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Spectrophotometric Determination of Urea in Sugar  
Cane Distilled Spirits

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Urea is an important precursor in the formation of ethyl carbamate, a known carcinogen in alcoholic beverages. Ethyl carbamate has recently been detected at high concentrations in sugar cane distilled spirits, but little is known about the concentration of urea in these beverages. The objectives of this study were to validate methodology for the determination of urea in sugar cane distilled spirits, to determine the levels in 68 samples from different regions within the state of Minas Gerais, Brazil, and to examine the relationship between the concentrations of urea and ethyl carbamate. The method, based on the reaction of urea with 1-phenyl-1,2-propanodione-2-oxime and spectrophotometric quantification at 540 nm, provided linear response from 0.5 to 15.0 mg/L. No purification of the sample was required. The limits of detection and quantification were 0.1 and 0.5 mg/L, respectively. Urea was detected in 69% of the samples at levels varying from 0.50 to 5.10 mg/L. There was no significant difference on the levels of urea in samples from different regions of the state. No significant correlation between the levels of urea and ethyl carbamate was observed for the samples analyzed.

**KEYWORDS:** Urea; sugar cane spirits; spectrophotometry; ethyl carbamate

## INTRODUCTION

Wines and other fermented and distilled beverages can occasionally have high levels of urea. According to Ough et al. (1) and Francis (2), heavily fertilized vineyards were probably the major cause of high urea levels in wines. However, urea is also formed as a consequence of yeast metabolism (3). Studies have indicated that arginine and citrulline are the main precursors (2, 4) and that the yeast strain and fermentation temperatures can also cause increased urea accumulation in wines (5).

The presence of urea in alcoholic beverages was of little significance until recent years, when it was found to react with ethanol, particularly at elevated temperatures, to form ethyl carbamate (EC). The concern with EC is based on the fact that in vivo and in vitro toxicity tests indicated that it is a genotoxic compound. It binds to DNA and is an animal carcinogen (6, 7). According to IARC (8), EC is a group 2A carcinogen. Since human exposure to carcinogenic compounds should be as low as reasonably achievable, legal limits have been established for EC in alcoholic beverages and other products. In Canada, the legal limit for EC in distilled spirits is 150  $\mu\text{g/L}$ , and the “no significant risk” level in California’s proposition 65 is 0.7  $\mu\text{g/day}$  (9). Recently, Brazilian health authorities established guideline levels for EC in sugar cane spirits (10). A limit of 150  $\mu\text{g/L}$  was fixed, and producers were given a period of 5 years to adequate their products. According to Labanca et al.

(11), sugar cane spirits contained EC at levels varying from 33 to 2609  $\mu\text{g/L}$  (mean level = 893  $\mu\text{g/L}$ ). Therefore, it is necessary to identify the precursors and to understand the mechanisms involved in the formation of EC in sugar cane spirits in order to intervene in the process and reduce levels to comply with the legislation.

The formation of EC in wines is well established. Urea, citrulline, and carbamyl phosphate can produce EC with ethanol, but urea is the primary precursor and therefore the best indicator of potential EC formation (12, 13). However, data are scarce for sugar cane spirits. According to Aresta et al. (14), EC can also be formed from the reaction of urea and ethanol, although other precursors have also been identified (among them, cyanide, copper, iron, and carbamyl phosphate). Urea can be added as a nutrient for the yeast and can also be formed through degradation

**Table 1.** Number of Samples of Sugar Cane Spirits Included in This Study<sup>a</sup>

regions	macroregions <sup>b</sup>	no. of samples
metropolitan	metropolitan	18
northwest	northwest	24
southeast	south of Minas “Alto São Francisco” “Triângulo Mineiro”	13
east	“Vale do Jequitinhonha” “Zona da Mata” “Rio Doce”	13
total		68

