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Classical and novel approaches to the analysis of honey and detection of adulterants

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Abstract

Honey is an extract of floral and secretions from a variety of bees. Some honey manufactures adulterate pure honey with industrial sugar, chemicals, and water either directly or indirectly. Many methods have been developed to detect honey adulterants including physicochemical analysis, microscopy, chromatography, immunoassay, thixotropicity, DNA metabarcoding, sensors, and spectroscopy. However, the most promising methods for the development of a portable test kit for honey adulterant detection are ELISA, electronic tongue, and NIR. The most sensitive and accurate method is NIR. These methods have shown satisfactory results when used individually or combined. Further research is still required to trial different combinations of methods to improve accuracy and the ability to detecting a wide variety of adulterants simultaneously. There is a need to develop a portable honey adulterant detection method, such as NIR spectroscopy using a smartphone.

Keywords: Honey, stingless bee honey, adulterants, portable honey adulterant kit, NIR spectroscopy, smart phone, electronic tongue

1. Introduction

1.1 Definition of honey

Honey is a yellowish liquid that acts as Newtonian fluid (Abu-Jdayil, Ghzawi, Al-Malah, & Zaitoun, 2002). It consists of secretions of bees and extracts of plant nectar. Several species of bees visit plant nectar, collect the extract and store them as food. The classification of honey is thus based on the source of the nectar. Based on color there are two types of honey; light and dark. The dark honey is considered more nutritious such as richer in minerals (Anthony & Balasuriya, 2016; White, 1978). Honey can also be classified as honeybee (*Apis mellifera*) and stingless bee (meliponini) honey (da Silva et al., 2013). The honeybee is bigger in size and it sting while stingless honey bee does not sting and is smaller in size (Jalil, Kasmuri, & Hadi, 2017). Honeybee honey is sweet in taste while stingless bee honey is a mixture of sweet and sour taste (Aziz, Giribabu, Rao, & Salleh, 2017). Stingless bee business is a potential and fast growing in Malaysia. In 2014 Malaysian researchers found five species of stingless bee; *Hypotrigona scintillans*, *Trigona laevicepts*, *Trigona thoracica*, *Trigona Terminata* and *Trigona itama*. Among this *Trigona itama* is the most widely used by farmers (meliponiculture) (Kelly, Farisya, Kumara, & Marcela, 2014).

1.2 Composition of honey

Honey is nutritious and has medicinal value. Sugars, amino acids, organic acids, and biologically active compounds in honey make it nutritious and medicinally beneficial (Ahmed, Prabhu, Raghavan, & Ngadi, 2007). In the honey, main constituents are carbohydrate (70-80% w/w) and water (10-20% w/w). Other varieties of minor components such as free amino acids, proteins,

phenolic compounds, minerals, vitamins and organic acids are also recorded in the honey (Ouchemoukh, Louaileche, & Schweitzer, 2007b). Amino acid content in honey is about 1% among which proline is dominant (50-80%) (Hermosín, Chicón, & Cabezudo, 2003). Carbohydrate content in honey by dry weight is recorded as 95% w/w and include mainly glucose and fructose (65-80% w/w), and saccharose/sucrose (disaccharides such as glucose and fructose bonded by glycosidic bonds) (de la Fuente, Sanz, Martínez-Castro, & Sanz, 2006). Propolis is one of the natural honey products that are waxy and resinous (Jalil et al., 2017). Physicochemical analysis revealed that propolis is rich in carbohydrates (49%) and crude fibre (44 %). It also consists of 23% moisture, 21% crude fat, 4 % ash and 3% crude protein (Ibrahim et al., 2016).

1.2.1 Honey composition standard

Codex Alimentarius (CODEX STAN 12-1981) standardized the composition of honey. Honey should have a moisture content not be more than 20%, sugar content not less than 60 g/100g, sucrose not more than 5 g/100g, free acidity not more than 50 milliequivalents acid/100g, diastase activity not less than 8 Schade units, hydroxymethylfurfural (HMF) content not more than 40 mg/kg, electrical conductivity not more than 0.8 mS/cm and water-insoluble content not more than 0.1 g/100g (Codex Alimentarius, 2001).

1.3 Honey quality

Honey quality is decided based on physicochemical parameters; water, sugar, HMF, acidity, ash (mineral content), density, electrical conductivity, invertase activity and diastase level (Bogdanov, 1999; Bogdanov & Gallmann, 2008; Olugbenga & Obasanmi, 2014; Pasias,

Kiriakou, & Proestos, 2017). The honey with high water content, low density, and high electrical conductivity easily ferments and degrades the quality resulting in a reduced shelf life. Water content indicates the honey density, extraction method and is also related to the maturity of the honey. The increase of water content decreases the honey density (Ouchemoukh et al., 2007b). Sucrose content of the authentic honey is less than 5% (Ouchemoukh, Louaileche, & Schweitzer, 2007a). Therefore, honey that contains more than 5% sucrose maybe unripe; sucrose is not converted completely into glucose and fructose by invertase enzyme (Ouchemoukh et al., 2007a).

Invertase activity, diastase, and HMF are quality indicators that indicate freshness and overheating of honey (Bogdanov et al., 1999; Pasias et al., 2017). Lower diastase content may also indicate that the honey contains naturally low amylase content (Ouchemoukh et al., 2007a). HMF also indicates the purity of honey; a higher HMF value indicates that the honey has been overheated, aged or stored under poor for too long. For instance, honey samples stored for more than 12-24 months contained 128-1131 mg/kg of HMF which is greater than the recommended standard (80 mg/kg). Honey should be consumed within one year of storage (Khalil, Sulaiman, & Gan, 2010).

Electrical conductivity (EC) increases as the mineral and acid content of the honey increases. Honey mineral contents were found significantly correlated ($P < 0.05$) to EC. Yemeni and Egyptian honey had 4.18 and 1.98 ms/cm EC, respectively. Saudi and Kashmiri honey had 0.53 and 0.67 ms/cm, respectively. Therefore, Saudi and Kashmiri honey is within the standard limit (not more than 0.8 mS/cm) while Egyptian and Yemeni honey exceeds the limit (>0.8 mS/cm) (El Sohaimy, Masry, & Shehata, 2015). The acidity of the honey is due to organic acids such as gluconic acid, esters, lactones and inorganic ions of chloride and phosphate. Besides that, the

extraction season varies the pH of a honey. The honey with pH below 3.5 is susceptible to spoilage (Bogdanov et al., 1999; El Sohaimy et al., 2015). El Sohaimy et al. (2015) found that the honey samples they tested were fresh as the acidic values (pH 4.1-4.6) comply with standard limits (pH 3.4-6.1) (Codex Alimentarius, 2001). When the acidic value exceeds the standard limit it indicates fermentation of honey sugar into organic acids. The acidity controls the microbial spoilage and maintains the honey flavor (Bogdanov & Gallmann, 2008).

1.4 Storage stability

During storage, honey is fairly stable. However, honey adulterated with water will deteriorate faster. Besides that honey adulterated with chemicals lower the medicinal value as well as may harm the consumers (Anthony & Balasuriya, 2016). Jiménez et al. (1994) investigated storage stability of honey for 2 years at 4-7ºC and 28ºC. The changes in pH, colour, sugar composition, water content, yeast and mould counts of honey were analyzed. Over two years of storage the color of the honey darkened, sugar content changed but the total yeast level increased significantly ($P<0.05$). However, there was no microbial growth and pH was found stable. Maltulose and turanose increased during storage while glucose, fructose, sucrose kojibiose, maltose, trisaccharides and isomaltose decreased. The yeast identified were *Schizosaccharomyces*, *Zygosaccharomyces*, and *Saccharomyces*. The moulds isolated were from the genera of Aspergillus, Fusarium, Alternaria, and Penicillium (Jiménez, Mateo, Huerta, & Mateo, 1994).

