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Spectrofluorimetric Analysis of Honey Samples for Quantification of Riboflavin: Statistical Evaluation of External Factors

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ABSTRACT

This study investigated the riboflavin content in honey samples collected from various geographical and climatic regions of Khyber Pakhtunkhwa. Quantification was carried out through spectrofluorimetric, utilizing riboflavin's native fluorescence with excitation and emission peaks at 464 nm and 525 nm, respectively. A calibration curve of fluorescence intensity against riboflavin concentration was established to determine its levels in the samples. Statistical analysis was performed to assess the influence of external factors such as altitude, bee species, floral source, and season on riboflavin concentration. Results revealed that honey from lower altitudes contained the highest riboflavin levels (1.156±0.08 µg g^{-1}), while autumn samples exhibited the maximum average concentration

(1.37±0.06 μg g^{-1}). Moreover, honey derived from Ziziphus flowers showed the highest riboflavin content (1.383±0.1 μg g^{-1}), and samples from small honeybee hives contained greater amounts (1.176±0.07 μg g^{-1}). The findings indicate that vitamin composition and nutritional value in honey are influenced by environmental and biological factors.
Keywords: Riboflavin, Honey, Spectrofluorometry, Geographical variation, Nutritional composition

INTRODUCTION

According to Pearson (1976), honey is a natural sweet substance produced by bees from the nectar of flowers through regurgitation and evaporation [1]. It mainly contains fructose and glucose, making it sweeter than table sugar, and is valued for its flavor and baking qualities [2, 3]. Historically, honey has been used across civilizations as food, medicine, and in religious rituals. Ancient texts from the Vedic, Greek, Roman, Christian, and Islamic traditions describe its nutritional and healing properties. In Hinduism and Ayurveda, honey has been used for over 4000 years in sacred rituals and medicinal formulations. Egyptian, Greek, Chinese, and Arab physicians also recognized its therapeutic effects for wound healing and disease treatment. The Quran refers to honey as a divine cure, praised for its healing qualities.

Throughout history, honey has served as an antiseptic, antibacterial, and antioxidant agent. Its medicinal use extended from ancient civilizations to modern times such as during the Balkan War in 1913 for wound treatment, and more recently, the use of *Manuka honey* to combat MRSA infections in hospitals [3].

Chemical Composition of Honey

Honey exists in several forms based on floral origin and production method. Blended honey is the most common commercial type, produced by mixing honeys from multiple sources. Poly floral honey comes from the nectar of various flowers, while nonfloral honey originates predominantly from a single flower type, giving it distinct flavor, color, and aroma characteristics—examples include acacia, lavender, orange blossom, and eucalyptus honeys [2]. Chemically, honey is a complex natural substance containing over 180 compounds [4–5]. Its main components are carbohydrates, proteins, organic acids, vitamins, minerals, phenolic compounds, volatile compounds, and enzymes. Honey is a complex natural substance primarily composed of carbohydrates, with glucose and fructose accounting for approximately 85% of its solid content [1]. Fructose is typically the dominant sugar, except in varieties such as rape and dandelion honey, where a higher glucose concentration leads to quicker crystallization [6–10]. Proteins constitute about 0.1% to 3.3% of honey, depending on the bee species [11], with proline being the most abundant amino acid and serving as an indicator of honey's maturity and purity [14]. Honey also contains essential enzymes like invertase, diastase, and glucose oxidase that contribute to its biochemical activity. The natural acidity of honey, approximately 0.57%, is due to organic acids such as gluconic acid, which influence its flavor, color,

and resistance to microbial growth [15–17]. Additionally, honey provides small amounts of B-complex vitamins (B1, B2, B3, B5, B6, B8, B9) and vitamin C, largely derived from pollen [5,18–20]. Its moisture content averages 17.2%, which plays a vital role in determining fermentation and crystallization tendencies [1].

Honey is also rich in minerals, including potassium, calcium, iron, magnesium, zinc, and manganese, with potassium being the most abundant element [21–25]. The mineral composition varies with the soil and the type of plants visited by bees. Phenolic compounds such as flavonoids and phenolic acids including quercetin, galangin, and caffeic acid are responsible for the antioxidant properties and serve as markers for identifying floral origins [26–27]. Furthermore, honey's aroma arises from more than 400 volatile compounds like furfural, benzaldehyde, acetone, and linalool derivatives [28–31]. Enzymes such as diastase, invertase, glucose oxidase, and catalase mainly secreted by bees play crucial roles in honey's formation and preservation [32–40]. Another important component, 5-hydroxymethylfurfural (HMF), forms through the acid-catalyzed dehydration of fructose during heating or prolonged storage. HMF content serves as an indicator of honey's freshness, with excessive levels signaling overheating or poor storage conditions [41–45].

Factors Affecting Composition of Honey

Honey is a complex natural substance containing around 181–200 different compounds [4,5]. It is mainly composed of sugars, water, proteins, organic acids, vitamins, minerals, phenolic and volatile compounds, enzymes, and trace particles from harvesting. Glucose and fructose make up about 85% of honey solids, with monosaccharides representing roughly 75% of total sugars [1]. The composition of sugars varies according to the botanical and geographical origin of honey, affecting its energy value, crystallization, and viscosity [6–8]. In most honeys, fructose predominates, except in rape and dandelion honeys, where glucose content is higher, leading to rapid crystallization [6]. Honey also contains a variety of sugars such as maltose, sucrose, and isomaltose, which are enzymatically hydrolyzed into simpler sugars [9,10]. The protein content of honey ranges from 0.1% to 3.3%, depending on the bee species [11]. These proteins mainly come from pollen, nectar, and bee secretions [12,13]. Proline is the dominant amino acid, accounting for 50–85% of total amino acids and serving as an indicator of honey's purity and maturity [14]. Honey also contains enzymes like invertase, catalases, diastases, and glucose oxidase that play essential roles in its biochemical properties. Honey exhibits mild acidity of about 0.57% due to organic acids such as gluconic acid, which is produced enzymatically from glucose [15,16]. Other acids like citric, formic, and clavulanic contribute to its flavor, color, and microbial stability [17]. In addition, honey contains several vitamins, mainly the B-complex group (B1, B2, B3, B5, B6, B8, and B9), along with vitamin C. These vitamins are primarily derived from pollen, and ascorbic acid is usually found in the highest concentration at about 0.31 ppm [5,18–20]. The average moisture content of honey is 17.2%, which plays a key role in preventing fermentation and controlling crystallization [1]. Honey is also rich in