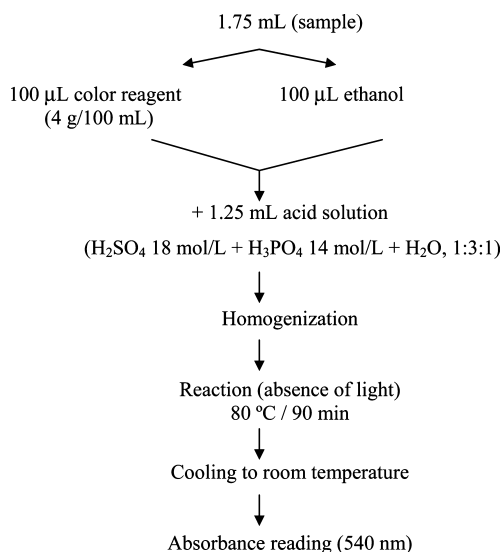
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<sup>a</sup> Samples were categorized according to area of production within the state of Minas Gerais, Brazil. <sup>b</sup> IBGE (25).

**Table 2.** Levels of Urea in Sugar Cane Spirits Spiked with Different Concentrations of Standard<sup>a</sup>

method	% recovery (CV <sup>b</sup> )/spiking levels (mg/L)			mean recovery (CV <sup>b</sup> )
	2.0	5.0	10.0	
original conditions boiling/120 min acid solution 1:3:1.25	89 (4.5)	104 (5.0)	100 (0.4)	97.4 (3.3)
modification A 70 °C/120 min acid solution 1:3:1	89 (6.2)	106 (4.0)	102 (0.8)	98.8 (3.7)
modification B 80 °C/90 min acid solution 1:3:1.25	94 (3.2)	104 (5.0)	104 (0.3)	100.5 (2.8)
modification C 80 °C/90 min acid solution 1:3:1	94 (4.3)	105 (3.2)	104 (0.4)	100.8 (2.6)

<sup>a</sup> Levels determined by the method described by Almy and Ough (5) with and without modifications during derivatization with 1-phenyl-1,2-propanedione-2-oxime. *n* = 3 <sup>b</sup> CV = coefficient of variation.

**Figure 1.** Flow diagram of the optimized method for the determination of urea in sugar cane spirits.**Table 3.** Linear Regression (Equations and Correlation Coefficients) of the Analytical Curves for the Analysis of Urea by the Optimized Method in Three Consecutive Days

days	linear regression/urea analytical curve	
	equation	correlation coefficient ( <i>R</i> <sup>2</sup> )
1	$y = 0.1684x + 0.0342$	0.9858
2	$y = 0.1638x + 0.0334$	0.9856
3	$y = 0.1638x + 0.0395$	0.9816

**Table 4.** Accuracy and Precision of the Optimized Method<sup>b</sup>

spiking (mg/mL) levels	recovery (%)	coefficient of variation (%)
1.0	99	8
5.0	106	2
10.0	108	2
reference value <sup>a</sup> > 0.1 mg/L	80–110	<15

<sup>a</sup> Accuracy and precision were determined by the recovery and coefficient of variation of urea in sugar cane spirit spiked with three different concentrations of standard. <sup>b</sup> Codex (27).

of arginine or other amino acids during fermentation (4, 15, 16). Since the reaction of urea with ethanol is faster at higher temperatures, it is likely that increased EC levels would be found in this distilled beverage compared to wine (12). The presence of urea in distillates could also result from problems during the distillation process or by contamination of the sugar added to the final product (15, 17).

Since urea is considered the main precursor of EC in fermented and distilled beverages, a fast and reliable method for the quantification of urea is necessary. Methods for the determination of urea can be grouped into three categories: enzymatic hydrolysis, color-forming reactions, and chromatographic separations. The

enzymatic method is based on the hydrolysis of urea and the subsequent analysis of the ammonium ions formed. However, the presence of urease inhibitors, prevalence of conditions that reduce the activity of enzymes, and low analyte concentrations can negatively affect the method. HPLC procedures have been described involving derivatization with 9-xanthinol, *o*-phthaldehyde, or 9-fluoreylmethyl chloroformate (18, 19). However, they require sophisticated equipment. Spectrophotometric procedures involve derivatization of urea to form colored products, which are quantified spectrophotometrically (2). The most widely used reagents are 1-phenyl-1,2-propanedione-2-oxime and diacetyl monoxime. Although spectrophotometric methods are time-consuming and labor intensive, they are relatively straightforward and well-established. Furthermore, they require equipment available in most laboratories (2, 20).

