Development of a method for determination of pesticide residues in honey using liquid chromatography tandem mass spectrometry

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Master Thesis

Institutionen för Livsmedelsvetenskap Publikation nr 335

Swedish University of Agricultural Sciences Uppsala 2011
Department of Food Science

Title:
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Key words: pesticides residues, honey, liquid chromatography, tandem mass spectrometry

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Course code:
EX 0425

“Education Level”:
Advanced a2e

Examiner:
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Place of publication:
Uppsala

Year of publication:
2011

Abstract

Honey is a product that may contain pesticide residues due to contamination from bees pollinating various plants as well as elimination of vermin inside the hive. Different methods are needed for analysis of pesticides, since the term includes a wide range of different substances. National Food Administration lacks a validated method for determination and quantification of pesticides in honey using liquid chromatography tandem mass spectrometry, LC-MS/MS. The technique is especially important in analysis of polar and non-volatile pesticides, where gas chromatography is not functioning optimally. After investigating what pesticides could be found in honey produced in Sweden, a number of four pesticides suitable for analysis with LC-MS/MS was compiled; boscalid, impidacloprid, tau-fluvalinate and thiacloprid. A validated multi method for pesticide analysis in fruit and vegetables using LC-MS/MS was utilized as reference method, as a new method was developed. Some of the alterations in the new method included addition of water, changed order of addition of salts, centrifuge settings and shakings of samples. Performing validation experiments at three levels; 0.01 µg/g, 0.02 µg/g and 0.1 µg/g, yielded recovery in the range from 69.4% to 91.8% with relative standard deviation <19%. These figures met, with one exception, the requirements for validation of new methods at National Food Administration and also held linear correlation coefficients higher than 0.97. Three labels of honey, with different origins, were used in the experiments. One of the honey labels formed emulsion during extraction. Absence of a separate ethyl acetate phase caused difficulties when creating a suitable extract for determination and quantification by LC-MS/MS. A special extraction procedure was developed for that specific honey label enabling extraction. As successful extraction procedures were developed and recovery tests yielded satisfying results, the aim to develop an analysis method for determination of pesticide residues in honey using liquid chromatography tandem mass spectrometry was fulfilled.

Keywords: pesticide residues, honey, liquid chromatography, tandem mass spectrometry

Sammanfattning

Honung är en produkt som kan innehålla pesticidrester på grund av kontamination av bin när de pollinerar olika växter och även från skadedjursbekämpning inne i bikupan. Olika tekniker behövs för analys av pesticidrester eftersom begreppet inkluderar en bred grupp av olika substanser. Livsmedelsverket saknar en validerad metod för bestämning och kvantifiering av pesticider i honung med hjälp av LC-MS/MS. Tekniken är speciellt viktig för analys av polära och icke-volatila pesticider, då analys med gaskromatografi inte är optimalt för dessa pesticider. Efter undersökning av vilka pesticider som kan påträffas i honung producerad i Sverige, sammanställdes en lista med fyra pesticider som kan analyseras av LC-MS/MS; boskalid, imidakloprid, tau-fluvalinat och tiakloprid. En validerad multimetod för pesticidanalys i frukt och grönsaker med användning med LC-MS/MS användes som referensmetod när en ny metod utvecklades. Några av ändringarna i den nya metoden inkluderade tillsats av vatten, ny ordningsföljd vid tillsats av salter, centrifuginställningar och skakningar av prover. Valideringsexperimenten genomfördes på tre nivåer; 0,01µg/g, 0,02 µg/g och 0,1 µg/g, vilket gav återvinning mellan 69,4% och 91,8% med en relativ standardavvikelse <19%. Dessa värden uppfyllde, med ett undantag, Livsmedelsverkets krav för validering av nya metoder och inkluderade även en linjäritetskoefficient högre 0,97. Tre sorters honung användes i experimenten. I en honungssort bildades emulsion under extraktionen. Avsaknad av separat etylacetat-fas genererade svårigheter att skapa ett lämpligt extrakt för bestämning och kvantifiering med LC-MS/MS. En speciell extraktionsprocedur utvecklades för denna honungssort vilket möjliggjorde extraktion. Då lyckade extraktionsprocedurer skapades och återvinningsförsöken genererade tillfredsställande resultat, uppnåddes målet att utveckla en analysmetod för bestämning av pesticidrester i honung med användning av LC-MS/MS.

Nyckelord: pesticidrester, honung, LC-MS/MS

Development of a method for determination of pesticide residues in honey using liquid chromatography tandem mass spectrometry- a simplified description

Honey is a popular product worldwide and it holds a consumption figure of 0.7 kilo per person and year in Sweden. To ensure health safety for consumers, monitoring of pesticide residue levels needs to be performed. The pesticides can either derive from various plants the bees have pollinated, but the derivation can also be elimination of vermin inside the hive. National Food Administration, responsible for the pesticide controls in Sweden, lacked a method for pesticide residue monitoring in honey using the technique LC-MS/MS. The aim of the study was therefore to develop a method for this purpose and acquire data for initiation validation through recovery experiments.
Three different labels of honey were used in the recovery experiments. When recovery experiments were performed on one of the honey labels used, it acted differently during the extraction process, resulting in difficulties performing the analysis. This problem was solved as an alternative extraction process was altered for this specific honey label. The aim of the study was achieved as a method for pesticide analysis in honey was developed and recovery experiments yielded satisfactory data for validation.

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Background

In a society utterly conscious about health, discussion has risen about pesticide residues in food and the impact on our health. A recent investigation found illegal pesticides in seven out of eight orange juices that could be bought in Sweden (Testfakta, 2010). This illustrates the significance of a well functioning control system of pesticide content in foods. By order of National Food Administration, the aim for this thesis was to develop and validate a method for analysis of pesticides in honey by LC-MS/MS.

Pesticides

Pesticides are a central concept in the area of food safety. They are substances or combination of substances aiming to avoid, moderate or eliminate any pest. The definition of pest also includes insects, fungi, weeds, different animals and prions (United States Environmental Protection Agency, EPA 2010).

To state the amount of pesticide residues allowed in a food, “Maximum Residue Limit”, MRL, is used. MRL is often given in mg/kg and is determined by field trials combined with toxicological risk evaluations. The field trials are performed according to “Good Agricultural Practice”, GAP, which supply guidelines for the trials as well as the assessment of the results. The minimum level of pesticide residues that can be determined by analysis is called “Limit of Quantitation”, LOQ. It is applied as threshold limit value when basic data from field trials is missing, if the pesticide was not intended for the specific food as well as if residue levels did not exceed LOQ during field trials (National Food Administration, 2010).

Legislation for approved MRL is, since 1 September 2008, joint for the member states within the European Union (EC Regulation No 396/2005) being the current statutory framework. This facilitates international trading of food and reinforces the safety assessment of pesticide intake for various vulnerable consumer groups such as small children and vegetarians (European Commission, 2010a). Analyses of pesticide residues in honey at National Food Administration has until 2010 only been performed within the control program of residues in animals and animal products. Those analyses have been executed mainly on samples of Swedish origin but also on samples taken at the border controls. When Regulation (EC) No 396/2005 updated various pesticide residue limits in the beginning of 2010, a number of pesticide residues found in honey was included. This resulted in the monitoring program of pesticide residues at National Food Administration expanding to also

include pesticide residue analyses of honey. The control program of residues in animals and animal products and the monitoring program of pesticide residues have different requirements concerning validation of new analytical methods, so it was not applicable to transfer an analytical method to the monitoring program of pesticide residues. In addition, no method was validated for pesticide analysis in honey using LC-MS/MS, hence the need for a development of such a method at National Food Administration.

The analytical method being developed in this study applied for four different pesticides; boscalid, imidacloprid, tau-fluvalinat and thiacloprid, which can all be found in honey. They were chosen according to the praxis of pesticide usage in farming in Sweden, as well as pesticides used in the bee hive to control vermin. For MRL for described pesticides, see Table 1.

