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ORIGINAL RESEARCH

Separating Mad Honey from Other Honeys with Grayanotoxin Analysis in LC-MS/MS

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Abstract

Objective: Honey samples were collected from 82 different pieces during the season when rhododendron flowers bloomed in Duzce areas in 2017. Since the majority of these honeys collected are sold as mad honey by local people, has been determined that it is the most suitable period to make their analysis.

Material-Method: To apply our sample preparation method, honey samples were prepared to be analyzed on the LC-MS/MS. The accuracy, repeatability and reliability of the analysis method were provided by us.

Results: The obtained results were calculated with the calibration plot drawn at ppb (ng/ml) level in LC-MS/MS. The grayanotoxin -III levels in mad honey were found to be % 36 for 0 ng/ml, %43 for 0.5-10 ng/ml, %15 for 10-50 ng/ml and %6 for 50≥ ng/ml.

Conclusion: Most of honey samples do not contain toxins. Some of them are safe to use in middle proportions and less of them dangerous to consume.

Keywords: Grayanotoxin-III, Mad Honey, LC-MS/MS

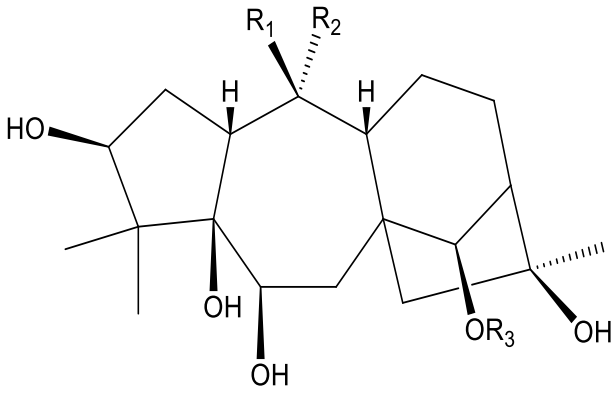
INTRODUCTION

Honey is one of the most important nutrients, which is consumed by people from past till today. Honey, which is among the rare nutritional source that is naturally sweet, has a wide range in taste depending on the nectars collected by the bees. As the multi-floral honeys, which are collected from the different kinds of flowers, are general, also the mono-floral honeys, which are formed by mainly collecting nectars from a single kind of flower, are known and consumed consciously. As an example to the mono-floral honeys, one of them is known as mad honey or bitter honey, which is both attention-grabbing and dangerous[1].

Mad honey is a natural nutrient, which is frequently, consumed in certain regions in our country, with its specific odour, bitterly – harsh taste, which causes a sense of slightly burning in throat, with its yellow colour, that is lighter than the other honeys, and late crystallization feature. Even though the poisoning due to andromedotoxine are phenomenal cases reported from the different countries world-wide, it is seen more frequently particularly in the settlements on the Black Sea coast of our country. It is consumed in small quantities in our country, generally the aim of self-medication for some medical effects of it[1, 2].

In some studies, it was suggested by the researchers that it may be related with Ericaceae family, through determining acetylandromedol (andromedotoxine), andromedenol, and andromedol, which are isolated from some species of Ericaceae plant family, in also andromedotoxine. The toxins, which are extracted from these plants by the bees, are mixed into the honey directly and causes poisonings, as they cannot be detoxified. Rhododendron, which is the scientific name of the forest roses within this plant family, means rose tree (In Greek rhodon = rose, Dendron = tree)[3, 4]. Meantime there are great number of and may different types of rhododendron in the world, particularly yellow rhododendron (*Rhododendron luteum*) and

rhododendron with purple flowers (*Rhododendron ponticum*) are very common in the forests on the north coasts of our country[4]. Acetylandromedol (andromedotoxine), andromedenol, and andromedol, which are the toxic compounds that are isolated from these species, are known also as grayanotoxin-I, grayanotoxin-II, and grayanotoxin-III, respectively. The grayanotoxines, which forms a group of toxic diterpens, are consisted of non-nitrogenous polyhydroxylecyclic hydrocarbons[5]. They are located in the nectar, flower, pollen, and leaf parts of the rhododendrons. Poisoning mostly occurs with the honeys produced from the nectars containing toxin. The type and amount of grayanotoxin, which is contained by the nectar, variety from one type to another. Today, more than fifty types of grayanotoxin are determined (Figure 1) and grayanotoxin-I, III, and IV are those having toxic characteristic compare to less toxic one is grayanotoxin-II. Grayanotoxin-III is the mostly found in the plant and honey, among those toxins[6].



Grayanotoxin	R_1	R_2	R_3	R_1=R_2
GTX I	-OH	CH_3	-H	-
GTX III	-OH	$-CH_3$	-Ac	-
GTX IV	-	-	-Ac	$=CH_2$

Figure 1. Structure of Grayanotoxins.