1.5 Production of honey

In the world, 1.5 billion kg honey is produced per year from 2005 to 2010. Worldwide twenty countries produce honey of which China is the largest producer (436000 Mt) followed by Turkey (88162 Mt). India is the 7th largest honey producer (6100 Mt) while Central African Republic is the least producer (1600 Mt) (FAOSTAT, 2016). Honey production is declining due to high labor costs and low profits from the honey business. Therefore, to overcome this decline pure honey is adulterated with chemicals and water (Anthony & Balasuriya, 2016). According to Codex Alimentarius, the honey intended for human consumption should not have any food ingredient other than honey thus must be free from food additives, organic and inorganic matters that are foreign to its original constituents (European Commission, 2001). Therefore, for honey to remain complied with international food standards honey adulteration need to be identified and enforced. For identification of honey that has been adulterated various methods need to be explored and developed. Thus the aim of this review is to explore the possibility of developing a portable test kit, which would detect adulterant of honey on the spot, for the consumers or regulatory authorities to check before buying or prior approval of honey to be sold in the market. Therefore, honey adulteration methods and potential honey adulterant detection methods are briefly described for exploring into a kit development possibility.

2 Adulteration of honey

Adulteration alters the quality and safety of honey. For instance, honey adulterated with chemicals lower the medicinal value as well as may harm the consumers (Anthony & Balasuriya, 2016). Honey adulterants are mainly starch syrup, inverted syrup, starch or inverted syrup fed to bees and low-quality honey added to high-priced honey. Adulteration methods (See Figure 1) of honey can be direct or indirect (Zábrodská & Vorlová, 2015). Direct adulteration is the direct

addition of a substance into honey. Indirect methods are when the honeybee is fed with honey, chemicals and industrial sugars (Figure 1) and thus detection of indirect adulteration is a challenge (Zábrodská & Vorlová, 2015) compared to direct contamination.

Honey is adulterated directly; adding industrial sugar or honey into ready-made honey (Figure 1). Main adulterants of honey are sugar such as the addition of high fructose corn syrups (HFCS), high fructose inulin syrups (HFIS), invert syrups (IS) and corn syrups (CS). Syrup or invert sugar constituents are same as the natural constituents in the honey thus these adulterants are not easily detected; a challenge for the scientists to discover a new method of distinguishing the differences of pure and adulterated honey (Mehryar & Esmaiili, 2011).

Most honey is produced from plants such as rice, wheat and beet (C3), and as well as maize and sugar cane (C4). Honey adulterated by plant sources are categorized as C3 and C4 as per their carbon metabolism. Plants that are categorized as C3 fix carbon dioxide via Calvin (C3 cycle) which has a low 13C/12C ratio to that of C4 plants fixing carbon dioxide using the Hatch-Slack (C4) cycle (Zábrodská & Vorlová, 2015).

3 Adulterant detection methods

Traditionally, honey adulterants are detected by physicochemical methods. Adulteration of honey by crystallized cane sugar, invert sugar syrup, and cane sugar syrup can be detected with chemical determinations including HMF, glucose, sucrose, fructose, and diastase (Codex Alimentarius, 1989; White, 1979). Geographically the honey can be categorized by physicochemical parameters such as HMF, fructose, sucrose, glucose, electrical conductivity, free acidity, moisture and color (Siddiqui, Musharraf, Choudhary, & Rahman, 2017). Also, the botanical origin of the honey can be identified by electrical conductivity (Bogdanov et al., 1999).

Besides, uni-floral honey has been characterized by electrical conductivity, water content, color, fructose, and sucrose (Bogdanov et al., 1999; Mateo & Bosch-Reig, 1998).

As the honey adulteration detection is complex more advanced methods of adulterant detection have been developed constantly. For example, oligosaccharides of the honey were adsorbed and fractionated by activated charcoal to prepare the samples for analysis. Then, high-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) was used to detect high fructose corn syrups (HFCS) and corn syrups (CS) adulterants in the sample which identified adulterants down to 5% (Morales, Corzo, & Sanz, 2008).

Methods used until 2014 for detection of adulterants from honey were summarized by Yilmaz et al (2014) as electrochemical analysis, enzymatic methods, thin layer chromatography (TLC), carbon isotopy, flow injection analysis, gas chromatography (GC), high-performance liquid chromatography (HPLC), anion-exchange liquid chromatography (LC), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), mid-infrared, near-infrared (NIR) transfectance spectroscopy, gas chromatography-mass spectrometry (GC-MS), high performance (HP) anion exchange chromatography with pulsed amperometric detection method (HPAEC- PAD), high performance thin layer chromatography (HPTLC), isotope ratio mass spectrometry coupled with an elemental analyzer, and low field nuclear magnetic resonance (Yilmaz et al. 2014). For interested readers could refer Yilmaz et al. (2014) paper for the details of the mentioned methods. Methods used to detect honey adulterants also include microscope combined with real-time PCR (Kast & Roetschi, 2017; Siddiqui et al., 2017), three-dimensional fluorescence spectroscopy (3DFS) coupled with multivariate calibration (Chen et al., 2014), electronic honey quality analyzer (Anthony & Balasuriya, 2016), fiber optic displacement sensor (FODS) (Bidin et al., 2016), electronic tongue (Gan et al., 2016), and

nuclear magnetic resonance (NMR) (Siddiqui et al., 2017). However, none of the methods to date could be used to identify all the adulterants in the honey simultaneously. Instead of going into details about all the honey adulteration detection methods, this work focused on methods that have the potential for developing a portable method for honey adulterant detection.

Wu et al. (2017) thoroughly reviewed sugar based adulterant detection methods including SCIRA, GC, HPAEC, HPLC, IR-based analysis, NMR, Raman spectroscopy and Q-TOF-MS that differentiate C3 plant honey adulterants, HFCS, C3 and C4 starch and rice syrups. However, the authors did not address the potential of these methods to develop a portable detection method that could be used on-site. The main difference between this present review and that of Wu et al is that the authors focused on sugar-based honey adulterants and detection methods while the present review focuses on potential portable honey adulterant detection methods using classical and advanced adulterant detection methods. This review also includes recent studies that have been published after the publication of Wu et al. (2017). Figure 1 illustrates the types of honey adulteration and the continuous development of honey adulterant detection methods and the need for to focus on portable honey adulterant detectors or kits.

4 Potential for the development of honey adulterant detection kits

Since available methods of adulterant detection in honey are complex and not portable to use for on the spot inspections a kit needs to be developed for a similar purpose which with one drop of honey may change the color and qualitatively detect if the honey is adulterated or not. For exploring the best fit method for honey adulterant analysis it is important to know the details of the pure honey compositions as stated in section 1.2, such as moisture content, sugar content and others. When the honey has adulterated some changes to this composition will occur which could

be used as indicators for developing methods for adulterants detection in honey. Honey adulterant detection methods are briefly discussed in section 4 to explore for a potential development of a rapid detection or portable honey adulterant detector. Table 1 summarizes the various honey adulterant detection methods and possibilities of portable test kit development.

4.1 Spectroscopy

Infrared (IR) spectroscopy can detect many adulterants in food and is regarded superior to other methods. Sample preparation is nil or minimal and sample size needed for the analysis is small. Moreover, the method is considered low-cost, fast, non-destructive and easy to use (Wu et al., 2017). Thus the equipment has potential to be portable to carry to the field for on-site analysis of adulterants from honey. Raman spectroscopic analysis is also a potential method to use on-site as the equipment can be made portable and is similar to IR spectroscopy in terms of low-cost, simple and rapid, requiring minimal sample preparation and is non-destructive. One advantage over IR is that the samples do not receive any interference by fluorescence (Wu et al., 2017).

The idea of IR spectroscopy to be made portable and miniaturized was recently designed and prototype released by a mobile company. On 6th January 2017, a UK online newspaper (dailymail.co.uk) published that Changhong released a breakthrough design of a smartphone (H2) that can detect chemical composition of a product. The phone possesses SCiO's material sensing technology that is a tiny NIR spectrometer built into the phone that emits a light and records the reflection where latter has a spectrum based on the product. These spectra are sent to the cloud for analysis and the detail of the materials is given to the owner of the phone. This technology can detect molecular properties of food and body metrics. The authenticity of the food can also be detected. Viagra and an identical imitation pill were used to demonstrate the

ability of the smartphone to distinguish fake Viagra (Macdonald, 2017). Similar technology can be used to identify the adulteration of honey for on-site inspections. Since, as previously described, NIR is the method that can detect most varieties of adulterants of honey, this could be a potential solution to design a test similar to the smartphone system described.