essential macro- and microelements, including potassium, calcium, iron, magnesium, and zinc [21,22]. The mineral content ranges from 0.04% in light honeys to 0.2% in dark varieties, with potassium being the most abundant element [21,25]. The mineral composition depends largely on the floral source and soil characteristics of the nectar-producing plants [13,22].

Phenolic compounds, including flavonoids and phenolic acids, are important bioactive components in honey that provide antioxidant properties and help in identifying floral origins [26–27]. Common phenolic compounds include quercetin, gallic acid, chrysin, and pinocembrin [5]. The flavor and aroma of honey are determined by more than 400 volatile compounds such as furfural, benzaldehyde, acetone, and linalool derivatives [28–31]. These compounds originate from nectar, bee metabolism, and post-harvest processing. Honey also contains several enzymes, including diastase, invertase, glucose oxidase, and catalase, which are mainly secreted by honeybees [32–36], although pollen and nectar can also contribute to enzyme content [37–40]. Another important compound found in honey is hydroxymethylfurfural (HMF), formed from the acid-catalyzed dehydration of fructose during heating or storage. The concentration of HMF serves as an indicator of honey freshness, and excessive levels suggest overheating or prolonged storage [41–45]. Overall, the chemical composition of honey depends on its botanical and geographical origin, soil characteristics, and environmental factors, as well as processing and storage conditions.

The study focuses on determining the concentration of Riboflavin (vitamin B_2) in honey samples collected from various locations in Khyber Pakhtunkhwa, Pakistan, using the spectrofluorimetric method. Riboflavin ($C_{17}H_{20}N_4O_6$) is a fluorescent compound with absorption maxima at 233, 267, 373, and 444 nm and fluorescence emission at 520 nm. It is slightly soluble in water and ethanol but insoluble in ether, benzene, acetone, and chloroform. Spectrofluorometry, a highly sensitive and selective analytical technique, measures fluorescence intensity, which is related to the lifetime of the excited state by the equation $I = I_0 e^{-t/\tau}$. The Riboflavin concentrations in honey samples will be analyzed using the proposed analytical method to ensure accurate quantification and reliable measurement. Following the analysis, a statistical assessment will be conducted to examine the individual and combined effects of various factors influencing Riboflavin levels in honey. This approach will help identify the key determinants affecting Riboflavin content and provide a comprehensive understanding of how different factors interact to influence their concentration.

LITERATURE REVIEW

Radzuan et al. [60] analyzed riboflavin content in green leafy vegetables—such as spinach, mustard green, and water morning glory—using a wet digestion and spectrofluorometric method, finding the highest concentration in spinach (4.2421 μg/g) and none in lettuce, concluding that leafy vegetables are natural sources of riboflavin. Abbas et al. [61] introduced a sensitive and selective spectrophotometric

technique for riboflavin determination in pharmaceutical formulations using a Cupric chloride–putrescine complex, with excellent precision and recoveries of 98–99%. Bonamore et al. [62] developed a low-cost fluorimetric method for riboflavin analysis in wine and beer based on fluorescence quenching by riboflavin-binding protein, achieving a detection limit of 15 ng/mL and results comparable to HPLC, making it suitable for routine beverage testing. Ghann et al. [63] designed an economical fluorimeter with blue LED excitation for riboflavin measurement, confirming good linearity (0.01–2.5 μg/mL) and accuracy (91.3–100.21% recovery) but no fluorescence enhancement by surfactants. Antakli et al. [64] established a precise RP-HPLC ion-pair method for seven water-soluble vitamins using DAD and FLD detectors, achieving high linearity ($R^2 > 0.9972$) and low detection limits (1.3–26.7 μg/L), suitable for food product analysis. Bartzatt et al. [65] proposed a simple and accurate spectrophotometric assay for riboflavin using sodium borate buffer (pH 7.52) with strong linearity ($R^2 = 1.000$) and sensitivity down to 30 ppm, effective for aqueous and tablet samples. Vinas et al. [66] optimized a reversed-phase LC method with fluorescence detection to analyze riboflavin (RF), FMN, and FAD, achieving very low detection limits (0.03–0.24 ng) and confirming RF as the dominant form of vitamin B_2 in various foods. Bartzatt et al. [67] also reported a spectrophotometric method using citric acid buffer at pH 5.03, showing excellent linearity ($R^2 = 0.9998$) and reproducibility across wide concentration ranges. Trang et al. [68] examined chromatographic behavior of nine water-soluble vitamins across reversed-phase columns, identifying Type-B-silica columns and LC-MS detection as the most effective for accurate vitamin analysis in complex matrices. Niazi et al. [69] developed a fluorescence-based method using EEM-PARAFAC for direct riboflavin detection in plasma, achieving strong predictive performance (RMSEP = 0.0059) and superior results compared to PLS. Seal et al. [70] used RP-HPLC for simultaneous vitamin quantification in wild edible fruits, finding high B_2 and B_3 levels and validating the method's precision for nutritional profiling. Yang et al. [71] analyzed fluorescence properties of NADH and riboflavin, identifying three major emission regions and confirming vitamin B_2 as a stronger fluorophore. Finally, Kadakal et al. [72] studied the thermal degradation kinetics of ascorbic acid, thiamine, and riboflavin in rosehip nectar via HPLC, revealing first-order degradation with activation energies between 36.38 and 55.30 kJ/mol, offering valuable data for food preservation research.

