Study on the *Penicillin*-content of botrytized wines and noble rotted berries in Tokaj-region

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Summary: Favourable parameters are offered to the formation of noble rot caused by *Botrytis cinerea* by the climatic conditions, soil circumstances and grape-varietes of Tokaj-region. As the result of noble rot, aszú-berries represent the most important raw material of the world-wide known Tokaji Aszú Wine. The objective of this study was to measure the **penicillin**-content of aszú-berries and Tokaj Wine Specialities. As the result of infection of *Botrytis* free way can be opened in front of other mould strains, especially *Penicillium* and *Aspergillus*. There is a possibility to the presence of *Penicillium chrysogenum* on the surface of aszú-berries during noble rotting process. *P. chrysogenum* was found to produce the so called Naturally Derived Penicillins such as Penicillin-V (phenoximethyl-penicillin).
According to our findings the Penicillin-V content was detected between 0.4 and 26 mg/l wine and 0–74 mg/kg aszú-berry. These values do net have therapeutic effect, but physiological benefit and it can be a basis to the qualification of the raw material of Tokaj Wine Specialities naturally together with the other component of noble rotted berries and botrytized wines.

Key words: noble rot, penicillin, Penicillium, Tokaji

Introduction

The most important base material of the famous Tokaji Aszú is the noble rotted grapes infected and modified by *Botrytis cinerea* under special conditions. Mechanism of noble rot and biochemical changes have been studied well, especially in the case of French and German botrytized wines *(Domerque*, 1957; *Dittrich*, 1987; *Donèche*, 1993). There are some studies published relating to the microbiota of botrytized grapes *(Minarik*, 1969; *Gandini*, 1973; *Magyar*, 1996; *Kalmár* et al., 1999; *Magyar & Bene*, 2001; *Bene & Magyar*, 2002), but the basic knowledge is missing about metabolates of concomitant saprophytic moulds beside *Botrytis*.

As a common result of the enzymatic activity of *Botrytis cinerea*, the degradation of cell walls of berries is realised, penetrable way is given for other mould strains. These microorganisms cannot infect the vegetable tissue such as grape-berries and do not proliferate, only when the hurts of berries allowing the juice with high sugar content to seep to the surface of the grape. This kind of hurts can be caused by many factors such as bee-stings or by *Botrytis*.

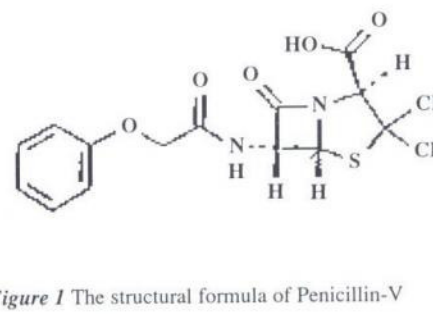
According to the domestic studies *Penicillium* and *Aspergillus* strains were commonly found in widely varying population *(Kalmár* et al., 1999; *Magyar & Bene*, 2001; *Bene & Magyar*, 2002).

There are a lot of *Penicillium* and *Aspergillus* species producing penicillin as secundary metabolite, especially *Penicillium notatum* and *Penicillium chrysogenum* were found to produce this antibiotic *(Clarke* et al., 1949). Penicillin is an important and useful antibiotic causing inhibition of bacterial growth. It involves a ß-lactam and a thiazolidine ring with side-chain on the 6th carbon atom. In small quantity, changes in morphological state of cells and deformed shapes are caused by penicillin. In higher volume, bacteria are under lysis by penicillin because of inhibition of peptidoglycane – scheme of cell-wall through more fulcrum *(Abraham*, 1981).

According to the side-chain precursors, there are a lot of so-called Naturally Derived Penicillins. Penicillin-F (pentenylpenicillin), Penicillin-K (heptylpenicillin), Penicillin-N, Penicillin-G (benzylpenicillin), Penicillin-V (phenoxymethylpenicillin) are the most significant of these penicillins from both the biochemical and the therapeutic standpoint.

The penicillins are proved to be very sensitive to acids and alkalines because of the dissolvation of ß-lactam ring. Only the Penicillin-V derivate is shown to have a capability of resisting higher acid content. The structural formula of Penicillin-V is shown in *Figure 1*.

Penicillia can be found in widely varying population on the surface of aszú-berries. There is a question to be solved,



fenoximetil-panicillin
$C_{16}H_{18}N_2O_5S$
Mol weight: 350.3

Figure 1 The structural formula of Penicillin-V whether any penicillin is produced or not by these *Penicillium*-strains under the special environmental conditions of aszú-berries. According to the small published works *(Arnstein & Morris,* 1960; *Chan* et al., 1976; *Flynn* et al., 1962; *Elander & Aoki,* 1982) in case of the presence of suitable precursor side-chains, the enzyme-system of *Penicillium chrysogenum* is capable of producing penicillin-derivates.