Table 1. Maximum Residue Limit levels for boscalid, imidacloprid, tau-fluvalinate and thiacloprid (European Commission, 2010b)

Analyte	MRL (mg/kg)
Boscalid	0,5
Imidacloprid	0,05*
Tau-fluvalinate	0,01*
Thiacloprid	0,2

Note. *= minimum level of analytical determination

Boscalid

Boscalid is a fungicide used in two pesticide products on the Swedish market; Cantus and Signum. The pesticide is applied on several different crops, for example rapeseed, turnip, plums, cherries and beans (Swedish Chemicals Agency, 2010). The toxicity is rated to be low, but animal tests with chronic and subchronic exposure of boscalid have shown effects on liver with altering of enzyme contents, enlargement of weight and histopathological effects. The tests also showed enlargement of weight and histopathological effects on thyroid. Studies on mice also showed effects on body weight with results giving decreased as well as increased numbers (United States Environmental Protection Agency, EPA, 2003).

Imidacloprid

Imidacloprid is an insecticide used on many plants for example potato, sugar beet, sunflower and corn (Medrzycki et al., 2003). Animal tests on acute toxicity showed high exposure leading to among others apathy, tremors and decreased motility (Yamamoto & Casida, 1999). The general verdict stated the acute toxicity to be low to moderate. Chronic and subchronic animal tests showed

effects on liver with a small enlargement of weight as well as some changes on enzyme activity.

Tau-fluvalinate

Tau-fluvalinate is an insecticide or miticide used to eliminate the varroa mite within the hive. It is applied through physical contact of the bee with a plastic banner treated with tau-fluvalinate. The toxicity is categorized in the moderate group, causing eye and skin irritation. Chronic exposure has shown to cause damage to skin and itching (Swedish Chemicals Agency, 1997).

Thiacloprid

Thiacloprid is an insecticide used in pesticide products such as Biscaya, CaLypso and Bariard (Cheminova, 2009). The pesticide is applied on varies crops, for example vegetables, pome fruit, potatoes and sugar beet (Food and Agriculture Organization of the United Nations 2010). The WHO has categorized thiacloprid in the "moderately hazardous" group and it is classified to be harmful after acute intake as well as inhalation in the majority of the European countries (Ma et al., 2009).

Honey

Honey is made of plant nectar, plant secretion or secretion by insects feeding on plants. Various compounds are ingested and then transformed to honey by *Apis mellifera* bees, commonly known as honeybees (National Honey Board, 2010). Storing of this energy dense product in the hive is essential for feed and heating during the colder months of the year (Swedish Beekeeper National Association, 2010). According to the injunction of honey by the National Food Administration, honey is divided into three groups; depending on origin, depending on method of production/presenting and bakery honey. There are several subgroups for methods of production and presenting, such as honey in honeycombs and filtered honey. Bakery honey may have undergone fermentation, been overheated or may hold a different taste, which makes it suitable for use in industrial baking as well as ingredient in the manufacturing of other foods (National Honey Board, 2010).

The exact content of honey varies since nectar is collected from different sources, but the composition may look like presented in Table 2 (Mattson et al., 2009). The sugar contains often of most fructose; approximately 40% whereas the glucose content is approximately 30%. But there are large variations; honey from rapeseed contains 55% glucose but the content is 11% in honey from heather. The amount of glucose determines how the crystallization proceeds. High percentages of

glucose quicken the crystallization, especially if the water content at the same time is low. The crystallization is often avoided if the glucose percentage is lower than 25%. Temperature also affects the forming of crystals in honey, where the forming happens most quickly at 14ºC. A variation of enzymes plays an important role in the transformation from nectar to honey. Invertase is used when sucrose is split into fructose and glucose. Starch is decomposed with help of diastase and glucose oxidase forms gluconic acid and hydrogen peroxide from glucose, oxygen and water. The most common minerals found in honey are different kinds of potassium salts. The vitamin content is not very significant, with different vitamin B's being the most common. The different compositions of honey affect taste, color and texture which explain the wide variety of diverse types of honey.

Table 2. Components of honey and example of their different contents

Component	Amount (%)	Contents
Sugar	79	Fructose
		Glucose
		Sucrose
		Other sugars
Water	18	
Other components	3	Minerals
		Enzymes
		Trace elements
		Aromatic compounds
		Vitamins
		Acids

Note. Modified after Mattson et al., (2009).

Honey is a common ingredient in food and often used as sweetener. It is also the main ingredient in mead, an alcoholic beverage where water and honey is fermented and possibly flavored with for example spices or fruit (Mjödhamnen, 2010). The consumption of honey in Sweden is approximately 0.7 kg per person and year with the level being more or less constant since 1990 (Swedish Board of Agriculture, 2010). In 2009, Sweden produced 3 400 tons of honey, while the world production reached a quantity of 1 511 257 tons. China dominated the world market with a production of 367 219 tons, followed by Turkey and Argentina (Food and Agriculture Organization of the United Nations, 2010a).

Studies show several beneficial physiological effects connected to intake of honey. Two studies showed positive effect on nocturnal coughing among children with decreased frequency, decreased severity and better quality of sleep for the child as well as the parent (Warren et al., 2007; Paul et al., 2007). A Cochrane study of 19 trials, including 2554 people, investigated if honey decreased healing time in acute and chronic wounds. The conclusion of the Cochrane study showed honey to

possibly increase healing rate in partial thickness burns, compared to some conventional dressings. Honey did not show increased healing rate when used on chronic wounds (Jull et al., 2009).

An important aspect of honey consumption is the issue of food safety. There are three health hazards referred to intake of honey; infant botulism, toxic honey and pesticides. Infant botulism is caused by a toxin produced by the bacteria *Clostridium botulinum*. The toxin affects breathing, when blockage occurs of the neural impulse to the striated muscles (Swedish Institute for Communicable Disease Control, 2010). The environment of the gastric system and the intestines are different in infants compared to adults, which explains why bacteria can grow and produce toxins (National Food Administration, 2010d) in infants. Whether honey really is the cause of infant botulism has been discussed and according to one source honey is only responsible for 5% of cases of infant botulism (Emmeluth et al., 2010).

Toxic honey is produced when nectar is collected from certain flowers containing toxins, for example various species of rhododendrons and laurels. The plants produce grayanotoxins, which may cause symptoms as nausea, dizziness, low blood pressure and vomiting (U.S. Food and Drug Administration, FDA, 2010). Another example is tutu (*Coriaria arborea)* bushes, where honey bees collect toxic honeydew from the sap sucking vine hopper. Poisoning can cause vomiting, dizziness, coma or even death (New Zealand Food Safety Authority, 2010).

Pesticides are transferred to the honey by the bees as they pollinate different plants, where pesticides have been applied. Beekeeping also contributes to accumulation of pesticides in honey, as it often includes application of various substances inside the hive to prevent and eliminate common vermin (Bogdanov, 2006).

Previous studies of pesticide residues in honey show various results with most of them reporting findings to be low in regard to MRL. The number of analyzed samples in seven studies ranged from 24 to 111, with detection of pesticide residues between 25%-100% of the samples. One study (García-Chao et al., 2010) performed in Spain found no pesticides in the samples. In a Turkish study (Yavuz et al., 2010) the majority of the 109 samples contained pesticide levels exceeding MRL. However there is no conformity in the evaluation of results between the authors (Garcia-Chao et al.,2010; Herrera et al., 2010; Blasco et al., 2008; Pirard, et al., 2007; Choudary et al.,2008; Blasco et al., 2003). National Food Administration has also performed pesticide analysis in honey. Controls carried out 1999, 2001, 2003, 2004, 2005, 2006, 2008 and 2009 found no samples above MRL or action level, a determined limit for substances lacking MRL (National Food

Administration 2010c; 2009b; 2007; 2006; 2005; 2004; 2002; 2000). The controls included analysis of 50 samples, with a few exceptions of sample quantity.

