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Detection of Oxytetracycline Residues in Honey Samples Using ELISA and HPLC Methods

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ABSTRACT

Background: Honey is being used as an ingredient in many foods, pharmaceuticals and cosmetics than ever before, so honey testing has become essential to maintaining its healthful characteristics and protecting public health. This study was designed to investigate the occurrence of oxytetracycline residue in honey samples. A number of 145 honey samples were collected from Ardabil provinces (Northwest region of Iran). ELISA and HPLC were used to qualify and quantify the contamination of the honey samples with oxytetracycline. The ELISA assay showed that out of 145 samples, 34 samples were positive for oxytetracycline residue. ELISA analyses demonstrated that the minimum and maximum level of oxytetracycline residue was 5.32 and 369.1ng/g respectively. HPLC analyses confirmed the ELISA findings, although the level of oxytetracycline which detected in honey samples using HPLC method was remarkably ($P<0.05$) lower than that of which detected by ELISA. Considering the relatively high contamination level of foods of animal origin with oxytetracycline and their high levels of consumption, it is likely that consumers experience a high risk of exposure to drug residues, especially through honey bees.

Introduction

Antibiotics are a vital component of treatment and elimination of disease in human, animals and plants. Antibiotic residues originating from agricultural use should be carefully monitored as they can adversely impact public health due to allergenic and carcinogenic factors, and may contribute to bacterial resistance.[1, 2] Honey is known as a food which is both natural and healthy. Annual world honey production is estimated at about 1.4 million tones. Asia is the largest producer of honey, accounting for about 40% of the global production.[3,4] Honey is one of many foods that are monitored for antibiotic residues worldwide. Honey producers, importers, exporters and regulators need simple, fast and effective ways to test honey for antibiotics, ensuring compliance with the maximum residue limits (MRLs) and minimum required performance limits (MRPLs) established for relevant countries.[5,6]

In 1990, the Commission of the European Union laid down the procedure for establishing maximum residue limits (MRLs) of veterinary drugs in foodstuffs of animal origin (EEC-Regulation 2377/90 and amendments). However, no MRLs have been fixed for bee products.[5] While some countries did not fix any MRL for oxytetracycline in honey, some others fixed some limits in order to make the situation clearer. Maximum residual limits (MRLs) of oxytetracycline in animal-derived foods according to the WHO and EU is shown in Table 1.[7,8]

Chloramphenicol, macrolides, tetracycline, sulfonamide, streptomycin, and nitrofuran residues have been commonly found in honey.[5,9] Antibiotic contamination in honey can be a result of improper treatment of hives to combat various diseases such as American foulbrood (AFB), European foulbrood (EFB) and nosemosis, a parasitic disease affecting adult bees. Antibiotics can also enter the honey supply as a result of antibiotics sprayed on fruit trees for the treatment of fire blight.[6]

Concerns about food safety especially foods with animal origin are increasing in developing countries where urbanization, increasing incomes and changing of life-styles are associated with greater dependence on marketed foods by an increasing number of people.[2]

During the last decade, different microbiological and chemical methods have been developed and validated for oxytetracycline and other antibiotics detection and quantitation. The microbiological assay is traditionally used for detecting antibiotic residues in food products.[10] There are two types of analytical methods including screening methods, normally utilizing immunoassay, and confirmatory methods performed

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based on gas-chromatography or liquid chromatography.[11,12] Moreover, an alternative method of corona discharge ion mobility spectrometry has also been introduced for determining the residues of veterinary drugs in chicken meat.[13]

The floral biodiversity of Iran is of great importance for honey production. Although Ardabil province has a small geographic area, it has rich biodiversity because of its geophysical characteristics, climate and flora.

Currently, there are no exact data about the amount of antibiotic residue in produced honey in Iran and also because of the lack of major quality assurance programs in the country, protecting public health against the adverse effects of antibiotics used in animal husbandry, is not possible.

This study was conducted aiming to evaluate the oxytetracycline residue in produced honey in Ardabil province (Northwest region of Iran), compared to international standards available in this field.

