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Detection and Quantification of Antibiotics Residues in Honey Samples by Chromatographic Techniques

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Abstract: In this work comparative evaluation of honey for the detection and quantification of antibiotics residues including tetracycline, streptomycin, gentamycin and penicillin. The detection of these residues were carried out by TLC method while the positive samples were quantified by an optimized HPLC method. A total of 100 samples were collected from market and categorized as branded and unbranded for comparative study. About 12.5% of branded sample and 19.96% unbranded samples were found positive. Tetracycline residue was found maximum in unbranded sample, while gentamycin was not detected in any tested sample by TLC method. From the quantification by HPLC the total streptomycin residue was determined 16.31µg/g in five positive unbranded sample while this residue was found to be minimum (3.6 µg/ml) in unbranded sample. Finally it was concluded that the unbranded honey had more contamination of antibiotic residues as compared with branded ones.

Key words: Honey · Antibiotic residues · TLC · HPLC

INTRODUCTION

Honey is a natural product having carbohydrates and other compounds. Carbohydrates are mainly in the form of fructose and glucose while also present in the form of maltose, sucrose and other complex carbohydrates are in traces [1]. Some vitamins are also reported in Honey samples [2] and several other compounds in honey are phenolic contents, vitamins, catalase, pinocembrin and pinobanksin chrysin, pinobanksin, vitamin C, catalase and pinocembrin. Trace amount of minerals was also found in honey. Honey having many nutritional and photochemical importance due to the presence of these compounds. Which shows antifungal, antibacterial and antioxidant activities. The quality of honey depends on the flowers variability and availability [3]. The use of Honey is spread all over the world and used as baking, in cooking, a spread on breads and also as an additive in various commercial beverages. Beside the usefulness of honey there are certain problems which when exist in honey, make them infected. The sources can be environmental and apicultural ones. [4, 5] in apicultural contaminations are aflatoxin, antibiotic residue and pesticides residue. Here our study specially focused on the detection and quantification of antibiotic residue. There are different diseases caused by bacterial and protozoan etc, European Foulbrood, American Foulbrood, which infect the honey bees, for curing these diseases the bee keepers mostly used different antibiotics. The antibiotics such as gentamycin, erythromycin, penicillin, tetracycline, streptomycin, ofloxacin and sulphonimides etc, are also reportedly used in bee keeping [6, 7]. These antibiotic residues have toxic acute and chronic effects on human health and also reduce the efficacy and quality of honey [8]. Actually half life of antibiotic residues are relatively long so that cause toxin infections in consumers. Different techniques were used for the detection and quantification of these antibiotics in honey. Mostly Biochip array Technology and Thin Layer Chromatography (TLC) was used for the detection [9] while other biological and Elisa method was also reported [10, 11].

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Spectrophotometric method was mostly used for the quantification purpose, while the latest research developed valid, simple and rapid method for antibiotic on HPLC, Mass spectrometry and LC/MS etc. [12, 13]. These techniques were found sensitive, reproducible, reliable and very useful for drug analysis because by the help of these techniques we can be able to quantify the presence of very low amount of drug in sample. So in our study the quantification of four major antibiotics residue including were carried out by High performance liquid chromatographic (HPLC) methods.

MATARIALS AND METHODS

Collection of Samples: A total of 100 samples were collected from market including forty samples were branded while other sixty samples unbranded and bring to the PCSIR Labs Complex Peshawar for the analysis of antibiotic residue.

Chemical and Reagents: Standard of tetracycline, penicillin, streptomycin and gentamycin were obtained from Sigma Chemical (Madrid, Spain), Methanol, CAN, o-phosphoric acid and Ethyl Acetate (Sigma Aldrich Germany). The chemicals used in TLC method were of analytical grade, while HPLC grade solvent were used for chromatographic separation. All the Solvents and deionized water were filtered through 0.45µm filter membrane and degassed for 20 minutes by ultra sonic cleaner.

Extraction Procedure for Detection on TLC: The antibiotic residues were extracted by the reported method [14]. Each 5g of sample was extracted with a mixture of ethyl acetate/water (80:20) by centrifugation at 3000rpm for 10 min and the supernatant was used for spotting on TLC plate for the detection of antibiotic residue.

Extraction Procedure for HPLC: The extraction of honey sample was subjected to a deproteinizing chemical procedure using ACN. A 2 g of honey sample was placed into a 10mL test tube and shaken intensively with 3mL ACN for 1 min. The mixture was centrifuged for 15 min at 5000 rpm. The supernatant was collected and dried under Nitrogen stream at 40°C. The residue was re-dissolved in methanol, filter through 0.45µm filter membrane and injected 10 µl to HPLC system.