The toxic impacts of grayanotoxin in the cell emerge through voltage-dependent Na channels. They increase the permeability of the sodium ions in excitable membranes and facilitate the entry of calcium into the cell. Initially toxin is linked to these channels in the opening stage of voltage-dependent channels. Then, the channels are modified and activation is hindered. Finally, the activation potential of the modified Na channels leads to the hyperpolarization of the channel. The excitable cells (nerve and muscle) remain as depolarized[7]. Although there may be cases, in which one teaspoon of honey may cause poisoning, the amount of the consumed honey may vary between 5 – 30 g in most of the poisoning cases, however same poisoning indications may not be present in everyone tasting the honey[8]. Toxic effects of the poisoning are rarely fatal, and mostly it does not exceed 24 hours. Depending on the amount of consumed honey, the symptoms may get more significant, and starts within average 1.5 – 3 hours after taking the honey[9]. The mostly seen symptoms are significant hypotension and bradycardia, which is seen approximately in more than %90 of the patients. Sweating, dizziness, and mental status alterations follow this. Syncope, diplopia, blurred vision, and hypersalivation are the other seen symptoms.

Due to the rapid development in tourism and trade today, it is possible to come across poisonings of this natural food in many parts of the world. Also in the early 1800s, Barton first described the symptoms of poisoning for a patient poisoned after mad honey in a published study[9, 10]. Bucak et al., one of the first researches conducted in our country about this nutrient called mad honey, holding honey, black net or bitter honey among the people[10]. Iberoglu et al., which examined 16 cases poisoned from mad honey and grayanotoxin was determined in these honeys between 1984 and 1986[11]. In the following years, although many cases of poisoning continue to be reported from Turkey, applications are considered to be much higher than those published due to mad honey poisoning.

Because of its widespread production and consumption in the Black Sea region, the occurrence of mad honey poisoning or the so-called popular involvement is common. Some of the honey produced in Duzce province was defined and sold by producers as mad honey. It is dangerous to

use because the amount of grayanotoxin is unknown. For these reasons, samples of honey produced in 2017 in Duzce province were collected and their grayanotoxin contents were analyzed[12].

MATERIALS AND METHODS

Chemicals and instruments

GTX-III standard was supplied as grayanotoxin III Hemi (ethyl acetate), MS grade methanol and acetic acid solvents obtained from Sigma-Aldrich (St. Louis, MO, USA). High quality ultra-pure water was supplied by Human Zeneer Navi Power I Integrate (Human Corporation, Korea). The grayanotoxin content and composition of mad honey were determined by using LC-ESI-MS/MS (Shimadzu, Kyoto, Japan).

Preparation of standards and samples

250 mg honey sample taken and added 2,5 ml dilution solution (methanol/water 1:4 + 0,1% acetic acid). Vortexed 2 minutes. Then diluted with same solution to 1 to 10. Vortexed 2 minutes and filtered with 0,45 µm filters. Filtered solution used to injection.

For calibration grayanotoxin standard solutions prepared concentrations between 1-200 ng/ml. Nine point of these concentration used for calibration (1, 2,5, 5, 10, 25, 50, 100, 250 and 500 ng/ml). The linearity of the method was confirmed by linear correlation which is R^2= 0,9999

Analysis method

We used linear gradient LC-MS/MS method for all grayanotoxins analysis. Analysis performed 100-mm x 4,6-mm, 5-mm particle C18 column. Column oven set to 45 °C. Mobile phases (A) water +0,1% acetic acid and (B) methanol +0,1% acetic acid. 0,3 ml/min flow and starting conditions with mobile phases %70/%30 respectively. From start to 2 min B was used %30; from 2 to 4,5 min B linear gradient to %52; from 4,5 to 7 min B linear gradient to %90. Thereafter, a linear gradient back to %30 for 3 min to equilibrate column for next injection. Injection volume was 10 µl.

ESI-MS/MS analysis was performed using multiple reaction monitoring (MRM) to detect the major product ions from the protonated molecules of Grayanotoxin III-3:25/4:76 (m/z 335.2→299.1, 317.1 and 91.1). The MS conditions were: nebulizer gas 15 psi, temperature 450 °C and collision energy −30 V.

RESULTS AND DISCUSSION

In LC-MS/MS MRM (Multi Reaction Monitoring) mode was used for analysis. Grayanotoxin-III peaks were detected nearly 4th minutes of analysis method (Figure 2). From these knowledge, 9 different concentrations of grayanotoxin-III standard were prepared, analyzed and calculated for calibration (Figure 3).

