



**CORVINUS UNIVERSITY OF BUDAPEST**

**INVESTIGATION OF ORIGINALITY OF TOKAJI ASZÚ:  
AMINES AND ORGANIC ACIDS IN GRAPES AND WINES**

**Ph.D. Thesis**

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## 1. INTRODUCTION

The authentication of agricultural products, in a narrow sense, foodstuffs are of vital importance in today's market competition. All products, which meet requirements in terms of "Protected Originality", "Protected Geographical Mark" or "Guarantee for Tradition and Speciality" categories are appreciated.

Besides the products of prominent Hungarian wine-growers, sometimes consumers can meet poor quality, unpleasant-tasting, possibly adulterated wines, which can do consumers and producers of quality wines a lot of harm. For this reason, methods suitable for determination of identity and origin of products for such a rare wine as Tokaji Aszú are especially important from economical and health points of view.

An increasing number of papers can be found in the literature, which deals with analytical methods to establish originality and identification of products (VOGELS et al., 1993; ARVANITTOYANNIS et al., 1999; CORDELLA et al., 2002). Although in many cases meeting these demands have not been solved so far.

The strategy of determining origin is to seek such constituents or groups of compounds, which characteristic of a product or products of various kinds. Many successful works have shown that it is possible to distinguish grape variety, vintage years, or geographical zones on the basis of chemical parameters such as trace elements, organic acids, amino acids and isotopic composition (SUHAJ and KORENOVSKÁ, 2005; ETIÉVANT et al., 1989; DAY et al., 1995; LATORRE et al., 1994).

In this regard, the study of amines has great importance because one part of the biologically active amines (biogenic amines) and primary aliphatic amines form in aszú berries during noble rot (SASS-KISS and HAJÓS, 2005; HAJÓS et al., 2000; SASS-KISS et al.; 2000).

Determining of geographical origin and establishing authentication of a product means a complicated task because both independent and interacting factors have an effect on chemical composition of products. Concerning Tokaji Aszú, it means that the characteristic type of these wine specialties is due to aszú grapes and the culture of centuries-old wine-making process, which appear in versatility of chemical constituents to a certain extent and relative similarity. All things considered, the quality of Tokaj wines (its chemical composition) made from aszú grapes can change in a wide range depending on vintages, viticultural region and what changes happen to aszú grapes from collecting, through wine-making technology, fermenting, during storage to drinking.

One part of biogenic amines, the polyamines (putrescine, speridine and spermine), which occur in living organisms, take part in cellular growth, regulation of nucleic acid and protein synthesis and stabilization of lipids. Biogenic amines occurring in food (Sauer kraut, cheese) and drinks can be classified into an other group, which are formed primarily from the decarboxylation of amino acids by the action of microorganisms. From physiological point of view, histamine, tyramine and phenylethylamine can be emphasized, which can cause allergenic reactions in humans above a certain concentration. These compounds form in all process (fermentation) during food processing, in which microbe (bacteria, yeast, mould) take part in. Biogenic amines can be indicator compounds of not only hygiene and technology but studying them, the formation of aszú grapes and the activity of *Botrytis cinerea* can be investigated.

As the result of noble rot, aszú grapes form the most important raw material of Tokaj wine specialities. Commonly, *Botrytis cinera* (gray mould) can be harmful to plants and so vine damaging green parts of plants and mostly yields. Fortunately co-existence factors of some grape varieties, soil and climatic conditions, just like in Tokaj region, *Botrytis cinerea* can spread only difficultly causing a slow concentration process in grape berries. The rich and characteristically unique and broad taste of aszú grapes is due to this noble rot.

The investigation of *Botrytis cinerea* affecting on grapes and wines was started by the colleagues of Central Food Research Institute in Departments of Analytical and Nutrition Science in 1997. As a colleague of Analytical Department of CFRI, I have been taking part in studies connected with grapes and wines since 2002.

## 2. OBJECTIONS

The goal of my work was to establish the foundations of a quality control method protecting the quality and origin of Tokaji Aszú to distinguish wines made from aszú grapes and normal wines and to differentiate Tokaji Aszú wines from foreign botrytized dessert wines.