Table 1. MRLs of oxytetracycline in animal-derived foods

Antibiotic	Target tissue	MRLs (µg/kg)
Oxytetracycline	Muscle	200*
	Liver	600*
	Kidney	1200*
	Milk	100*
	Eggs	400*
	Honey	25**

* According to the WHO
** According to the EU

Material and Methods

Honey samples

A total number of 145 fresh honey samples (weighing 50g each) were obtained from beekeepers from different regions of Ardabil province from January, 2010 to November, 2011. Honey samples were transferred to food chemistry laboratory of the faculty of veterinary medicine, University of Tabriz under appropriate conditions and were analyzed immediately after preparations.

Analysis of oxytetracycline residue

ELISA assay

The ELISA technique was performed according to manufacturer's instructions [RIDASCREEN Oxytetracycline ELISA kit (R 1501), r-biopharm, Germany]. Briefly, five grams of each honey sample was agitated using vortex-mixer with 50 ml of phosphate buffered saline (PBS). This extract was subsequently centrifuged at 3290 g for 30 min at room temperature in order to eliminating pollen and other impurities. According to the procedure described by manufacturer, 50 µl of considered antibiotic standards was prepared in PBS and the extracts of honey were added to the bottom of each well in duplicate. At the last step, in the presence of considered antibiotics, the addition of the stop reagent (which contains 1 M sulphuric acid) led to a colour change from blue to yellow. The measurement was made by photometry at 450 nm (Multiskan plus MK2 spectrophotometer from Elvetec Service, France). Limit of detection (LOD) and limit of quantification (LOQ) were 0.02 ng/g and 0.05 ng/g; respectively. The recovery rate was >80% for all samples.

HPLC analysis of honey samples

Sample preparation

To confirm and determine the accurate level of oxytetracycline contamination in ELISA positive samples, the HPLC measurement was conducted according to previously described method with minor modification.[13] Briefly, 10 g honey sample was weighted and transferred into a 50 ml falcon tube. Then 20 ml PBS (pH 7.2) was added and samples were divided into small pieces, followed by homogenization using Ultra Turrax T25. The extraction procedure was followed by addition of 20 ml PBS, vortexing for 2 min and sonicating for 15 min. The tubes were centrifuged at 1400g for 10 min and the supernatants were transferred to fresh tubes and 3 ml trichloroacetic acid (15%) was added to each tube. Following 2 min vortexing the samples were centrifuged at 1400g for 10 min and the supernatant was subjected to solid phase extraction.

Solid phase extraction (SPE)

To perform the SPE, prior to extraction, the cartridge C18 (3 ml, 5 mg, J.T. Baker, The Netherlands) was conditioned with distilled water and methanol subsequently. The samples from chemical extraction were passed through the cartridge. The columns were washed with distilled water and dried under N2 stream. Ultimately, the bound compound to the cartridge was eluted with methanol. The eluted samples were dried under gentle stream of N2 and dissolved in mobile phase for HPLC analyses.

HPLC

oxytetracycline level in extracted samples was determined using HPLC, according to previously described method.[15] The chromatographic system consisted of an auto sampler (Autosampler Triathlon type 900, Germany) and dual pumps (Wellchrom HPLC pump, K-1001, KNAVER Germany). Twenty microliters of the extracted sample was injected in to an ODS C18 (250 _ 4.60 mm, 5 lm, Phenomenex) column. The mobile phase consisted of a mixture of water–methanol (60:40, v/v) eluted at a flow-rate of 1.0 ml/min. OXYTETRACYCLINE was detected by the means of an UV detector (RF-10AXL KNAUER, Germany), set at wavelength of 276 nm. oxytetracycline levels were quantified by measuring the areas under the peaks and comparing them to the relevant peaks generated by oxytetracycline in HPLC method using an external standard. Figure 1 shows the

chromatogram of mixed standard solution of oxytetracycline (100 ng/g).

The limit of detection (LOD) for tetracycline was established by determining the signal to noise ratio at 3 and was found 2.5ng/g. To obtain calibration curve for oxytetracycline, different concentration of external standard ranging from 0 to 100 ng/ml (r^2 = 0.9992) were used. The mean recovery and RSD for each sample were obtained with three times spiking of 5 ng/g.

HPLC chromatogram of one honey sample containing oxytetracycline residues is shown in Figure 2.

