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A Simple and Fast Method for Arginine Determination in Grape Juice

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ABSTRACT

Arginine in grape juice can be metabolized by wine yeasts and malolactic bacteria to precursors of ethyl carbamate, a known carcinogen. The aim of this study was to develop a simple, fast and accurate method for determining arginine in grape juice with Sakaguchi reaction following the separation of arginine with Amberlite IR120 strong cation-exchange resin. Measurement was done under optimized conditions as follows: sample (mg) to cation-exchange resin (g) ratio of 0.683, sample pH 2.0, sample flow rate 0.5 mL/min, use of distilled water rinsing to avoid interference of other compounds adsorbed on the resin, and elution with 1 mol/L of NaOH solution. This method can be applied in a quick and accurate quantification of arginine in grape juice for controlling the ethyl carbamate level in wine.

Key words: grape juice, arginine, ion-exchange, ethyl carbamate, Sakaguchi reaction

INTRODUCTION

Arginine, one of the most abundant amino acids in grape juice, is closely related to the ethyl carbamate (EC) level in wines(1-4). Ethyl carbamate, also known as urethane, occurs naturally in wines and some other fermented foods and beverages, and is classified as a possible human carcinogen(5,6). In 1988, the American wine industry has established a voluntary target for EC below 15 μg/L in table wines and below 60 μg/L in fortified wines. Currently, a discussion is going on to establish maximum limits for EC in the Codex Alimentarius Commission (CAC).

Arginine is degraded to urea and ornithine by the enzyme arginase (EC 3.5.3.1) via urea-circle pathway. Some urea is assimilated by the yeast, and some is released into the fermentation medium with excess arginine in grape juice. Accumulated urea in the medium can spontaneously react with ethanol to form ethyl carbamate(1,7-9).

Citrulline, formed by arginine metabolism via the arginine-deiminase pathway by wine malolactic bacteria, is another significant precursor of ethyl carbamate in wine(10-12). Urea and citrulline accumulation in the fermentation medium mainly depends on arginine level and type of yeast and malolactic bacteria strains. Ough (1989)(13) reported that if arginine concentration in juice is higher than 1000 mg/L, ethyl carbamate concentration will be potentially above 15 μg/L in wine, the current voluntary limit in the United States.

To avoid potential health hazard of ethyl carbamate in wine, determination of arginine levels in grape juice is an essential step to realize ethyl carbamate HACCP control. A simple and accurate method for determining arginine levels in grape juice is the prerequisite for winemakers to take proper measures.

Reported methods for arginine analysis involve HPLC(2,14-16), amino-acid analyzer and capillary electrophoresis(17). These methods are mostly slow and not commonly available to small-scale wineries. On the other hand, the Sakaguchi reaction to determine arginine is simple, specific and sensitive, and has been used for determining trace amount of arginine in biological materials. In our experiment, 8-hydroxyquinoline was selected as the Sakaguchi reagent because the chromogenic product formed had much higher apparent molar absorption than those with other Sakaguchi reagents. The chromogenic product was formed by the reaction between arginine and 8-hydroxyquinoline and its absorbance was measured at 500 nm. The reaction took place at 0°C and excess urea was added when the color developed. The color stability lasted for 4 min, which is sufficient to finish the measurement.

However, the composition of grape juice is so complicated that arginine cannot be directly measured by Sakaguchi reagents due to interference of other compounds. Therefore, separation of arginine from grape juice is a very important pretreatment for quantita-

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tive analysis. Ion exchange is commonly used to separate amino acids based on their isoelectric points. Amino acids are ampholytes with pH-dependent net charges. Arginine has the highest isoelectric point (pI = 10.8) among amino acids commonly found in grape juice. In acidic solution, Arg^{2+} and Arg^{+} can be adsorbed by strong cation-exchange resins and eluted from the resin by alkaline solution(18). In fact, some interferential cations other than arginine, such as potassium, lysine, histidine, proteins and charged phenolics in the grape juice, are also adsorbed by strong cation-exchange resins. In addition, ion exchange resins have a limited adsorption capacity, thus overflow of targeted component can happen due to large sample volume or insufficient resin. To avoid this, it is necessary to estimate the adsorption capacity of the resin, particularly when there are high concentrations of interferences.