Experimental work

Spectrofluorophotometer (RF-5301 PC, Shimadzu, Japan) having 150-watt Xenon lamp as excitation source with 1.0 cm quartz cell was used throughout the experimental work for fluorescence measurements. The emission and excitation slit was 4 nm for fluorimetric operation. Required chemicals were weighed using electrical digital balance (Shimadzu ATY 224) and stirring of the reaction mixture was carried out with the help of magnetic heating stirrer (Heidolph). Borosilicate glassware and apparatus were used for respective operations.

Collection of Honey Samples

The honey samples collection was made in the years 2017-2018. A total of twenty (20) honey samples from different agro-ecological zones were collected by taking into consideration the altitude of sampling area, season in which the honey was made, flowers of the area in the respective season, climatic conditions, bees' types and size etc. The sample collection was carried out using standard protocol. Fundamentally, any protocol which is used by common consensus can be a standard. An ideal standard protocol should fit broad user needs for information and should be easy to use. Such a protocol should fit the criteria as, equipment that is inexpensive, readily obtainable, and simple to build and deploy - techniques that can be used in a variety of field situations, straightforward, rapid data collection and analysis methods, acquisition of data which are reproducible, unbiased, and include variance estimation, guidelines that can be used by individuals with diverse training background. Standardization is most important for individuals who plan to compare their data with results from other studies or across years within the same study. All the samples were carried in glass stoppered vials from the concerned beekeepers and were kept in the dark cupboard to prevent photodecomposition at ambient temperature until needed for laboratory analyses. A spectrofluorimetric method was employed to determine riboflavin in all the collected samples. In which the fluorescence intensity of riboflavin (RF) was measured at 525 nm following excitation at 464 nm.

Analysis of honey samples for the determination of riboflavin

Riboflavin was determined by following the steps given below.

Calibration curve

This was constructed by plotting Fluorescence Intensity (FI) of a series of standard Riboflavin (RF) solutions versus concentration.

Preparation of a stock solution of RF

Riboflavin Stock Solution (100 µg/ml) was prepared by dissolving 0.01 g of riboflavin in 50 ml of distilled water. Then, a few drops of glacial acetic acid, because riboflavin is stable in it. Then transferred it to a 100 ml volumetric flask and diluted it up to the mark. This stock solution was stored at 4ºC and was covered with a black plastic bag to prevent photodecomposition.

Preparation of a standard solution of RF for the calibration curve

A riboflavin working standard in the range of 0.005-0.3 µg ml^{-1} was prepared using the dilution formula $C_1V_1 = C_2V_2$, and the FI of each solution was measured using a spectrofluorometer. The range of 0.005 -0.3 µg ml^{-1}. These solutions were protected from light by covering them with a black plastic bag. Then the fluorescence intensity was measured through a spectrofluorometer.

The calibration data and plot of FI vs concentration are given in table 1 and shown in figure 1.

Sample preparation and measurement of FI

(i) Riboflavin of analytical grade.

(ii) Glacial acetic acid.

(iii) Distilled water obtained from a distillation plant.

Preparation of the sample solution

In a small beaker, 5.0 g of each sample was taken. Dissolved it in 50 ml of distilled water, added a few drops of Glacial acetic acid, and diluted up to the mark in a 100 ml volumetric flask. Covered the sample solution with a black plastic bag to prevent photodecomposition of RF.

Fluorescence measurement

Each sample was taken in a quartz cuvette and placed in the sample holder of the pre-calibrated spectrofluorometer, and FI was measured at emission wavelength 525 nm following excitation wavelength at 464 nm. The measurement was done in triplicate.

Calculation of the concentration of RF

The concentration of RF in each sample was calculated from the average FI using the straight-line equation of the calibration curve, which is given below.

$Y = 2513.2x + 13.17$ (1)

Here, Y is the fluorescence intensity of the sample X in the above equation 1 is concentration, calculated by rearranging equation 1 as

$X=(Y-13.17) / 2513.2$ (2)

To calculate the concentration of RF in μg g^{-1} of honey, the following equation was used.

Conc (μg g^{-1}) = (X (μg mL^{-1})) (100 ml)/5g (3)

Conc (μg g^{-1}) = X (μg g^{-1}) (4)

RESULTS AND DISCUSSION

A calibration curve or working curve was constructed by plotting Fluorescence Intensity (FI) of a series of standard Riboflavin (RF) solutions versus concentration in the range of 0.005 -0.3 μg ml^{-1}. The results are given in table 1 and shown in figure 1.

Table 1: Calibration curve for quantification of RF in honey samples

Concentration (μg ml^{-1})	FI
0.005	15.153
0.008	22.795
0.01	29.497
0.03	92.374
0.05	139.844
0.08	228.754
0.1	276.864
0.2	533.608
0.3	747.449