Analytical methods of pesticide detection and quantitation

There are several methods and techniques to determine the pesticide content in honey samples; gas chromatography, liquid chromatography and enzyme-linked immunosorbent assay (ELISA). For gas chromatography and liquid chromatography, a variety of detectors can be used. Detectors commonly used with liquid chromatography are UV-Visible, fluorescence, electrochemical, multifunctional and tandem mass spectrometry, which will be described below.

UV-Visible detectors

This kind of detection uses the phenomenon of the analyte absorbing light, either in the UV or in the visible region. When the eluant has passed the column it goes through a flow cell, where it is irradiated by light of specific wavelength and bandwidth. The light entering the flow cell is compared to the light leaving, whereby absorbance and any possible background noise are detected. A reference system is used, often with light going through a reference flow cell containing mobile phases that is static. Advantages of these kinds of detectors are among others reliability and cheapness. One disadvantage is when the absorbance of the analyte is not strong enough to be used analytically or if the absorbance wavelength of the analyte synchronizes with several other interfering compounds (Venn, 2008).

Fluorescence detectors

Fluorescence occurs after absorbance of light in a molecule, as internal energy is lost by emitted radiation when the molecule goes back to the ground state. Different kinds of instruments can be used, as the light source may be static or use variable-frequency pulses. As pH often affects the fluorescence response, it is possible to increase the fluorescence by altering pH. Advantages with fluorescence detectors are the sensitivity at the same time as is it not heavily affected by instrumental parameters such as flow rate and pump pressure. Another advantage is the selectivity as double wavelength is chosen together with the fact that a minority of the compounds yield fluorescence. One disadvantage with these kinds of detectors is the fact that only at low

concentrations is there linearity between fluorescence and concentration (Venn, 2008).

Electrochemical detectors

The principle for these detectors is detection of compounds that oxidize or reduce at a chosen voltage. Electrons from the oxidation pass from the working electrode to the solution by the counter-electrode and during reduction adding of an extra electron take place. The detectors can be divided into dynamic and equilibrium ones. One advantage of theses detector is the sensitivity, as it is probably the best compared to all other HPLC detectors. One disadvantage is the bad robustness, as the system need time to stabilize and also to maintain a good condition of the electrodes (Venn, 2008).

Multifunctional detectors

There is an opportunity to connect two different detectors in case several features are needed for the analysis. One disadvantage of this system is the amount of space needed for two detectors, something which has been taken care of by production of multifunction detectors. One example is UV-fluorescence detectors (Venn, 2008).

Tandem mass spectrometry detectors

The tandem mass spectrometry detectors are composed of a collision cell, also called interface, connected to two mass analysers (Venn, 2008). The ions, entering the MS/MS from the chromatography column, pass through the first quadrupole Q1. The Q1 selects an ion of a specific mass to enter Q2, where collision gas (N_2 or Ar) split the ions into product ions. The product ions of all masses enter the third quadrupole, Q3, where only one size of ions is let through for the conclusive detection (Harris, 1999). One advantage with MS/MS is its selectivity as the background noise is decreased when specific compound-dependent fragments of ions are formed (Venn, 2008). The high specificity of theses kinds of detectors is an important feature in the aspect of identification, as it functions even for pesticides with similar retention time. Because of the high-performance of tandem mass spectrometers, they are the detectors of choice today for pesticide analysis. Some disadvantages are the heavy expenses for purchase as well as maintenance.

Aim

To certify honey being a safe food to consume, it is of great importance to control the pesticide content. Since 2010 honey is included in the monitoring program of pesticide residues at National Food Administration, which has resulted in a need of validated analytical methods of pesticide residues in honey. Because of the features of different pesticides, various techniques are required to perform the analyses. Polar and non-volatile pesticides are preferably analyzed using liquid chromatography tandem mass spectrometry, hence the aim of this thesis was to develop and validate a method for analysis of pesticides in honey using liquid chromatography tandem mass spectrometry.

Materials and Method

Chemicals and standards

Methanol (gradient grade for liquid chromatography), sodium sulphate (grade pa) and sodium bicarbonate (grade pa) were obtained from Merck (Darmstadt, Germany). HPLC grade ethanol was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland). Pesticide standards; boscalid, imidacloprid, tau-fluvalinate and thiacloprid, were purchased from Dr. Ehrenstorfer (Augsburg, Germany) (see Table 3 for specified concentrations and purity). Appropriate volumes of stock standard were mixed and diluted with methanol to acquire stock mixture. The stock mixture was diluted to different concentrations with methanol to receive working standards, which were used on the instrument. Stock standards, stock mixtures and working standards were stored at 4ºC in refrigerator and used within the time of expiry of twelve months.

Table 3. Pesticides used in the experiment and their respective concentration in standard solution

Pesticide name	Concentration (µg/ml)	Purity (%)
Boscalid	1215	99.5
Imidacloprid	920	98.0
Tau-fluvalinate	991	93.0
Thiacloprid	1199	98.0

Honey samples

Three labels of honey were used with origin in different Swedish provinces; Hälsingland, Östergötland and Blekinge. Honey label "1" and "2" were used in experiments developing the extraction method, honey label "2" was used in recovery experiments and honey label "3" was used for an alternative extraction method development. The first honey label was collected 20081029 and the last label was collected 20101005. After processing of the honey at Svensk Honungsförädling AB, samples were sent to National Food Administration. Analyses were performed to determine whether the honey samples contained a number of pesticides, but not the pesticides included in this thesis. The results showed no pesticides above MRL or above action level, a determined limit for pesticides lacking MRL. The honey samples were collected for utilization in this study. The blank samples were examined to confirm absence of pesticides included in validation. The honey was stored in sealed plastic containers, in a dark place which held room temperature.

Matrix fortification

Ten grams of honey was fortified with 0.2 ml of standard solution, containing selected pesticides. Five replicates were prepared with concentrations of 0.01µg/g, 0.02µg/g and 0.1µg/g. The samples were incubated for 30 minutes before extraction followed.

Extraction procedure

As reference method the National Food Administration's multi method for analysis of pesticides in fruit and vegetables using LC-MS/MS was used (National Food Administration, 2010a). An additional step was added to the reference extraction procedure, as 10 ml of water was first added to the samples of 10 ± 0.1 g honey put in falcon tubes. The samples were shaken for 30 seconds and vortexed 30 seconds at medium intensity on a Genie vortexer (Scientific Industries, Inc, Bohemia,

NY, USA). This step is not included in the written version of the reference procedure but has been applied in pesticide analysis of raisins using that procedure. By personal communication, advice was given to use water addition in this study as well, since honey and raisins share the feature of high sugar content. After addition of water to the honey samples, the reference procedure was utilized as followed.

Sample of 10.0 g honey was put in falcon tubes whereby adding of 3 g sodium bicarbonate ($NaHCO_3$), 10 g sodium sulphate (Na_2SO_4) and 20.0 ml ethyl acetate. The sample was shaken 30 seconds and then put in a Bandelin Sonorex RK 100 (Berlin, Germany) ultra sonic bath for 3 minutes. Before being centrifuged in room temperature using a Hettich Rotanta/T (Hettich-Zentrifugen, Tuttlingen, Germany)at 4182 n/min^{-1} for 3 minutes, the sample was turned a couple of times. The extract was filtered through a 0.2 µm PTFE syringe filter. The extract held a sample concentration of 0.50 g sample/ml for analysis with LC-MS/MS.

