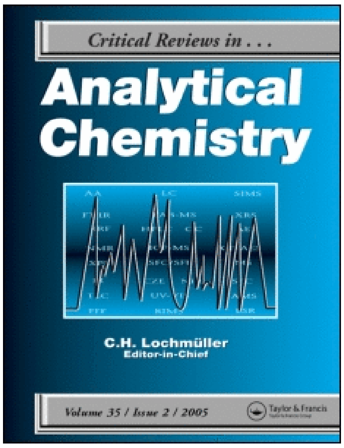

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

Analytical Methods for the Determination of Organic Acids in Honey

Inés Mato[a]; José F. Huidobro[a]; Jesús Simal-Lozano[a]; M. Teresa Sancho[b]
[a] Facultad de Farmacia, Departamento de Química Analítica, Nutrición y Bromatología, Área de Nutrición y Bromatología, Universidad de Santiago, Galicia, Spain [b] Facultad de Ciencias, Departamento de Biotecnología y Ciencia de los Alimentos, Área de Nutrición y Bromatología, Universidad de Burgos, Burgos, Castilla y León, Spain

To cite this Article Mato, Inés , Huidobro, José F. , Simal-Lozano, Jesús and Sancho, M. Teresa(2006) 'Analytical Methods for the Determination of Organic Acids in Honey', Critical Reviews in Analytical Chemistry, 36: 1, 3 — 11

To link to this Article: DOI: 10.1080/10408340500451957

URL: http://dx.doi.org/10.1080/10408340500451957

Critical Reviews in Analytical Chemistry, 36:3–11, 2006

ISSN: 1040-8347 print / 1547-6510 online
DOI: 10.1080/10408340500451957

REVIEW

Analytical Methods for the Determination of Organic Acids in Honey

Inés Mato, José F. Huidobro, and Jesús Simal-Lozano
Facultad de Farmacia, Departamento de Química Analítica, Nutrición y Bromatología, Área de Nutrición y Bromatología, Universidad de Santiago, Galicia, Spain

M. Teresa Sancho
Facultad de Ciencias, Departamento de Biotecnología y Ciencia de los Alimentos, Área de Nutrición y Bromatología, Universidad de Burgos, Burgos, Castilla y León, Spain

Although organic acids represent less than 0.5% of honey's constituents, they make important contributions to organoleptic, physical, and chemical properties of honey. They could be used as fermentation indicators, for the treatment of Varroa infestation, and to discriminate among honeys according to their botanical and/or geographical origins. This article reviews the current literature related to the analytical methods (enzymatic, chromatographic and electrophoretic) that have been applied recently to the determination of honey's organic acids. The advantages and disadvantages of all the procedures described are also discussed. This review has been written to make the study of these interesting honey component easier.

Keywords organic acids, honey, analytical method review

Although honey is comprised of organic acids in a small proportion (they only amount to less than 0.5%), the study of these components is interesting because they make important contributions to both its organoleptic properties, such as color and flavor, and to its physical and chemical properties, such as pH, acidity and electrical conductivity (1, 2). They also have antibacterial and anti-oxidant activities; together with other substances (3–5), they could be used as fermentation indicators, or for the treatment of Varroa infestation (6–8). They could also be deployed to discriminate between honeys according to their botanical and/or geographical origins (9–12).

Despite their increasing importance in honey, there is relatively little information available about these organic acids and their analysis. In a previous article, the current literature related to the significance of non-aromatic organic acids in honey was reviewed (13). Therefore, we have also decided to comment the current analytical methods that allow the determination of honey organic acids. In this review, enzymatic, chromatographic and electrophoretic methods have been presented to identify and quantify the most important organic acids in honey, individually or as a group simultaneously. The advantages and disadvantages of all the procedures described are also discussed.

ENZYMATIC METHODS

Enzymatic methods have been used, mainly, for the quantification of citric, malic, formic, D- and L-lactic, oxalic and total D-gluconic acids in honeys. However, it would be possible for the determination of other acids such as acetic, L-ascorbic and succinic acids (14).

All enzymatic methods are based on the measurement of the increase or decrease in absorbance of the coenzymes NADH (nicotinamide-adenine dinucleotide, reduced form) or NADPH (nicotinamide-adenine dinucleotide phosphate, reduced form), which absorb light in the long wavelength region, generally at 340 nm.

Tourn et al. (15) applied enzymatic tests, without previous clarification, to quantify citric and malic acids in 5 samples of honey (3 of honeydew, and 2 of multifloral honeys) and 1 sample of royal jelly. According to these authors, the enzymatic procedure showed noticeable advantages with respect to other methods, because of its specificity, sensitivity, rapidity and simplicity. These authors provided neither data of precision nor of recovery of their proposed enzymatic procedure.

Address correspondence to José F. Huidobro, Facultad de Farmacia, Departamento de Química Analítica, Nutrición y Bromatología, Área de Nutrición y Bromatología, Universidad de Santiago, 15782 Santiago de Compostela, Galicia, Spain. Fax: +34 81 594612. E-mail: qnhuidob@usc.es

Stoya et al. (16) determined formic acid content by applying an enzymatic analysis to honeys coming from hives untreated against varroase (natural content of formic acid in honey) and to honeys coming from hives that had been treated with formic acid against Varroa infestation (residual formic acid, after treatment). In this paper, only data of recovery are shown, with an average recovery percentage of 96.0 ± 6.5%. These researchers concluded that honeys from treated beehives contained from 54 to 82 times more formic acid than honeys from untreated beehives. Those levels of formic acid decreased asymptotically with time, so that between 5 to 8 months after the treatment, the levels of formic acid in honeys from treated beehives were more or less similar to those found in honeys from untreated beehives.