In the year of 1999, *Kalmár* et al. have isolated *Penicillium notatum* on the surface of noble rotted grapes. Furthermore, there are different volatile phenols, anise aldehydes, other cinnamic acid derivates and cystein, valine aminoacids inside aszú-berries and in botrytized wines, which contain derivates of ß-lactam antibiotics. We have mentioned above that penicillins are found to be very sensitive to acids and alkalines, only the Penicillin-V (phenoxymethylpenicillin) can resist to a higher acid-level. In this way because of the acid-content of noble rotted berries and botrytized wines, Penicillin-V remained the only penicillin-derivate to be studied. We did not find any data on the penicillin-content of botrytized wines and noble rotted berries in the world's literature of enology, although if may occur theoretically.

In this work we have studied the Penicillin-V content of aszú-berries and botrytized wines from the Tokaj-region. To establish a complex qualifying method of noble rotted grapes and botrytized wines, deeper knowledge is inevitable concerning the metabolites of concomitant mould strains beside *Botrytis*.

Material and method

Samples of wines and berries were taken from various wine-making companies in Tokaj. Origin of wines are shown in *Table 1*, samples of berries are listed in *Table 2*.

Preparing of samples

100 ml of wine-sample was extracted with 100 ml of dichloride-methane in a bolting-funnel. Before extraction to avoid formation of emulsion there is often a need to double or triple dilute the sample with water. After splitting up of phases, lower phase involving Penicillin-V was collected and evaporated in 60 °C under vacuum in a rotator.

Table 1 The list of wines studied from Tokaj-region

Code	Appellation of wines	Production area
1.	Raw Aszú 2002	Bodrogkeresztúr
2.	6 puttonyos Aszú 1993	Bodrogkeresztúr
3.	Aszúeszencia 1999	Bodrogkeresztúr
4.	Nature Eszencia 2002	Bodrogkeresztúr
5.	Dry Szamorodni 1985	Bodrogkeresztúr
6.	5 puttonyos Aszú 1998	Bodrogkeresztúr
7.	5 puttonyos Aszú 2001	Tokaj
8.	6 puttonyos Aszú 2001	Tokaj
9.	Dry Szamorodni 1998	Tolcsva
10.	Nature Esszencia 2002	Tolcsva
11.	Nature Esszencia 2000	Mád
12.	Dry Szamorodni 2000	Mád
13.	5 puttonyos Aszú 1923	Tarcal

Table 2 The list place of sampling and storage circumstances of aszú-berries

Code	Year	Place of sampling	Treatment with sulphiting
ASZB1	2002	Bodrogkeresztúr	–
ASZB2	2002	Tarcal	–
ASZB3	2002	Tarcal	+
ASZB4	2001	Mád	+
ASZB5	2001	Tarcal	+
ASZB6	2002	Bodrogkeresztúr	+
ASZB7	2002	Tarcal	–

Then the residue was dissolved in 1 ml ethanol of HPLC-quality and used to the measuring of Penicillin-V content.

In case of aszú-berries, firstly noble rotted berries were measured and diluted with 100 ml sterile water. Then there was a revealing method with a mixer without hurting the grape-seed. The juice was steeped in 1 hour, then strained twice. The quantity was completed to 200 ml and we continued the preparing of samples in the same way as described above in the case of wines.

Analysis

The prepared samples were measured with High Performance Liquid Chromatography.

Machine: HPLC equipment typed HP 1050

Detector: UV

Integrator: HP 3396A

Column: Spherisorb ODS C-18, 200 x 4.5 mm

λ: 215 nm (UV)

Flow: 1 ml/min

Temperature: 30 °C

Separation: isochratic

Mobile phase: methanol/water/phosphate-buffer = 400:500:100

Time of analysis: 30 min

Preparation of phosphate-buffer: 68 g KH_2PO_4 free from water was double diluted with water to 1000 ml, the pH was put into 3.5 with ccH_3PO_4 then the solution was membrane filtered (0.45 µm).

Setting up the calibration – line

Penicillin-V (Sigma) chromatographic standard (1 mg is equivalent with 1685 NE) was diluted with 12% water – ethanol solution to make 10–50–100 µg/l solutions, then the sample-preparing described above was followed. According to the three times repeated chromatogrammes, retention time

(T_R) of Penicillin-V was determined 8.8 min under the special sample-preparing conditions, which were described earlier (in case of 18 measurings, 8.3 and 9.2 min were detected as extreme value).

The equation of calibration-line:

$$x = 9.74447 * 10^{-5} y - 0.6991$$

x: Penicillin-V; µg/l

y: integration area of retention time under convenient peak

Verifiable border: 1 µg/l Penicillin-V.

Results

Penicillin-V content of botrytized wines

Figure 2 displays the results of our work in case of wines. The penicillin-V numbers are mean-values of three measurings and indicated in mg/l.

According to our findings, the Penicillin-V content can be measured in the range of 0,4–26 mg/l, which is very varied. There are considerable differences among wines.