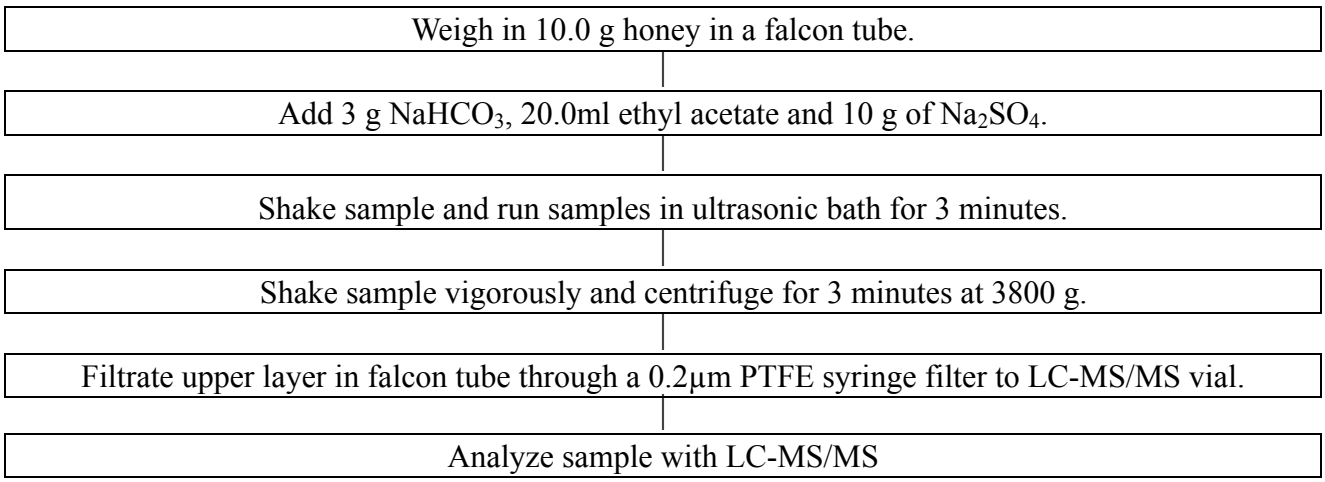


Figure 1. Scheme of the extraction procedure

The samples were vortexed utilizing a Genie vortexer (Scientific Industries, Inc, Bohemia, NY, USA). A Bandelin Sonorex RK 100 (Berlin, Germany) was used for the ultra sonic baths. Centrifugation was performed on a Hettich Rotanta/T (Hettich-Zentrifugen, Tuttlingen, Germany) and when using chilled centrifugation a Hereaus Multifuge 3 S-R (Hereaus, Buckinghamshire, England) was used.

LC-MS/MS instrumentation and operating conditions

The analysis was performed on LC-MS/MS with electrospray-interface (ES +) using an API 4000 QTrap (Applied Biosystems, USA). The settings of the LC-MS/MS were established according to a reference method used for pesticide analysis of cereals (National Food Administration, 2010b).

A Genesis C_{18} column, 4µm, 100 x 3 mm was used with injection volume of 5 µl. The LC operated with mobile phase A, consisting of methanol and mobile phase B consisting of 10/90 methanol/10mM ammonium format solution, pH 4. The gradient used, ranged from 5 to 100% of mobile phase B (Table 4).

Table 4. Gradient for positive electrospray ionization (National Food Administration, 2010b)

Time (min)	% A	% B
0	0	100
15,8	95	5
21	95	5
24	0	100

Note. A: mobile phase with methanol. B: mobile phase with 10/90 methanol/10mM ammonium format solution, pH 4.

The source temperature of the mass spectrometers was 400°C, and a Turbo V^{TM} spray source conducted positive electrospray ionization (ESI). Capillary voltage was set at 5500 V. Acquired ion transitions in LC-MS/MS for the different pesticides are shown in Table 5.

Table 5. Ions acquired by LC-MS/MS during analysis of boscalid, imidacloprid, tau-fluvalinate and thiacloprid

Compound	Precursor ion (m/z)	Product ion 1 (m/z)	Product ion 2 (m/z)
Boscalid	343	271	307
Imidacloprid	256	209	*
Tau-fluvalinate	503	208	181
Thiacloprid	253	126	99

Note. * = no transition to product ion 2 acquired

Quantification

To calculate the pesticide concentration of the sample, the peak area of the sample was compared to the peak area of the standard in an equation.

C_s= standard concentration, µg/ml A_s= standard peak area

C_{sa}= sample concentration, g/ml A_{sa}= sample peak area

Pesticide concentration in sample (µg/g) = A_{sa} x C_s / A_s x C_{sa}

For recovery, the sample concentration determined by analysis is compared to known spiked concentration of sample.

C_{found}= sample concentration assigned through analysis, µg/g

C_{spike}= spiked sample concentration, µg/g

Recovery (%) = (C_{found}/C_{spike})* 100

Integration, quantification and confirmation calculations were performed using Analyst software version 1.5.

Result and Discussion

Development of extraction method

A number of experiments were performed to examine if altering various steps of the reference method would increase the recovery levels. The experiments resulted in a modified procedure of extraction.

Solubility of honey

Solubility of honey was examined adding volumes of 5 ml, 10 ml and 15 ml of water to add to the honey, before starting of the extraction process. The recovery results did not differ substantially, but

to ensure enough water for the solving of honey, an aliquot of 10 ml water was chosen for the extraction procedure. A try to heat the solution to 35-40 ºC before performing the extraction was performed, as heating has been used in several pesticide studies of honey (Jiménez et al., 2008; García-Chao et al., 2010; Sánchez-Brunete et al. , 2008). The recovery results were not noticeable better for the heated samples, so this step was not included in the extraction procedure further on. Another reason to not include the heating step was to avoid any possible degradation of pesticide residues when using the extraction in the future, as additional pesticides would be included in analysis using the method.

Adding of salts

Addition of Na_2SO_4 to the samples was examined in order to determine whether the recoveries were affected by salt. It is of great importance to be able to motivate every step in the extraction procedure and to keep the procedure as efficient and cheap as possible. Since the results implied a small increase in recovery values (approximately 7 percentage points) when adding Na_2SO_4, it was decided to keep it in the extraction procedure. Salt addition is seen in a high number of other studies, either Na_2SO_4 (Jansson et al., 2004) or $MgSO_4$ (Kamel, 2010; Lehotay, 2005; Payá et al., 2007). But there are also studies excluding this step (Xu et al., 2009; Hiemstra et al., 2007). In the study by Hiemstra et al. (2009) it was decided to exclude the addition of Na_2SO_4 in the original method, but if recovery values did not reach 70 %, method B was applied which included addition of Na_2SO_4. This together with previous results (personal communication) have shown increased recovery with adding of Na_2SO_4, a decision was made to keep this step in the extraction process. Another step being modified was the order and the procedure of adding $NaHCO_3$, ethyl acetate and Na_2SO_4 to the samples. During the first tests ingredients were added in the order of: $NaHCO_3$, Na_2SO_4 and ethyl acetate. The order was changed to: $NaHCO_3$, ethyl acetate and Na_2SO_4 as this noticeably improved the recovery with approximately 20 percentage points. With the new order of addition, precautions were also made to ensure that $NaHCO_3$ covered the whole surface of the matrix to achieve more thorough interaction of the salt with the matrix. It was also ensured by stirring with a spoon that the added Na_2SO_4 did not contain any lumps, which would aggravate solving in the falcon tubes.

Centrifuge settings and shaking of samples

To make the extraction process more efficient and thereby gaining improved recovery, a few extra

steps were added to the procedure. After adding water to the honey samples, the falcon tubes were shaken for 30 seconds and then run in the Genie vortex for 30 seconds. This was performed to ensure satisfactory solving of the honey. Another step added to the method was shaking of falcon tubes for 30 seconds after 3 minutes of ultrasonic bath before placing them in the centrifuge. This was performed to improve the extraction as the pesticides migrated to the ethyl acetate phase. The setting of the centrifuge was modified to enable better separation. The method suggested using 4182n/min^{-1}. During the tests this figure was changed to 3500 n/min^{-1} as this gave the best separation.