4.2 Electronic tongue

Food for mankind relies on perception through our senses that help judge the quality and acceptability of the product. Biomimetics involves mimicking human senses to design such things as an electronic tongue and is an emerging technology that will advance science. Nanotechnology is used to minimize the size of these instruments (Twomey, de Eulate, Alderman, & Arrigan, 2009). The performance of these sensors is enhanced with computers and its software using calibration techniques (Ghasemi-Varnamkhasti, Mohtasebi, & Siadat, 2010; Lenau, 2009). The electronic tongue mimics the gustatory systems of the mankind. The effectiveness of the sensor depends on the absorption and catalysis of the materials into ions.

A taste sensor is a low selective sensor which identifies components in a solution mixture. The identification is through pattern recognition and multivariate calibration by computer software for data processing. The sense of taste contributes to ‘umami’, sweet, bitter, sour and salty tastes which are the basic tastes identified in different areas of the human tongue with specific receptors on the tongue, papillae. Once the food enters the mouth the information from the olfactory receptors are combined to judge the taste of the food. Sensing principles applied in the electronic tongue include voltammetry and potentiometry which are electrochemical methods. The electronic tongue takes the fingerprint of the food and then chemometrics tools attached to it are used to process the data. Methods to prepare a taste sensing system include the use of

materials that have electrochemical sensing properties and semiconductors. For example, radical lanthanide bisphthalocyanines are intrinsic semiconductors that can be used to improve the sensitivity of taste sensors and electrical measurements (Ghasemi-Varnamkhasti et al., 2010).

The electronic tongue detects and identifies the complex material in the liquid, even if the different components are very similar by pattern-recognition and by multivariate calibration technique and qualitatively and quantitatively identify the target materials (Vlasov, Legin, & Rudnitskaya, 2002). These sensors are in the early stages of its technology but their applications in the food analysis are already established.

In recent years the electronic tongue has been used to analyze various beverages, water, and food components after modification of the sensor to the target analysis (Deisingh, Stone, & Thompson, 2004). These applications include analysis of sensory attributes of beer (Rudnitskaya et al., 2009), analysis of palatability, sourness and bitterness of nutritive drinks (Kataoka, Miyanaga, Tsuji, & Uchida, 2004), analysis of tomato taste (Beullens et al., 2008), salt prediction from minced meat (Labrador et al., 2010), umami taste flavor of food (Yang et al., 2013) and identification of honey (Wei, Wang, & Liao, 2009). Although many food analyses have been carried out using an electronic tongue, few studies have applied this to the analysis of honey. For instance, electronic tongue was used to analyze geographical and floral origins of honey (Wei et al., 2009), physiochemical characteristics and botanical origin of honey (Escriche, Kadar, Domenech, & Gil-Sánchez, 2012; Major et al., 2011), and adulterants of honey (Gan et al., 2016).

Electronic tongue, α-Astree ET, with seven potentiometric chemical sensors and an Ag/AgCl standard electrode was used to analyze honey effectively for its geographical and floral origins (Wei et al., 2009). Another electronic sensor was used in 2011 to analyze honey. This

commercial electronic tongue (αAstree, Alpha M.O.S) was employed to identify physicochemical characteristics and botanical origin of honey; chestnut, acacia, and honeydew. The equipment was equipped with seven potentiometric sensors that contained an Ag/AgCl reference electrode. The physicochemical analysis (acidity, water content, invert sugar, total sugar, and electrical conductivity) was quantified using Artificial Neural Network (ANN) modeling and the reference value for these parameters was obtained from the traditional methods. The botanical classification was obtained from Principal Component Analysis (PCA), Canonical Correlation Analysis (CCA) and ANN modeling. ANN modeling was found to be the best (100% accurate). The authors concluded that the electronic tongue could be a potential tool to characterize honey (Major et al., 2011). In 2012 a potentiometric electronic tongue with metals and metallic compounds was developed to analyze honey. The sensor successfully identified the botanical origin and physiochemical parameters of honey. The data obtained was modeled using PCA and ANN. The authors suggested developing a new system of the electronic tongue for the honey sector (Escriche et al., 2012).

In 2016 adulterants of honey were tested using an electronic tongue. Gan et al (2016) analyzed honey samples using sensors (electronic nose and tongue) and spectra and compared and concluded that the most effective method to analyze honey to be an electronic tongue. Adulterant and pure honey are divided into 3 groups and the adulterant honey is easily distinguished from pure honey. The electronic tongue (ET) was also found to be more sensitive to minerals, mono and disaccharides, amino acids, and phenols in the honey and the gustatory difference was easily observed by pure and adulterated honey using the ET. The adulterant was more accurately identified when the ET-Partial Least Squares Discriminant Analysis (ET-PLS-DA) model and ET-PCA models were combined. However, many more research studies are required as few

studies have to date have focused on honey adulterant analysis using electronic tongues (Gan et al., 2016).

More research on the taste sensor systems needs to be explored as they are in the early stages of development. Scientists are now trying to advance and expand the technology of the electronic tongue (Twomey et al., 2009). Since honey may be adulterated with multiple adulterants a multisensory system such as electronic tongue is suitable for honey adulterants detection. This electronic tongue development could focus on wholly on honey and how to detect added adulterants. Therefore, an electronic tongue is to be developed for all the adulterants of honey and the equipment must be miniaturized and portable for on-the-spot inspection.

4.3 Immunoassays

Immunoassays are based on antibody and its antigen interaction and are an analytical technique having the concept of immunology. The antibody, a glycoprotein, is produced in the body when it is exposed to a foreign body substance, antigen. In a favorable environment, these antigens induce antibodies production. Immunoassay is used to detect foreign bodies (antigens) in a sample matrix and these antigens could be a protein or a smaller molecule. The antibody is used to locate and capture the antigens in the sample matrix. The antibodies can be used as probes. When the antibody reacts with its antigen the antigen-antibody complex is formed and measured to identify and quantify the amount of foreign body in the samples. In enzyme immunoassay, an enzyme label is used that can change the color of the sample matrix for easy detection and quantification (Hsieh & Ofori, 2017). Honey adulterant kit development based on honey proteins and enzymes is discussed in sections 4.3.1, 4.3.2 and 4.3.3.

4.3.1. Honey protein

Honey contains very low amounts of protein (0.1-0.5%). Honey protein originates from hypopharyngeal glands and salivary glands of bees and from the enzymatic reaction of pollen and saliva of the bee (Baroni, Chiabrando, Costa, & Wunderlin, 2002). Early researchers (1900s) reported that honey contains protease, albumin, peptone, and globulin. Specific protein found in most honey is royal jelly protein (Šimúth, Bíliková, Kováčová, Kuzmová, & Schroder, 2004; Won, Lee, Ko, Kim, & Rhee, 2008). For example, Korean and European honey contain glycoprotein as a major protein (MRJP1, identical to apalbumin-1), one of the royal jelly proteins (Won et al., 2008). New Zealand honey was found to contain proteins such as apalbumins, arabinogalactan protein (AGPs) and apisimin (Gannabathula et al., 2017). In 2013 a review was published regarding the extraction methods of honey protein using mass spectrometry (Chua, Lee, & Chan, 2013). Honey from different regions was investigated for the presence of royal jelly protein using Western-blot that used polyclonal antibodies. The protein identified was apalbumin-1 with the size of 55 kDa, the most dominant protein among royal jelly proteins (Šimúth et al., 2004).

Honey protein can also be isolated and identified using LC-MS/MS (Liquid chromatography-mass spectrometry/mass spectrometry) after separating them using electrophoresis; SDS-PAGE. For instance, electrophoresis is used to detect protein (19 protein bands) in Australian honey using silver stain containing methylamine, followed by SDS-PAGE (Marshall & Williams, 1987). Honeybee protein is used as chemical markers to identify the floral origin of honey as the protein is common regardless of the type of honey. A combination of SDS-PAGE and immunoblot assays with anti-pollen antibodies raised from pollen extracts were used to identify the floral origin of honey (Baroni et al., 2002).

