concentration. The initial recorded concentrations were within expected ranges (>98% recovery). These results indicate that the reaction time required to achieve full thiochrome conversion is no more than that of the initial vortexing procedure (15 s). Neutralization of the samples immediately following the vortexing step provided long-term (24+ h) stability of the thiochrome esters, which allowed an automated sample injection system to be used (Figure 5).

The amount of potassium ferricyanide required to be added to derivatize all potential thiamin and phosphate esters was also investigated. Different amounts of derivatization solution were added to 1 mL of standard solution or spiked (125 μg/L of each vitamer)

Table 1. Accuracy, Precision, Limit of Quantitation (LOQ), Limit of Detection (LOD), and Linearity of Calibration of Described Thiamin/Riboflavin Vitamer Methods

					linearity	
analyte	accuracy (av % spike recov)	precision (% RSD)	LOQ (ng/mL)	LOD (ng/mL)	range (ng/mL)	calibration (R^2 value)
thiamin diphosphate (TDP)	97.8	2.4	5.0	1.5	5−500	0.9998
thiamin monophosphate (TMP)	98.2	2.5	5.0	1.5	5−530	0.9997
thiamin	98.4	3.5	2.0	0.6	2−550	0.9998
flavin mononucleotide (FMN)	97.6	2.7	5.0	1.5	5−520	0.9999
riboflavin	98.4	2.8	2.0	0.6	2−550	0.9999

pale ale beer samples. The addition of increasing volumes of derivatization reagent to standard solutions of thiamin vitamers caused a lowering of the measured thiochrome response and the optimal volume was found to be 300 μL (Figure 6). When comparing these results to a beer sample (Figure 6B), 300 μL of derivatization reagent was required to achieve the highest possible response; while larger volumes did not negatively affect the fluorescent response. This experiment was also repeated with stout and wheat beers to investigate any potential interference in more complex beers. The same effect as shown in Figure 6B was recorded for the stout and wheat beers (data not shown).

Accuracy, Precision, Limit of Detection (LOD), Limit of Quantitation (LOQ) and Linearity of Calibration. Table 1 shows the accuracy, precision, limit of detection (LOD), limits of quantitation (LOQ) and linearity of calibration of the above-described methods. The accuracy of the methods described in this research was determined by spiking a variety of different samples (five from each beer style/beverage type) with 25 ng/mL and 50 ng/mL of a vitamin standard that contained all of the thiamin and riboflavin vitamers measured in this research. This experiment was performed in duplicate. The spike recoveries were calculated and averaged for both thiamin and riboflavin methods and are reported in Table 1. To ensure this accuracy was maintained throughout analysis of all the samples, one in 10 samples were spiked with 50 ng/mL of a mixed thiamin/riboflavin vitamer standard. All calculated spike recoveries were maintained within a 95−105% recovery range.

The precision (relative standard deviation) of these methods were calculated by performing multiple analyses (10 times) on the same sample. This was also performed on spiked and standard samples that contained 50 ng/mL of each vitamer, and the average of this is reported in Table 1. As with the accuracy determination, the precision of the methods described here was continually monitored during analysis with a tolerance of only 5% RSD. If this was exceeded, the samples were retested. The LOD and LOQ were calculated for each vitamer on the basis of 3σ and 10σ respectively, using the regression lines for the calibrated standards (Table 1).

Thiamin, Riboflavin and Their Respective Vitamers in Fermented, Alcoholic Beverages. A range of fermented alcoholic beverages were analyzed employing the method described above. The alcoholic beverages analyzed included a range of commercially available beers, ciders, and white and red wines. The beers were classified based on the style guidelines of the Australian International Beer Awards (AIBA), being lager, ale, stout/porter and wheat beers. The mean concentration of FMN and riboflavin was relatively similar across the four different beer styles; however, the mean concentrations of FMN in the cider and white wine samples were significantly ($p < 0.001$) higher than the mean values obtained from any of the beer styles (Table 2). Furthermore, the mean riboflavin levels were significantly lower ($p < 0.001$) in the cider and white wine samples (approximately 15 and 20% of the mean riboflavin found in the beers). Graham et al.[19] showed that the riboflavin content of fermenting wort did not significantly change over the course of the fermentation, suggesting that brewing yeast either has a limited ability to utilize externally supplied riboflavin (i.e., lacks a riboflavin uptake mechanism) or has a limited requirement for externally supplied riboflavin (i.e., is sufficient in nascent production of riboflavin). This may explain the differences in riboflavin vitamers among the beers, and the fact there is no statistical difference between any of the beer styles when comparing FMN and riboflavin concentrations ($p = 0.168$ and $p = 0.608$ respectively). The differences in riboflavin levels between beers, ciders and wines might be influenced by the raw materials (grain-based wort, apple juice, and grape juice respectively) used to produce the fermented beverages containing significantly different base levels of riboflavin.[52,53]

Table 2. Riboflavin Vitamer Concentrations in Alcoholic Beverages (μg/L)