### **The study was performed in two directions:**

In one part of the study, *grape varieties* were studied to follow up changes during noble rot of grapes:

- For this purpose the effect of *Botrytis cinerea* and other microorganisms settling on grape berries on amine and acid composition of grape varieties of various kinds was studied with comparing intact (normal, dried) and infected grape berries (gray- and green rotten).
- Besides the main varieties (Furmint, Hárslevelű), other indigenous varieties and varieties intended to replant were investigated observing susceptible of grapes to noble rot.
- The effect of vineyards was investigated.

In the second part of the study, *botrytized and normal wines* were analyzed originating from different places.

- Amine and organic acid composition of many Tokaji Aszú and foreign botrytized wines as well as other Hungarian botrytized and normal (non-botrytized) wines were studied.
- Data were analyzed by multivariable statistical methods to investigate the difference and similarity among the chemical composition of wine samples.

### 3. MATERIALS AND METHODS

Two vintages (2003, 2004) were studied in the case of **grape samples**. In 2003 intact, sun-dried and aszú grapes were from the characteristic varieties of Tokaj wine region such as Furmint, Hárslevelű and mixture of these two varieties. Samples derived from five different vineyards of Mád, Tarcál and Tolcsva. Altogether sixteen samples were obtained from vintage 2003. In 2004, besides the intact, dried and aszú grapes studied previously, gray and green rotten grapes were analyzed. In addition to the main varieties (Furmint, Hárslevelű), further three grape varieties (Yellow Muscat, Zéta and White Grape) were sampled from three vineyards (Sátoraljaújhely, Tarcál és Mád) of the wine region in vintage 2004.

*Sample preparation of grapes:* Intact and rotten grapes (25 g) were homogenized with Ultra-turrax homogenizer and centrifuged at 10000 rpm for 10 min then the supernatant was filtered through a membrane. Because of high dry weight of Aszú, dried grapes, and green rotten grapes, these samples (12 g) were extracted with 20 mL of 10% perchloric acid. Because of high dry weight of Aszú, dried grapes, and green rotten grapes, these samples (12 g) were extracted with 20 mL of 10% perchloric acid. After homogenization, centrifugation, and filtration, the supernatant was analyzed by HPLC similarly to intact grapes. Three replicate extractions from the grape samples of each variety were performed. The concentration of amine compounds in grapes was calculated on a dry matter basis of samples. The dry weight was gravimetrically determined according to standard MSZ EN 12145:1998.

The **studied wines** took part in the 6th International Wine Competition VinAgora 2002 (Budapest) and the 1st International Wine Competition VinAgora Botrytis 2004 (Tarcál). In 2002 Tokaji Aszú wines (32) and Tokaji Eszencia (4 samples) from different vintages and producers were collected. Besides seven foreign wines were also analyzed, which come from four countries (Germany, Slovakia, France and Austria) and were produced from noble rotten grapes similarly to Hungarian wines. All together forty-three samples were analyzed and two replicates were performed. Hungarian non-botrytized white wines were purchased in local stores and opened before analysis. In the case of Vinagora Botrytis 2004, amine and organic acid composition of Hungarian botrytized wines (botrytized white and red wines from Eger /5 samples/, Tokaj wine specialities /Tokaji Aszú: 21 samples and Tokaji Szamorodni: 5 samples/) and many foreign botrytized wines /24 samples/ were studied. Foreign wines came from nine countries. Wines were injected directly into the column after filtration without any further sample preparation.

The measurement of amines was performed with Alliance Waters 2690 HPLC chromatographic system equipped with a Waters 474 fluorimetric detector ( $\lambda_{\text{ex}}=345\text{ nm}$ ,  $\lambda_{\text{em}}=455$ ). Amines were separated and quantified with ion pair formation (octanesulfonic acid) on reverse phase column ( $\mu$ Bondapak C18, 300 mm X 3.9 mm, 10  $\mu\text{m}$ ; from Waters). For separation of amines a previously elaborated (SASS-KISS and HAJÓŠ, 2005) gradient elution program was modified. Post-column derivatization of amines was performed with OPA solution.

In grape samples, for the separation of **organic acids** Rezex ROA Organic acid (Phenomenex) ion exchange column was used with 0,005 N sulphuric acid under isocratic conditions. The flow rate of eluent was 0,6 ml/minute. Before analysis grape samples were homogenized with Ultra-turrax homogenizer, diluted and filtered. Wine samples were diluted, filtered and injected to the column with any further sample preparation. The organic acids of wines were separated on ODS-AQ (YMC European GMB) reversed phase column (250 x 4.6 mm I.D., S-5 $\mu\text{m}$ ) with 0,02 M phosphate buffer (2,75 g/L  $\text{KH}_2\text{PO}_4$ , pH 2,7) under isocratic conditions. The flow rate of mobile phase was 0,7 ml/minute. Organic acids were detected by UV detector at 214 nm in both cases.