Few tests have been used to identify adulterated and pure honey using the honey protein. Biological tests developed for identification of pure and adulterated honey include immunological methods such as the development of anti-bee serum and rabbit anti-serum (White, 1957). The major protein of honey, apalbumin-1 was proposed as a marker for immunochemical testing to detect adulterants in honey (Šimúth et al., 2004). In light of these suggestions, it is feasible to develop a test kit for honey adulteration test similar to pregnancy test kits. The principle of pregnancy test kits is the detection of human chorionic gonadotropin (hcG) with the use of antibodies. The hcG rises rapidly during early pregnancy and thus is easily detected in the urine of a pregnant woman (Gnoth & Johnson, 2014). However, the drawback is honey protein is present in low concentrations unlike hcG in pregnant women. Therefore, unless a better option using the protein as a marker is developed, a honey adulterant test kit based on lateral flow devices such as the pregnancy test kit, may not be practical.

4.3.2. Honey enzymes

Enzymes found in honey include glucose oxidase, amylase, α-glucosidase, β-glucosidase (Won et al., 2008) and proteases (Rossano et al., 2012). Proteases were first discovered from honey in 2012. Bidimensional zymography (2-DZ), a very sensitive method for enzyme identification as it detects the enzyme in the order of nanograms, was used to analyze proteases where proteases were isolated using isoelectric focusing (IEF) and by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Proteolytic enzymes affect the quality and nutritional value of honey as they aid in the degradation of honey proteins (Rossano et al., 2012). Using enzymes few scientists have developed biosensors to quantify compositions of honey such as fructose, phenols, and glucose.

Fructose in honey was quantified using an amperometric biosensor based on D-fructose dehydrogenase that is immobilized on the electrode surface. The biosensor was developed using a CNTP electrode that is modified using 3,4-dihydroxybenzaldehyde, an electropolymerized film. The probe was then optimized by optimizing pH, temperature, enzyme immobilization and a lifetime of the probe. The biosensor reading was proportional to D-fructose content and the detection limit was 1 $x10^{-6}$ mol/L. After analyzing the fructose content of honey the biosensor was validated using a commercial enzymatic kit (Antiochia, Lavagnini, & Magno, 2004).

A label-free potentiometric biosensor was developed to quantify total phenols in honey. The sensor was immobilized with tyrosinase via a covalent bond on a solid contact transducer surface. This transducer had two layers in which first layer itself had two layers; the first layer consisting of poly(vinyl)chloride carboxylated, potassium permanganate and graphite and to this layer 2^{nd} layer was deposited using a mixture of graphite and poly (vinyl chloride) carboxylated. The second layer was immobilized with tyrosinase enzyme using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride. The biosensor detection limit was 7.3 $x10^{-7}$ M. The results obtained from the biosensor reading was validated using the Folin-Ciocalteu method (Draghi & Fernandes, 2017).

The screen-printed carbon electrode was used to quantify glucose in honey. Bulk and surface modified screen-printed carbon electrodes were prepared using multiwalled carbon nanotubes and palladium and the surface of the electrodes were immobilized with glucose oxidase. The electrodes were characterized in a 7.5 pH solution by hydrodynamic chronoamperometry and cyclic voltammetry. Gold nanoparticles were also added into the electrodes biolayer. The detection limit of the electrode was 0.07 mM glucose. The electrode (GOx/Pd-MWCNT-SPCE)

was used to quantify glucose in multi-floral honey and the results obtained were validated using commercial equipment for glucose quantification (Guzsvány et al., 2017).

Although biosensors have been developing for honey composition detection, to our knowledge no biosensor was developed to quantify adulterants in honey. Honey enzymes may be used to develop a biosensor that could detect adulterants of honey. However, to determine the authenticity of honey, active enzymes in the honey would be needed. Honey enzymes do become inactive during storage or when honey is heated (Šimúth et al., 2004) and therefore may not have the adequate enzyme to depend on for biosensor development. Another alternative approach could be to use an enzyme from another source to immobilize onto a biosensor that could detect honey adulterant. For example, fructose dehydrogenase was immobilized on carbon nanotubes to develop a nano-biosensor to detect fructose in honey with a detection limit of 1×10^{-6} mol/L (Verma, 2017; Zhao et al., 2007). Nanotubes may be immobilized with various enzymes that are able to detect adulterants simultaneously, this way one biosensor could be used to detect several adulterants.

4.3.3. Enzyme-linked immunosorbent assay

Enzyme immunoassay is named as enzyme-linked immunosorbent assay (ELISA) and is pioneered by Engvall and Perlmann (1971) in which reactants are bound to a 96-well plastic microtiter plate and is separated by unbound materials (Hsieh & Ofori, 2017). An ELISA test kit method was successfully used in the detection of streptomycin residues in honey (Cara, Dumitrel, Glevitzky, Mischie, & Silaghi-Perju, 2013). Streptomycin, an aminoglycoside antibiotic, is used to protect bees from a variety of brood diseases during apiculture. MaxSignal ® Streptomycin ELISA test kit has 10 mg/kg as a detection limit and was used to test

399 streptomycin in honey samples. The ELISA plate wells were coated with a conjugate protein of
400 streptomycin. The streptomycin is detected based on a competitive reaction; anti-streptomycin
401 antibody is added into the reaction and this competes with free streptomycin and the conjugate
402 streptomycin antibody and the unlinked antibodies get removed during washing. The ELISA test
403 depends on the reaction between an antigen and an antibody (Cara et al., 2013). A similar ELISA
404 test kit could be produced for the detection of adulterants in the honey. For this, specific
405 adulterants would need to be targeted for the development of an appropriate kit. Using ELISA
406 method, for each adulterant specific kit may be developed.
407

4.4. Microscopy

Microscopic analysis on honey could reveal some adulterants present in it in addition to the geographical and botanical origin of the honey.

4.4.1. Microscopic detection of pollen grains

Palynology (early branch called Melissopalynology) is the study of pollen grains and spores in geological deposits (Ohe, Oddo, Piana, Morlot, & Martin, 2004). For survival honeybee require natural resources such as resin, nectar, water, and pollen (Seedley, 2001). Pollen contains protein, minerals, vitamins, and fats and is considered as a protein source of honey (Haydak, 1935). Honey contains pollen grains, and honeydew elements such as algae, fungal spores, and wax tubes. The pollen grain is from nectar which gets into honey as honeybee collect nectar. These pollen and honeydew elements are fingerprints to locate the geographical and botanical origin of honey (Ohe et al., 2004). The pollen can be identified under microscopic observation. Although pollen analysis is traditionally used for quality analysis of the honey it is tedious (Hermosín et al., 2003).

For pollen analysis, the honey samples are mixed, 10 g of honey in 20 ml warm water (40ºC), and centrifuged twice at 2000 rpm for 10 min. The sediment is dried, mounted on a slide with glycerine gelatine and stained with fuschin- alcohol solution. The slides are then observed under the microscope for the pollen identification (Kerkvliet, Shrestha, Tuladhar, & Manandhar, 1995; Louveaux, Maurizio, & Vorwohl, 1970).

For the details of how pollens are detected and quantified under the microscope refer to Louveaux et al (1970). Pollen grains are not identified to genus or species level by this method but identified to in shape and morphological characteristics. The pollen grain is analyzed based

on frequencies; 45% of pollen grain means very frequent, 16-45% is frequent, 3-15% is rare and less than 3% pollen grains found means sporadic. Predominant pollen means that the sample contains more than 45% of pollen grains, secondary pollen means 16-45%, 3-15% means important minor pollen and minor pollen is when pollen grain present in the sample is less than 3%. The pollen grains are expressed in percentages if equal or greater than 1200 pollen grains identified. Pollen grains of 1% are referred if greater or equal to 1200 pollens are identified in the sample materials. Pollen studies reveal the geographical origin of the honey but not the country of origin. The pollen spectrum of honey provides information about forest and floral agricultural conditions. The pollen also detects the botanical origin of honey. The frequencies of pollen types in honey can be used to identify the botanical origin (Louveaux et al., 1970).