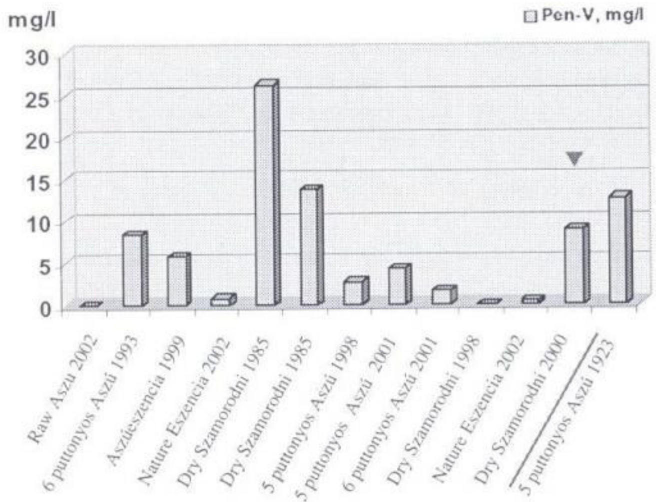


Figure 2 The Penicillin-V content of botrytized wines in the Tokaj-region

Regarding the fact, that one-time therapeutic dose of penicillin-medicine is 1 million NE (which is equivalent with approximately 600 mg/l), the measured values are considered 0,5–4% of therapeutic quantity in one litre Tokaj Wine Speciality. Antibiotics taking in the human system in this way possess 50% of biological usability. Furthermore antibiotics have an antimicrobial activity (they have an either inhibitory or killer effect on actual pathogen or not) and a pharmacokinetic property (they are capable of getting to the place of bacterial contamination or not). Actually the measured values are enough to inhibit many microorganisms (for example in the case of *Streptococcus pneumoniae* the minimum inhibitor concentration is 2 mg/l), although it is not a guarantee for reaching the centre of infection. There fore the therapeutic values are multiplied by 300 than the minimum inhibitor concentration. Regarding the cultural wine consumption, glass of wine contains Penicillin-V although in very slight quantity. In this case it was found again that Tokaj wines in the first place are considered to be consumer goods, but with the involvement of compound like penicillin the wine will have a favourable physiological effect. Naturally we do not talk about medicines in the case of Tokaj Wines, but Tokaj Wine Specialities containing penicillin strengthen the protective capability of human system and enhance the formation of balance between human and its bacterial environment.

Penicillin-V quantity of noble rotted berries

Figure 3 displays the measured values of Penicillin-V content in case of aszú-berries. The penicillin-V numbers are mean-values of three measurings and indicated in mg/kg.

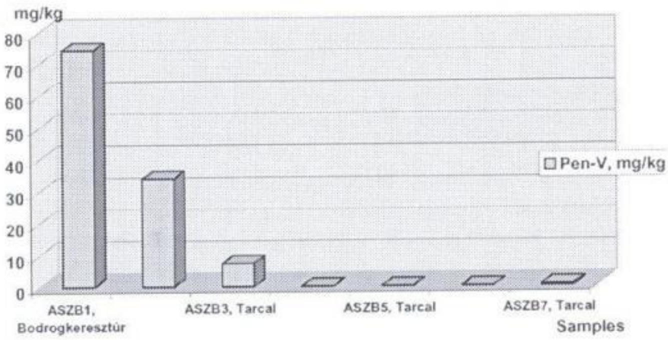


Figure 3 The Penicillin-V content of noble rotted berries

According to our findings, Penicillin-V content can be measured in the range of 0–74 mg/kg. There are many possibilities to explain the enormous difference among the measured values.

Firstly there is a need to enhance the number of samples, because more information is inevitable about year, production area, magnitude and presence of *Penicillium* and *Aspergillus* strains beside *Botrytis cinerea* during the noble rotted process.

Furthermore the occurence of *Penicillium*-strains is very varied and the presence of penicillin-producing species is unpredictable.

Actually the acid of noble rotted berries can destroy the ß-lactam ring of penicillin and during the storage it is increased in case with sulphur-dioxide treatment.

When berries do not reach the overmatured state, the acid content is very high and the higher presence of *Penicillium*-strains does not develop penicillin producing activity.

To sum up, a penicillin content of 74 mg/kg is considerable and 12% of therapeutic dose of penicillin-medicine, in this way aszú-berries have a favourable effect on human system.

Conclusions

The unique feature of Tokaj Wine Specialities is determined by the „Aszú-character" derived from noble rot and the „Tokaj-type" come from ageing. Noble rotted grapes are the result of the special metabolic activity of *Botrytis cinerea*. Beside *Botrytis* other mould species like *Penicillium* and *Aspergillus* are commonly found and they can produce penicillin as secunder metabolite in widely varying quantity.

In the future, presence and concentration of penicillin-V in aszú-berries and botrytized wines could be a distinctive parameter in judgement of identification.

Further research is in progress concerning the effect of year and production area on penicillin-V content. As next step the effect of penicillin-content on organoleptic properties has to be elaborated too.

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