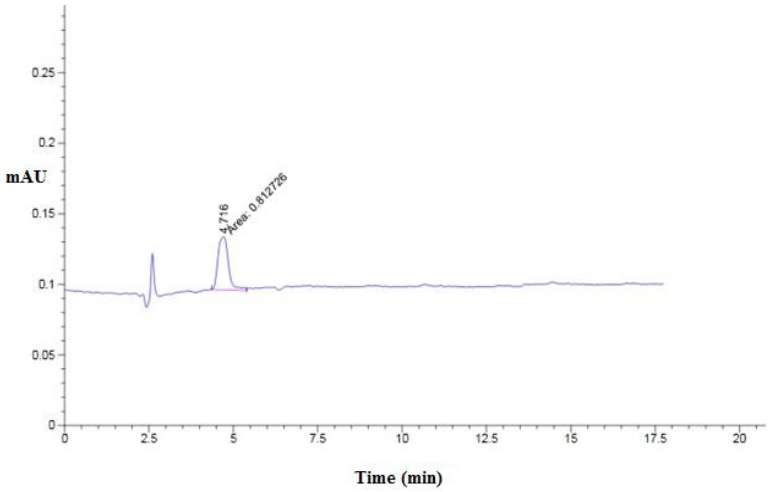

Figure 1. The chromatogram of mixed standard solution of oxytetracycline (100 ng/g).

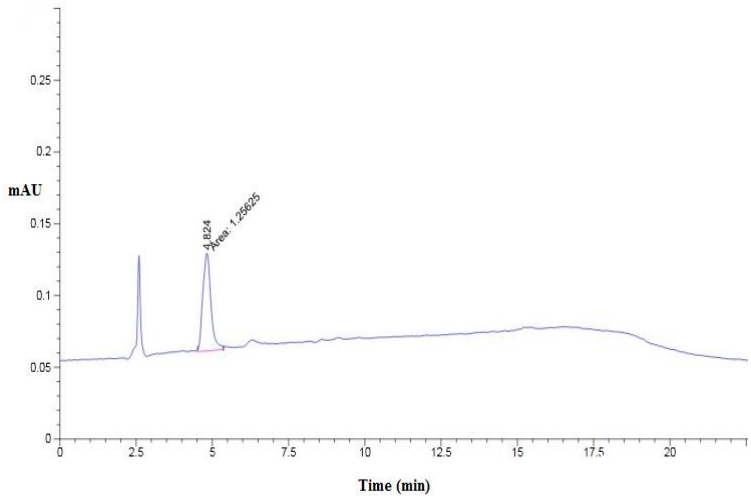

Figure 2. The HPLC chromatogram of one honey sample containing oxytetracycline residues.

Statistical analysis

The Student t-test was used to analyze the difference between samples subjected to both ELISA and HPLC. Statistical differences at $P<0.05$ were considered significant.

Results

ELISA assay

ELISA method showed that 34 (23.44%) out of 145 honey samples were positive for oxytetracycline residue (Table2). The levels of contamination of honey samples with oxytetracycline are shown in Figure 3. Minimum and maximum detected level of oxytetracycline was 5.3 and 369.1 ng/g in examined honey samples.

Discussion

Recently the occurrence of antibiotic residues in honey due to broad use of antibiotics for the treatment of different diseases is a major concern for public health. This study reports for the first time the residue of oxytetracycline (which is mostly used by the bee keepers for the treatment of different diseases) in honey samples in Northwest region of Iran using both ELISA and HPLC methods.

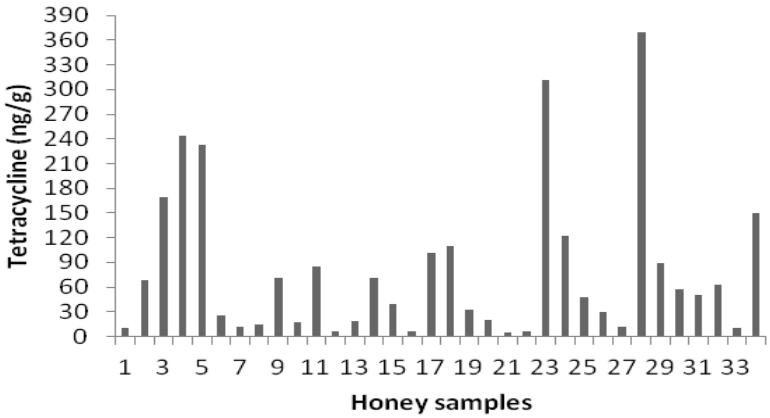

Figure 3. oxytetracycline concentrations in the honey samples analyzed by ELISA assay (n = 34).