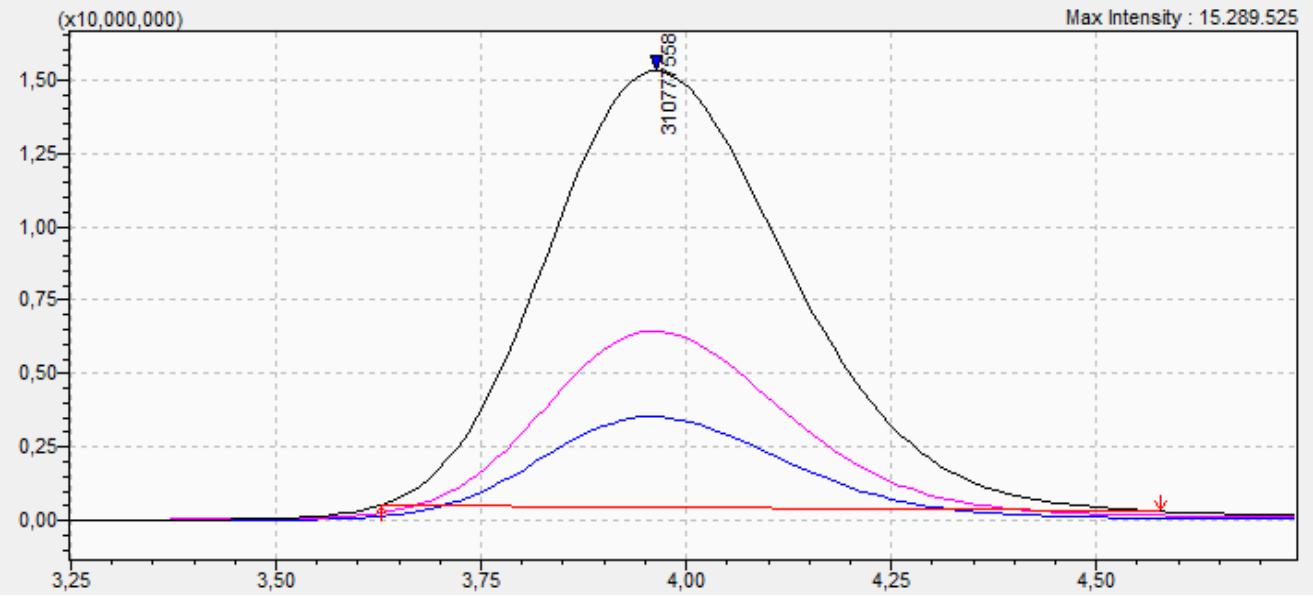


Figure 2. Peak of the grayanotoxin-III in LC-MS/MS MRM mode.

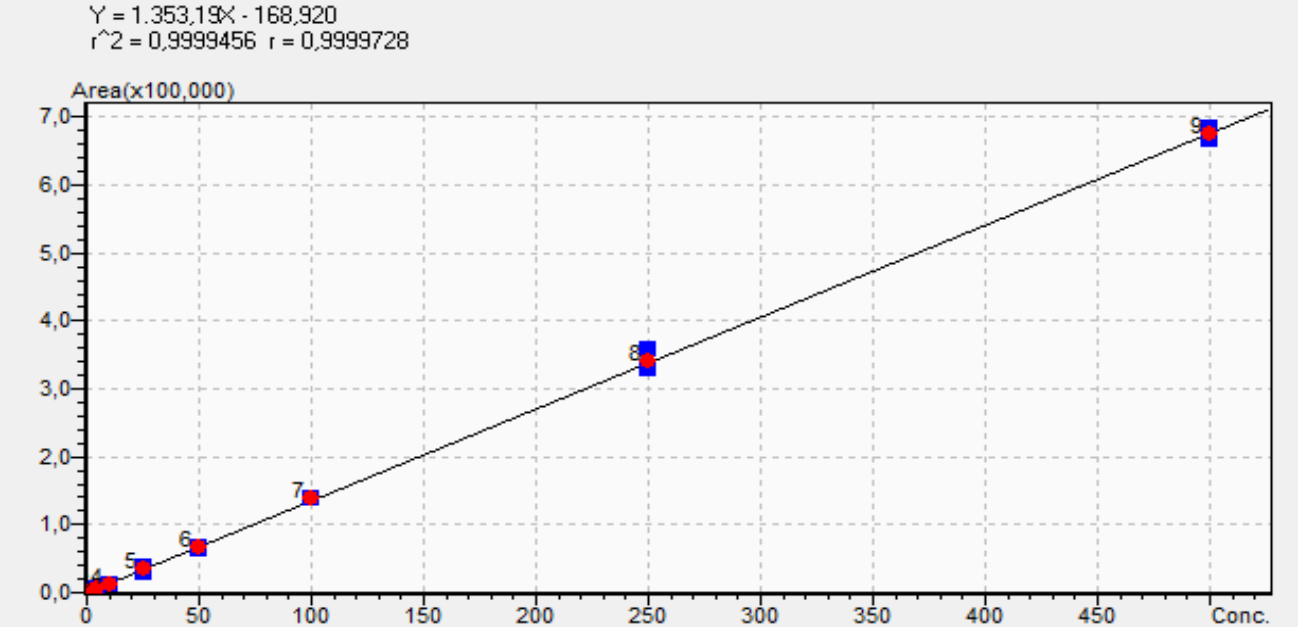


Figure 3. Nine point of calibration curve with linear correlation.

