Section: Research Paper

Quantitative Estimation of Pyrrolizidine Alkaloids in Apis honey by Spectrophotometric method

Bhushanam M[1], Madhusudhan S[2], Dakshayini P N[1]*, Arun Jyothi Mathias[3] and Abhinandini I D[4]

[1]Department of Zoology, Maharani Cluster University, Bangalore (Karnataka), India

[2]Department of Biotechnology, Maharani Cluster University, Bangalore (Karnataka), India

[3]Department of Microbiology, Maharani Cluster University, Bangalore (Karnataka), India

[4]Department of Zoology, GFGC, Channapatna (Karnataka), India

ABSTRACT

Pyrrolizidine alkaloids (PAs) are phytotoxins, produced by more than 6,000 plant species. Bees forage on flowers of plants producing PAs, which leads to contamination of honey with the toxic compounds, through honey is known for its nutritional and medicinal properties. Pyrrolizidine alkaloids are toxic plant secondary metabolites produced as defense against herbivores by a wide variety of plants, mainly of the *Boraginaceae, Asteraceae* and *Fabaceae* families. Consumption of products contaminated with PAs may lead to hepatotoxic, carcinogenic, genotoxic and teratogenic effects in animals and humans. PAs are found in bee pollen when bees collect nectar and pollen from PA-producing plants, they enter the honey. In plant pollen, the PA content is in some cases higher than the one in leaves. PAs are a difficult class of toxins to investigate due to the large taxonomic diversity of PA-producing plants. Therefore the present investigation was aimed at extraction, isolation and purification of PAs by a simple and inexpensive method for the quantitative determination of PAs in *Apis* honey samples that makes the method applicable for the determination of PAs from food sources at toxic levels for consumers. By using only one solvent extraction of alkaloids from honey, the recovery percentage of senecionine (PA) is low (50.10%). By successive extractions, two times, with the same solvent, the senecionine retrieval percentage increases to 79.0%.

Keywords: *Apis* honey, toxins, Pyrrolizidine alkaloids and spectrophotometric method.

INTRODUCTION

The use of natural honey as food and medicine by mankind has been in existence from time immemorial. Raw honey is the most ancient sweetener, and it was noted to have been in use throughout the world several million years ago (Crane E, 1975). Natural honey (NH) is a sweet, flavourful liquid food of high nutritional value (White and Doner,1980 & Bogdanov *et al.*,

2008) and immense health benefits (Ajibola *et al.,* 2007). NH is produced by honey-bees as blossom honey by secreting nectars of flowers, and honeydew honey (forest honey) by secreting the exudates of plant sucking insects (Aphids). NH is widely embraced by all ages, and its use transcends the barriers of culture and ethnicity. The use of honey is even advocated and embraced by all religious and cultural beliefs. Food safety has become an essential food quality attribute, not only because of the major role played by foodstuffs within a human healthy diet, but also because of public concern (Liang *et al.,* 2019).

Pyrrolizidine alkaloids are one of the most common groups of natural toxins, produced by a wide variety of plants as a chemical defence against herbivores (Boppré , 2011). Plants containing PAs often grow undesired in agricultural production systems, posing a risk of contamination of feeds and crops (Edgar *et al.,* 2011). Bees often forage on the flowers of plants producing PAs, which leads to contamination of honey with the toxic compounds (Chung and Lam, 2017). The toxicity of PAs in humans is documented in a series of case reports of intoxication following ingestion of PAs containing herbal medicines and teas, and outbreak cases including deaths caused by the consumption of grain contaminated with PAs containing weeds (European Food Safety Authority, 2017). However, not all PAs exert toxicity. Only 1,2-unsaturated alkaloids are pro-toxins which can be converted into toxic metabolites in the liver. The conversion is triggered by the cytochrome P450 monooxygenases located primarily in hepatocytes. As a result, reactive electrophilic pyrrolic metabolites capable of binding to proteins and nucleic acids are created (Edgar *et al.,* 2011 & Lucatello *et al.,* 2016). PAs can be a cause of acute and chronic intoxication. Acute poisoning with PAs in humans is more associated with liver damage (European Food Safety Authority, 2011). In the case when the small amounts of dehydro PAs are regularly delivered via diet, cancer, pulmonary arterial hypertension, and cirrhosis are more likely to occur (Edgar e*t al.,* 2015). Humans can ingest PAs unintentionally via consumption of various products. Like the grains contaminated with PA-producing plants, vegetable harvests with similar-looking weeds (e.g. ragwort), herbal preparations, teas, honey, pollen contaminated with PAs (Boppré, 2011) and other food such as milk or eggs (Mulder *et al.*, 2015 & Mulder *et al.*, 2016).