Pollen studies can be used for identification of the geographical and botanical origin of honey. Floral origin of the honey is traditionally identified by pollen analysis (Hermosín et al., 2003). Honey samples from Algeria were identified by pollen quantity and pollen spectrum. The pollen grains present in samples were rich, greater than 45% (80,000 to 24, 832, 000) (Ouchemoukh et al., 2007a). In another study, pollen detection of honey samples from Austria, Canada, Germany, Pakistan, Saudi Arabia, America, and Australia were carried out and revealed that pollens were from 15 plant species. The pollen spectra identified nectarless and nectariferous sources bees visited. The pollen identifies climate, geographical location of the beehive and their vegetation. The composition of the pollen exposes the floral origin. For example, *Eucalyptus fibrosa* pollens were detected mostly from Saudi Arabian, Pakistani, Germans and Austrian honey (Bibi, Husain, & Malik, 2008).

In summary, pollen detection provides information about the geographical and botanical origin of the honey and it does not provide information about the adulterants of the honey. This method

453 along with the physicochemical analysis of the honey can be used for identification of honey
454 quality for trade purposes. The pollen detection is through microscopy which is tedious and in
455 need of replacement with a more efficient method that could be made portable. There is
456 potential to use pollen detection as an indicator of honey adulteration as honey from a particular
457 region will have expected pollen profiles that are likely to be altered with adulteration.

458 **4.4.2. Microscopic detection of adulterants**

459 A microscopic (polarization microscope) procedure was used to detect cane sugar and acid-
460 hydrolyzed can sugar syrup, honey adulterants fed to honey bees. Cane sugar contains particles
461 from the cane stem such as epidermis cells, single rings of ring vessels, sugar cane starch, and
462 sclereid (Kerkvliet et al., 1995). Supplementary tests used with the microscopic characterization
463 of the honey can include HPLC to analyze glucose, sucrose, fructose, and HMF. Moreover, the
464 honey pH, water content and electrical conductivity can also be measured. This combination of
465 methods was used to identify 10 adulterated honey samples from Nepal and Philippines
466 (Kerkvliet et al., 1995). Chemical analysis of these honey highlighted that samples were
467 adulterated or heated.
468 Another combination of methods is the use of microscopy and real-time PCR assay to detect
469 sugar, adulterants of honey using *Saccharomyces cerevisiae* (baker's yeast) as an indicator of the
470 sugar adulterant (Kast & Roetschi, 2017; Siddiqui et al., 2017). *S. cerevisiae* (baker's yeast) is
471 added into the sugar paste prepared to feed the honey bees. However, this yeast does not multiply
472 in the honey and will only multiply in the presence of sugar adulterants in the honey. Within 10
473 days after the honey bees are fed with baker's yeast, if the yeast has multiplied in adulterated
474 honey, they can easily be detected by microscopy and real-time PCR assay.

In summary instead of the use of only microscopic analysis for adulterants, it is more accurate to combine it with other methods such as physicochemical analysis, HPLC, and PCR. Microscopic methods may be most useful in developing countries where alternative methods are impractical due to high cost.

4.5. DNA metabarcoding

Botanical and entomological origin of honey has been identified using DNA metabarcoding (Prosser & Hebert, 2017). Three gene regions were used to analyze pollen components; mitochondrial cytochrome c oxidase subunit (COI) to identify bee species and to classify entomological source of honey (Prosser & Hebert, 2017; Yao et al., 2010), nuclear ITS2 (for honey pollen signature identification to discriminate plant species) (Yao et al., 2010) and pollen-free plant material plastid gene (rbcLa) to recognize any plant DNA in honey (Prosser & Hebert, 2017). Nuclear ITS2 is present in all pollen grains while plastid markers are not always present (Bell, Burgess, Okamoto, Aranda, & Brosi, 2016). Prosser and Hebert (2017) discovered that the indirect adulteration of honey with low quality of honey can be detected by DNA metabarcoding without a study of pollen. This study revealed that flavored or dark-colored honey is not accurately identified by this method and this could be due to interference from secondary metabolites on PCR. A change in the buffer used during DNA extraction may solve the problem of PCR inhibition. The authors suggested the potential use of this method require various modifications in the future for the detection of adulterants in honey (Prosser & Hebert, 2017).

The current limitation to the DNA metabarcoding is that the genetic markers used for identification affect the taxonomic resolution of the assay (Prosser & Hebert, 2017). The other drawback of DNA metabarcoding is when honey is purified by filtration to remove impurities the

pollen is also removed. The method does not work on creamed, flavored and darker honey. For darker and flavored honey, a new genetic marker needs to be developed. The accuracy and reliability of DNA metabarcoding depend on the effectiveness of the genetic markers. The genetic markers for problematic analysis could be developed through research on a wider analysis of honey authentication. Also for better identification, a combination of melissopalynology and DNA metabarcoding is recommended.

DNA metabarcoding could be a potential method that could identify all the adulterants in the honey, however, there are challenges in developing a portable kit; need more research and expertise in the different field that requires the kit to become reality.

4.5.1. Thixotropicity

Thixotropicity (stickiness or rheology) of honey could be explored for the possibility of developing a honey adulterant test kit. The following section provides details on this methodology. Honey adulterated with carbohydrates was identified based on the nitrogen content of the honey. For example, nitrogen content less than 10 mg/100g honey in Venezuela honey was considered adulterated with carbohydrates (Anklam, 1998; Olivier, 1987). Another method to detect adulterated carbohydrates in honey is by rheological methods. For instance, adulterants such as fructose and saccharose syrups in honey can be detected using rheological methods (Yilmaz et al., 2014).

Yilmaz et al (2014) adulterated natural honey with saccharose and fructose syrups at different levels (0-50% by weight) and then tested these honey by creep, dynamic and steady shear tests. For steady shear analysis, the samples were sheared between 0.1-100 s^{-1} at 25ºC. The viscosity was analyzed as a function of shear rate. The dynamic shear analysis was conducted in the strain

519 range of 0.1-100% using the amplitude sweep test at 1 Hz and the linear viscoelastic region
520 (LVR) was determined. The frequency of the sweep test was also investigated between 0.1-10
521 Hz at 25ºC using 1% strain, and this strain is obtained from amplitude sweep test. The storage
522 modulus and viscous or loss modulus are viscoelastic parameters. Temperature sweep test at a
523 $50s^{-1}$ shear rate and 1 Hz and between 5-50 ºC was also conducted to test any variation between
524 dynamic and steady shear parameters. Creep and recovery tests were conducted using constant
525 stress at 0.1 Pa within the LVR. Within a given time at steady state, the viscoelastic material
526 deformation was analyzed and then stress applied and released to examine for recoverable shear.
527 This method was validated using parameters such as linearity, sensitivity, and repeatability.
528 Statistical analysis was conducted to see the different adulterant levels on dynamic and steady
529 shear parameters and bivariate correlations were conducted to check the relatedness between
530 sugar composition and Pearson's test was carried out to analyze rheology parameters of the
531 adulterated honey and principle component analysis (PCA) was used to categorize honey
532 between sugar composition and rheological parameters (Yilmaz et al., 2014).