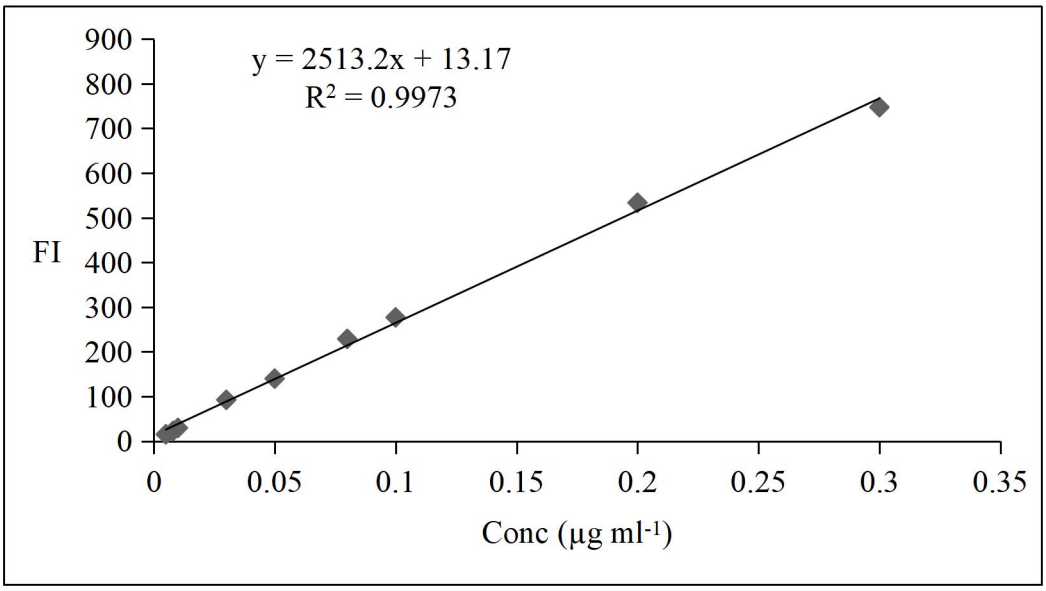

Figure 1. Calibration curve of FI vs RF concentration (µg ml^{-1})

Figure 1. shows that the curve has good linearity with a correlation coefficient (R^2) = 0.9973 and can be successfully applied for the determination of RF in honey samples.

Quantification of RF in honey samples

All the collected samples were analyzed by spectrofluorimetric measurement and subsequent calculation of RF in (µg g^{-1}). The results are given in Table 2.

Table 2. Concentration of RF (µg g^{-1}) in honey samples

Sample No	Altitude from sea surface (ft)	Flowers	Season	Bee size	Conc of RF (µg g^{-1}) ±SD
1	1497	Ziziphus	Jan-18	Small	2.74±0.00014
2	2478	Ziziphus	Nov17	Medium	1.34±0.0004
3	3487	Multifloral	Jul-17	Small	1.2±0.00005
4	3407	Multifloral	Apr-18	Small	1.32±0.00006
5	5433	Commercial	Dec-17	Unknown	0.255±0.000001
6	4194	Brassica	Apr-18	Medium	0.075±0.000006
7	2353	Ziziphus	Oct-18	Medium	1.4±0.000018
8	3655	Brassica	Apr-18	Medium	0.306±0.000003
9	2310	Multifloral	Apr-18	Medium	0.246±0.000002
10	1805	Ziziphus	Dec-17	Small	0.81±0.0000087
11	3655	Acacia	Jun-17	Medium	1.2±0.00022
12	2310	Acacia	Jun-17	Medium	0.912±0.00002
13	1897	Ziziphus	Dec-17	Medium	1.74±0.00022
14	1869	Acacia	Jul-17	Small	0.654±0.000002
15	1890	Acacia	Jun-17	Medium	1.38±0.00012

16	1517	Ziziphus	Dec-17	Big	1.32±0.000015
17	1880	Acacia	Jun-17	Big	0.505±0.00004
18	1805	Multifloral	Apr-18	Medium	0.216±0.00006
19	1880	Acacia	Jun-17	Medium	1.04±0.00005
20	4190	Ziziphus	Jan-18	Small	0.332±0.00001

Effects of different factors on the concentration of RF in honey samples

The effect of different factors on the concentration of RF in honey samples was investigated. The studied factors include the altitude of the sampling area, the season of honey formation, the types of flowers, and the size of honeybees.

Effects of the altitude of the sampling area on RF concentration

The effect of the altitude of the sampling area on the RF concentration in the honey samples was investigated by determining the concentration of RF in samples collected at altitudes in the range of 1497-5433 feet height from sea level. The results are given in table 3 and shown in figure 2.

Table 3. Effects of altitude of sampling area on RF concentration

Altitude	Range (feet)	Average conc. Of RF (μg g^{-1})
Altitude 1	1400-1900	1.156
Altitude 2	1900-2500	0.975
Altitude 3	3400-3700	1.007
Altitude 4	4100-5433	0.221

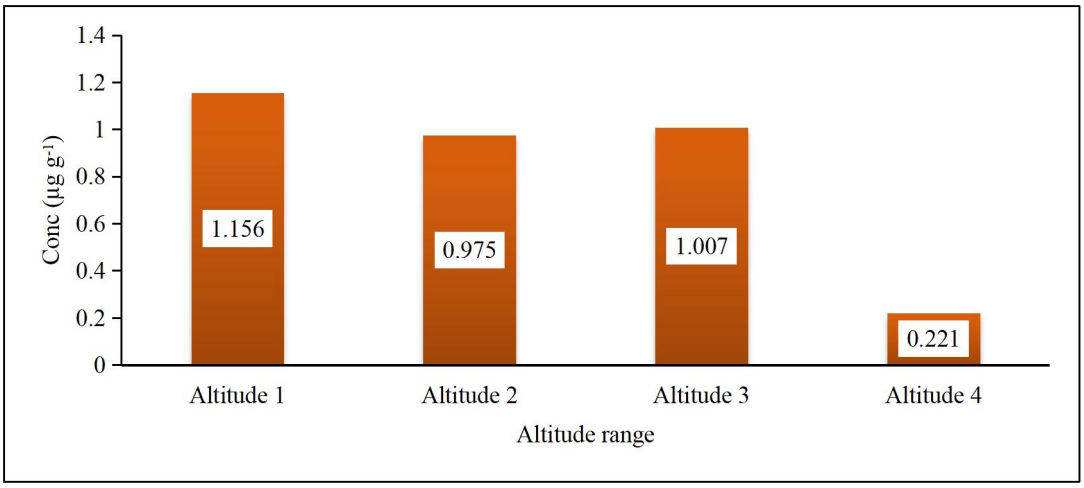

Figure 2. Effects of altitude of sampling area on RF concentration

It is clear from the data in table 3 and figure 2 that the concentration of RF generally decreases with an increase in altitude of the sampling area. The RF concentration is maximum in the altitude range of 1400-1900 feet, in the range 1900-2500 and 3400-3700 feet it remains nearly constant, and then in the range of 4100-5433 feet it decreases. We were unable to find and collect an authentic sample from the area at altitudes in the range of 2500-3400 feet and 3700-4100 feet.