Description of the developed extraction procedure

The modified version of extraction procedure to be applied in the method was as followed. An aliquot of 10 ml of water was added to the honey sample in a falcon tube, whereafter the sample was shaken 30 seconds and vortexed for 30 seconds at medium intensity on a Genie vortexer (Scientific Industries, Inc, Bohemia, NY, USA). To the sample 3 g of $NaHCO_3$ was added. Then an aliquot of 20.0 ml ethyl acetate was added, followed by 10 g of Na_2SO_4. The sample was shaken 30 seconds and vortexed for 30 seconds at medium intensity on a Genie vortexer, before running the sample in a Bandelin Sonorex RK 100 (Berlin, Germany) ultrasonic bath for 3 minutes. The sample was shaken 30 seconds and then centrifuged using a Hettich Rotanta/T (Hettich-Zentrifugen, Tuttlingen, Germany) for 3 minutes, 3500 n/min^{-1}. Filtration was performed with a 0.2µm PTFE syringe filter after which the extract was analyzed with LC-MS/MS. See Figure 2 for scheme of the extraction procedure.

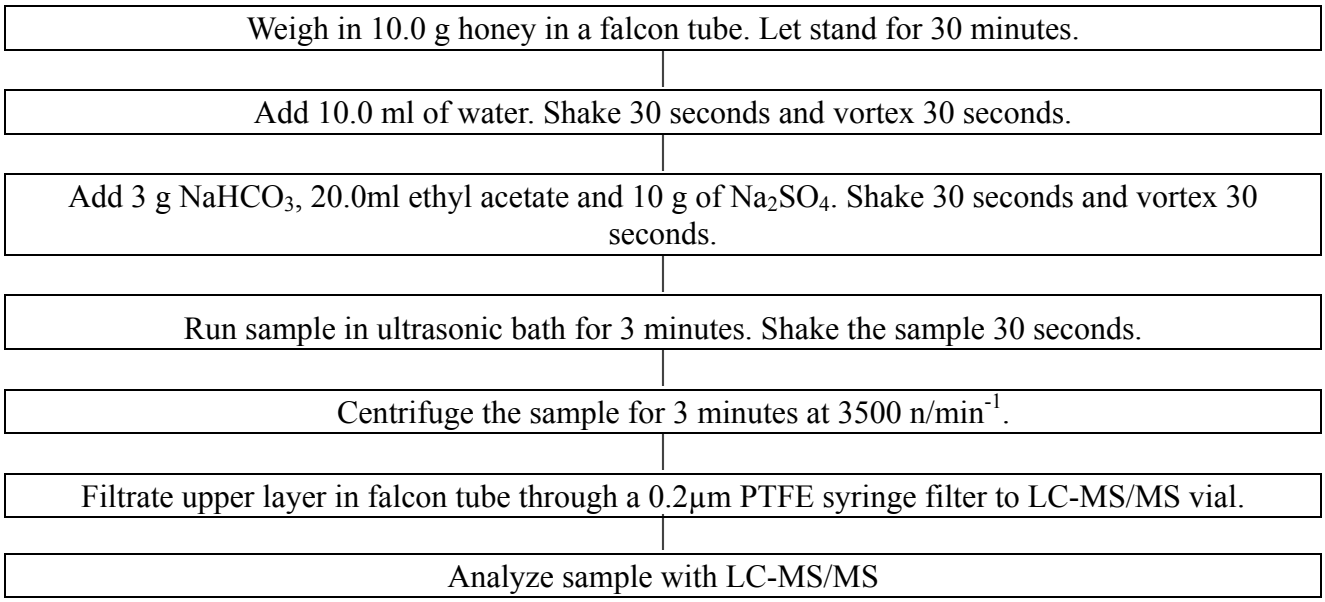


Figure 2. Scheme of the extraction procedure

Development of extraction procedure for honey label "3"

The method was performed on different honey labels, whereby one label of honey acted differently during extraction. This label of honey had a bit of a different appearance compared to the other two labels of honey when stored in the plastic containers. While the other two labels had a few bubbles on the surface, this label carried a centimeter deep layer of small bubbles. The smell of this specific honey label was much stronger and distinct and it had a softer texture which made it easier to spoon compared to the other two labels which were more firm. During extraction, instead of forming several phases after all ingredients had been added, the ingredients in honey label "3" were tightly bound together in one gel formed phase. This resulted in no suitable final extract for determination and quantification of the pesticides in LC-MS/MS. Different tests were performed on the specific honey label, trying to find a method to enable extraction. Primary Secondary Amine, PSA, was added, with hope that it would bind to the sugar in the honey and thereby facilitate separation. This did not succeed. Glass wool was put in the tip of a pasteur pipette and the gel was then added to the pipette to examine if this filtration would give a separate ethyl acetate phase for final determination. It did not work out as the gel was too tightly bound to be separated. A plastic syringe with glass wool, as well as a 0.2 μm PTFE syringe filter, was also used trying to filtrate the gel, but it did not succeed.

In another experiment the samples were chilled to 4°C and then centrifuged at that temperature. In the samples with added Na_2SO_4 satisfying forming of different layers occurred, while in samples without Na_2SO_4 no separation occurred. When repeating the experiment, the same results as the first time was not achieved. A test was also performed where Na_2SO_4 was added after centrifugation, followed by shaking, vortexing, chilling (4°C) and then centrifugation again. However, this did not result in any suitable extract to use for final determination and quantification.

A more intense centrifugation, 4000 n/min^{-1} at 4ºC during 20 minutes, was performed on samples with or without Na_2SO_4 added during extraction. Both extraction methods resulted in a separate ethyl acetate phase. Samples without Na_2SO_4 had more than twice as much separated ethyl acetate phase to use as extract. Compared to the sample with Na_2SO_4, this more intense centrifugation was used again to examine if addition of Na_2SO_4 after extraction also would yield a separate ethyl acetate phase. Extraction was performed in two samples where Na_2SO_4 was added, the samples were shaken for 30 seconds and run in the Genie vortex for 30 seconds. The samples were chilled and then centrifuged at 4000 n/min^{-1}, 4 ºC during 20 minutes. Satisfying forming of different layers

was formed in both samples. An additional test was performed to examine whether an additional ultrasonic bath for 3 minutes would increase the volume of the separate ethyl acetate phase. The ultrasonic bath was performed before chilling of the samples and it resulted in more than twice as much volume to use as extract than the samples without the use of an extra ultrasonic bath. As the altered extraction method resulted in satisfactory volume of separate ethyl acetate phase for this specific label of honey, it was selected as a modified version of the developed method. See Figure 3, for scheme of the modified extraction.