The **identification** of organic acids (tartaric acid, malic acid, citric acid, shikimic acid, fumaric acid, acetic acid and lactic acid) and amines (putrescine, i-butyl-mine, cadaverine, tyramine, histamine, 2-methyl-buthyl-amine, agmatine, 3-methyl-buthyl-amine, n-penthyl-amine, spermidine, phenylethylamine) found in grapes and wines were accomplished with comparing the retention time of amine standards with compounds present in samples and by addition of standard solution to the samples.

To **evaluate results**, analysis of variance, *t*-test, correlation analysis and from multivariable statistical methods principal component analysis (PCA), and linear discriminant analysis (LDA) were performed by Microsoft Excel and the Minitab statistical program.

#### 4. RESULTS, SUMMARY

Under appropriate conditions the different species of *Botrytis cinerea* can cause noble rot on grape berries in the Tokaj wine region of Hungary. The mould is responsible for advantageous changes in grapes, which yields wines of different composition.

In the first part of the study the effect of microbiota on infected grape berries was investigated by comparing amine and organic acid composition of noble, gray and green rotten grapes. Beside the main grape varieties (Furmint, Linden Leaf, Yellow Muscat), a less wide-spread variety (Zéta) and grapes recommended for propagation in Tokaj in the near future (Kövérshőló) were studied.

Statistically significant difference was found between intact and Aszú grapes in amine (putrescine, tyramine, 2-methyl-butylamine, agmatine, 3-methyl-butylamine, spermidine, phenylethylamine) and organic acid content (tartaric acid, malic acid, acetic acid, shikimic acid, fumaric acid) independently of the place of origin and grape variety.

In my research work I succeeded in applying classification models based on Multivariate Statistical Methods to differentiate infected grape berries from the same growing location on the using amine and acid concentration values. From Multivariate Statistical Methods, the recognition of possibly differentiated groups was accomplished by Principal Component Analysis and the presumed goodness of the classification was checked by Linear Discriminant Analysis.

Investigating grape berries from the same growing location, significant difference was found between Aszú and green rotten grapes in the content of putrescine and i-butyl-amine and between Aszú and grey rotten grapes in the concentration of tyramine, agmatine and spermidine.

Independently of the place of origin, significant difference was found in tyramine, agmatine and spermidine and tartaric acid, malic acid and the unknown acid compound content of noble rotten and grey rotten samples. Applying Principal Component Analysis, intact, noble rotten and grey rotten grapes were successfully separated (independently of the place of origin) on the basis of amine and organic acid composition.

Studying grape varieties, Furmint possessed the highest amine and organic acid content from both intact and infected grapes. Hárslevelú, Yellow Muscat, Kövérshőló and Zéta varieties did not show statistically provable difference.

In the second part of the study botrytized and normal wines of different origin were analyzed. Botrytized wine samples, which won silver and gold medals, were taken at wine



competitions (6<sup>th</sup> International Wine Competition VinAgora 2002, 1<sup>st</sup> VinAgora Botrytis 2004). Normal wines (non-botrytized) were purchased in local stores.

According to earlier results, it was concluded Tokaji Aszú wines possess characteristic amine composition. The characteristic composition of Aszú wines were illustrated with spider web diagrams, which shape proved to be similar. On the other hand, foreign botrytized wines were easily differentiated from Aszú wines due to their different shape.

Normal, Tokaji Aszú and foreign botrytized wines were separated from each other using mainly primary aliphatic amines (i-butyl-amine, tyramine, 2-methyl-butyl-amine, 3-methyl-butyl-amine, agmatine, n-pentyl-amine, phenylethylamine and two unknown compounds) as variables in Principal Component Analysis.

Using Multivariate Statistical Methods, it was established that amine compounds are more suitable for authenticity than organic acids. The separation of Tokaji Aszú and foreign botrytized wines was improved with amines supplying with organic acids as variables.

