# Chapter 32
# Nonaromatic Organic Acids of Honeys

**María Teresa Sancho, Inés Mato, José F. Huidobro, Miguel Angel Fernández-Muiño, and Ana Pascual-Maté**

## 32.1 Introduction

The composition of stingless bee (Meliponini) honey, also called pot-honey, has been researched since the 1960s (Gonnet et al., 1964 *apud* Souza et al. 2006). Despite having particular organoleptic properties and being highly appreciated in tropical areas, stingless bee honeys are not commonly available for purchase by consumers in most parts of the world.

Stingless bees have been widely studied by several researchers (Wille 1979; Kerr 1987; Camargo and Menezes Pedro 1992, 2007; Roubik 1995; Heard 1999; Michener 2000). As food commodities, some pot-honeys have been described as delicate and with delicious flavors (Kent 1984; van Veen et al. 1990), as well as honeys with sweet and sour flavors (Vit et al. 2010).

Many researchers have studied the physical and chemical properties of stingless bee honeys, as reviewed by Souza et al. 2006. With regard to acidity, scientists have reported that in general, pH of these honeys ranges from 2.0 to 4.7, whereas the values of free acid may be close to 200 meq/kg (Souza et al. 2006; Persano Oddo et al. 2008; Sgariglia et al. 2010). Although high values of free acid have been sometimes related to honey fermentation, the high acidity shown by stingless bee honeys has not been characteristically associated with spoilage of this food, and therefore, a high free acid could be a normal parameter of pot-honeys. In fact, several researchers have pointed out that an organic acids profile could be a better parameter than free acidity to determine *Apis mellifera* honey spoilage (Mato et al. 2006a).

M.T. Sancho (✉) • M.A. Fernández-Muiño • A. Pascual-Maté
Department of Biotechnology and Food Science, Faculty of Sciences, University of Burgos, Burgos, Spain
e-mail: mtsancho@ubu.es

I. Mato • J.F. Huidobro
Department of Analytical Chemistry, Nutrition and Food Science, Faculty of Pharmacy, University of Santiago de Compostela,Santiago de Compostela, Spain

P. Vit et al. (eds.), *Pot-Honey: A legacy of stingless bees*,
DOI 10.1007/978-1-4614-4960-7_32, 

Stingless bee honeys are included neither in the revised codex standard for honey (CODEX 2001) nor in the European council directive 2001/110/EC relating to honey (OJEC 2002). Current studies on this food are needed because these standards would provide the consumers with a guarantee of food safety and food control by responsible laboratories.

This chapter reviews the importance and methods of analysis of nonaromatic organic acids of honey, based mainly on data obtained for *Apis mellifera*, compared to *Tetragonula carbonaria* and *Melipona favosa*, as well as its relationship to other parameters of this food.

## 32.2 Importance of Nonaromatic Organic Acids in Honey

More than 30 different nonaromatic organic acids have been identified in honey (Mato et al. 2003), most of them added by bees (Echigo and Takenaka 1974). Along with the concentration of sugars and hydrogen peroxide, nonaromatic organic acids are responsible for the excellent resistance of honey against microbial spoilage (White 1979a). Gluconic acid is the predominant nonaromatic organic acid in honey (Stinson et al. 1960), instead of malic or citric acids as previously thought (Nelson and Mottern 1931). Gluconic acid in equilibrium with gluconolactone is present in all honeys, in concentrations much higher than others (White 1978). Besides gluconic acid, other nonaromatic organic acids commonly present in honey are malic, citric, lactic, succinic, fumaric, maleic, formic, acetic, oxalic, and pyruvic, among others (Mato et al. 2003). Malic acid was one of the first acids identified in honey (Hilger 1904) and has been usually considered the second in importance after gluconic acid (Cherchi et al. 1994). Citric acid is a tricarboxylic acid, and the relationship between the acid forms and salt depends on honey pH, total citric acid content, and citric acid dissociation constants (Mato et al. 2000). The content of citric acid has been considered potentially useful to differentiate between nectar and honeydew honeys (Talpay 1988).