Effects of the season of honey formation on RF concentration

The effect of season of sampling on the RF concentration in the honey samples was investigated by determining the concentration of RF in samples collected in different seasons: spring, summer, autumn, and winter. The average concentrations of RF in different seasons of the year are given in table 4 and shown in figure 3.

Table 4. Effects of season of honey formation on RF concentration

S.NO	Season	Concentration (µg g^{-1})
1	Spring	0.433
2	Summer	0.984
3	Autumn	1.37
4	Winter	1.2

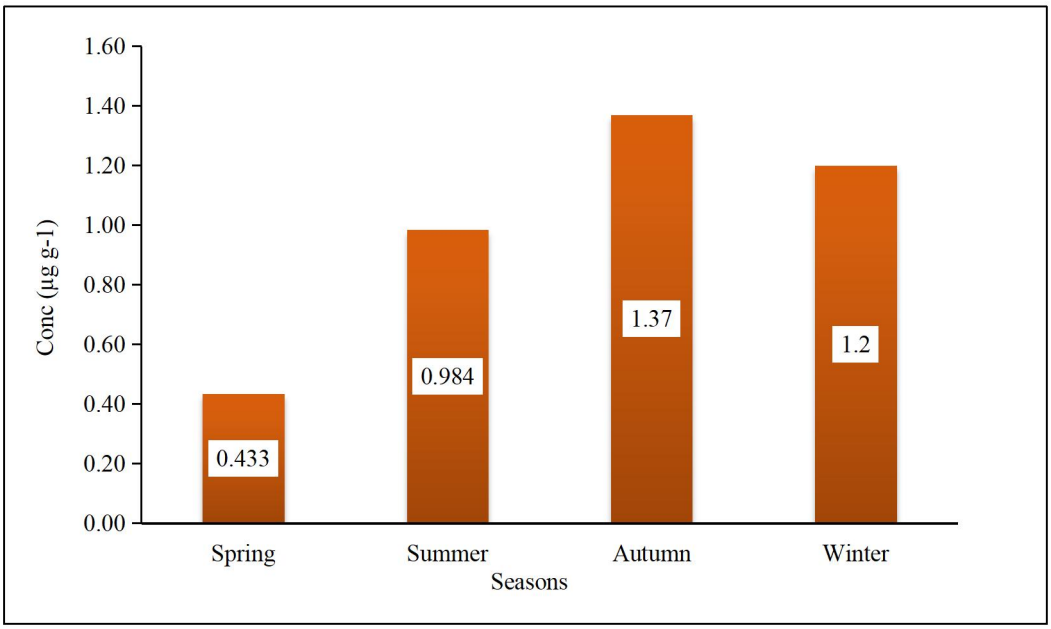

Figure 3. Effects of season of honey formation on RF concentration

It is clear from table 4 and figure 3 that the concentration of RF increases from spring through summer to autumn, then remains nearly constant through the winter season. The highest concentration of RF in autumn is because the honey is made by bees that collect nectar from flowers, which are produced in the summer season.

Effects of types of flowers on RF concentration in honey samples

The effect of types of flowers on the RF concentration was investigated by determining the concentration of RF in samples that were collected at different times of different flowers. The honey samples were of ziziphus, acacia, brassica, and multifloral. The results are given in table 5 and shown in figure 4.

Table 5. Effects of types of flowers on RF concentration in honey samples

S.NO	Types of flowers	Average conc (µg g^{-1})

1	Ziziphus	1.383
2	Acacia	0.949
3	Multifloral	0.745
4	Brassica	0.202

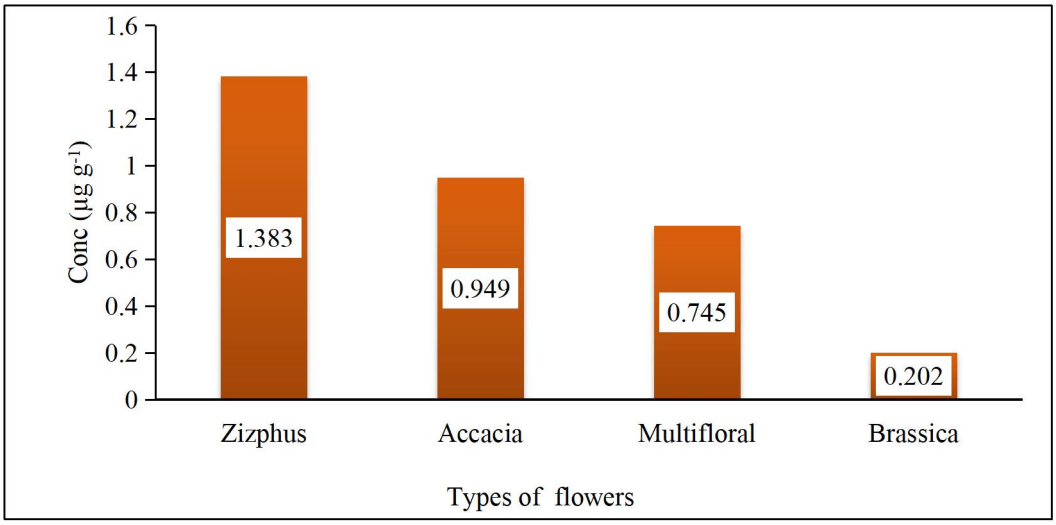

Figure 4. Effects of types of flowers on RF concentration in honey samples

It is clear from table 5 and figure 4. that the RF concentration is higher in ziziphus honey than in acacia, multifloral, and is much less in honey extracted from brassica flowers. The effect of the size of bees on the RF concentration in the honey samples was investigated by determining the concentration of RF in honey samples that were of different sizes of bees, like small, medium, and large.