533 Rheological properties vary during the manufacturing processes such as mixing, filtering,
534 heating, bottling and hydraulic transport. For example, the viscosity of the honey is one of the
535 rheological parameters influenced by the quality, processing steps, and honey processing
536 equipment design. Viscosity also depends on moisture content, colloids and crystals and other
537 materials in the honey. The rheological parameters also depend on time, stress, shear rate and
538 temperature which are also important factors during the manufacturing processes and its
539 equipment design. Moisture content and the temperature of the honey influences the viscosity.
540 The viscosity decreases with moisture increase up to 19% moisture with less effect with a further
541 increase in moisture content. The viscosity decreases with increase in temperature up to 30°C

542 with less effect at higher temperatures. Viscosity also varies depending on the botanical origin of
543 the honey (Yanniotis, Skaltsi, & Karaburnioti, 2006).
544 The physicochemical analysis reveals that the adulterated honey is brighter in color while the
545 pure honey is more reddish. The pH of the adulterated honey decreases when the adulterant level
546 increases while the water activity of the adulterated honey increases compared to that of the pure
547 honey. The steady shear stress and viscosity values decrease with increase in the adulteration
548 level (Yilmaz et al., 2014). The adulterants in the honey can be detected between the
549 temperatures of 5-50ºC. Steady shear analysis revealed that the samples adulterated with 10%
550 sucrose and fructose syrups could be detected between 5-20°C. Dynamic shear properties
551 revealed that the adulterated honey is not elastic but viscous in nature. Also adulterated honey
552 decreased in resistance to deformation and the Newtonian model parameters that describe shear
553 properties of the samples could be used to detect a 10-50% level of adulterated sugar content in
554 honey. The creep-recovery analysis is suggested to be a potential approach to detect fructose and
555 saccharose as the adulterated honey structure is easily deformed and this behavior can be picked
556 up by creep-recovery analysis. The method is repeatable and the limit of detection of rheological
557 parameters for adulteration ratio in honey is more than 4%. The study results revealed that the
558 behavior of the natural honey such as its flow, creep and viscoelasticity was notable and pure.
559 However, when the honey is adulterated with syrup, the viscosity loss and storage modulus
560 values and deformation was prominent compared to natural honey. HPLC-RID was used to find
561 the composition of the syrups. A significant correlation ($P<0.05$) was found between sugar
562 composition and the rheology parameters (dynamic shear, creep and steady shear) of the honey
563 when Pearson's correlation test was conducted (Yilmaz et al., 2014). The study concluded that
564 these rheological parameters; creep and dynamic and steady analysis is a novel approach for

565 detecting fructose and saccharose syrups as adulterants of honey (Yilmaz et al., 2014). The use
566 of rheological parameters in combination with HPLC-RID is a promising method for the
567 identification of fructose and saccharose syrup adulterated honey. However, the method is a
568 challenge to make it portable for on-sight use as there is no portable rheological method.
569 Honey crystallization is based on storage time, temperature as well as the botanical origin
570 (Smanalieva & Senge, 2009). Below 30ºC honey is known to crystallize (Venir, Spaziani, &
571 Maltini, 2010). Although glucose and fructose content in the honey are approximately same, due
572 to the lower solubility of glucose the latter crystallizes (Venir et al., 2010; Young, 1957).
573 Parameters that affect honey crystallization are fructose and glucose concentration and water
574 content (chemical composition) as well as mechanical processing and the storage temperature
575 (Smanalieva & Senge, 2009). Crystallization affects the rheological properties of honey and the
576 crystallization rate of the honey can be determined by the ratio of fructose and glucose, F/G. The
577 glucose (α-D-glucose monohydrate) crystallizes below 50ºC (α-D-glucose anhydrous) while
578 remains stable in the anhydrous forms between 50-80ºC and above 80ºC (β -D-glucose
579 anhydrous form) (Venir et al., 2010; Young, 1957). The crystal size produced in the honey is
580 determined by the F/G ratio and its storage condition. To remain stable, the F/G ratio must stay
581 above 1.33 and below this value, it crystallizes (White, 1978). In natural honey the F/G ratio is 1-
582 1.2 and the addition of adulterated glucose or fructose will change this ratio (Puscas, Hosu, &
583 Cimpoiu, 2013). Rheological properties of honey are affected by temperature and the natural
584 honey making process. The temperature and the F/G ratio is used to determine the size of the
585 crystal formation in the honey (Smanalieva & Senge, 2009). Smanalieva and Senge (2009) tested
586 39 German honey to identify the botanical origin. Flowing behavior of honey depends on
587 botanical origin and temperature (Smanalieva & Senge, 2009). Natural honey behavior is

588 generally considered as Newtonian (Abu-Jdayil et al., 2002). However, all non-floral German
589 honey demonstrated the non-Newtonian flowing behavior (Smanalieva & Senge, 2009)
590 indicating they may be adulterated. However, for creamed honey in its natural form, the flow
591 behavior is non-Newtownian (Karasu, Toker, Yilmaz, Karaman, & Dertli, 2015). Honey from
592 *Eucalyptus spp* also exhibits non-Newtonian behavior (Trávníček & Přidal, 2017). Adulterated
593 honey with fructose and saccharose is Newtonian (Yilmaz et al., 2014).
594 Adulterants in honey lower viscosity. For example, when saccharose and fructose syrup were
595 added to natural honey, viscosity decreased and the decrease was enhanced as the concentration
596 of the adulterants increased. Shear stress of the adulterated honey decreases as the adulterant
597 content increases which results in the decrease of viscosity of the honey. These results highlight
598 that the adulterants of honey, saccharose and fructose syrups, can be successfully detected by
599 steady shear rheological analysis. The temperature range that honey adulteration can be detected
600 using rheology is reported to be 5-20ºC. Honey is a viscous liquid that is non-elastic in nature
601 and possesses liquid-like behavior. The resistance to deformation of the adulterated honey is low
602 compared to the natural honey. Thus total resistance to deformation could be a good indicator for
603 adulterant (10-50%) detection in the honey. The authors indicate that with the inclusion of
604 adulterants in honey, the viscoelastic nature of the honey changes as the deformation is enlarged.
605 The authors suggest that viscosity is also a good indicator to detect fructose and saccharose
606 adulteration in honey. The researchers concluded that the adulteration between 0-50% level with
607 saccharose or fructose was detected by a change of creep, flow and viscoelastic behavior of the
608 pure honey (Yilmaz et al., 2014).
609 In summary, using thixotropicity honey adulterants such as carbohydrates; glucose, fructose and
610 saccharose syrups, could be detected using viscoelastic and flow behavior, change of creep, shear

stress, crystal formation, and nitrogen content. The storage time, temperature, and solubility are factors that effect in the detection of adulterants in honey. However, the sensitivity of the adulterant detection using viscoelastic behavior is questionable although it is good detecting the presence and absence of carbohydrate adulterants in honey. For quantification, more advanced detection methods are required. Further study is still needed to explore the thixotropicity of honey with other adulterants before selecting a method that could be considered feasible for a honey adulterant kit. Adulterants of fructose, saccharose syrup and glucose could be identified after developing a carbohydrate adulterant detection kit.

5. Conclusion

In conclusion, for honey adulterant detection there are many methods that have been developed. However, none of the methods are portable to use for on-site inspection. The methods used for honey adulterants include physiochemical analysis, microscopic methods, ELISA methods, rheological analysis, chromatographic methods, PCR, DNA-metabarcoding, sensors, and spectroscopic methods. The most promising methods among these for the development of a portable test kit are ELISA test kits, sensors such as an electronic tongue and NIR spectroscopy. These techniques may be very effective particularly if coupled with appropriate statistical analyses. These promising methods also need to be further researched for various honey adulterant detection and need to be miniaturized as portable honey adulterant detectors or kits, ideally compatible with smartphone technology. Convenience and growing applications through smart phones would suggest that in the future, some analytical tests could be conducted using this technology.