Honey gluconic acid comes mainly from the action of bee glucose-oxidase on nectar or honeydew glucose. Part of this acid is also produced by *Gluconobacter* spp., bacteria that are common in a bee's gut and stay throughout the ripening of honey. In aerobic environments with high glucose concentrations, *Gluconobacter* spp. microorganisms produce large amounts of gluconic acid (Ruiz-Argüeso and Rodríguez-Navarro 1973). The variation in the amounts of gluconic acid depends on the time required to completely transform the nectar or honeydew into honey; the longer it is, the greater the addition of glucose oxidase by the bee, and the greater therefore the amount of gluconic acid. Other factors that also influence the process are the strength of the colony and the quality and quantity of nectar coming into the hive (White 1979b). The origin of the other nonaromatic organic acids in honey is not fully known. They may come directly from nectar or honeydew, and some of them are produced from nectar and honeydew sugars by the action of enzymes secreted by worker bees and added to honey at ripening (Echigo and Takenaka 1974). Many honey nonaromatic organic acids are intermediates of such enzymatic

pathways as Krebs cycle and others, being oxidized throughout the mentioned pathways (Echigo and Takenaka 1974; White 1979b; FAO 1990).

Honey organic acids have been proposed as potentially useful to characterize the botanical and geographical origin of honeys (Steeg and Montag 1988; Talpay 1989; Cherchi et al. 1994; Anklam 1998; Del Nozal et al. 1998; Mato 2004; Kaskoniene and Venskutonis 2010). 2-Methoxybutanedioic and 4-hydroxy-3-methyl-*trans*-2-pentenedioic acids were described as possible markers of *Knightia excelsa* (Proteaceae) honeys (Wilkins et al. 1995). In *Erica* sp. (Ericaceae) honeys, *cis*,*trans*-abscisic acid and *trans*,*trans*-abscisic acid (Ferreres et al. 1996), as well as high concentrations of quinic acid (Del Nozal et al. 1998), were found as possible markers, being the concentrations of *cis*,*trans*-abscisic acid about ten times higher than those found in honeys of other botanical origins (Gheldof et al. 2002). Low concentrations of pyruvic acid and high quantities of both malic and succinic acid were typical of *Quercus* sp. (Fagaceae) honeys, whereas high citric acid concentrations were described as a possible marker of *Thymus* sp. (Lamiaceae) honeys (Del Nozal et al. 1998). In *Castanea sativa* (Fagaceae) honey, high levels of formic acid were found, contrary to the low levels of formic acid described in *Eucalyptus* spp. (Myrtaceae) honey (Suárez-Luque et al. 2006).

Acetic acid has been proposed as possible indicator of honey fermentation, when its levels are excessively high (Mato et al. 2003). Such osmophilic yeasts as *Saccharomyces* spp., *Zygosaccharomyces* spp., *Torula* spp. and others, produce alcohols and eventually organic acids from honey sugars (Gonnet 1982). These yeasts come from flowers, soil, air, or the equipment used for honey extraction and processing, and are very sensitive to heat, so many companies pasteurize their honeys in order to prevent fermentation (Piana et al. 1989). For unpasteurized honeys, the possible usefulness of nonaromatic organic acid profile as a fermentation indicator should be researched (Mato et al. 2003).

Among other parameters such as phenolics, peptides, aminoacids, Maillard reaction products and enzymes, and nonaromatic organic acids, also contribute to antioxidant capacity observed in honeys (Gheldof et al. 2002). Such honey organic acids as citric, malic, and others act as metal ion chelators, and are considered as synergists of primary antioxidants enhancing antioxidant activity (Gheldof et al. 2002; Wanasundara and Shahidi 2005).

There is evidence that some acidic components of honey show antibacterial activity (Russel et al. 1988; Wahdan 1998). Acidic substances identified to date as antibacterial in honeys are mainly aromatic organic acids; such as ferulic and caffeic acids (Wahdan 1998), benzoic acid derivatives (Russel et al. 1988; Weston et al. 1999), and acids of royal jelly (Isidorov et al. 2011). Possible relationships between honey acidity and antibacterial activity have been studied, as well as between honey pH and antibacterial activity (Yatsunami and Echigo 1984; Bogdanov 1997). Honey antibacterial activity was significantly correlated with free acid and total acidity, showing the acidic fraction of several honeys with the greatest non-peroxide antibacterial activity (Bogdanov 1997; Kirnpaul-Kaur et al. 2011). In an acidic medium, honeys show better antibacterial activity (Bogdanov 2011).