Methods for the determination of urea in distilled beverages such as sugar cane spirits are scarce. Polastro et al. (15) determined urea in sugar cane spirits at 527 nm after extraction and purification with ion exchange and reaction with diacetyl monoxime and thiosemicarbazide in acid media. The method was selective and sensitive; however, the chemical structure of the chromophore produced has been the subject of some debate (2, 21). The use of an enzymatic method is not recommended as ammonium ions are present in sugar cane spirits at levels up to 0.036 nmol/L (15). Several methods have been described in the literature for the determination of urea in wine (5, 22–24). Urea is usually extracted from wine by ion-exchange resin Dowex or Amberlite (2). The most widely used derivatization agent is 1-phenyl-1,2-propanedione-2-oxime in acid conditions and the absorbance taken at 540 nm (5, 24). It would be interesting to determine if it is adequate for the analysis of sugar cane distilled spirits.

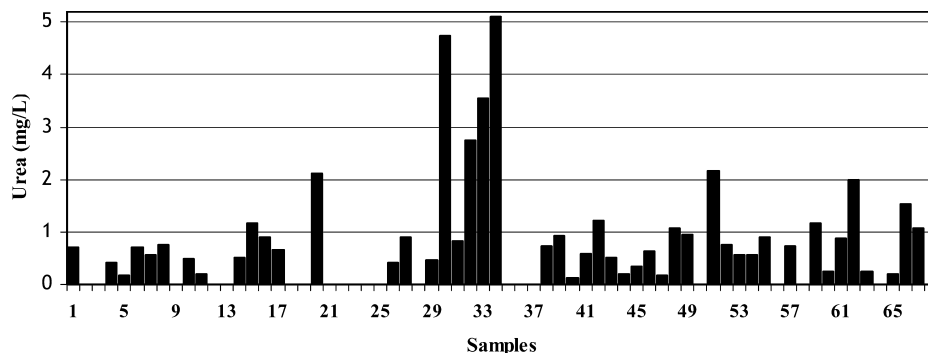
The objectives of this work were to optimize and validate a spectrophotometric method for the quantification of urea in sugar cane spirits, to determine the levels of urea in products from different areas within the state of Minas Gerais, Brazil, and to determine if there is a correlation between the levels of urea and ethyl carbamate in the respective products.

## MATERIALS AND METHODS

**Materials.** Sixty-eight samples of sugar cane spirits were purchased at stores in Belo Horizonte, MG, Brazil, from May 2003 until March 2004. The samples comprised closed bottles of approximately 750 mL of sugar cane distilled spirit produced in different regions of the state of Minas Gerais. Urea was determined in the samples, and the results were analyzed individually and also grouped in four geographical regions: metropolitan, northwest, southeast, and east. The last region included some macroregions established by IBGE (25), as indicated in Table 1.

Urea and 1-phenyl-1,2-propanedione-2-oxime were purchased from Sigma Chemical Co. (St. Louis, MO). The other reagents were of analytical grade. A UV–visible spectrophotometer was used (Shimadzu model 160A, Kyoto, Japan).

**Optimization of the Method for the Determination of Urea in Sugar Cane Spirits.** The spectrophotometric method described by



**Figure 2.** Levels of urea (mg/L) in samples of sugar cane spirits purchased randomly at stores in Belo Horizonte, MG, Brazil from May 2003 to March 2004.

**Table 5.** Descriptive Statistical Analysis of the Levels of Urea in Sugar Cane Spirits<sup>a</sup>

descriptive statistics	urea levels (mg/L)
average	0.71
standard error	0.12
median	0.49
mode	0.00
standard deviation	1.01
interval	5.10
minimum	0.00
maximum	5.10
total number	68.0

<sup>a</sup> Spirits purchased randomly in stores of Belo Horizonte, state of Minas Gerais, Brazil, from May 2003 until March 2004.