Applying Principal Component Analysis, Tokaji Szamorodni wines and botrytized wines from Eger were successfully distinguished from Tokaji Aszú due to their different amine content. I established that Tokaji Szamorodni wines have significantly lower i-butyl-amine, 2-methyl-butyl-amine, agmatine, two unknown amine compounds, 3-methyl-butyl-amine, n-pentyl-amine and phenylethylamine content than Aszú wines. Furthermore I found significant difference between Tokaji Aszú wines and botrytized wines from Eger in the concentration values of putrescine, i-butyl-amine, cadaverine, unknown 2 amine compound, 2-methyl-butyl-amine, agmatine, 3-methyl-butyl-amine and phenylethylamine.

Cluster Analysis was applied to investigate similarities among foreign botrytized wines. A few foreign wines grouped according to place of origin (the same producer, country) because of their characteristic amine composition.

Summarising the results, an objective evaluation method can be elaborated for quality control in order to protect the authenticity and origin of Tokaj wine specialties against denigrator, illegal adulterations and personates. On the basis of amine and organic acid concentration values, data bank and multivariate model have been worked out, which make it possible to distinguish unknown wine samples.

## 5. NEW SCIENTIFIC RESULTS

1. During the separation of amines, two new compounds, 2-methyl-buthyl amine and 3-methyl-buthyl-mine were identified with comparing the retention time of amine standards with compounds present in samples and by addition of standard solution to the samples.

2. It was the first time to investigate and compare of amine and organic acid composition of noble, green and gray rotten grapes, as well as newly planted and varieties intended to replant in Tokaj.

Studying many species it was verified that among grape varieties Furmint possess the most amine and acid content. Other grape varieties (Linden leaf, Yellow Muscat, Kövérszőlő, Zéta) did not show statistically significant difference.

It was established on the basis of amine composition of studied grape samples that the production of primary aliphatic amines are characteristic of other mould species (*Penicillium*) besides *Botrytis cinerea* species. Meanwhile primary aliphatic amines do not play role in separating grape samples.

Independently of the place of origin, significant difference was found in tyramine, agmatine and spermidine and tartaric acid, malic acid and the unknown acid compound content of noble rotten and grey rotten samples. Statistically significant difference was found in the putrescine and i-buthyl-amine content of aszú and green rotten and tyramine, agmatine and spermidine content of aszú and gray rotten grapes coming from the same viticultural region.

3. Applying principal component analysis, intact, noble and gray rotten grapes were successfully grouped on the basis of amine and organic acid composition independently of the place of origin.

I succeeded in separating the infected grape samples (noble, gray and green rotten) originating from the same location.

Furthermore it was established that environmental conditions (vintage, region) have an effect on amine composition of intact grapes at a less extent than on noble rotten grapes.

4. It was confirmed that amine compounds are suitable for the study of originality of Tokaji Aszú wines.

Applying principal component analysis, normal (non-botrytized), Tokaji Aszú and foreign botrytized wines were separated from each other.

Tokaji Aszú wines were distinguished also from Tokaji Szamorodni and botrytised wines from Eger.

Wine samples were separated from each other mainly by using primarily primary aliphatic amines as variables (i-buthyl-amine, tyramine, 2-methyl-buthyl amine, 3-methyl-buthyl-amine, n-buthyl-amine, phenylethylamine and two unknown amine compounds).

Analyzing foreign wines, it was verified that spider web diagram as a simple and alternative method besides principal component analysis is suitable for differentiating Aszú wines and foreign dessert wines.

5. Organic acid compounds themselves are less suitable for studying originality of Tokaji Aszú, although investigating amines and acids together, the separation of foreign and Hungarian wines produced from botrytized grapes were better.
6. The basis of a quality control method was elaborated to protect quality and authentication of Tokaji Aszú, which make it possible to differentiate unknown Hungarian and foreign wines on the basis of data bank of Tokaji Aszú and the elaborated multivariate model.

## **PUBLICATIONS AND PRESENTATIONS**

### **ARTICLES:**

**KISS, J., SASS-KISS, Á.** (2005) Protection of originality of Tokaji Aszú. Amines and organic acids in botrytised wines by HPLC, *Journal of Agricultural and Food Chemistry*, Vol. 53, 10042-10050 p. (impact factor: 2,327)

**KISS, J., KORBÁSZ, M., SASS-KISS, Á.** (2006): Study of amine composition of botrytized grape berries. *Journal of Agricultural and Food Chemistry*, Vol. 54, 8909-8918 p. (impact factor: 2,327)

**SASS-KISS, Á., KISS, J.** (2007): Eredetvizsgálat aminokkal. A Tokaji aszú ujjlenyomata. *Élet és Tudomány*, LVIII. évf., 8, 240-243.