Stingless bee honeys have been used in traditional and Mesoamerican aboriginal medicine (Vit and Tomás-Barberán 2004; Vit et al. 2004; Sgariglia et al. 2010).

Pot-honeys show high free acid values, and antibacterial activity is found in them by many scientists (DeMera and Angert 2004; Dardon and Enríquez 2008; Irish et al. 2008; de Almeida et al. 2009; Rodríguez-Malaver et al. 2009; Vit et al. 2009a; Boorn et al. 2010; Sgariglia et al. 2010). Therefore, it would be very interesting to study antibacterial activity of stingless bee honeys in relation with their levels of organic acids.

## 32.3 Honey Components and Parameters Related to Nonaromatic Organic Acids

Honey contains less than 0.5% of organic acids. Nevertheless, they are a group of constituents that contribute to several properties of this food, such as its color, aroma, taste, pH, acidity, and, to a lesser extent, electrical conductivity.

Color is an optical property of honey, described as the result of different degrees of absorption of light at different wavelengths by honey compounds (FAO 1990). The color of honey varies widely, from nearly colorless to almost black. This variability depends heavily on its origin and thus on its composition. Dark honeys tend to have higher acidity and higher organic acids contents (White 1979b; Crane 1990) than light honeys.

Aroma and flavor of honey are mainly due to a complex mixture of substances that are highly dependent on the botanical origin, but also influence the processing and storage conditions of this food (Anklam 1998). Among these substances organic acids are important, in particular for the taste of honey (Louveaux 1985; Crane 1990; Bogdanov 2009).

Honey acidity depends mainly on the presence of organic acids (White 1979b). Lactones are internal esters of organic acids and do not contribute to honeys' active acidity (Bogdanov 2009). Lactones hydrolyze over time, therefore increasing honey free acid. Total acidity is the sum of free acid and lactones. Honey pH depends on the amount of ionized acids, as well as the content in such minerals as potassium, sodium and calcium (White 1979b). Small oscillations in the range of pH in relation to the large swings in the free acid values were attributed to the buffer properties of honey, due to such mineral salts as phosphates, carbonates and others (Bogdanov 2009).

Electrical conductivity is a physical property of honey mainly related to the content of mineral salts, and to a lesser extent to the content of organic acids, proteins, sugars, and polyols (Crane 1990). It was found that the electrical conductivity was directly proportional to ash content and acidity of honey (Vorwohl 1964).

## 32.4 Methods of Analysis of Nonaromatic Organic Acids in Honey

The most important and frequently employed methods to determine honeys' nonaromatic organic acids are enzymatic assays, chromatographic techniques, and electrophoretic procedures (Mato et al. 2006b). Enzymatic assays are based on spectrophotometric

measurements, usually at 340 nm, of the increase or decrease in absorbance of the reduced form's coenzymes nicotinamide adenine dicucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH), after the reaction of organic acids with specific enzymes. Enzymatic methods are precise and accurate. In addition, their specificity is excellent, allowing quantification of the D/L isomers of several organic acids. Furthermore, enzymatic procedures require very simple equipment, normally available in every quality control laboratory. Unfortunately, the stability of the enzymatic kits is not very long, and enzymatic procedures are tedious and time-consuming, allowing the determination of only one organic acid each time. Enzymatic analyses were commonly used to determine nonaromatic organic acids in *Apis mellifera* honeys (Tourn et al. 1980; Stoya et al. 1986, 1987; Hansen and Guldborg 1988; Talpay 1988, 1989; Sabatini et al. 1994; Mato et al. 1997, 1998a, b; Mutinelli et al. 1997; Cossu and Alamanni 1999; Alamanni et al. 2000; Bogdanov et al. 2002; Gheldof et al. 2002; Pulcini et al. 2004; and Vit et al. 2009a, b, among others). In respect of honeys produced by stingless bees, total D-gluconic, citric, and L-malic acids were quantified enzymatically in honeys from Australian *Tetragonula carbonaria* (Persano Oddo et al. 2008) and Venezuelan *Melipona favosa*.