**Table 6.** Levels of Urea in Sugar Cane Spirits Categorized according to the Region of Production<sup>b</sup>

regions	n	urea levels (mg/L)	
		range	mean $\pm$ sd <sup>a</sup>
metropolitan	18	nd–1.17	0.40 $\pm$ 0.37
northwest	24	nd–5.10	1.02 $\pm$ 1.52
southeast	13	nd–2.00	0.59 $\pm$ 0.62
east	13	nd–2.17	0.70 $\pm$ 0.60
total	68	nd–5.10	0.71 $\pm$ 1.01

<sup>a</sup> Levels are from production regions in the state of Minas Gerais, Brazil, from May 2003 until March 2004. <sup>b</sup> Mean levels ( $\pm$  standard deviation) in samples from different regions are not significantly different (Anova, 5% probability). <sup>c</sup> nd = not detected ( $< 0.1$  mg/L).

Almy and Ough (5) was used. Two 1.75 mL portions of the sample were placed in separate test tubes. An ethanolic solution of 4% 1-phenyl-1,2-propanedione-2-oxime (0.100 mL) was added to one test tube, and the same volume of ethanol was added to the other (blank). The tubes were vortexed, and 1.25 mL of a mixture of 18 mol/L  $\text{H}_2\text{SO}_4$ , 14 mol/L  $\text{H}_3\text{PO}_4$ , and  $\text{H}_2\text{O}$  was added. The tubes were vortexed, sealed with PTFE-lined screw caps, placed in boiling water for 2 h without light, and brought to room temperature within 30 min in the dark. The absorbances of the samples and blanks were determined at 540 nm, and the blank readings were subtracted from the readings from samples to eliminate interference from the sample.

During optimization of the method, different compositions of the acid solution and different temperatures/times of reaction were tested. The samples were spiked with urea at three different spiking levels (2.0, 5.0, and 10.0 mg/L).

**Validation of the Optimized Methodology.** The selected method was validated for the determination of urea in sugar cane spirits. The parameters tested were linearity, detection limit for the equipment, detection and quantification limits of the method, specificity, accuracy, and precision according to procedures established by Eurachem (26) and Codex (27).

**Statistical Analysis.** All of the experiments and analyses were performed in triplicate. The results were submitted to analysis of

variance (ANOVA), and the means were compared by the Duncan test at 5% probability. The existence of significant correlation between the levels of urea and of previously reported ethyl carbamate of the same samples (28) was determined by Pearson correlation.

## RESULTS AND DISCUSSION

**Optimization of the Method for the Determination of Urea in Sugar Cane Distilled Spirits.** During optimization of the method described by Almy and Ough (5), some modifications (A–C) were undertaken in the preparation of the acid solution and also in the temperature and time necessary for the formation of the derivative. Analysis of the recoveries of urea using the different procedures at three spiking levels is indicated in **Table 2**. The recoveries and the coefficients of variation are acceptable when compared to values established by Codex (27). However, modification C provided the highest recoveries and the lowest coefficients of variation. Besides the improvement in the performance of the method, the modifications made were useful in reducing the time required for the derivatization from 120 to 90 min, and the acid solution was easier to prepare and reproduce. Therefore, the optimized conditions, as described in **Figure 1**, were validated for the analysis of urea in sugar cane spirits.

The purification of the sample, described by Almy and Ough (5) as an essential step for the removal of interfering compounds in the determination of urea in wines, was not required in the analysis of sugar cane distilled spirits. This could be confirmed by the readings of the blanks, which were not significant (blank absorbance values were lower than 0.002). Furthermore, tests performed with different mono- and disaccharides (sucrose, glucose, fructose, galactose, manose, and lactose at concentrations of 1 g/L), which could be added to sugar cane spirits (10), did not provide any reading at 540 nm after derivatization with 1-phenyl-1,2-propanedione-2-oxime. According to Francis (2), the most appropriate method for the analysis of urea will depend on the sample matrix. The less complex matrix of the sugar cane distilled spirit compared to wine can warrant simpler procedures during the analysis of urea.