TLC Analysis of Antibiotic: The spot of each sample with targeted antibiotic were loaded standard on TLC plate with the help of micro syringes by automatic TLC spotter [15]. Then developed in methanol/water (95:5) and the detection was carried out by comparing the Rf value of sample with that of standard under UV light of 254nm.

HPLC Analysis of Antibiotics: The determination of antibiotic residue in honey samples were carried out according to a described procedure [16]. A Hitachi (D-2000 Elite system manager) with a dual pump (L-2130), auto sampler L-2200 and UV-Visible detector L-2420 was used for the quantification of targeted antibiotic residue, in which the separation was achieved using Column oven L-2300 and column Intersil ODS-3 C18 (GL Sciences Inc. Tokyo Japan 5µm, 250×4.6 mm). All solvents were filtered through 0.45 µm sartolon polymide membrane by filtration assembly of (Rocker-300 Model Taiwan) and degassed by ultrasonic cleaner Ceia (Model CP-104 Italy). The determination of these compounds were performed using different mixture of an aqueous mobile phase (A) Acidified water and organic mobile phase (B) methanol/ACN with a flow rate of 1 ml/min. Streptomycin, tetracycline and the other two antibiotic residue were quantified by a modified method [17, 18]. The compounds were detected at 210-240 nm. The quantification was achieved by comparison of the peak area of the sample with that of the external standard. The identical chromatogram was quantified by the peak area of sample with that of standard in same retention time.

RESULTS

The branded and unbranded honey samples were evaluated for the presence of streptomycin, tetracycline, penicillin and gentamycin antibiotic residues. These antibiotic residues were detected by TLC method and the results are tabulated in (Table 1) which shows that a total number of 5 samples out of 30 branded and 9 samples out of 49 unbranded were found to be positive. The contamination of tetracycline was maximum in unbranded sample, which was about 8.3%, streptomycin and penicillin were 6.66 and 5%, while in branded 5, 6 and 1% of samples were contaminated by penicillin, streptomycin and tetracycline. However, gentamycin has not detected in any sample. The positive samples were proceeds for quantification by HPLC. The retention time of tetracycline was 5.63, 2.60 for streptomycin

Table 1: Detection of antibiotic residues by TLC method.

Samples	No of samples	Penicillin G	Streptomycin	Gentamycin	tetracycline	Total
Branded	40	2* (5.0%)	2 (2.5%)	0(0%)	2 (5.0%)	6(12.5%)
Unbranded	60	3 (5.0%)	4(6.66%)	0 (0%)	5 (8.3%)	12(20%)
Total	100	5(5.0%)	6(6.0%)	0 (0%)	7 (7.0%)	18(18%)

* Positive sample

Table 2: Quantification of antibiotic residues from Peak Area of HPLC

Samples	Branded/unbranded	S1 µg/ml	S2 µg/ml	S3 µg/ml	S4 µg/ml	S5 µg/ml	TCS µg/ml
Penicillin G	Branded	1.42	3.12	---	---	---	4.54
	Unbranded	1.76	3.43	4.86	---	---	10.05
Streptomycin	Branded	1.42	---	---	---	---	1.42
	Unbranded	6.65	2.04	1.12	2.21	---	12.02
Gentamycin	Branded	---	---	---	---	---	---
	Unbranded	---	---	---	---	---	---
Oxytetracyclin	Branded	2.13	1.54	---	---	---	3.67
	Unbranded	1.12	2.11	3.32	6.42	2.34	16.31

S = Positive Sample --- = Not Detected

and 10.96 for gentamycin, which was identified by previous literature and quantified by external standards. The quantification of these positive samples was carried out by comparing the peak area of sample with that of targeted standard. The maximum contamination of tetracycline was calculated 16.31µg/g from total five positive unbranded samples (Table 2). Streptomycin residue was 12.02µg/g in total four positive samples, while the contamination of these residues in total positive branded sample was recorded 10.5, 4.54 and 3.67µg/g for streptomycin, penicillin and tetracycline respectively.