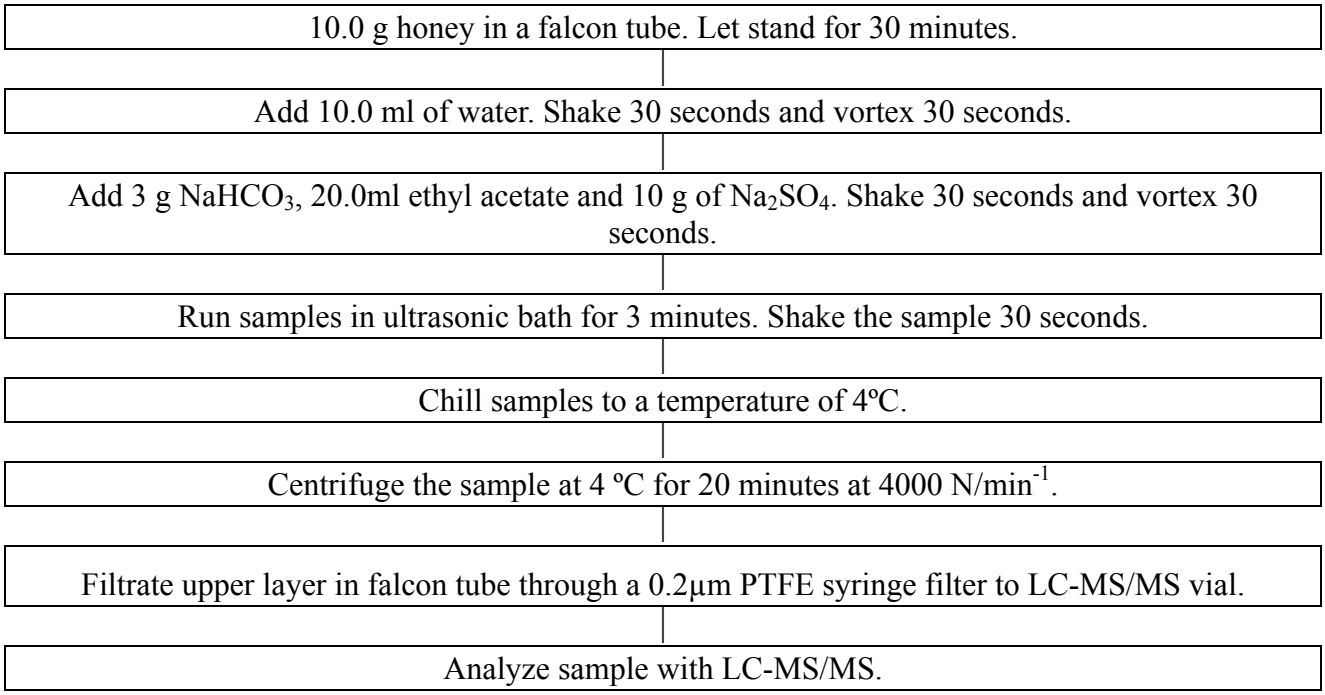


Figure 3. Scheme of extraction for honey label "3".

The discovery regarding formation of emulsion for a specific label of honey was important, although it would have been desirable if the method was applicable to all honey labels. In total three different honey labels were used in the experiments, so it is difficult to make any assumptions how common this phenomenon is. The difficulty with forming of emulsion that may occur during extraction is also described in another study (Kujawski et al., 2008). The study suggests a couple of procedures which may help when gel formation occurs; adding of Na_2SO_4, using ultra sonic bath, freezing of samples to separate ice from liquid organic phase, adding of solutions as ethanol and methanol to decrease the tension of surface and careful shaking to limit surface where liquids interact. Several of these proposed procedures, were used when trying to find an alternative extraction method in this thesis. Information was retrieved about the honey samples but apart from different origin of production, no specific information could explain the differences in "behavior" during extraction. Since honey can contain more than 180 different substances (Crane, 1985), it

may be difficult to determine the exact content of the present three labels. Honey label “3” had a more intense smell and a softer texture than the other labels, which may indicate that its content differ significantly. Since the texture was a bit softer for honey label “3”, it presumably contains slightly lower percentages of glucose than the other labels. Honey label “3” probably holds a higher water content as well, as viscosity has strong correlation to water content (Crane, 1985).

Estimation of matrix effect of honey

When performing analysis in LC-MS/MS, the matrix of the sample often alters the signals giving other results than when performed in solvent. The concentration of standard in solvent and blank matrix is equal but the acquired signals given by the LC-MS/MS differentiate due to the matrix effect. To determine matrix impact on recovery rates of the analytes, signals from standards in methanol were compared to signals from standards in blank honey matrix for every pesticide. The quantification software program executed a division where signal from standard in solution was the numerator and signal from standard in blank matrix was the denominator. The difference of levels for the standards in solvent compared to the standards in honey matrix is illustrated in Table 6.

Table 6. Matrix effect at 0.01 µg/g in honey samples for boscalid, imidacloprid, tau-fluvalinate and thiacloprid. Relative signal intensity of standard solutions in solvent compared to standard in blank matrix as reference (n=1)

Analyte	Relative signal intensity (%)
Boscalid	90.9
Imidacloprid	86.0
Tau-fluvalinate	66.6
Thiacloprid	100.6

The figures point toward honey causing a slight ion enhancement in the analysis for three of the four pesticides. The matrix effect seems to be most pronounced in tau-fluvalinate, where analysis with standard in solvent generates approximately 67% of the signal from analysis performed with standard in matrix. Thiacloprid seems to be least affected by matrix effect, as the relative signal intensity was almost equal (101%) using standard in solvent or matrix. This gives an indicator of how honey affects the recovery results for respective pesticide, even though using more replicates would generate even securer data of the matrix effect.

Procedures to evaluate the matrix effect are of high significance in development and validation of a new method, otherwise the results might be connected to an amount of uncertainty (Taylor, 2005). Estimated values for matrix effects vary in different matrices (Kruve et al., 2008), from time to time with the use of the same matrix and pesticide (Jansson et al., 2004) and between different pesticides (Jansson et al., 2004). This emphasizes the importance of evaluating the matrix effect for every pesticide included in the method and to continue estimating this effect on the results for every single run in the LC-MS/MS. If the method will be expanded in the future with pesticides available on the international market, it can be valuable to do the same evaluation of the matrix effect on these new pesticides. Since only a small number of tests were performed during the development of this method, the estimation of matrix effect for the included pesticides is only approximated figures.

There are several other techniques to balance the matrix effect.

Isotopically labeled internal standard is a technique where labeled analogues are used to compensate for the matrix effect (Barr et al., 2002). They have similar retention time as the analytes (Zrostliková et al., 2002), but differentiate in ion mass and/or fragment ions (Barr et al., 2002). The technique might be very expensive, since internal standard is needed for each analyte and representative isotopically labeled internal standards does not exist for all pesticides (Zrostliková et al., 2002).

Improved clean-up during chromatography can also reduce matrix-related effects on the results (Pascoe et al., 2001). A precolumn is used to collect interfering matrix material from sample, before the analytes passes through the main column. To avoid components in the matrix with similar retention time as the analytes to interfere, it is recommended to use a different kind of precolumn from the main column.

Echo-peak calibration, named because of the two peaks formed, uses injection of an unknown sample and a known standard solution very close to each other in time (Zrostliková et al., 2002). To balance the matrix effect, the two compounds should have retention times quite similar. This technique also work in the analysis of several pesticides, as a precolumn is used that is switched on and off.

Among all the techniques available to compensate for the matrix effect, this thesis used external matrix-matched standard to balance the effect. It is used for most of the pesticide analysis performed at the National Food Administration (SANCO, 2009). It purports a qualitative evaluation

of the matrix effect and with the matrix matched standard used in the determination and quantification in LC-MS/MS, the effect on recovery is abolished.

Recovery

Recovery experiments were performed at three levels; 0.01, 0.02 and 0.1µg/g for the four pesticides. The lowest two levels included experiment using 5 replicates whereas at 0.1 µg/g, data was included from a previous experiment using the same conditions and 10 replicates were used. The recovery levels and relative standard deviations for the experiments are shown in Table 7. All analytes hold recovery levels from 69 % to 92%, with corresponding relative standard deviations <20%. Guidance documents for monitoring of pesticide residue analysis within the European Union (SANCO, 2009) set mean recoveries for initial validation in the range of 70-120%, a requirement met for 11 of 12 different pesticide levels. The pesticide levels were lowest for tau-fluvalinate, especially at 0.01µg/g where more than 20 percentage points differed from two of the other pesticides.

Table 7. Recovery levels (± and relative standard deviation) for boscalid, imidacloprid, tau-fluvalinate and thiacloprid extracted from honey samples with different concentrations

Analyte	0.01µg/g (n=5)	0.02µg/g (n=5)	0.1µg/g (n=10)
Boscalid	86.6 ± 8.7	89.2 ± 3.6	77.9 ± 7.8
Imidacloprid	90.7 ± 11.5	78.0± 10.7	73.6 ± 18.4
Tau-fluvalinate	69.4 ± 9.8	71.0 ± 9.1	70.6 ± 18.9
Thiacloprid	91.8 ± 15.2	77.5 ± 8.4	74.3 ± 16.0

As the software program executes determination and quantification of pesticides, created LC-MS/MS chromatograms are manually checked to guarantee that correct peaks are chosen for quantification. Figure 4 shows three chromatograms of boscalid. The two upper chromatograms are from a sample at 0.01µg/g and standard at representative level, which satisfactorily show distinct peaks at equal retention time. The chromatogram on the bottom left shows a blank sample. It does not contain any large peaks at the retention time of boscalid and a cluster of really small peaks are spread out during spectrum of retention time. The y axis is set to be representative to the size of the peaks in the blank sample, showing peaks even though there heights are small. Figure 5 shows the same chromatogram of the blank sample, but the y axis is set to be representative to the y axis of the sample and standard of 0.01 µg/g.