Even though the toxicity of PAs has been well documented and high concentrations of PAs have been detected in various products, there is no official limit for the maximum allowable level of PAs in food and feed. In India, the production and consumption of honey is systematically increasing. Honey is consumed in its pure form or an ingredient of breakfast cereals, sweets, or baked products. However, it has been proved that honey can be contaminated with PAs, and in some cases, high concentration of the alkaloids have been detected (Dübecke *et al.,* 2011 ; Huybrechts and Callebaut, 2015 & Lucatello *et al.,* 2016). To increase consumer protection by minimising dietary exposure to these toxins, it was suggested that all honeys need to be assessed for their content of PAs (Edgar *et al.,* 2002 & Orantes *et al.,* 2013). In 2017, European Food Safety Authority (EFSA) report was suggested that new and sensitive analytical methods enabling PAs determination should be developed. Hitherto, analytical methods on the LC-MS/MS analysis were emphasised. For the purification of extracts mostly cation exchange cartridges were used combined with ammonia in methanol elution of PAs. However, the purification of the extracts is not efficient, especially when LC-MS is used for the instrumental analysis, and many problems concerning the appropriate identification and quantification of PAs can occur. That is why a new sensitive analytical method providing effective clean-up of honey

extracts has been developed. Based on the EFSA recommendations and on the results of the occurrence of PAs in honey of European origin reported by other authors (European Food Safety Authority, 2011; Griffin *et al.,* 2013 & Martinello *et al.,* 2014). , the ten most often detected alkaloids were selected for their study. The compounds designated were Senecionine- type PAs: jacobine, retrorsine, senecionine, and seneciphylline; Lycopsamine-type PAs: lycopsamine, intermedine, and echimidine; and Heliotrine-type PAs: heliotrine and lasiocarpine. Senkirkine was also included, as it was found that together with echimidine, echimidine N-oxide, heliotrine, lycopsamine, retrorsine, senecionine, and seneciphylline; it constituted around75%–90% of the total PAs measured in honey (European Food Safety Authority, 2016). The N-oxides are reduced with the zinc dust, hence the final determined concentration reflects the content of both free base and N-oxide forms. On the basis of the detected PAs concentrations it can be stated that most of the analysed honey samples should not pose any potential risk to the consumers.

MATERIALS

The methodology of Umme Amara *et al.,* (2017) and Oana *et al.,* (2018) were employed in the present research investigations.

Standard solution

Standard solutions in methanol were prepared from a solution of senecionine (100 µg/mL; 0.3 ml).

Sample preparation

The polyfloral *Apis* honey samples of 30 g were taken as triplicates of two sets. Each of it was mixed with 150 ml of methanol 50% and acidified with citric acid to pH 2 to 3.

Standard solution of senecionine (100 µg/mL; 0.3 ml) was added to each sample, mixed, and filtered. For one honey sample, the residue was again mixed with 150 mL of acidified 50% methanol and filtered.

The filtrate solutions were reduced to about 30 mL each and purified by liquid-liquid extraction once with 30 mL chloroform and then with 30 mL ethyl ether. The samples were filtered, alkalinized with 25% ammonia solution to pH 9-10, and extracted one time with 30 mL chloroform. The chloroform solutions were reduced to about 5 mL using a rotary evaporator and brought to dryness under nitrogen. The residue was dissolved in 3 mL of methanol and passed through 0.2 µm adaptive syringe filters.

The same process was repeated twice with *Apis* honey samples and the filtrates were combined. The filtrate solutions were reduced to about 30 mL each and purified by liquid-liquid extraction two times with 30 mL chloroform and two times with 30 mL ethyl ether. The samples were filtered, alkalinized with 25% ammonia solution to pH 9-10, and extracted two times with 30 mL chloroform. The chloroform solutions were reduced to about 5 mL using a rotary evaporator and brought to dryness under nitrogen. The residue is dissolved in 3 mL of methanol and passed through 0.2 µm adaptive syringe filters.

Simultaneously, the control samples (honey) were processed in the same way, but without the addition of standard solution.

The samples were noted as H1 (honey extracted once) and H2 (honey extracted twice).

Reagents

Oxidation reagent: 20 mL methanol was mixed with 0.20 mL hydrogen peroxide 30% containing sodium pyrophosphate 5 mg/mL as a stabilizer, 0.20 mL ethylene glycol and 20 mg butylated hydroxytoluene Diglyme: Diethylene glycol dimethyl ether containing 5 mg/mL butylated hydroxytoluene. Acetic anhydride was redistilled and the fraction boiling between 136 to 139 °C was collected.