### ***Others:***

**SASS-KISS, A., KISS, J., MILOTAY, P., KEREK, M., TOTH-MARKUS, M.** (2005): Differences in anthocyanin and carotenoid content of fruits and vegetables. *Food Research International*, Special Issue: International Congress – Pigments in Foods, 38, 1023-1029 p. (impact factor: 1,332)

### **PROCEEDINGS:**

**KISS, J., SASS-KISS, A.** (2003): Különböző botrytisálódott borok biogén amin tartalmának vizsgálata. XXVI. Kémiai Előadói Napok, Szeged, 79-83. (lecture)

**KISS, J., SASS-KISS, Á.** (2004): Tokaj-hegyaljai szőlőfajták amin-és sav-összetételének vizsgálata HPLC-vel. XXVII. Kémiai Előadói Napok, Szeged, 84-88. (lecture)

**HAVADI, B., KISS, J., SASS-KISS A., ADÁNYI, N., VÁRADI, M.** (2006): Classification of wine samples on the basis of elemental and amine composition. Trace elements in the food chain. International Symposium on Trace Elements in the food chain (proceedings), Budapest, 246 p. (poster)

***Others:***

**KISS, J.** (2002): CID detektoros ICP spektrométer optimalizálása szelén és arzén meghatározására. MÉTE XIV. Országos Tudományos Diákköri Konferencia, Szeged, 26. (Supervisor: Dr. Fodor Péter) (lecture)

SASS-KISS, A., **KISS, J.**, MILOTAY, P., KEREK, M., TOTH, M. (2004) Anthocyanins and carotenoids in varieties of fruits and vegetables. 3<sup>rd</sup> International Congress on Pigments in Food, Quimper, France, 396-399 p. (poster)

**ABSTRACTS:**

***International conferences***

**KISS, J.**, SASS-KISS, Á. (2003): Study of Biologically Active Amines in Botrytised Wines By HPLC. 5<sup>th</sup> Balaton Symposium on High-Performance Separation Methods, Siófok, 157 p.

**KISS, J.**, SASS-KISS, A. (2004): Biogenic amines in botrytised wines. 2<sup>nd</sup> Central European Congress on Food, Budapest, 231 p. (poster)

VÁRADI, M., ADÁNYI, N., **KISS, J.**, SASS-KISS, Á. (2004): Determination of biogenic amine level in vegetable juices by using biosensor. 2<sup>nd</sup> Central European Congress on Food, Budapest, 231 p. (poster)

**KISS, J.**, SASS-KISS, Á. (2005): HPLC Study of Tokaj Aszú Wines and Tokaj Grape Varieties. 6<sup>th</sup> Balaton Symposium on High-Performance Separation Methods, Siófok, P-100. (poster)

**KISS, J.**, SASS-KISS, A. (2004): Biogenic amines in botrytised wines. 2<sup>nd</sup> Central European Congress on Food, Budapest (proceeding, CD-ROM) (poster)

VÁRADI, M., ADÁNYI, N., **KISS, J.**, SASS-KISS, Á. (2004): Determination of biogenic amine level in vegetable juices by using biosensor. 2<sup>nd</sup> Central European Congress on Food, Budapest (proceeding, CD-ROM) (poster)

HAVADI, B., **KISS, J.**, SASS-KISS A., ADÁNYI, N., VÁRADI, M. (2006): Classification of wine samples on the basis of elemental and amine composition. Trace elements in the food chain. International Symposium on Trace Elements in the food chain (abstracts), Budapest, 50 p. (poster)

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### *Hungarian conferences*

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**KISS, J.**, SASS-KISS, A. (2003): Botrytisálódott borok biológiailag aktív amin tartalmának vizsgálata HPLC-vel. Lippay János – Ormos Imre – Vas Károly Tudományos Ülésszak, Budapest, 34. (poster)

**KISS, J.**, SASS-KISS, Á. (2004): Különböző dűlőkről származó szőlőszemek amin- és savösszetételének vizsgálata. Elválasztástudományi Vándorgyűlés, Hévíz (poster)

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