6. Abbreviations

Differential scanning calorimetry (DSC)

Fiber optic displacement sensor (FODS)

Electronic tongue (ET)

High-Performance Liquid Chromatography (HPLC)

Isotope ratio mass spectrometry (IRMS)

Nuclear magnetic resonance (NMR)

Polymerase Chain Reaction (PCR)

Fourier transform infrared spectroscopy (FTIR)

Gas chromatography-mass spectrometry (GC-MS)

Near Infrared Spectroscopy (NIR)

3-dimentional fluorescence spectroscopy (3DFS)

High-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD)

Thin layer chromatography (TLC)

Gas chromatography (GC)

649 Mid-infrared NIR transflectance spectroscopy

650 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

651 Food and Agriculture Organization (FAO)

652 Hydroxymethylfurfural (HMF)

653 High fructose inulin syrups (HFIS)

654 Invert syrups (IS)

655 Corn syrups (CS)

656 Liquid chromatography coupled to isotope ratio mass spectrometry (HPLC-IRMS)

657 Back propagation neural network (BP-ANN)

658 Dianhydrides of fructose (DFAs)

659 Beet Invert syrup (BI)

660 Principal component analysis (PCA)

661 Soft independent modeling of class analogy (SIMCA)

662 Partial least squares regression (PLS)/(PLSR)

663 Discriminant partial least squares (DPLS)

664 Partial least squares linear discriminant analysis (PLS-LDA)

665 Competitive adaptive reweighted sampling (CARS)-PLS-LDA

666 Error of cross-validation ($RMSE_{CV}$)

667 Wavelet transformation (WT)

668 Standard normal variate transformation (SNV)

669 Least square support vector machine (LS-SVM)

670 Support vector machine (SVM)

671 Back propagation artificial neural network (BP-ANN)

672 K-nearest neighbors (KNN)

673 Support vector machine discriminant analysis (SVMDA)

674 Interval Partial Least Squares (iPLS)

675 High-performance thin-layer chromatography (HPTLC)

676 High fructose corn syrup (HFCS)

677 Glucose syrup (GS)

678 Saccharose syrup (SS)

679 n-Decyl alcohol (DA)

680 Oleic acid (OA)

Dioctyl phosphate (Bis[2-ethylhexyl]hydrogen phosphate (DOP)

Trioctyl methyl ammonium chloride (TOMA)

Oleyl amine (OAm)

Artificial Neural Network (ANN)

Canonical Correlation Analysis (CCA)

Electronic tongue-Partial Least Squares Discriminant Analysis (ET-PLSDA)

Electronic tongue- Principal Component Analysis (ET-PCA)

Bidimensional zymography (2-DZ)

Isoelectric focusing (IEF)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Enzyme-linked immunosorbent assay (ELISA)

Human chorionic gonadotropin (hcG)

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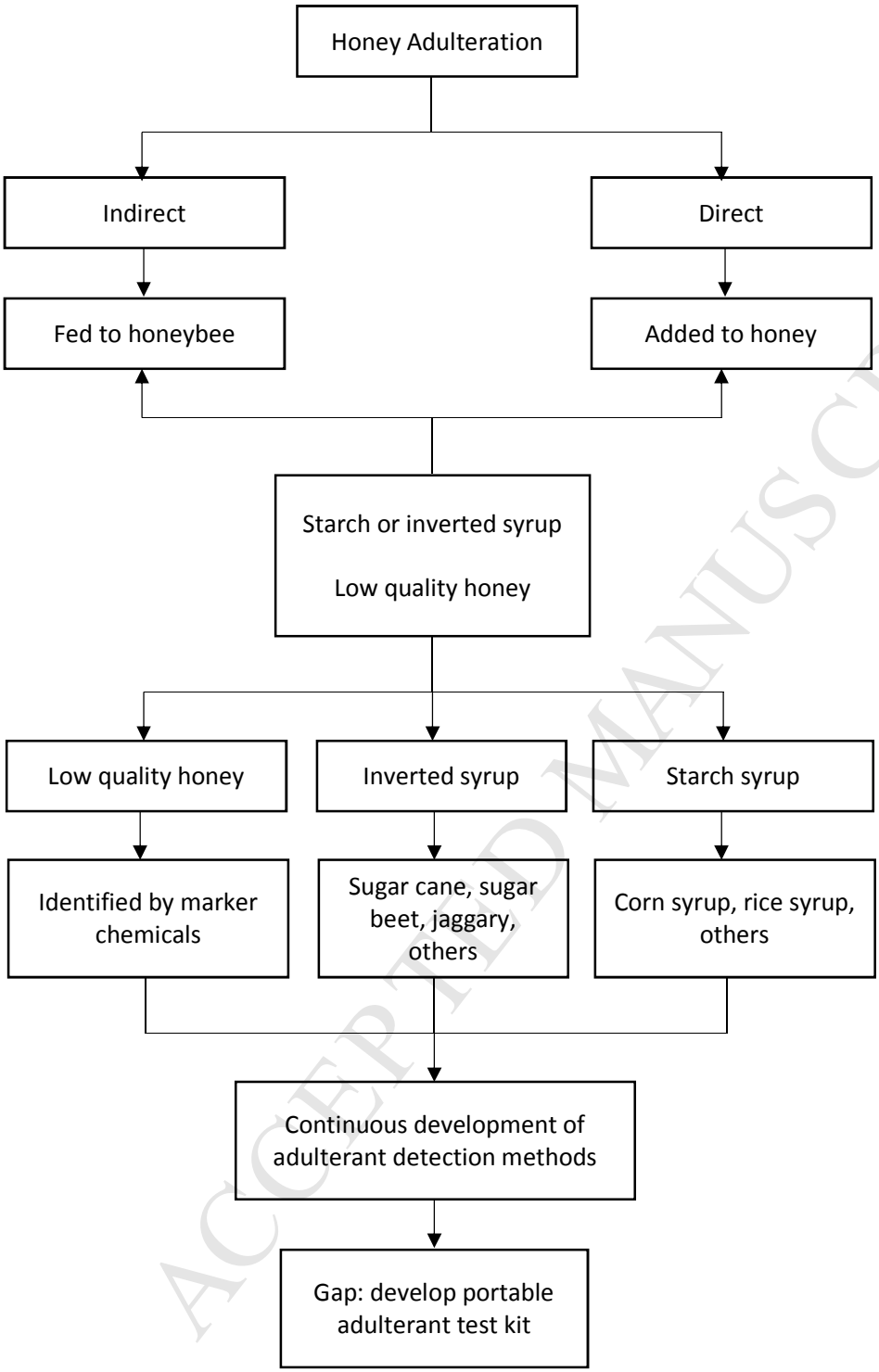

Figure 1. Honey adulteration and detection research gap

Table 1. Summary of potential adulterant detection methods as portable kits

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
NIR transflectance spectroscopy (sample scanned: 1100-2498 nm)	Beet invert syrup (BI) high fructose corn syrup (HFCS)	83	Irish honey and adulterant solution adjusted to 70° Brix with distilled water 18 honey samples adulterated; 8 with BI (7,10,14,21, 30,50, 70% w/w; n=56), 10 with HFCS (10, 30, 50, 70% w/w; n =40)	Raw spectra pre-treated: multiplicative scatter and second derivative spectra analyzed: unusual spectra detected using PCA model adulterants identified and quantified using SIMCA adulterants predicted using PLSR	Correlation coefficient of determination; BI = 0.79, HFCS = 0.72	Most accurate prediction; BI- with a multiplicative scatter correction pre-treatment, HFCS- second derivative calculated from pre-treatment	BI and HFCS: 20% w/w	After miniaturizing the equipment possible to develop a portable test kit	(J. D. Kelly, Petisco, & Downey, 2006)

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
FT-NIR spectrometer (sample scanned between 10,000-4000 cm^{-1})	HFCS, Maltose syrup (MS)	102	Natural and adulterated honey adjusted to 60 °Brix with distilled water honey adulterated with HFCS (n=180) and MS (n=180) at 10%, 20% and 40% w/w	PLS-LDA and CARS-PLS-LDA used to analyze data PLSR model predicted and quantified adulterated honey	Actual and predicted adulterant level at coefficient of determination of data sets ranged: 0.902-0.992 and 0.901-0.981, respectively	Accurate for adulterant detection; PLS-LDA-88.5% CARS-PLS-LDA 92%	NIR combined with CARS-PLS-LDA classified MS detection better compared to HFCS. The prediction for MS adulteration was satisfactory and non-satisfactory for HFCS	After miniaturizing the equipment possible to develop a portable test kit	(Li et al., 2017)

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
NIR Transflectance spectroscopy (1100– 2500 nm)	High fructose corn syrup (HFCS; 40% fructose, 33% glucose)	4	artifisanal Robinia honey adulterated with HFCS (0-40%; n =40)	PLSR model developed based on pre-treated spectra Leave-one-honey out cross-validation used to quantify adulterated honey	$RMSE_{CV}$ = 1.48 Coefficient of determination (R^2_{CV}= 0.987	1300-1800nm spectral intervals identified adulterants and water	NIR combined with aquaphotomic is satisfactory to use for adulterant detection	After miniaturizing the equipment possible to develop a portable test kit	(Bázár et al., 2016)