Modified Ehrlich's reagent: 4 mL boron trifluoride in methanol 14% is diluted with 36 mL absolute ethanol and 0.8 g 4-dimethylaminobenzaldehyde are added.

METHOD

For the preparation of the calibration curve, volumes of the working standard solutions, corresponding to 2.5–20 μg senecionine, were evaporated under nitrogen jet; 0.5 mL oxidizing reagent was added and the test tubes were left in the boiling water bath for 20–30 min. 1 mL diglyme and 0.1 mL acetic anhydride were added, and the tubes were heated again in the water bath for 1 minute. After cooling the tubes at room temperature, 1 mL of modified Ehrlich reagent was added, and the tubes were heated in a water bath at 55– 60 °C for 4–5 minutes. The samples were transferred in volumetric flasks and acetone was added up to 10 mL. For assessing the alkaloid content, 0.4 ml of each sample was evaporated under nitrogen stream and the same procedure was followed as described in the preparation of the calibration curve. All measurements were performed on a spectrophotometer, at 565 nm versus a blank.

All assays were performed in triplicate. Statistical analysis was performed and the results were expressed as mean ± standard deviation (M±SD) and percentage (%).

RESULTS AND DISCUSSION

An eight point linear calibration curve of senecionine in the 0.25 to 2 μg/mL range, with good linearity was obtained (Fig.1).

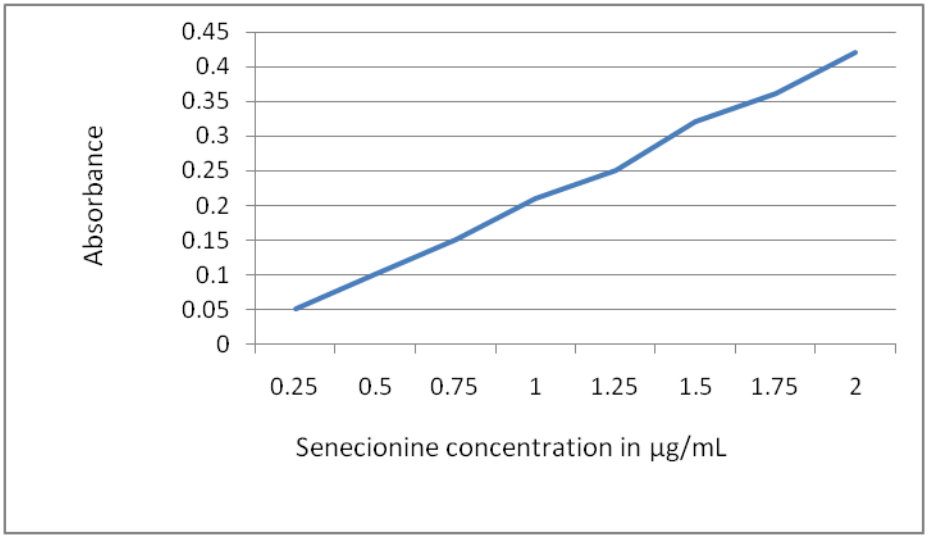

Fig. 1. The standard calibration curve for senecionine

The results of the quantitative determinations are presented in Table 1. By twice washing the residue obtained after filtration with acidified methanol 50%, the recovery percentage of senecionine increased to 79.00% in honey.

Table 1. Recovery of senecionine from honey

Sample	Senecionine Recovery (M±SD)	Senecionine Recovery (%)
H1	0.501±0.06	50.10%
H2	0.790±0.002	79.00%

n= 5, significant at p>0.05

By using only one solvent extraction of alkaloids from honey, the recovery percentage of senecionine was low (50.10%). By successive extractions, two times, with the same solvent, the senecionine retrieval percentage increased to 79.0%. Similar findings were recorded by Natasha *et al.*, (2019).

Apiarists in many countries regularly use a number of PA-containing plants for honey production (Edgar *et al.*, 2002 & 2011). Kempf *et al.* (2011) have demonstrated that honey from many of the plants contain significant levels of PAs ranging from 56 to 87 per cent.

Among 50 honey samples analysed by Ewelina and Krzysztof (2018), 32% were positive for the presence of at least one of the alkaloids ranging from 1.4 to 5.2µg / kg.

CONCLUSION

Because of the health hazards raised by the consumption of food contaminated with PAs, the present investigations developed a simple and inexpensive method for the quantitative

determination of PAs in honey that makes the method applicable for the determination of PAs from food sources at toxic levels for consumers.

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