Table 6. Effect of the size of bees on RF concentration in honey samples

S.NO	Bees size	Average conc (µg g^{-1})
1	Small	1.176
3	Large	0.913
4	Medium	0.896

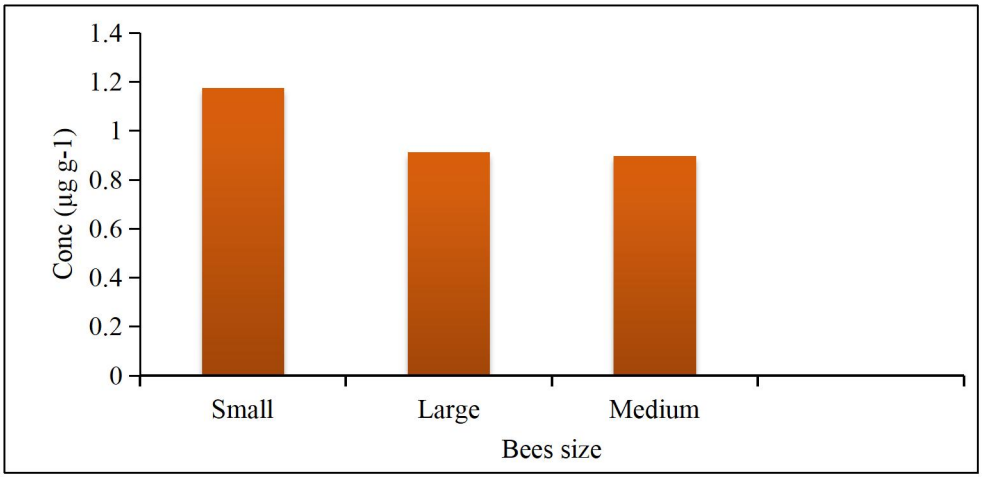

Figure 5. Effect of the size of bees on RF concentration in honey samples

It is clear from table 6 and figure 5 that the RF concentration is higher in honey samples of small-sized bees, and then the RF concentration remains nearly constant for large and medium-sized bees.

Statistical analysis. For statistical evaluation of the individual effect of different variables like altitude of sampling site, season of honey formation, Bee size, and flower type on the concentration of RF (μg g^{-1}), SPSS V. 23 was used.

Effect of altitude on RF concentration in honey The effect of the altitude of the sampling site on RF concentration in honey was investigated. Here, the dependent variable is RF concentration (μg g^{-1}), whereas altitude is considered the independent variable. The results are given in table 7 and shown in figure 6.

Table 7. Effect of altitude on RF concentration in honey

Model	Coefficients		t-value	P-value
	B	Std. Error		
(Constant)	1.674	0.360	4.654	0.000
Altitude	-0.271	0.125	-2.170	0.044

a. Dependent variable: RF Concentration (μg g^{-1})

b. Independent variable: Altitude

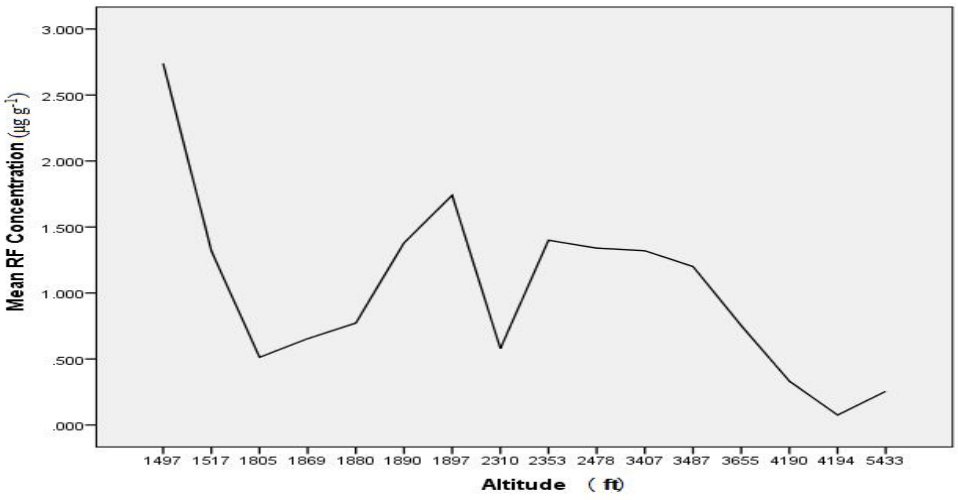

Figure 6. Effect of altitude on RF concentration in honey

In the results given in Table 7, the coefficient of altitude = -0.271 indicates that there is a negative effect of altitude on RF concentration, i.e., for a one thousand ft increase in altitude from sea level, an average of 0.271 μg g^{-1} decrease will occur in RF concentration. The p-value = 0.044 indicates that the effect of altitude on the RF concentration in honey is statistically significant.

Effect of the season of honey formation on RF concentration

To study the effect of season on RF concentration in honey, RF concentration was taken as the dependent variable while the season (i.e., winter, spring, summer, and autumn) was considered as the independent variable. Since season is a categorical variable, the concept of dummy variable is used in regression. As there

are four categories of the season, three dummy variables, winter, spring, and summer, were included in the regression, and the left-over category, i.e., autumn, was taken as the base category. The results are given in table 8. and shown in figure 7.