Upon the results of honey samples we came across different concentration of grayanotoxins. Nearly half of the samples have none toxins even the physical properties are pointed to mad honey. Other half of the samples have low and safe to eat concentrations of toxins. In 5 of the honey sample we calculated high amount of grayanotoxin-III and those are very dangerous to consume (Figure 4). Concentrations of grayanotoxins depends on *Rhododendron* blossoms. It affected by weather conditions such as cold and rainy weathers. If spring season become hot and dry, more *Rhododendron* blossoms appear and bees collect more nectar from them. For these reasons grayanotoxins levels in honeys changing every year. Due to the lack of standardization and changing conditions every honey samples should analyze before consumption. Most of beekeepers looking for strong odor and light yellow colour for mad honey but in some samples they need more informations. For measured samples contains different amounts of grayanotoxins between 0 – 70 ng/ml (Table 1).

Looking at the different analysis methods in the literature, the sample preparation part of our method takes less time and eliminates the preparation steps such as cartridges and similar with less amount. This situation can be called more useful as it shortens the preparations before analysis. The results we obtained match the literature and confirm the ranges determined as dangerous doses. Even if the results obtained from honey samples differ depending on the season, a common value can be revealed by contributing to such studies. Due to recent updates for the sample preparation and method section, it has seen that our method is more advantageous than other methods in order to get more practical and short results in poisoning cases[13-16].

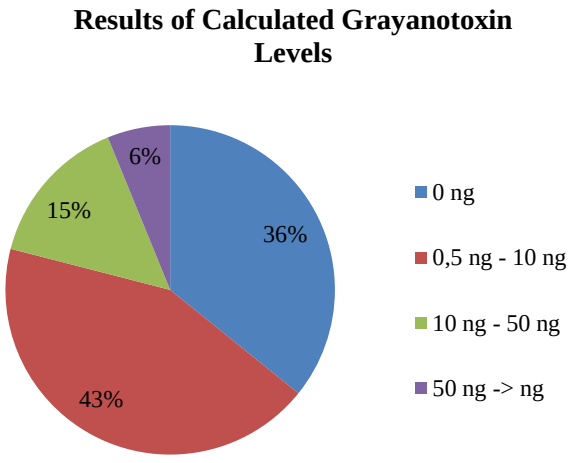


Figure 4. Percent representation of results.

Table 1. Concentration results of collected honey samples.

Sample	Concentration (ng/ml)	Sample	Concentration (ng/ml)	Sample	Concentration (ng/ml)
1	9.028	28	0.915	55	0.893
2	28.256	29	1.436	56	0
3	38.248	30	3.496	57	0
4	44.526	31	0.963	58	54.875
5	32.289	32	2.473	59	51.868
6	48.520	33	0	60	0
7	33.489	34	0	61	0
8	26.597	35	0	63	0
9	66.498	36	0	64	2.197
10	16.259	37	61.169	65	3.497
11	24.596	38	0	66	1.178
12	4.267	39	0	67	0.924
13	2.462	40	0.983	68	0
14	1.637	41	1.455	69	0
15	5.420	42	2.784	70	0
16	3.196	43	2.258	71	0
17	0	44	0	72	1.909
18	0	45	0	73	2.478
19	0	46	0	74	0
20	2.268	47	0	75	57.329
21	1.267	48	0	76	0
22	1.479	49	0	77	0
23	4.563	50	0	78	0
24	5.839	51	27.938	79	3.407
25	9.120	52	22.289	80	6.316
26	7.427	53	1.258	81	6.489
27	5.249	54	11.256	82	8.159

In conclusion, they seem physically same but in chemical composition some of them are not mad honey. Local people use mad honey for medication and they take grayanotoxins unknowingly. Calculated grayanotoxin amounts define of the purposes of their honey usage. In this study we collect samples from Duzce and near villages in the mad honey season. Some samples do not contain grayanotoxins even though strong odor and light yellow colour. Some samples contain high values of grayanotoxins even without physical differences. Mad honey usage is highly risky and not recommended for consuming but for medication purposes its promising for hypertension.

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