	FMN			riboflavin		
alc bev	range	mean	SD	range	mean	SD
lager ($n = 74$)	<5−142	34.4	28.7	73−737	307.0	129.5
ale ($n = 67$)	<5−151	37.0	33.7	110−644	300.2	127.4
stout/porter ($n = 31$)	5−122	45.4	30.8	116−757	322.0	134.3
wheat beer ($n = 32$)	<5−136	41.7	30.3	109−615	326.9	118.0
cider ($n = 23$)	6−115	64.0	37.9	3−107	49.8	24.4
white wine ($n = 38$)	<5−79	54.6	21.1	19−178	58.2	33.6
red wine ($n = 51$)	7−83	57.0	17.0	119−226	162.7	26.4

While no statistical difference could be found among the various beer styles with regard to riboflavin, statistical differences were determined among the thiamin vitamer results ($p < 0.001$) of all alcoholic beverages examined. On further investigation (using Dunn's all pairwise multiple comparison method), the greatest significant differences among the beer styles lay between lager and stout/porter, wheat and stout/porter, and lager and ale, where in all instances, for the second of each of the pairs, the mean result was significantly higher for the TDP data ($p < 0.001$). The TMP and thiamin data showed that all other styles contain significantly ($p < 0.001$) higher levels of TMP and thiamin when compared to the lager style beers (Table 3). Since lager beers are typically filtered and pasteurized, it is possible that the higher level of processing involved in these beers may actually lower the thiamin, TDP and TMP content. It was also noticed that a

Table 3. Thiamin Vitamer Concentrations in Alcoholic Beverages (μg/L)

	TDP			TMP			thiamin		
alc bev	range	mean	SD	range	mean	SD	range	mean	SD
lager (n = 74)	<5−36	11.9	8.0	<5−29	7.1	6.1	<2−249	35.7	50.3
ale (n = 67)	<5−55	20.1	13.4	<5−154	20.7	25.5	<2−411	88.3	96.7
stout/porter (n = 31)	<5−129	34.1	30.1	<5−139	21.9	23.5	<2−310	104.4	85.5
wheat beer (n = 32)	<5−55	15.4	12.6	<5−30	11.7	6.8	<2−433	130.7	98.3
cider (n = 23)	nd[a]	nd	nd	<5−40	6.5	8.3	<2−391	86.4	105.0
white wine (n = 38)	nd	nd	nd	<5−10	2.5	2.4	<2−368	40.4	69.3
red wine (n = 51)	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a] Not detected.

majority of the ale, stout/porter and wheat beers were in fact bottle conditioned. The higher levels of thiamin in these beers in comparison to the lager style may be attributed to the higher concentrations of the thiamin vitamers. On further investigation into the potential source of the thiamin, it was found that there was a significant difference ($p = 0.013$) between the bottle and nonbottle conditioned beers where those that were bottle conditioned had a higher mean result. This suggests that a longer yeast contact time associated with bottle conditioning could potentially result in an increase in the thiamin content of the alcoholic beverage.

When comparing the vitamin levels in beers with wines and ciders it can be noted that no TDP and only very minor quantities of TMP were detected in the ciders and white wines. No thiamin, TMP or TDP was detected in red wines. This is most likely due to the presence of anthocyanins in red wine interfering with the oxidation of thiamin to thiochrome by the use of the alkaline potassium ferricyanide solution.[54,55] Several authors[54−57] have reported using a potassium ferricyanide solution to measure the reducing power of anthocyanins in a variety of fruits. The fact that this reagent is used to measure reducing power suggests that the anthocyanins present in the red wine samples are being oxidized by the ferricyanide. This would lead to a shortfall in the availability of oxidizing reagent required to convert the available thiamin to thiochrome. This could also explain the limited detection of this vitamin in red wine and why this was only observed in the red wine and not the white wines (skins removed that contain the anthocyanins). Therefore this method is not appropriate for thiamin quantification in red wine, and further research is required to determine a method to first successfully remove the anthocyanins prior to derivatization. More ferricyanide solution could be added, however this would further dilute the sample, making it harder to accurately quantify the levels of thiamin in red wine. The lowered levels of thiamin vitamers determined in the white wine and ciders may be attributed to the different raw materials used in production. The lower levels of thiamin present in the wines and ciders may also be due to the potential presence of sulfite ions.[58−62] Sulfur dioxide is frequently used in both wine and cider production as an antioxidant to minimize color formation associated with oxidation of phenolic compounds,[63−66] or as a preventative antibacterial compound.[65] However, the presence of sulfites can cleave thiamin to 2-methyl-5-sulfomethylpyrimidine at room temperature.[55,56]

This research has established a highly reproducible, simple and low cost method for analyzing both thiamin and riboflavin and their respective vitamers in a variety of fermented alcoholic beverages. However this method would require further work to be able to accurately quantify thiamin levels in red wine. This study also found that generally lager style beers contain less thiamin and thiamin esters than other beer styles, probably due to the enhanced processing used to make these beers. The riboflavin concentration remains relatively constant across the different beer styles. The lowered levels of thiamin and riboflavin vitamers in ciders and wines may be most likely attributed to different raw materials and the potential use of preservatives such as sulfur dioxide.

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