Table 8. Effect of sampling season on RF concentration in honey

Model	Coefficients		t	Significance
	B	Std. Error		
(Constant)	1.370	0.436	3.141	0.006
Winter	-0.171	0.504	-0.339	0.739
Spring	-0.937	0.516	-2.316	0.048
Summer	-0.386	0.495	-2.780	0.044

a. Dependent variable: RF concentration (μg g^{-1})

b. Independent variables: season

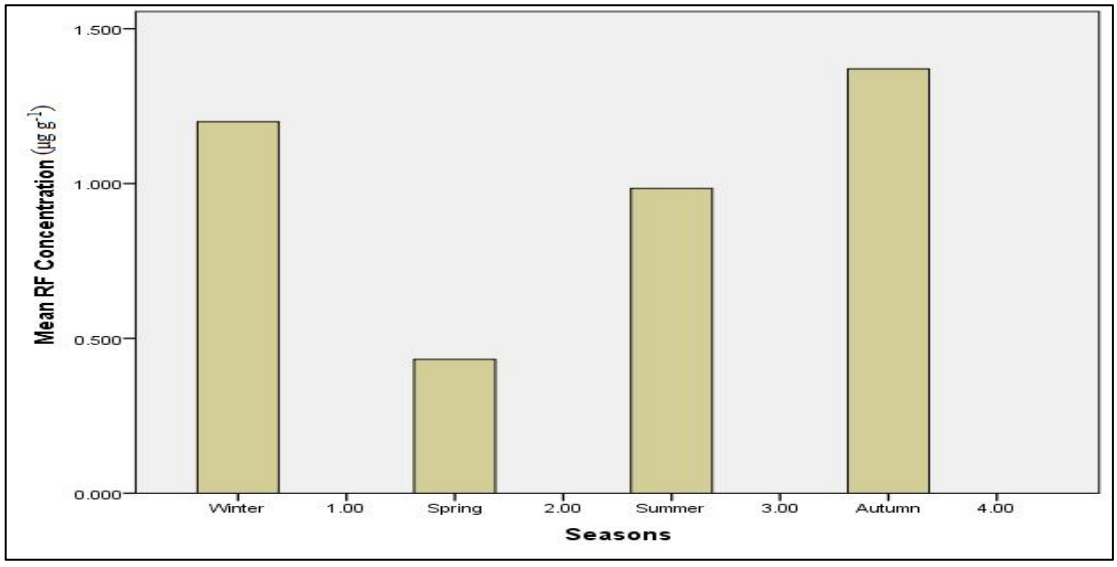

Figure 7. Effect of sampling season on RF concentration in honey

Table 8 shows that the mean riboflavin (RF) concentration in honey during autumn is 1.370 μg g^{-1}, serving as the reference season. Compared to autumn, RF concentration decreases by 0.171 μg g^{-1} in winter (not significant), 0.937 μg g^{-1} in spring (significant, $p = 0.048$), and 0.386 μg g^{-1} in summer (significant, $p = 0.044$). This indicates that riboflavin levels are highest in autumn and significantly lower in spring and summer.

Effect of bee Size on RF concentration in honey

To examine the effect of bee size on riboflavin (RF) concentration in honey, RF concentration was taken as the dependent variable, while bee size (small, medium, big) served as the independent variable. As the bee size is a categorical variable, dummy variables were used in the regression model. Two dummy variables, medium and big, were included, with small bee size treated as the reference category. The regression results are presented in table 9 and illustrated in figure 8

Table 9. Effect of bee size on RF concentration in honey

Model	Coefficients		t	P-value
	B	Std. Error		

(Constant)	1.176	0.275	4.277	0.001
Medium	-0.333	0.337	-0.990	0.033
Large	-0.263	0.550	-0.479	0.638

a. Dependent variable: RF Concentration (μg g^{-1})

b. Independent variable: Bee size

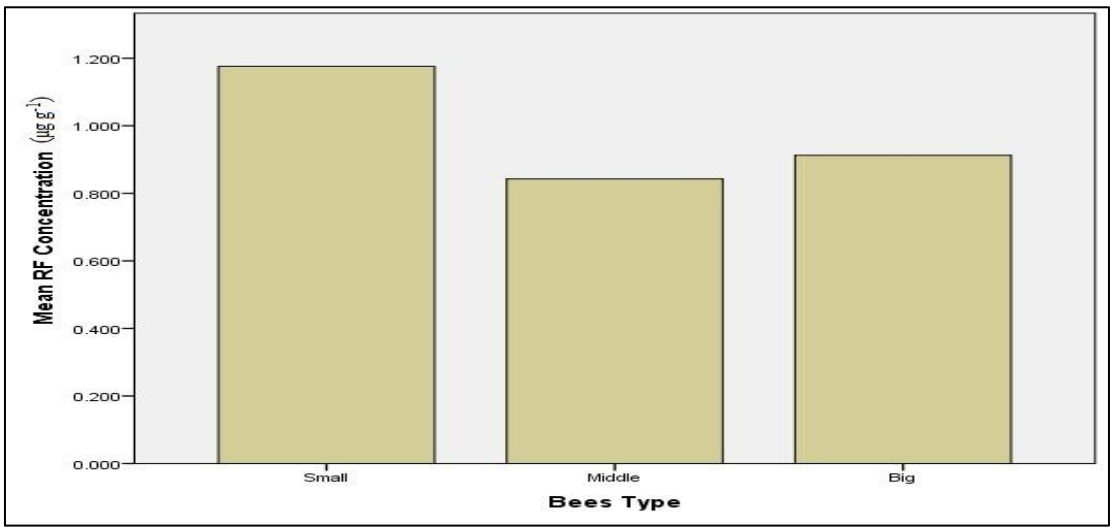

Figure 8. Effect of bee type on RF concentration in honey

Table 9 presents the regression analysis of RF concentration based on bee size. The results indicate that the average RF concentration in honey produced by small-sized bees is 1.176 μg g^{-1}, and this finding is statistically significant. The coefficient for medium-sized bees is -0.333, suggesting that RF concentration decreases by 0.333 μg g^{-1} compared to small-sized bees, with a statistically significant P-value of 0.033. In contrast, the coefficient for big-sized bees **is** -0.263, indicating a reduction of 0.263 μg g^{-1} relative to small-sized bees; however, this result is not statistically significant (P-value = 0.638**).**