Therefore, the direct analysis of the sugar cane distilled spirit without need for a purification step greatly reduces the overall analysis time, which is a major constraint in the analysis of large numbers of samples.

**Method Validation.** The linearity of the response of the equipment, during absorbance measurements at 540 nm of standard solutions at concentrations of 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, and 15.0 mg of urea per liter of 40% ethanol on three consecutive days, is indicated in **Table 3**. On the basis of the correlation coefficients obtained ( $0.9816 \leq R^2 \leq 0.9858$ ),



the response was linear from 0.1 to 15.0 mg/L. Furthermore, the linearity was confirmed by the method of least-squares.

The limits of detection of the equipment and of the method for the analysis of urea were both 0.1 mg/L. The limit of quantification (LOQ) of the method was 0.5 mg/L as it provided 94% recovery and coefficient of variation of 11%. These percentages are within the limits established by Codex (27) (i.e., percent recovery between 80 and 110% and coefficient of variation smaller than 15%). This LOQ is lower compared to values obtained for wines (1.0 mg/L) using the same method and also when using diacetyl monoxime/thiosemicarbazide as the coloring agent (2). Although the LOQ reported by Clark et al. (19) (0.003 mg/L) for the determination of urea in urine samples was lower, the limits of quantification in our procedure were sufficient for the intended application.

The method was accurate (Table 4), as recovery of urea from samples spiked with three different levels of urea (1.0, 5.0, and 10.0 mg/L) provided values within the limits established by Codex (27). The method was also precise, as the coefficients of variation for the recoveries were  $\leq 8\%$  for every spike level.

**Levels of Urea in Sugar Cane Distilled Spirits.** The levels of urea in the samples of sugar cane distilled spirits analyzed are indicated in Figure 2. Urea was not detected (levels  $< 0.5$  mg/L) in a large number of samples (31%). 50% of the samples contained urea at levels ranging from 0.5 to 1.0 mg/L, 15% from 1.0 to 3.0 mg/L, and only 4% of the samples showed levels higher than 3.0 mg/L. According to descriptive statistical analysis (Table 5), the highest level of urea was 5.10 mg/L, the mean was 0.71 mg/L, and the median was 0.49 mg/L.

When grouping the samples according to the region of production within the state of Minas Gerais (Table 6), no significant difference was observed among mean values for the different regions. This result suggests that the differences in sugar cane cultivation practices and distilled spirit production did not affect the levels of urea in the final product. These samples showed no significant differences regarding alcoholic degree; however, the levels of copper were significantly higher in samples from the metropolitan region compared to the others (28).

The levels of urea found in samples produced in the state of Minas Gerais are lower than values reported by Polastro et al. (15) for samples from different parts of Brazil. These researchers found urea in every sample analyzed at levels varying from 0.18 to 73.2 mg/L with an average of 18.9 mg/L.

**Correlation between Levels of Urea and Levels of Ethyl Carbamate in Sugar Cane Distilled Spirits.** No significant correlation (Pearson, 5% probability) was observed between the levels of urea and the levels of EC described by Labanca et al. (11) for the same samples. The sampling approach probably contributed to this result as several variables were not controlled, among them beverage production, conditions and time of storage of the samples, and aging time, among others.

On the basis of these results, the spectrophotometric method developed was accurate, precise, sensitive, straightforward, and easy to perform, and therefore, adequate for the analysis of urea in sugar cane distilled spirits in industry laboratories. Urea was detected in 69% of the samples at levels varying from not detected ( $< 0.05$ ) to 5.10 mg/L. The widespread levels detected could reflect the lack of standardization, typical of the small scale, in traditional production of sugar cane distilled spirits in the state of Minas Gerais. Further studies are needed on the levels of urea throughout the production of sugar cane distilled

spirits in order to ascertain its role in the formation and accumulation of ethyl carbamate. This information will be relevant in the identification of critical control points, which should be controlled to warrant the quality and safety of the product.

## ABBREVIATIONS USED

EC, ethyl carbamate; nd, not detected; IARC, International Agency for Research on Cancer; LOQ, limit of quantification.

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