The purpose of this work was to develop a simple, accurate and rapid arginine separation method for measuring arginine in grape juice with Sakaguchi reaction. The developed method could have the application potential in routine quantification of arginine for both grape growers and winemakers in order to monitor and control the ethyl carbamate level in wine.

MATERIALS AND METHODS

I. *Juice Sample*

Grape samples were harvested from Yangling (Shaanxi Province), Yantai (Shandong Provice), Yinchuan (Ningxia Province) and Shacheng (Hebei Province) and transported immediately to the laboratory in August and September 2006. Grape samples were kept frozen at -22°C, and the juice was clarified by centrifugation at 1100 ×g for 10 min at room temperature prior to analysis.

II. *Ion Exchange Resins and Pretreatment*

A strong cation-exchange resin (Amberlite IR120, Aldrich Chemical Company, Milwaukee, USA, 2.0 equiv/kg resin (Na^{+}), particle size 0.4-1.2 mm and moisture content 46-52%) was chosen for this study based on a preselection of resins provided by several producers (results not shown). New resin was pretreated with 2 mol/L of HCl and 2 mol/L of NaOH for 4 hr, respectively, and then washed with distilled water.

Resin was regenerated by pretreatment with 2 mol/L of HCl for 2 hr and washing with distilled water. Pretreated resin was stored in distilled water before use.

For separating arginine, pretreated resin was packed in chromatographic column (D 1.0 × 30 cm). Sample (10.0 mL, pH 2.0) was loaded to the column at the rate of 0.5 mL/min, then rinsed with borate buffer (0.2 M, pH 8.0 and 9.0) or distilled water. Finally, the sample was eluted with 1 mol/L of NaOH solution.

III. *Model Juice Composition*

A model juice was formulated as described by Austin (2000)(19) with minor modifications. The model juice had the following composition (in 100 mL of deionized water): 11 g of fructose, 11 g of glucose, 600 mg of tartaric acid, 300 mg of malic acid, 50 mg of citric acid, 30 mg of catechin, 200 mg of potassium chloride, 100 mg of arginine, 25 mg of histidine, 13 mg of alanine, 11 mg of lysine, 9 mg of glutamine, and 7 mg of serine. Arginine (purity ≥ 99%) was purchased from Xi'an Runde Bio-tech Co., Ltd, Xi'an, Shaanxi Province, China.

IV. *Analysis of Arginine by Sakaguchi Reaction*

Determination of arginine was based on Sakaguchi reaction as described by Gilboe (1956)(20). Absorbance was measured with an UV-1700 spectrophotometer (Shimadzu Corporation, Japan). All reagents and solutions were cooled on ice before determination. Afterwards, 1.0 mL of 0.02% 8-hydroxyquinoline and 1.0 of mL 10% NaOH were added sequentially to a tube containing 5 mL of sample (0-12 μg/mL arginine). The solution was mixed thoroughly and then 0.5 mL of 0.4% sodium hypobromite was added to develop the color. After mixing, 1.0 mL of 40% urea was added within 30 sec, the mixture was kept in water bath at 0°C for 2 min before its absorbance was read at 500 nm. Standard solution from 1 to 12 μg/mL arginine was prepared. Arginine concentration was determined based on the standard curve and dilution rate.

V. *Static Adsorption Capacity of Strong Cation-exchange Resin*

Model juice (15 mL, pH 2.0) was added to a conical flask containing 5 g of pretreated strong cation-exchange resin. Then the flask was placed on a shaker at 95 rpm at room temperature. Arginine levels in the solution in the conical flask were measured every 10 min.