Organic acids of honeys have been widely determined by chromatographic techniques. At first, these compounds were analyzed by paper and on-column ion exchange chromatography (Stinson et al. 1960). Gas chromatography–mass spectrometry (GC-MS) and gas chromatography–flame ionization detector (GC-FID) were applied to analyze honey nonaromatic organic acids with a previous derivatization process, due to the fact that most of these acids are not volatile (Echigo and Takenaka 1974; Wilkins et al. 1995; Horváth and Molnár-Perl 1998; Pilz-Güther and Speer 2004; Sanz et al. 2005), albeit recently, 29 organic acids were analyzed by GC-MS in honeys and other food commodities, using a procedure based on continuous solid-phase extraction without prior derivatization (Jurado-Sánchez et al. 2011).

Many researchers analyzed honey nonaromatic organic acids by high-performance liquid chromatography with ultraviolet detection (Cherchi et al. 1994, 1995; del Nozal et al. 1998, 2003a, b; Alamanni et al. 2000; Suárez-Luque et al. 2002a, b; Serra-Bonvehí et al. 2004; Hrobonová et al. 2007), although ionic chromatography with conductivity detection was also used to determine some nonaromatic organic acids in honeys (Pérez-Cerrada et al. 1989; Defilippi et al. 1995; del Nozal et al. 2000), as well as anionic exchange chromatography with UV detection (del Nozal et al. 1998) or constant voltage amperometric detection (Casella and Gatta 2001). Liquid chromatographic methods allow the simultaneous determination of several organic acids, showing a good versatility, reproducibility, and sensitivity. However, there are many interferences that must be removed by pretreatment of honey samples, or by using several columns in series, thus liquid chromatographic methods to determine honey nonaromatic organic acids are tedious and time-consuming.

Capillary electrophoresis with ultraviolet detection is another method that was successfully employed to quantify nonaromatic organic acids in honeys (Boden et al. 2000; Navarrete et al. 2005; Mato et al. 2006a; Suárez-Luque et al. 2006). Capillary electrophoresis is a rapid and low cost procedure that allows the simultaneous determination of several nonaromatic organic acids with a very simple preparation of the honey sample. The drawbacks of this method, if compared with

other procedures, are its lower reproducibility and sensitivity. Nevertheless, capillary electrophoresis is a very promising technique that should be intensively studied for future analysis of honey compounds. Its application to analyze non-aromatic organic acids of pot-honeys could contribute to their characterization, which would be very interesting to promote and improve the commercialization of stingless bee honeys.

## 32.5 Nonaromatic Organic Acids in Pot-Honey

The content of D-gluconic, L-malic, and total citric acids was analyzed in eight samples of pot-honey produced by *Tetragonula carbonaria*, (Persano Oddo et al. 2008, as *Trigona carbonaria*, but see Rasmussen and Cameron 2007), and seven samples of *Melipona favosa* from Venezuela (Fig. 32.1). In all these pot-honeys, the quantities of L-malic and total citric acids were in general similar to those of *Apis mellifera* honeys described in the literature. As usual, D-gluconic acid values were one thousand times higher than L-malic and total citric acid concentrations. The quantities of D-gluconic acid in *Trigona carbonaria* honeys were in the same range of levels of D-gluconic acid of *Castanea* sp., *Thymus* sp., *Arbutus* sp. and honeydew honeys from *Apis mellifera* (Pulcini et al. 2004). The values of D-gluconic acid were about ten times higher in *Melipona favosa* samples (Fig. 32.1a), which might be indicative of a very high glucose oxidase activity at ripening (Persano Oddo et al. 2008), and could contribute to characterize *Melipona favosa* pot-honeys. Conversely, the concentrations of both L-malic and total citric acid were about ten times lower in honeys from *Melipona favosa* than in samples from *Trigona carbonaria* (Fig. 32.1b, c). It is interesting to highlight the fact that the *Melipona favosa* honey (sample 2) with the highest quantities of both L-malic and citric acid was the sample with the lowest concentration of D-gluconic acid. In contrast, the *Melipona favosa* sample with the lowest value of citric acid was the sample with the highest quantity of D-gluconic acid. In pot-honey from *Trigona carbonaria* it was observed that, in general, samples with the highest contents of D-gluconic acid contained the lowest quantities of total citric acid and vice versa. Most studies of pot-honey characterized the honey produced by different bee species of stingless bees (Vit et al. 1994; Souza et al. 2006; Persano Oddo et al. 2008; Sgariglia et al. 2010). It should be very interesting to research the nonaromatic organic acid profiles of these honeys, of particular interest the possible identification of the acid(s) responsible for the high free acid of pot-honey.