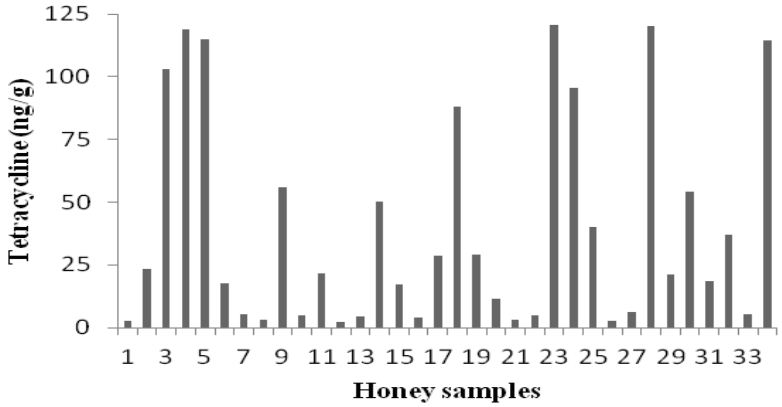

Figure 4. oxytetracycline concentrations in the honey samples analyzed by HPLC method (n = 34).

Table 2. Oxytetracycline residue in honey samples: Comparison between two analysis methods.

			Concentration (ng/g)		
Type of analytical methods	Sample size(n)	Positive samples (%)	Mean± SEM	Min–Max	Exceed legal limit (%)(EU*)
ELYSA	145	34(23.44)	56.39±8.50	5.32-369.10	22(64.70)
HPLC	145	34(23.44)	39.73±7.27	2.10-120.60	8(23.52)

*The European Agency for the Evaluation of Medicinal Products, MRL for OXYTETRACYCLINE in honey is 25 ng/g.
Means ± SEM in the column with different letters are significantly different (P < 0.05).

There are several international reports of antibiotic residues in honey samples, some of them are mentioned below:

Reybroeck (2003) reported evidence of many antibiotic residues specially streptomycin, tetracycline, sulfonamides, B-lactam antibiotics and chloramphenicol in considerably high amounts in honey samples, in the period 2000-2001.[16]

In Switzerland a number of 75 samples of honey which obtained commercially were analysed. A number of 13 samples (17%) out of 34 samples which were from Asian countries, contained chloramphenicol residues. Concentration of chloramphenicol measured in honey between 0.4 and 6.0 µgkg-1, with six samples containing approximately 0.8–0.9 µgKg-1 (just below the Swiss limit) and two containing approximately 5 µgkg-1.[17]

In a study in which 251 honey samples collected across Greece were analysed by liquid chromatography to detect tetracycline-derived residues, 29% of the samples had tetracycline residues. Majority of samples contained residues from 0.018 to 0.055 mg/kg of honey while some others had residues higher than 0.100 mg/kg.[18]

In another study aimed to assess oxytetracycline residue levels in honey after treatment of honeybee colonies using two methods of oxytetracycline application (in liquid sucrose and in powdered icing sugar), the samples of honey were extracted up to 12 weeks after treatment and following metal chelation and analysed by HPLC showed that the application of oxytetracycline in liquid form results in very high residue levels in honey with residues of 3.7 mg/kg, eight weeks after oxytetracycline application.[19]

Nectar and honey samples collected from bee hives during the peak flowering seasons of rubber (March to April) and banana (December to January) plantation crops in southern part of Tamil Nadu were analysed for antibiotic residues. Nectar and honey samples showed 4-17 and 11-29 µg/kg of streptomycin, 2-29 and 3-44 µg/kg of ampicillin and 17-34 and 26-48 µg/kg of kanamycin respectively.[20]

In another study by Vidal et al. (2009), in which 251 honey samples was analyzed. 19% of the samples have found to be contaminated by the residue of tetracycline while the other antibiotic including streptomycin, sulfonamides and ciprofloxacin residue was in trace amount.[21]