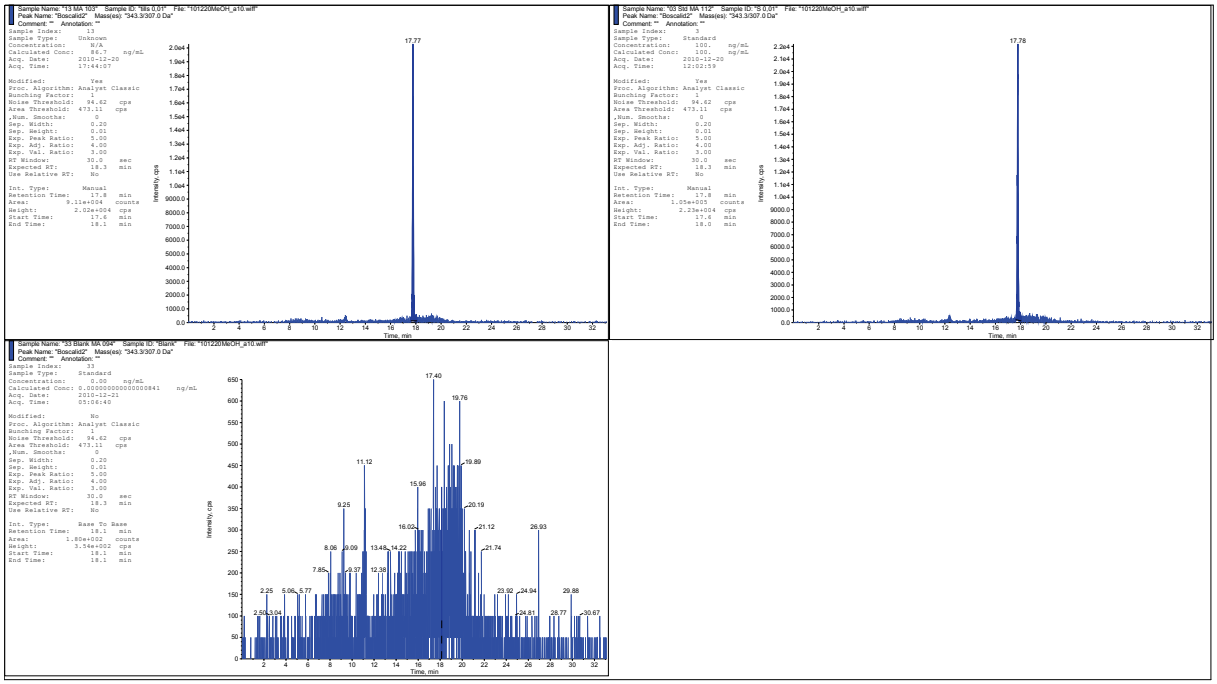


Figure 4. LC-MS/MS chromatograms of boscalid from validation experiment. Upper left: sample at 0.01µg/g, upper right: standard at 0.01µg/g and down left: blank.

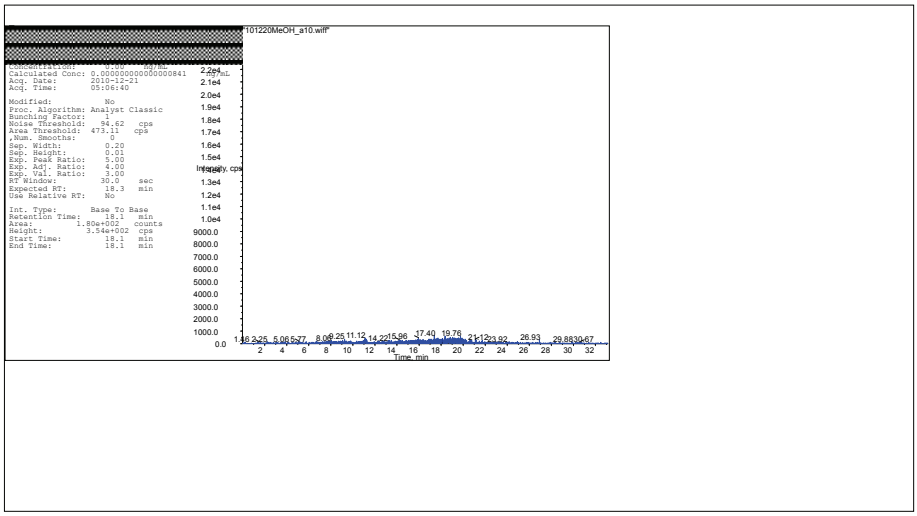


Figure 5. LC-MS/MS chromatogram of boscalid in blank sample. Same blank is used as in figure 3, with axes of coordinates graded similar to samples of figure 3.

An experiment was done where analysis was performed without honey in the samples in order to examine whether recovery would change distinctly by absence of honey and also to determine whether any analysis would show any interfering peak in the extract. The recovery in those samples where between 59.2 % and 83.4% (Table 8). Only two of the four pesticides had accepted recovery range of 70-120%, according to European Union guidelines for method validation for pesticide

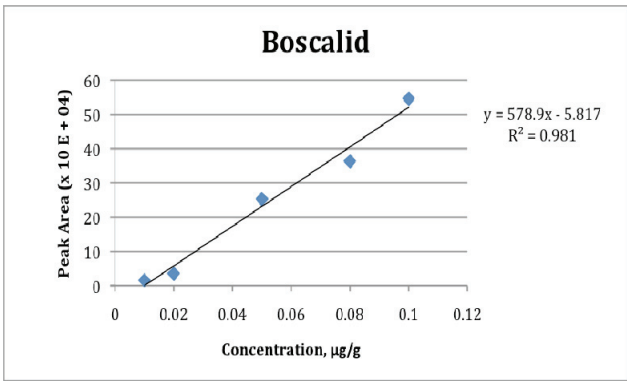
residue analysis (SANCO, 2009). Drawing any more solid conclusions from this experiment should not be performed though, since the results were compared to standard in blank matrix. The samples did not contain any honey, which gave results not totally representative.

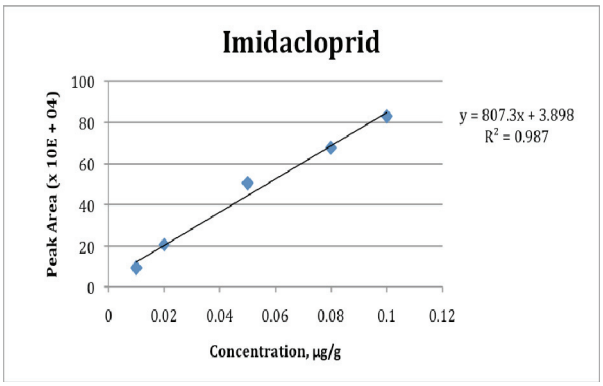
Table 8. Recovery results (± and relative standard deviation) at 0.10 µg/g level for boscalid, imidacloprid, tau-fluvalinate and thiacloprid, obtained by developed extraction procedure in samples without honey (n=2)

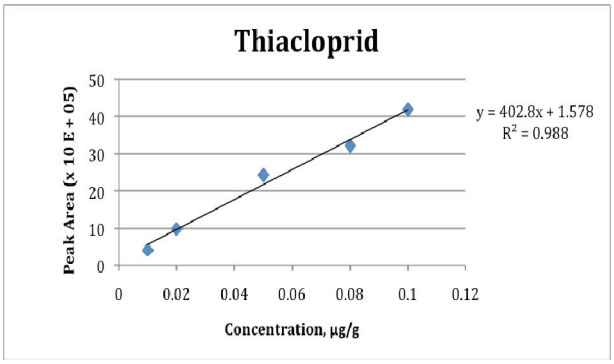
Analyte	Recovery (%)
Boscalid	73.0 ± 3.6
Imidacloprid	59.2 ± 4.8
Tau-fluvalinate	83.4 ± 11.2
Thiacloprid	68.1 ± 9.3

Linearity and Limits of Qualification

To determine whether a detection method holds a linear response between amount of analyte and acquired response from the LC-MS/MS, calibration curves with pure compounds were produced. According to the validation guidelines (National Food Administration, 2009a), the linearity of the method is considered satisfying (0.95- 1.00). This requirement was fulfilled for all of the pesticides (Figure 6). The r^2-values are, according to the validation guidelines of the National Food Administration, helpful for the quantification but not a requirement. These results support the results from the recovery experiments as it confirms the accuracy of the method.