Static adsorption capacity of resin (mg/g) can be expressed as:

$$\frac{(C_0-C)V}{m}$$

Where C_0 is arginine concentration in model juice before adsorption (μg/mL), C is arginine concentration in model juice after adsorption (μg/mL), V is the volume of the model juice (mL), and m is the amount of resin used (g).

VI. *Validation of Analysis with an Amino-acid Analyzer*

Accuracy of the new method was checked by running the same sample through a Beckman 121MB Amino-acid Analyzer (Beckman Instruments, Fullerton, CA, USA). The resin was eluted with 2.0 mol/L of ammonium hydroxide solution, followed by analysis of arginine by ninhydrin solution.

VII. *Statistical Analysis*

The SAS package(21) was used to perform multiple range tests to compare sample means. All experiments were conducted in quadruples, and results were expressed as mean values ± standard deviation.

RESULTS AND DISCUSSION

I. *Static Adsorption Capacity of Amberlite IR120 Resin*

The adsorption capacity of Amberlite IR120 increased with time, and adsorption equilibrium was obtained after 20 min (Figure 1). The maximum static capacity of Amberlite IR120 to adsorb arginine was 0.683 ± 0.020 mg/g. Based on the estimated arginine content in the model juice and the maximum adsorption capacity of the resin, we could calculate quantity of the resin to be used for a given sample.

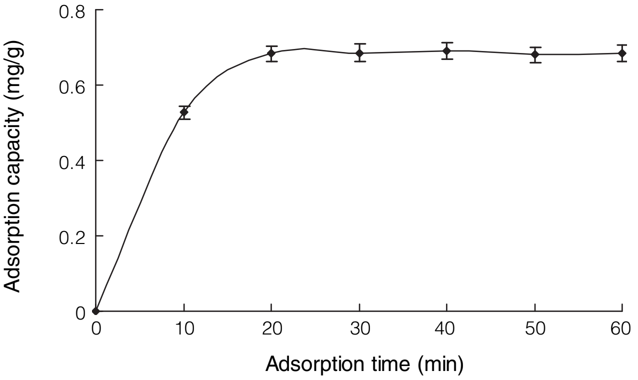

Figure 1. Static adsorption curve of arginine by Amberlite IR120 resin. Fifteen milliliter of model juice (pH 2.0) was mixed with 5 g pretreated Amberlite IR120 resin. The mixture was shaken at 95 rpm at room temperature. Data are expressed as means ± standard deviation (n = 4) $P \leq 0.05$.

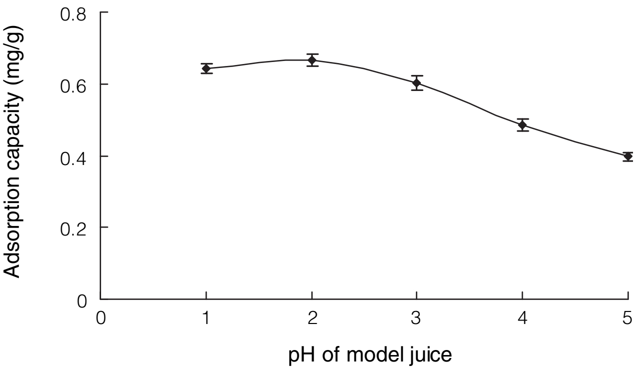

Figure 2. Influence of model juice pH on the adsorption capacity of Amberlite IR120 resin. Fifteen milliliter of model juice was mixed with 5 g pretreated Amberlite IR120 resin. The mixture was shaken at 95 rpm at room temperature. Data are expressed as means ± standard deviation (n = 4) $P \leq 0.05$.

