Quantitative Determination of L-Arginine by Enzymatic End-Point Analysis

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An enzymatic end-point method for the quantitative determination of L-arginine was evaluated with samples of synthetic wine and natural grape juice. The enzymes arginase, urease, and glutamate dehydrogenase were used in this simple assay, similar to those described for many metabolites by Boehringer-Mannheim. In synthetic wine, recovery of L-arginine ranged between 98.3 and 104.4% and the precision as coefficient of variation was between 0.4 and 1.47% in the concentration range of the method, 0−100 mg/L L-arginine. The recovery of L-arginine in a grape juice with added L-arginine after clarification with polyvinylpolypyrrolidone ranged between 100 and 101.3%, and the coefficient of variation was 0.6%. The method has low material costs of ∼0.43 U.S.$ per assay, and the time course of the reaction facilitates measurement of several samples concurrently. The results of this evaluation indicate that the enzymatic assay is a preferred method over colorimetric methods for the manual determination of L-arginine.

Keywords: *Arginine; enzymatic analysis; determination; quantification*

INTRODUCTION

L-Arginine is a basic amino acid of considerable interest for human nutrition and health (*1*) and is present in significant amounts in many agricultural products. Its concentration is used to measure the fruit maturity of peanuts (*2*) and grapes (*3*). Especially in the wine-making process, knowledge of arginine concentrations in musts and wines is important. Low arginine concentrations have been related to fermentation problems (sluggish fermentations) and to sensory imperfections (*4*). Furthermore, arginine degradation by yeast and lactic acid bacteria present in wine leads to excretion of carcinogenic ethyl carbamate precursors (*5*−*7*).

A large variety of methods for the quantitative determination of arginine have been described in the past. Application of HPLC (*8*), amino acid analyzers (*9*), capillary electrophoresis (*10*), voltammetry (*11*), or fluorometric methods (*12*) is often inappropriate for smaller laboratories in the food industry because they are time-consuming, expensive, or require skilled labor. Therefore, relatively easy to perform colorimetric reactions such as the Staron-Allard and Voges-Proskauer methods (*13*) and especially the Sakaguchi reaction and its developments (*14*−*16*) are still widely used, despite their lack of specifity. In fact, ornithine, ammonia, and citrulline interfere with the Voges-Proskauer method and ornithine and ammonia with the Staron-Allard method (R. Mira de Orduña, unpublished results). Glycine, TRIS (*15*), and ammonia (*17*) (R. Morenzoni, E. J. Gallo Inc., personal communication) interfere with the Sakaguchi method. The interferences are significant because ornithine, ammonia, and citrulline are metabolites of the arginine catabolism of wine microorganisms. Additionally, the determination with colorimetric methods can be further complicated in samples originating from solutions that undergo color changes over time at the wavelength of the chromophore.

The application of the enzyme arginase for the determination of L-arginine has been reported early on (*18*). L-Arginine is degraded by arginase to ornithine and urea, and the latter can be hydrolyzed by urease to yield ammonia and CO_2. Several methods have been proposed using this approach. Urea can be determined colorimetrically (*19*), or ammonia can be measured by potentiometric biosensors (*20*), conductance sensor systems (*21*), or colorimetrically by the Berthelot reaction (*22*). However, biosensors may have limitations because of their stability, and they are not readily available. Application of colorimetric determinations such as the Berthelot reaction on plain samples involves again the risk of interferences, unless determination is preceded by cumbersome separation steps. This is valid as well for the method described by Hutzler (*23*), which uses the unspecific reaction of fluorodinitrobenzene with biogenic amines that have been produced from the enzymatic decarboxylation of amino acids. Therefore, a test would be desirable that is performed solely by enzymes, comparable to those described for many metabolites by Boehringer Mannheim (*24*) and requiring only a standard laboratory spectrophotometer. One attempt to present such a method for L-arginine is known to the author (*25*). However, the method described is slow (60 min per sample) and is useless if urea or ammonia is present in the sample.

The aim of this work was to elaborate and evaluate a simple and cheap enzymatic end-point method for the quantitative routine determination of L-arginine with the enzymes arginase, urease, and glutamate dehydrogenase.