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
spectrometer NIR spectroscopy (XDS^{TM} Optipro be analyzer reflection type) with Chemometrics	Jaggery syrup	4	honey samples mixed with jiggery syrup at various ratios and total 160 spectra collected using the XDSTM Optiprobe	PLSR was used to build a calibration model	Calibration error = 0.00751	coefficient of determination (R^2) = 0.9924	The honey adulteration was predicted satisfactorily	After miniaturizing the equipment possible to develop a portable test kit	(Kumaravelu & Gopal, 2015)
NIR filter-based technique (NIR transflectance method at different wave length)	jaggery syrup	56	Indian honey adulterated at different ratio with jiggery syrup	PCA used to compress data PSLR model developed	adulterants predicted at standard error of calibration of 4.55	R^2 = 0.81	determined adulterants successfully	After miniaturizing the equipment possible to develop a portable test kit	(Mishra, Kamboj, Kaur, & Kapur, 2010)

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
NIR transflectance spectroscopy (10,000-4000 cm^{-1})	Fructose:glucose mixtures	68 authentic 67 adulterated Total 135 spectra	Natural and adulterated honey samples were set to 70 Brix adulterated honey samples prepared in distilled water at different level (7, 14, 21, and 28% w/w)	Spectra pretreated using SNV and WT Spectra data compressed using PCA and WT Five classical modeling used to detect adulterants: LS-SVM, SVM), BP-ANN, LDA and KNN	WT better in variable selection compared to PCA. Best model was LS-SVM. The recognition ratio of 95.2% and the area under the receiver operating characteristic curves (AUC) of 0.952 by WT-LSSVM model	LSSVM model is better in generalizing than others; SVM, BP-ANN, KNN, and LDA good accuracy (95.1%) and better generalization using WT-LS-SVM	WT-LS-SVM model suitable for the adulterant detection	After miniaturizing the equipment possible to develop a portable test kit	(Zhu et al., 2010)

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
sensors (Electronic Tongue, ET; a-Astree ET with seven potentiometric chemical sensors- with an Ag/AgCl standard electrode) and spectra (NIR- a FTNIR system; Mid Infrared spectrum, MIR- FT-IR equipped with an Attenuated Total Reflection (ATR)	Rice syrup Corn syrup	35 pure honey total 259 samples (105 pure and 154 adulterated honey)	Pure honey adulterated with syrup (5%, 10%, 20%, 40%) For ET: 120s for each evaluation and data recorded every 1s. Mean value recorded between 110-120 s. NIR: samples scanned at 10000-4000 cm^{-1} MIR: samples scanned at 4000-650 cm^{-1}	Adulterants determined using PLSDA and LS-SVM model -raw data pretreated; for sensor- SNV smoothing, auto-scale and derivatives Sensor and spectra (NIR, MIR) results optimized using SVMDA and iPLS data dimensions reduced using PCA	ET-PLSDA model; The total discriminant accuracy of calibration =98.43% prediction = 100% NIR: LS-SVM model total accuracy = 95.1%.	Total accuracy for calibration and prediction sets: above 96% in NIR, MIR and ET by PLSDA model.	ET is more suitable for detecting honey adulteration Spectra is more accurate than sensors Sensor-sample needed pretreatment but sensor better than traditional methods. ET, NIR and MIR successfully detected adulterants in honey.	Further work needed as few studies on ET use to detect adulterants and compared to sensors spectra are more accurate	(Gan et al., 2016)

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	precision	accuracy	Detection limit	Ability as portable test kit	References
Honey protein: ELISA kit developed using polyclonal anti-apalbumin1 antibody for immunochemical quantification of apa1 in honey	Marker: apalbumin1 (apa1), the major royal jelly (RJ) protein Adulterant: corn syrup, high-fructose corn syrup	N = 46	Honey samples vortexed at room temperature with water (1:1, w/v) for 5 min Diluted samples filtered through a 0.8 µm membrane filter to obtain Pollen-free honeys	honey and bee pollen contain RJ proteins of molecular mass from 3 to 90 kDa Western-blot analysis using polyclonal anti-apa1 antibodies	apa1 used as a protein standard for ELISA analysis apa1: 55 kDa protein with N-terminal amino acid sequence N-I-L-R-G-E =	Results is effected based on the substrate type	The limit of detection for apa1 was 2 ng mL^{-1} concentration of apa1 in honey below 50 µg g^{-1} would be indicative of the presence of industrial glucose syrups in honey or dilution of floral honey with the honey obtained by feeding the honeybee colony with sucrose syrup	more research on other adulterants need to be tested. Possible for the development of portable kit	(Bilikova, Kristof Krakova, Yamaguchi, & Yamaguchi, 2015)

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
Honey enzyme: detection of foreign amylase addition based on the comparison of diastase determination using the Schade and Phadebas procedures, Diastatic activity was determined spectrophotometrically by Schade and Phadebas method	Marker: activity of diastase (α-, β-, γ-amylase) Adulterants: sucrose, hydrolysed starch; HFCS- increase of adulterant decrease diastase number- addition of foreign amylases (eg bakery mould amylases) compensate this decrease and mask it	N =15	The model samples of honeys with addition of foreign amylase (Aspergillus oryzae) were analysed	hydrolytic activity is expressed in g of starch/100g honey when hydrolysed for 1h at 40C Adulterant detection is based on the substrate specificity of enzymes	Diastase number quantify the general enzyme activity in honey, expressed as amylase activity on standardised substrate. Amylase activity differ in substrate specificity selection of substrate is the principal factor that affect the result	Results for same honey differed in both methods and thus reliability is poor The results also differed within the results of Schade test- variability of substrate also observed	Diastase level varied in the samples and variation existed in both Schade and Phadebas method although in general there is a correlation between the results.	Potential method to make portable but further research required to improve the detection accuracy	(Voldřich, Rajchl, Čížková, & Cuhra, 2009)

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
DNA metabarcoding to detect indirect adulterants, and identify botanical and entomological origin of honey	Markers: gene regions; ITS2, rbcLa, COI Adulterants: indirect adulterant- low quality honey fed to bees	7	Honey prepared for DNA extraction DNA extraction PCR amplification Sequencing	Plant and insect sources identified in 5 samples Two samples only identified botanical or insect sources Two samples misrepresented	fail to identify when honey is rich in polyphenolic compound	honey crystallization effect the analysis	Satisfactory to identify plant and insect sources Detail study need to be done on only focusing to identify and quantify honey adulterants	Sample preparation tedious. Various field of research need to be joined to provide portable method for adulterant detection in honey- not easy	(Prosser & Hebert, 2017)

Detection technique	Adulterant marker	No of samples	Sample preparation	Measurement range	Precision	Accuracy	Detection limit	Ability as portable test kit	References
Thixotropicity: rheological analysis; rheometer, HPLC-RID	Fructose and saccharose syrups	Not stated	saccharose or fructose syrups mixed with water and adjusted to 75 ºBrix at 60°C adulterated honey prepared by mixing syrup with natural honey (0, 10, 20, 30, 40 and 50%, w/w). Then samples stirred and centrifuged at 2500 rpm for 3 min	Steady, dynamic and creep tests conducted using a stress or strain controlled rheometer equipped with a peltier system. sugar composition of adulterants analysed using HPLC-RID	Change in viscosity, flow and creep behavior of natural honey was clear adulterated honey showed decreased viscosity, storage, loss modulus values and obvious deformation	significant correlations were observed between sugar composition and rheology parameters Suggesting that these parameters could be prominent indicators for presence of saccharose or fructose syrups	Use of steady, dynamic and creep analysis satisfactorily detect adulterants	The equipments that test the rheological parameters need to be made portable and possibly made as one equipment testing all the parameters simultaneously to use this method. Currently not easy to apply	(Yilmaz et al., 2014)