II. *Influence of Model Juice pH on Adsorption Capacity*

Figure 2 shows the results in a series of model juice at pH 1.0 to 5.0 mixed with 5 g of pretreated Amberlite IR120. It can be observed that as pH rose from 1.0 to 2.0, adsorption capacity gradually increased. However, adsorption capacity decreased with further increase of pH from 2.0 to 5.0.

The net charge on arginine depends on pH of the solution. Arg^{2+} is the main cation in a pH below 2.17, while Arg^{+} is the main cation in a pH between 2.17 and 9.04(18). Selectivity coefficient of Arg^{2+} is usually much higher than that of Arg^{+}. In addition, Arg^{2+} is competing with other univalent and bivalent cations. Absorption capacity decreased with the increase of pH. However, due to the competition between H^{+} and Arg^{2+} (22), the exchange capacity was lower than that at pH 2.0 in the pH 1.0 model juice. Therefore, the optimal pH for adsorption was 2.0.

III. *Influence of Flow Rate on the Dynamic Adsorption Capacity*

Sixteen grams of pretreated Amberlite IR120 was packed in a chromatographic column and the model juice (pH 2.0) flow rate was increased from 0.5 to 2.0 mL/min (Figure 3). The results show that the maximum adsorption capacity, 0.676 ± 0.015 mg/g, occurred at the flow rate of 0.5 mL/min. At the flow rate of 1 mL/min, the adsorption capacity was 0.643 ± 0.019 mg/g, slightly lower that of 0.5 mL/min. When the flow rate was changed to 1.5 or

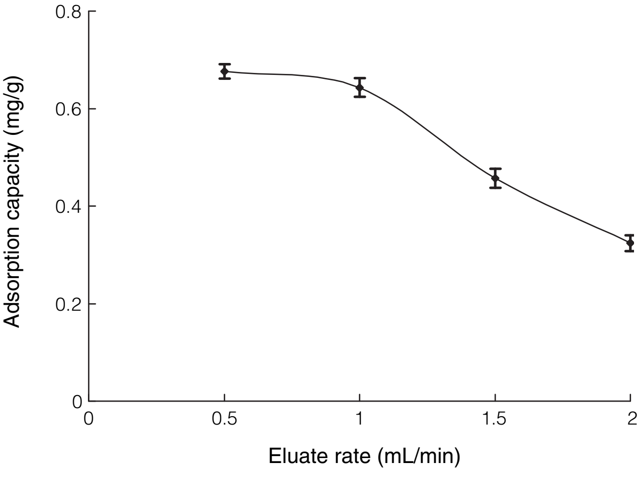

Figure 3. Influence of the model juice flow rate on the adsorption capacity of Amberlite IR120 resin. Sixteen gram pretreated Amberlite IR120 resin was packed in the chromatographic column (D 1.0 × 30 cm). The pH of model juice was 2.0. Data are expressed as means ± standard deviation (n = 4) $P \leq 0.05$.

2.0 mL/min, adsorption capacity decreased significantly. If the flow rate was too high, some arginine cations could not be adsorbed on the resin. If the flow rate was too slow, adsorption time became much longer. As the result, efficiency decreased although adsorption capacity increased. Therefore, 0.5 mL/min sample flow rate was selected in subsequent studies.

IV. *Exclusion of Interference with Buffer Rrinsing*

Some pigments, saccharides and other amino acids in grape juice are potential interferences to arginine determination. To eliminate the potential interference, column loaded with sample was rinsed with borate buffer or distilled water.

Arginine loss caused by buffer or distilled water rinsing to remove interferences adsorbed on Amberlite IR120 resin. About 0.05% of arginine was flushed out with distilled water, about 0.08% with the pH 8.0 buffer, and about 0.2% with the pH 9.0 buffer.

Austin (2000)(19) reported that the method of separating arginine with 500 mg Varian Bond Elut LRC SCX column and rinsing with borate buffer removes about 5% of the arginine content at pH 8.1 and about 15% at pH 8.2. Obviously, our separation method had significantly higher arginine recovery.