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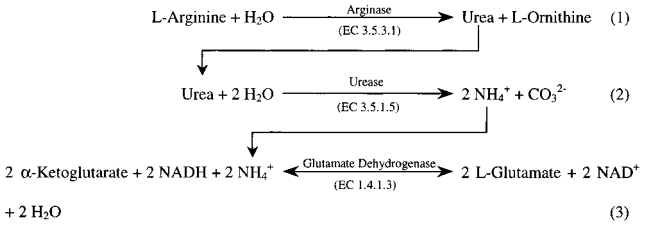
MATERIALS AND METHODS

Principle.



The decrease in concentration of NADH, as measured by the change of extinction at 340 nm, is proportional to the amount of arginine originally present. Two moles of ammonia is formed from 1 mol of L-arginine.

Chemicals. L-Arginine (Arg), triethanolamine (TEA), polyvinylpolypyrrolidone (PVPP), α-ketoglutaric acid (α-KG), urease (EC 3.5.1.5), and arginase (EC 3.5.3.1) were from Sigma. NADH, ADP, and glutamate dehydrogenase (Gl-DH; EC 1.4.1.3) were from Roche (previously Boehringer Mannheim). For the preparation of standards, anhydrous analytical grade arginine (dried for 24 h at 105 °C) was used. A modified synthetic wine containing several amino acids [after Liu et al., without tannins (*26*)] and pure commercial grape juice (Grapetise, Pacific Beverages, Bayswater, VIC, Australia) were used as media. Clarification of grape juice was carried out with 10% (w/v) PVPP.

Instrumentation. A UV−vis spectrophotometer (Cary 1, Varian Inc.) was used for kinetic studies, whereas a simple laboratory spectrophotometer (300−900 nm; Nova Tech, Auckland, New Zealand) was used for all other measurements. Disposable half-micro cuvettes (maximum 2 mL) were used throughout the work.

Preparation of Buffer and Enzymes. Buffer was prepared by adding the following amounts of solutes per liter of water and adjusting to pH 8.5 with orthophosphoric acid: TEA, 32 g (0.21 M); α-KG, 2.4 g (13 mM); NADH, 0.1 g (0.14 mM); ADP, 1 g (2.1 mM) (concentrations in final buffer in parentheses). At 4 °C, buffer containing TEA and α-KG is stable for at least 1 month, and buffer with added NADH and ADP is stable for 3 days. Gl-DH was used pure at a concentration of 200 U/mL. Urease and arginase were dissolved in 50% glycerol (15 and 20 mg/mL, respectively) to give concentrations of 1500 and 940 U/mL, respectively. All enzyme solutions are stable for at least 3 months. The maximum concentration of L-arginine allowed in the sample is 100 mg/L.

Procedure and Calculations. The assay procedure is described in Table 1, and measurements were carried out at 25 °C. Under these conditions the reaction proceeds stoichiometrically, and 2 mol of NAD is formed from 1 mol of L-arginine. The absorbance difference is calculated as $\Delta A_{Arg} = \Delta A_1 - \Delta A_2$. The absorbance difference of a blank assay (ΔA_{blank}) containing 0.1 mL of water instead of sample was subtracted from ΔA_{Arg} for each measurement. The following formula was used to calculate arginine concentrations in samples:

$$c_{L-Arg}\,[mg/L] = (\Delta A_{Arg} - \Delta A_{blank})\frac{V \times MW}{v d \epsilon 2}$$

In this formula V = final volume in cuvette (1.53 mL), v = sample volume (0.1 mL), MW = molecular weight of the substance to be assayed (174.205 g/mol), d = light path (1 cm), and ϵ = absorption coefficient of NADH [6.3 l/(mmol × cm) at 340 nm]. It follows that

$$c_{L-Arg}\,[mg/L] = (\Delta A_{Arg} - \Delta A_{blank}) \times 211.53$$

RESULTS

Figure 1 shows two examples of absorbance time courses measured to determine arginine in synthetic wine samples with the enzymatic assay. At an L-arginine concentration of 100 mg/L in the sample, the final absorbance A_2 reached after addition of arginase was obtained within 12 min. It was possible to reduce the duration of the assay to <5 min by increasing the enzyme concentrations in the assay to 10 U/mL for glutamate dehydrogenase and to 15 and 8 U/mL for urease and arginase, respectively. The absorbance A_2 was stable for at least 20 min after reaching the final value as shown in Figure 1 for synthetic wine.