Effect of flower type on RF concentration in honey

The effect of flower type on RF concentration in honey was studied, in which the dependent variable is RF concentration, whereas flower type (i.e., ziziphus, multifloral, brassica, and acacia flowers) is considered as the independent variable. Since flower type is a categorical variable, the concept of a dummy variable is used in regression. As there are four categories of flower type, therefore, 3 dummy variables, multifloral, brassica, and acacia are included in the regression, and the left-over category, i.e., ziziphus is taken as the base category. The results are given in table 10 and shown in figure 9.

Table 10. Effect of flower type on RF concentration in honey

Model	Coefficients		t value	Significance
	B	Std. Error		
(Constant)	1.242	0.219	5.663	0.000

Multifloral	-0.497	0.380	-1.307	0.021
Brassica	-1.052	0.490	-2.144	0.048
Acacia	-0.294	0.335	-0.876	0.394

a. Dependent Variable: RF Concentration
b. Independent variable: flower type

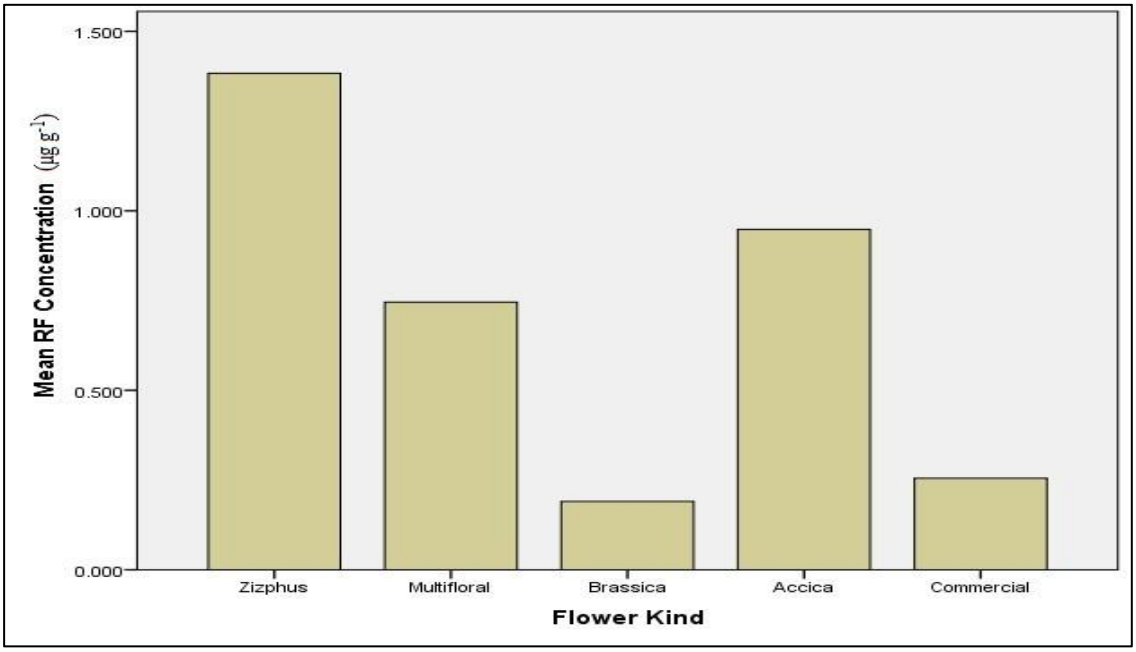

Figure 9. Effect of flower type on RF concentration in honey

Table 10 shows that honey from ziziphus flowers had the highest average RF concentration (1.242 µg g^{-1}), which was statistically significant. In comparison, multifloral and brassica honeys showed significantly lower RF concentrations by 0.497 µg g^{-1} and 1.052 µg g^{-1}, respectively. However, acacia honey showed a slight decrease of 0.294 µg g^{-1} in RF concentration compared to ziziphus honey, but this result was not statistically significant.

CONCLUSION

This study investigated honey samples collected from diverse geographical and climatic regions of Khyber Pakhtunkhwa to determine their riboflavin content. Since riboflavin is naturally fluorescent, its quantification was carried out using a spectrofluorimetric method. Riboflavin exhibits a characteristic fluorescence spectrum, showing maximum excitation at 464 nm and an emission peak at 525 nm. The study evaluated the influence of several external factors, including the altitude of the sampling site, type of honeybee, floral source of nectar, and season of collection, on riboflavin concentration. The results showed that honey obtained from lower-altitude regions contained a higher concentration of riboflavin,

averaging 1.156 ± 0.08 μg g^{-1}. Similarly, samples collected during the autumn season had the highest mean riboflavin content of 1.37 ± 0.06 μg g^{-1} compared to other seasons. The floral source also affected riboflavin concentration. Honey produced from nectar collected from *Ziziphus* flowers exhibited the highest riboflavin level, averaging 1.383 ± 0.1 μg g^{-1}. In addition, samples collected from hives of smaller honeybees showed a higher riboflavin concentration of 1.176 ± 0.07 μg g^{-1}. The findings indicate that riboflavin levels in honey are significantly influenced by environmental and biological factors. The study further suggests that other vitamins and nutritional constituents of honey may also vary depending on these external conditions.

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