Some pigments, saccharides, acidic acids and neutral amino acids were washed out with distilled water and the pH 8.0 and pH 9.0 borate buffer, whereas the alkaline amino acids, such as lysine (pI = 7.6) and histidine (pI = 9.7) may still be adsorbed by the resin. However, Sastry(23) reported that lysine and histidine in grape juice could not interfere with the arginine assay results. Therefore, we chose distilled water as the rinsing agent.

V. *Effect of NaOH Concentration on Elution*

Arginine is removed from the column by an alkaline solution, usually sodium hydroxide or ammonia. Since ammonia may interfere with the Sakaguchi method(24), arginine was removed from the resin with sodium hydroxide. Sixteen gram of pretreated Amberlite IR120 was loaded with 10.0 mL of model juice (pH 2.0), and then eluted with NaOH solution at the rate of 2.0 mL/min. Arginine content in the eluate was then determined. Figure 4 shows the ending arginine content in the eluate with NaOH at two different concentrations, 1 mol/L and 2 mol/L. When the resin was eluted with 1 mol/L of NaOH, about 290 to 310 mL of NaOH, recovery of arginine was 97.7%. When the resin was eluted with 2 mol/L of NaOH, about 230 to 250 mL of NaOH was used and the recovery of arginine was 98.3%. Considering material and waste disposal costs, we chose 1 mol/L NaOH though the recovery of arginine was 0.5% lower than that of 2 mol/L NaOH.

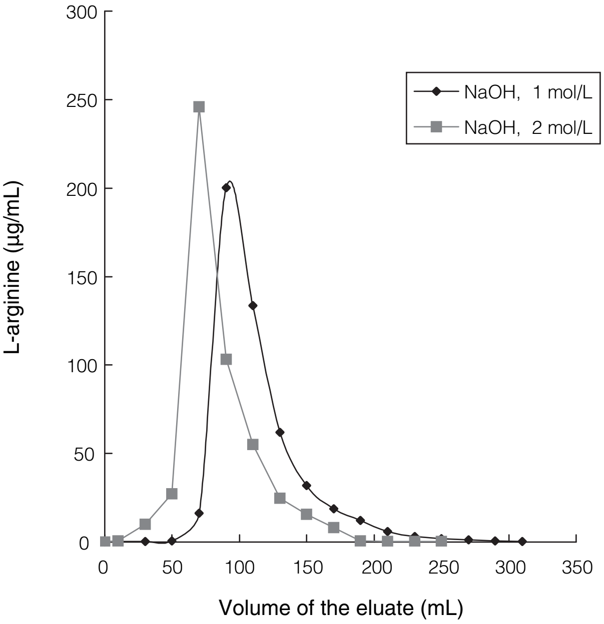

Figure 4. The elution curves of model juice in Amberlite IR120 column with NaOH solution. The weight of Amberlite IR120 resin was 16 g. Model juice (10.0 mL, pH 2.0) was loaded to the column at the rate of 0.5 mL/min, and rinsed with distilled water, and then eluted with NaOH solution at the rate of 2.0 mL/min.

Table 1. Arginine levels in sample grape juice

Sample No.	Initial arginine (μg/mL)	RSD (%)	Pure arginine added (μg/mL)	Measured (μg/mL)	Recovery (%)
1	633.25 ± 10.03	1.58	600	1186.03	92.1
2	736.28 ± 11.01	1.50	800	1471.76	91.9
3	1148.90 ± 24.98	2.17	1000	2094.86	94.6
4	939.32 ± 20.15	2.15	1000	1875.63	93.6
5	1314.11 ± 23.98	1.77	1500	2824.46	100.7
6	751.80 ± 8.47	1.13	800	1520.82	96.1
7	595.09 ± 10.74	1.80	600	1153.20	93.0
8	269.17 ± 6.52	2.42	300	530.22	87.0

RSD, relative standard deviation. Data are expressed as means ± standard deviation (n = 4) $P \leq 0.05$.