In our previous study, enrofloxacin, penicilin, chloramphenicol, gentamicin, tylosin, tetracycline and sulfonamide residues in honey samples (taken from Alamot region of Qazvin province, Iran) were investigated by ELISA analysis method. Results showed that the range of antibiotic radiuses value was 0.0-72.1 ng/g, among them enrofloxacin was detected in 20.7% of examined honey samples. The highest and lowest mean contamination (ng/g) belonged to enrofloxacin (10.8±1.6) followed by Penicillin (4.4±2.9) and chloramphenicol (0.1±0.1). The highest percentage of honey samples (71.85%) contaminated with antibiotic residues belonged to samples which have been collected in autumn.[22]

The present study showed that honey samples collected during 2010-2011 contained varying amounts of oxytetracycline residue (2.1-120.6 ng/g). It is clear from the results that 34 out of 145 examined honey samples were contaminated with oxytetracycline.

oxytetracycline Contamination of honey samples and its values in the present study are considerable compared with other country honey samples; the results of this comparison are shown in Table 3.

Table 3. Comparison of oxytetracycline residue in honey samples between the present study and other countries

Country	Author, Year	Analysis methods	contamination Percent	range
India (produced with country)	Reybroeck, 2003	HPLC	2.77	-
India (imported)	Reybroeck, 2003	HPLC	29.58	-
Greece	Saridaki-Papakonstadinou et al., 2006	HPLC	29	0.018-0.055 mg/kg
Switzerland	2007	HPLC	1.7	5 – 2076 µg/kg
Turkey	Gunes et al., 2009	HPLC	-	-
Pakistan	Muhammad Zai et al., 2013	HPLC	7	1.12-2.13 µg/kg
Iran (Current study)	Mahmoudi et al., 2012	ELYZA, HPLC	23.44	2.10-120.60 µg/kg

One of the interesting findings of this study was significant differences between the detected level of oxytetracycline residue by ELISA and HPLC methods. Interestingly, the level of oxytetracycline which detected with HPLC method was remarkably lower than that of ELISA. HPLC assays are now the preferred method for analysis of many substances of biological interest. Such method has good sensitivity and simultaneous assay of various tetracyclines in honey[23] as well as in other samples, such as milk and meat.

In HPLC method possible cross reaction between oxytetracycline itself and its metabolites maybe occurs while ELISA method is able to detect cross reactions.[24] The results indicate the presence of oxytetracycline residues in honey samples produced from Ardabil province. It was concluded from our study that oxytetracycline were extensively used by beekeepers for treating diseases in bees, also some honey samples contained more intentioned drug residues than MRLs. These antibiotic residues may result drug resistance, digestive and allergic effects in consumers. It would also changes organoleptic specifications in some honey samples.

Conclusion

The present study showed that honey samples collected from Northwest region of Iran contained different amounts of oxytetracycline residues. Therefore, the honey samples did not have desired conditions because of presence of oxytetracycline residues more than

Maximum Residue Limits (MRLs). Other studies are necessary to evaluate other drug residues in honey samples and to evaluate the hazards of these residues in relation with daily intakes and other related factors.