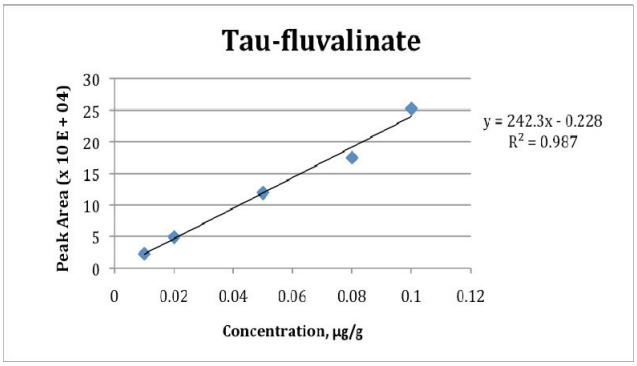


Figure 6. Calibration curves for boscalid, imidacloprid, tau-fluvalinate and thiacloprid.

Results obtained by using reference method with extra adding of water

National Food Administration's validated multi method for analysis of fruit and vegetables (National Food Administration, 2010b) was used as reference method as the new method was developed. An additional step was added to the reference method as 10 ml of water was added to honey and the samples were shaken for 30 seconds and vortexed for 30 seconds, whereafter the procedure followed the reference method. An experiment was performed using the old multi method to observe the recovery and to evaluate if the new method had increased the recovery (Table 9).

Table 9. Recovery (± and relative standard deviation) at 0.10 µg/g level for boscalid, imidacloprid, tau-fluvalinate and thiacloprid, obtained by reference procedure with extra adding of water compared to the new modified extraction procedure

Analyte	Reference procedure with extra water (n=2)	New procedure (n=10)
	Recovery (%)	Recovery (%)
Boscalid	62.0 ± 2.5	77.9 ± 7.8
Imidacloprid	54.5 ± 1.3	73.6 ± 18.4
Tau-fluvalinate	76.7 ± 5.4	70.6 ± 18.9
Thiacloprid	54.2 ± 0.0	74.3 ± 16.0

The analysis seemed to generate better results using the new method, with tau-fluvalinate being the only exception; 70.6% obtained by the new method and 76.7% obtained by old method. Since only two replicates were used when testing the old method, the results should be interpreted with caution. But during the development of the method and testing of different variables, a number of

tests were performed with recovery showing significantly lower numbers which points to the importance of this more efficient extraction to gain satisfying recovery results. It is likely to presume that additional shakings of falcon tubes with ethyl acetate would increase recovery results as pesticides migrate from the water phase to the ethyl phase during shaking.

Discussion of general method aspects

Significance of pesticide residue analysis methods

To have adequate methods to determine pesticide content in honey is important in more aspects than just the perspective of food safety. The industry with the beekeepers use the image of honey as a pure and healthy product to sell, which gives them an interest to control the pesticide levels and keeping them to a minimum. Another reason showing the magnitude of valid methods for pesticide detection in honey is protection of the honey bee colonies. The pollination carried out by the bees is important for the eco system and to enable an effective food production. There has been suspicion whether pesticides partly account for the widespread death of honey bees during the last years, which have caused some countries to ban the use of certain neonicotinoids such as imidacloprid (Benjamin, 2008). Studies have shown imidacloprid to affect the communicative capacity, mobility (Medrzycki et al., 2003) and foraging behavior (Yang et al., 2008) of the honey bees. With increasing knowledge about the environmental and health effects of the pesticide usage, accurate analysis methods for pesticide determination is essential for monitoring of harmful levels.

Origin of data

The recovery results are retrieved from one experiment, except on the level of 0.1µg/g where 5 replicates, of 10 in total, derived from an experiment performed before the validation experiment. The two experiments shared the same conditions and since the experiment, performed before the validation experiment, generated results within the allowed limits of 70-120%, a decision was made to include these figures. This is the reason for there being 10 replicates at the level of 0.1 µg/g and 5 replicates at the other levels.

Imidacloprid

According European Union guidelines for method validation for pesticide residue analysis (SANCO, 2009) for MS/MS is identification of compounds valid when identification is performed using two or more product ions. To examine whether a compound is present or not in the sample, this is not required. Using MS/MS determination in this method gave identification of two ions in samples for boscalid, tau-fluvalinate and thiacloprid. Problems with different instrumental settings gave only one identified ion for imidacloprid. The signals were satisfying regarding retention time, peak shape and size for this ion which could confirm identity for this ion. When using this method in future pesticide analysis, some adjustment could be needed on the instruments to gain additional transitions.

Extraction

Solvent extraction, also known as liquid-liquid extraction, was applied in the method to acquire an extract suitable for determination and quantification by LC-MS/MS. Ethyl acetate was used as extraction solvent as this is applied in the reference method. The reference method, developed by the National Food Administration, has yielded satisfactory results in previous analysis and has been approved as an official reference method within the European Union. Pesticide analysis using the reference method has, with satisfying recovery, been performed on raisins, which share some resemblance with honey concerning the high content of sugar. This was yet another reason to assume that the reference method, with some alterations, would be a good starting-point for development of the new method. Using ethyl acetate as extraction solvent has been performed in other studies as well. One study (Frenich et al., 2005) compared using ethyl acetate, acetone and two mixtures of them (1:1 and ethyl acetate: acetone, 3:1) as extraction solvent in LC-MS/MS analysis of vegetables. The results showed highest recovery levels for ethyl acetate and lowest levels for acetone.

Future

A high amount of honey consumed in Sweden is being imported, 2007 a quantity of 2 725 000 kilos of honey was imported to Sweden (Swedish Board of Agriculture, 2008). This large quantity emphasizes the need expanding this method to also include pesticides used in other countries.

Honey is a food varying in composition, such as enzymes and water content. As seen in the

development of an analytic method in this study, this means a possibility of specific honey labels may not act according to plans when the extraction is being performed. An additional method was established to enable extraction for “special honeys”, but there might be a risk of unexpected events as the method will be used on additional honeys. This is something to keep in mind in the future as the method will be applied on several different labels and origins of honey.

Conclusion

With a need to develop a method for pesticide analysis in honey using liquid chromatography tandem mass spectrometry, a new method was developed using National Food Administration’s multi method for pesticide determination in fruit and vegetables as reference. Samples were extracted according to the new extraction procedure yielding recovery in the range of 69.4% to 91.8% with a relative standard deviation <19 % and satisfactory linearity. Results were within the acceptable range for validation standards for all but one sample. One label of honey formed emulsion during extraction, which obstructed development of suitable extract for determination by LC-MS/MS. A modified extraction procedure was developed resulting in satisfying phase separation and thereby analysis of the specific label.

Acknowledgements

First I want to express my gratitude towards Tommy Wåglund, my supervisor at the Chemistry division 1, National Food Administration. You made it possible for me to carry out this project and I have enjoyed working with you. I appreciate your patience and positive attitude throughout the whole process as I have gained a lot of knowledge and experience with your guidance.

I want to show my appreciation towards Lena Dimberg, my supervisor at Department of Food Science, Swedish University of Agricultural Sciences. You have offered helpful support and been an encouragement to me during the work of this thesis.

Finally, I want to thank all the people at the Chemistry division 1, National Food Administration, for being very welcoming and kind during my time at your division. It was a great time, sharing your company.

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