**Acknowledgments** To Dr. Tim Heard from CSIRO, Brisbane Australia for providing the *Tetragonula carbonaria* honey, and for English proof reading of the manuscript. To Prof. Patricia Vit from the Food Science Department, Faculty of Pharmacy and Bioanalysis, Universidad de Los Andes, Mérida, Venezuela, for providing the samples of *Melipona favosa* honey, and editorial care. To Prof. João MF Camargo from the Biology Department, Universidade de São Paulo, Ribeirão Preto, Brazil, for the identification of the *Melipona favosa* bee.

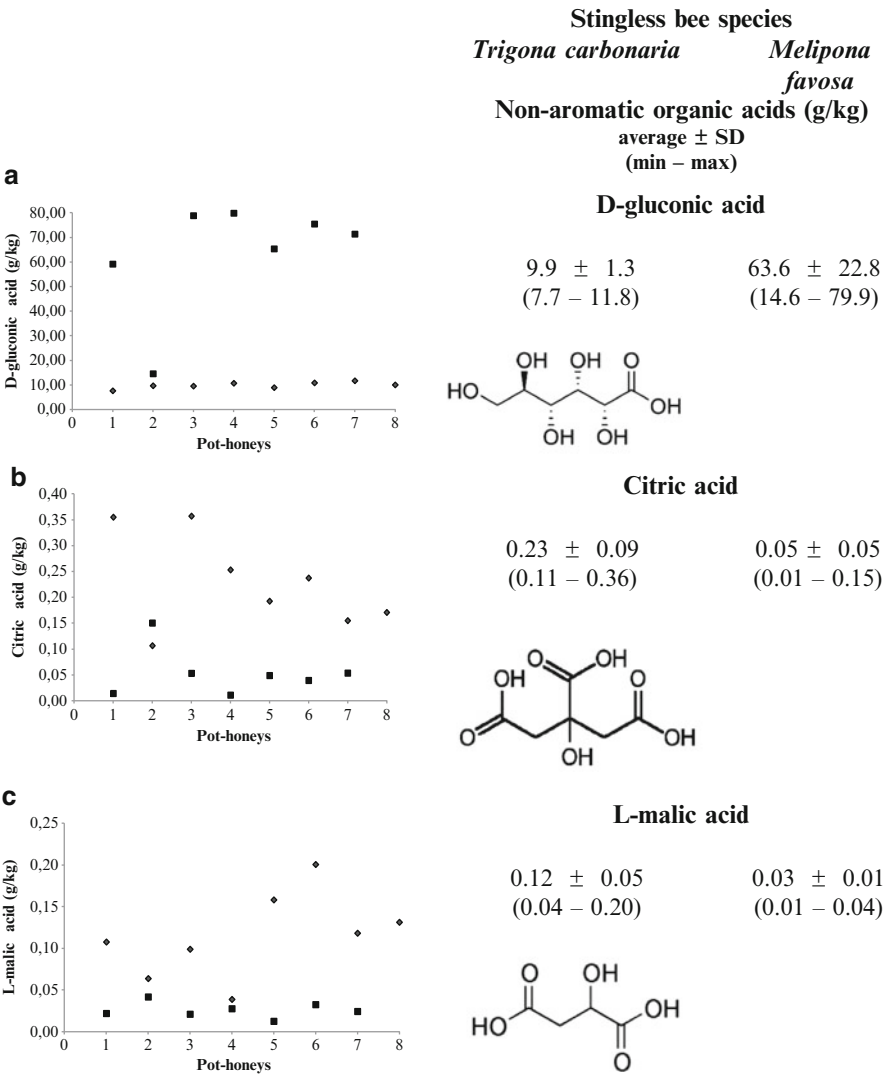


**Fig. 32.1** Content of nonaromatic organic acids in pot-honey. (**a**) D-Gluconic acid, (**b**) citric acid, and (**c**) L-malic acid contents in pot-honey of *T. carbonaria* (*filled diamond*) from Australia and *M. favosa* (*filled square*) from Venezuela

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