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References

1. Wassenaa TM. The use of antimicrobial agents in veterinary medicine and implications for human health. *Crit Rev Microbiol*. 2005;31:155-169.
2. Baggio A, Gallina A, Benetti C, Dainese N, Manzinello C, Piro R. Official control on imported honey in north-easter Italy. *Apiacta.* 2004;39:5-7.
3. Food and Agriculture Organization (FAO), 20005; Retrieved from: http://faostat.fao.org/site/570/default.aspxr.
4. Ferguson J, Baxter A, Young P, Kennedy G, Elliott C, Weigel S, Gatermann R, Ashwin H, Stead S, sharman M. Detection of chloramphenicol and chloramphenicol glucuronide residues in poultry muscle, honey, prawn and milk using a surface plasmon resonance biosensor and Qflex_ kit chloramphenicol. *Anal Chim Acta.* 2005; 529: 109-113.
5. Hammel YV, Mohamed R, Gremaud E, LeBreton MH, Guy PA. Multi-screening approach to monitor and quantify 42 antibiotic residues in honey by liquid chromatography–tandem mass spectrometry. *J Chromatogr A*. 2008;1177:58-76.
6. Henzelin AB, Perroud MC, Le Breton M H, Hammel YA, Germain I, Bebius A. Contaminants and Residues in Food: Strategies (if any) to Screen and Analyse Veterinary Drug Residues in Food from Animal Origin", 5th International Fresenius Conference 2007, http://www.biocop.org/content_pdf/Contaminants__Residues_in_Food_5th_Fresenuispp t.pdf.
7. WHO. Joint FAO/WHO Expert Committee on Food Additives (JECFA). Evaluation of certain veterinary drug residues in food (Fiftieth report of the Joint FAO/WHO Expert Committee on Food Additives). *WHO Tech Rep Ser*, No. 888,1999.
8. The European Agency for the Evaluation of Medicinal Products, EMEA/MRL/ 023/95. http://www.emea.eu.int/pdfs/vet/mrls/Tetracyclines(3)02395.pdf. 2003.
9. Kilinc B, Cakli S. Screening for antibiotic residues in the trout by the four plate test, Premi test and ELISA test. Eur. *Food Res Technol*. 2008;226:795-799.
10. Gaudin V, Maris P. Development of a biosensor-based immunoassay for screening of chloramphenicol residues in milk. *Food Agric Immunol.* 2001;13:77-86.
11. Jafari MT, Khayamian T, Shaer V, Zarei N. Determination of veterinary drug residues in chicken meat using corona discharge ion mobility spectrometry. *Anal Chim Acta*. 2007;581:147-153.
12. Myllyniemi AL, Rannikko R, Lindfors E, Miemi A, Backman C. Microbiological and chemical detection of incurred penicilline G, oxytetracycline, enrofloxacin, and ciprofloxacin in bovine and porcine tissues. *Food Addit Contam*. 2000;17:991-1000.
13. Okerman L, Van Hoof J, Debeuckelaere W. Evaluation of the European four-plate test as a tool for screening antibiotic residues in meat samples from retail outlets. *J AOAC Int.* 1998;81:51-56.
14. Lee MH, Lee H, Ryo PD. Public health risks: chemical and antibiotic residuesdreview. Asian-Australasian *J Animal Sci*. 2000;14:402-413.
15. Mesgari Abbasi M, Nemati M, Babaei H, Ansarin M, Nourdadgar AOS. Solid-Phase Extraction and Simultaneous Determination of Tetracycline Residues in Edible Cattle Tissues Using an HPLC-FL Method. *Iranian J Pharm Res*. 2012;11:781-787. Reybroeck W. Residues of antibiotics and sulfonamides in honey on the Belgian market. *Apiacta*, 2003;38:23-30.
16. Ortelli D, Edder P, Corvi C. Analysis of chloramphenicol residues in honey by liquid chromatography–tandem mass spectrometry. *Chromatographia.* 2004;59:61-64.
17. Saridaki-Papakonstadinou M, Andredakis S, Burriel A, Tsachev I. Determination of tetracycline residues in Greek honey. *Trakia J Sci*. 2006;4:33-36.
18. Thompson HM, Waite RJ, Wilkins S, Brown MA, Bigwood T, Shaw M, Ridgway C, Sharman M. Effects of European foulbrood treatment regime on oxytetracycline levels in honey extracted from treated honeybee (*Apis mellifera*) colonies and toxicity to brood, *Food Add Cont Part A*. 2005;22: 573-578.
19. Solomon RDJ, Satheeja SV, Vimalan J. Prevalence of antibiotics in nectar and honey in South Tamil Nadu, India. *Integ Biosci.* 2006;10:163-167.
20. Vidal JL, Luiz MA, Gonzalez R. Antibiotic, pesticide and microbial contaminants of honey: Human Health Hazards. *J Agric Food Chem*. 2009; 57:1760-1767.
21. Mahmoudi R, Norian R, Pajohi MR. Antibiotic Residues in Iranian 1 Honey by ELISA. *Int J Food Properties*. 2014. In press.
22. Kochansky J. Analysis of oxytetracycline in extender patties. *Apidologie*. 2000;31:517-524.
23. Lopez MI, Feldlaufer MF, Williams AD, Chu PS. Determination and Confirmation of Nitrofuran Residues in Honey Using LC-MS/MS, *J Agri Food Chem*. 2007;55:1103-1108.