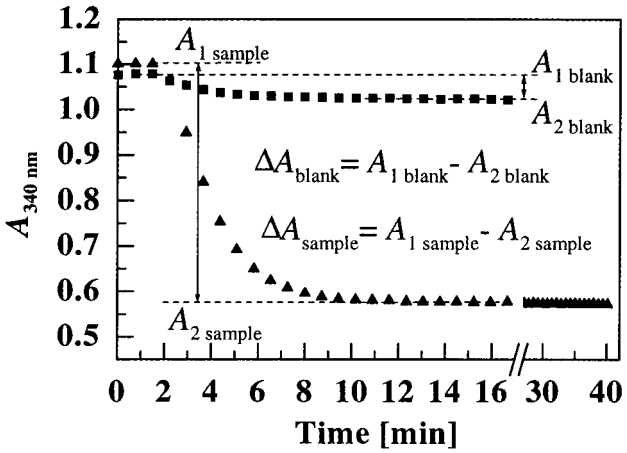


Figure 1. Enzymatic determination of L-arginine in synthetic wine: blank (0 mg L^{-1} arginine), ■; sample (100 mg L^{-1} arginine), ▲. Time course of absorbances at 340 nm is shown. Reaction was started by arginase addition at t = 2 min.

Table 1. Enzymatic Determination of L-Arginine: Addition of Buffer, Enzymes, and Substrate to the Cuvette

add to cuvette	concn in cuvette
buffer, 1.4 mL	TEA, 0.19 M α-KG, 12 mM NADH, 0.13 mM ADP, 1.9 mM
sample, 0.1 mL	arginine, max, 38 μM
Gl-DH, 0.01 mL	Gl-DH, 7.8 U/mL
urease, 0.01 mL	urease, 9.8 U/mL
mix, wait for constant absorbance, and read A_1	
arginase, 0.01 mL	arginase, 6 U/mL
mix, wait for constant absorbance, and read A_2	

Accuracy and precision of the enzymatic method were determined by measuring the L-arginine concentration in several samples of synthetic wine, adjusted to L-arginine concentrations of 0−150 mg/L. The linear regression of a standard response curve generated with data from 5-fold determinations in the concentration range 0−100 mg/L of L-arginine had the equation

$$c_{L-Arg}\,[mg/L] = [\Delta A_{340nm} - 0.059(\pm 0.002)]/0.005(\pm 2.8 \times 10^{-5})$$

(method of least squares, standard errors in parentheses, n = 25). Under the conditions described, L-arginine concentrations exceeding 100 mg/L in the sample led to a nonlinear response curve and, therefore, a maximum L-arginine concentration of 100 mg/L in the sample was established. Table 2 shows the recovery and repeatability results of the enzymatic method from these determinations. The recovery of L-arginine in the synthetic wine samples ranged from 98.3 to 104.4%, and the precision (% CV) ranged from 0.4 to 1.47%.

Table 2. Precision of the Enzymatic Method and Recovery of L-Arginine in Synthetic Wine: Results of L-Arginine Determinations in Synthetic Wine, Adjusted to L-Arginine Concentrations of 25−100 mg/L

arginine added (mg/L)	n	mean (mg/L)	% CV[a]	recovery (%) min	recovery (%) max
25	5	25.7	1.47	101.0	104.4
50	5	50.9	1.12	100.5	103.4
75	5	76.2	0.5	101.1	102.5
100	5	98.6	0.4	98.3	99.1

[a] Coefficient of variation.

Table 3. Precision of the Enzymatic Method and Recovery of L-Arginine in Grape Juice (after PVPP Clarification): Results of L-Arginine Determinations of Grape Juice Samples, an L-Arginine Standard Solution, and a 1:1 Mixture of both Grape Juice and L-Arginine Standard

sample[a] (dilution factor)	mean (mg/L)	% CV[b]	recovery (%) min	recovery (%) max
grape juice (1:10)	58	0.67		
grape juice (1:20)	28.85	0.46		
0.5 g/L arginine standard (1:10)	50.7	0.64	100.6	102.1
1:1 grape juice/standard (1:10)	54.63	0.6	100.0	101.37

[a] $n = 5$ for all samples. [b] Coefficient of variation.