DISCUSSION

The major problem which persists in honey is the occurrence of antibiotic residues which is present due to broad use of antibiotics for the treatment of different disease. In this study we focused on the detection of those major antibiotic residues i.e. tetracycline, penicillin, streptomycin and neomycin which are mostly used by the bee keepers for the treatment of different diseases. The detection of these antibiotic residues was carried out by thin layer chromatography [19], while the quantification of these compounds was done by HPLC method [20]. There are different other peak in each HPLC chromatograms of honey sample having tetracycline, penicillin and streptomycin, which is not concern with our research, so not identify (Fig. 1-3). About 50 honey samples were screened for antibiotic residue, in which 3% contaminated by tetracycline, 4% by penicillin, 3% by streptomycin, while gentamycin and neomycin was not found in any sample. It has been also reported that the residue of streptomycin was found in 4 out of 248, tetracycline in 2 out of 72, sulfonamides in 3 out of 72 samples. No residues of lactam antibiotics and chloramphenicol were found. While in imported honey samples streptomycin was detected in 51 out of 102 samples, tetracycline in 29 out of 98 samples,

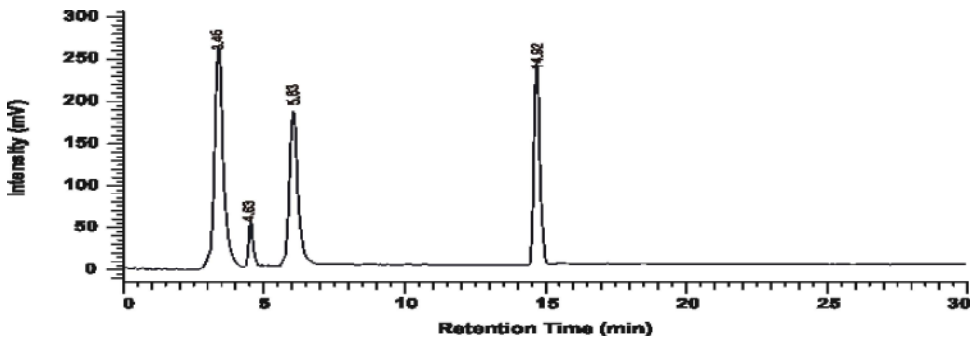

Fig. 1: HPLC Chromatogram of Honey sample: 5.63 Tetracycline Residue, other peaks at 3.45, 4.63, 14.92 not identified

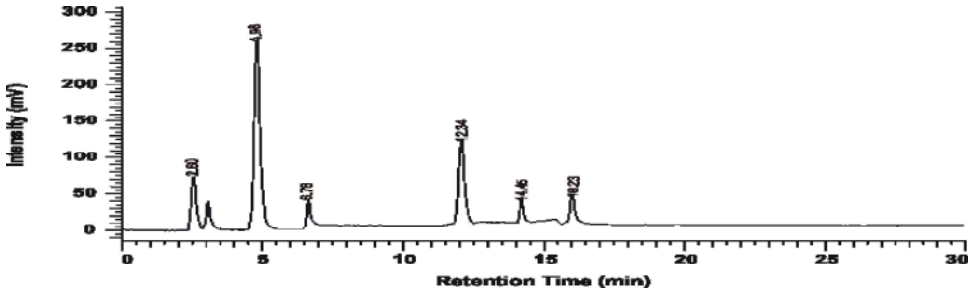

Fig. 2: HPLC Chromatogram of Honey sample: 2.60 Streptomycin Residue, other peaks at 4.98, 6.78, 12.34, 14.45 and 16.23 not identified.

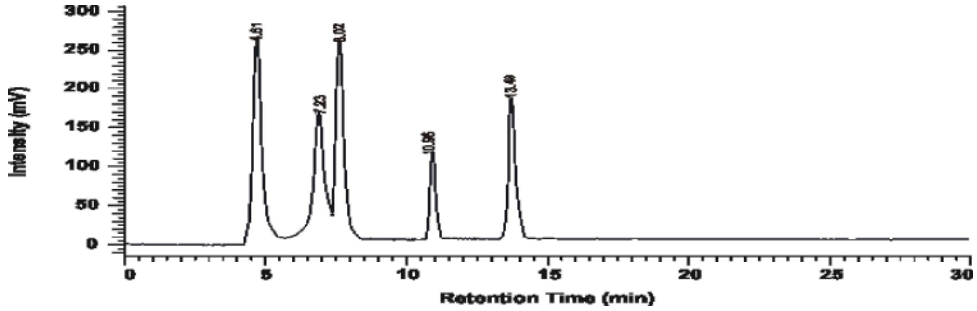

Fig. 3: HPLC Chromatogram of Honey sample: 10.96 Gentamycin Residue, other peaks at 4.61, 7.23, 8.02, 13.49 not identified.

sulfonamides in 31 out of 98 samples, chloramphenicol 40 out of 85 samples [21, 22]. Another study by Vidal [23], in which 251 honey samples were analyzed. 19% of the samples have found to be contaminated by the residue of tetracycline while the other antibiotic residue was found in trace amount namely streptomycin, sulfonamides and ciprofloxacin. It was concluded from our study that streptomycin and tetracycline were extensively used by the bee keeper for curing the diseases in bees.

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