VI. *Separation and Determination of Arginine from Grape Juice*

According to the optimized parameters above, when the eluate volume reached 300 mL, the colorimetric reaction of arginine with Sakaguchi reagents was done. If no color was detected, the elution procedure ended. Arginine concentration in the sample was proportional to the arginine concentration in the NaOH fraction (Table 1).

The separation method was reliable, as the relative standard deviation was from 1.13 to 2.42% and the recovery ranged from 87.0 to 100.7%.

As shown in Table 1, arginine contents in grape juice samples ranged from 269.17 ± 6.52 to 1314 ± 23.98 μg/mL, and arginine contents in 2 of 8 samples were higher than 1000 μg/mL. Obviously, arginine contents of the sample juice varied drastically, probably resulted from vineyard site, soil, irrigation and fertilization practices, weather, scion and rootstock, etc(2,7,14). Different arginine levels resulted in different levels of ethyl carbamate in wine as confirmed by determination of potential levels of ethyl carbamate in wine fermented by the corresponding grape juice (data not shown). Furthermore, potential levels of ethyl carbamate in wine fermented by sample 3 and sample 5 were higher than 15 μg/L, the voluntary limit in the United States, whereas ethyl carbamate in wine fermented from sample 8 was not detected. In winemaking, knowledge of the initial arginine concentration in grape fruit is as an important indicator for grape growers to take necessary measures to control of arginine content n grape juice and consequently, to control the ethyl carbamate level in wine.

VII. *Validation of the Methodology*

Arginine contents of the samples were validated with an amino-acid analyzer. There was no significant difference at 0.05 probability level between amino-acid analysis and the separation method that used Amberlite IR120 resin and Sakaguchi reagent (Table 2). Besides, the recovery of the separation method was between 87.0% and 100.7% (Table 1), and the recovery of amino-acid analyzer method was between 87.6% and 104.2%. The results were close to each other, thus confirmed accuracy of our method. The relative standard deviations of the separation method and the amino-acid analysis were 1.50-2.17% and 1.26-1.80%, respectively, confirming reproducibility of the separation method. HPLC method needs sample cleanup and derivatization(15,16), and capillary electrophoresis method needs complicated capillary and sample preparation(17). In contrast, the sample for our separation method did not need the above-mentioned cleanup, derivatization, and preparation. Our method is simple, efficient and suitable particularly for small and middle size grape growers and winemakers to monitor arginine content in grape juice.

Table 2. Validation of Amberlite IR120 separation method for analysis of arginine by evaluating the same sample with an amino-acid analyzer

Sample No.	Evaluated using Amberlite IR120 separation and Sakaguchi reaction (μg/mL)	Amino-acid analyzer (μg/mL)
1	633.25 ± 10.03[a]	642.42 ± 8.25[a]
2	736.28 ± 11.01[a]	732.83 ± 9.23[a]
3	1148.90 ± 24.98[a]	1131.63 ± 15.89[a]
4	939.32 ± 20.15[a]	930.49 ± 16.78[a]

t test was used to ascertain statistically significant differences ([a]statistically significant difference at a confidence interval of 0.05).
Data are expressed as means ± standard deviation (n = 4) $P \leq 0.05$.

CONCLUSIONS

We developed a method for separation of L-arginine from grape juice with strong ion-exchange resin for simple and fast determination with Sakaguchi reaction. The maximum arginine adsorption capacity of Amberlite IR120 was 0.683 ± 0.020 mg/g. Interference of other adsorbed compounds can be excluded by rinsing with distilled water. Desorption was done with 1 mol/L NaOH The method provided good recovery and reproducibility. Therefore, the method could be applicable in routine determination of arginine in natural grape juice. This method gives the winemakers a tool to screen out juice with excessive arginine content, and to evaluate viticultural practices for control of ethyl carbamate content in wine.

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