Evaluation of recovery and repeatability was further carried out using a commercial grape juice with unknown arginine concentration that was clarified with PVPP and spiked with an L-arginine standard solution. The results of this evaluation are shown in Table 3. The L-arginine concentration in the juice was measured with 1:10 and 1:20 diluted juice samples. The L-arginine concentration of a 1:10 diluted standard solution (0.5 g/L) of L-arginine was determined, as well. Measurement of a 1:1 mixture of both juice and L-arginine standard solution (1:10 diluted) showed that L-arginine recovery in the spiked juice ranged between 100 and 101.3% of the theoretical value calculated from the L-arginine concentrations determined in the grape juice and the L-arginine standard. The precision of the determinations with spiked grape juice was 0.6 (% CV).

Unclarified samples of grape juice led to creeping absorbance decreases and reaction times of >30 min. The increase of enzyme concentrations in the assay alone did not lower reaction times significantly, but previous clarification of the juice with PVPP reduced the assay duration to 16 min.

DISCUSSION

A coupled enzymatic method including arginase, urease, and glutamate dehydrogenase (Gl-DH) has been evaluated for the determination of L-arginine. Although the application of the three enzymes for the determination of arginine is not new, no simple and readily applicable method is described in the literature. Additionally, data about specifity and accuracy of methods using these enzymes is scarce.

In this work, a synthetic wine and grape juice were chosen for the evaluation of the method because they contain several substances known for their inhibitory effects on arginase and Gl-DH. Citric acid (*27*) and several amino acids (*28*) including L-lysine, L-valine, and especially L-proline, which is abundant in grape juices and wines, have been shown to inhibit arginase. Tannins in fruit juices and their products inhibit Gl-DH (*24*). The present work has shown the suitability of the enzymatic method for the determination of arginine in both media used. Accuracy and precision were satisfactory compared to values found for many enzymatic determinations described by Bergmeyer (*29*). Utilization of a microassay leads to low material costs of ∼0.43 U.S.$ per assay, including the cuvette. The method is rapid, and because the absorbance at the end-point is stable for 20 min, numerous samples can be analyzed concurrently. The time for analyzing grape juice was increased compared to synthetic wine despite clarification with PVPP. However, the assay time could be considerably reduced by increasing the concentrations of enzymes in the assay. Increased enzyme concentrations alone could not replace clarification with PVPP. Samples with different composition from the ones used in this work should be prepared according to instructions given by Boehringer Mannheim for the analysis of ammonia and urea (*24*).

The enzymes urease and glutamate dehydrogenase are specific for their substrates urea and ammonia, respectively (*30*). Besides L-arginine, canavanine is known to be hydrolyzed by arginase (*27*). Its occurrence is confined to some legume species (e.g., *Canavalia ensiformis*, the jack bean), and if present in the sample, the method may give false-positive results.

When added to pure buffer containing Gl-DH and urease, arginase causes a significant decrease in absorbance ($\Delta A \approx 0.06$). This may originate from arginine or canavanine impurities in the utilized commercial urease, which is prepared from jack beans. It is therefore important to determine the decrease in absorbance of a blank, as described in the methods, and to subtract it from the value of each measured sample prior to calculation of arginine concentrations. As observed from the coefficients of variation at different arginine concentrations (Table 2), the precision of the method decreases with lower arginine concentrations. This has to be considered especially when the ratio of ammonia and urea to arginine in the sample is high, because dilution of the samples has to be carried out according to the concentrations of all three substrates.

The simplicity of the presented method enables analytical laboratories in agriculture and the food industry to carry out routine arginine determinations in-house, avoiding the cost and delay of analysis by outside services. The results of this evaluation suggest the enzymatic assay should be considered as a preferred method over colorimetric methods for the manual determination of L-arginine in foodstuffs.

ABBREVIATIONS USED

A, absorbance at specified wavelength; Arg, L-arginine; *c*, concentration; CV, coefficient of variation; Gl-DH, glutamate dehydrogenase; α-KG, α-ketoglutaric acid; *n*, number of measurements; PVPP, polyvinylpolypyrrolidone; TEA, triethanolamine; TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; U, enzymatic unit (quantity of enzyme that leads to formation of 1 μmol of product per minute under the conditions specified by the supplier).

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Supporting Information Available: Figures 2 and 3 showing the interference of several substrates with the determination of L-arginine by two colorimetric methods as cited in the Introduction and Figure 4 showing the standard response curve generated from synthetic wine samples adjusted to several L-arginine concentrations (shown under Results as formula). This material is available free of charge via the Internet at http://pubs.acs.org.

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