Stoya et al. (17) determined D-(−) and L-(+)-lactic acid contents by using an enzymatic method both in honeys untreated against varroase (natural content of these acids) and in honeys coming from hives that had been treated with D-(−) and L-(+)-lactic acids against Varroa infestation (residual acids, after treatment). In this paper quotients between both isomers (D-(−)/L-(+)) are shown for each sample. In the vast majority of samples, the mentioned quotient was lower than 3, although there were honeys with a quotient higher than 20. Average recovery percentage for D-(−)-lactic acid was of 83 ± 13%, and for L-(+)-lactic acid was of 96 ± 6%. These researchers found that in honeys coming from treated hives, only L-(+)-lactic acid contents were increased 10 times in comparison with normal levels. In contrast, D-(−)-lactic acid levels in honeys from treated and untreated beehives were similar. About 7 or 8 weeks after treatment, L-(+)-lactic acid levels decreased asymptotically to usual levels.

Hansen and Guldborg (18) analyzed formic acid contents of Danish honeys. The detection limit was 5–10 mg formic acid/kg honey. No additional validation data was shown. Some samples came from hives untreated against varroase, and other samples came from hives that did not suffer from varroase but were treated with formic acid, and harvested 8–9 months after treatment. These researchers concluded that after this time, formic acid levels in honeys from treated beehives were similar to the natural levels in honeys from untreated beehives.

Talpay (19) quantified citric acid contents in 153 floral honeys, 97 honeydew honeys, and 18 samples coming from bees that had been artificially fed with sugar. The enzymatic test was applied directly, obtaining a precision with coefficients of variation (RSD %) between 1.61% and 1.72% and a recovery percentage of 92.0 ± 6.8%. In this work, the author suggested that maybe citric acid content might be useful to distinguish between floral, honeydew honeys, and sugar-fed honeys. Citric acid levels were significantly lower in floral honeys than in honeydew honeys. However, no differences were found between honeys from floral origin and from artificial sugar-fed origin.

Talpay (20) determined formic acid content of 306 honeys from different botanic and geographic origins. The enzymatic test that was used showed a precision with a coefficient of variation (RSD %) of 4.09%, and a percentage of recovery of 97.6 ± 1.7%. This author concluded that chestnut (*Castanea sativa*), eucalyptus (*Eucalyptus* sp.), ling heather (*Calluna* sp.), and bell heather (*Erica* sp.) honeys, in this decreasing order, were markedly different from honeydew and other floral honeys in relation to the formic acid content. No statistical analysis was performed.

Sabatini et al. (21) employed enzymatic tests to determine formic, D-lactic and L-lactic acids contents in 42 unifloral honeys from 7 different botanical sources. These authors highlighted that the honey solution was previously clarified with activated charcoal to minimize the influence of honey color and to remove certain instability of the spectrophotometric measurement. No result of both precision and recovery was given. The conclusion of this work was that, with regard to formic acid contents, there were important differences between light and dark honeys.

Mato et al. (22) set up an enzymatic micro-method to determine total D-gluconic acid in honey, which includes free D-gluconic acid and D-glucono-δ-lactone, because the latter has not been able to be quantified separately. To be sure of the complete decomposition of D-glucono-δ-lactone, these authors took honey solution to a pH of 10.5 for 10 minutes at room temperature. Afterwards, the pH was dropped to 7.8, which is the optimum pH for enzymatic activity. This method combines precision (RSD% ≤0.30%), good recovery (99.8 ± 0.40%), specificity, simplicity and low cost. Gheldof et al. (5) have applied this method for studying the relationship between anti-oxidant activity and gluconic acid (as an indicator of organic acid of organic acid concentration). In this paper, no significant correlation between gluconic acid level and anti-oxidant activity was detected. This method was also used by Pulcini et al. (23), who analysed total D-gluconic acid of 112 Italian unifloral honeys. In these honeys, the precision of results (RSD%) were between 0.59% and 3.93% and mean recovery was 97.9 ± 1.12%).

Mutinelli et al. (7) determined honey's oxalic acid content by using an enzymatic method. Detection limit was 3 mg oxalic acid/kg honey. No additional validation data were shown. In this work, the researchers analysed the levels of oxalic acid in honeys from untreated beehives and from treated beehives against Varroa infestation with oxalic acid. No statistical differences were found between pre- and post-treatment content of oxalic acid.

Mato et al. (24) applied an enzymatic micro-method for the direct determination of L-malic acid of honey, studying both precision and accuracy. RSD% was ≤3.5% and the average recovery was 100 ± 3.5%. Furthermore, these authors carried out several assays for the clarification of the samples by using different clarifying agents: polyvinylpolypyrrolidone [PVPP], Carrez, Carrez with NaOH, Carrez with KOH, Carrez with polyvinylpolypyrrolidone [PVPP], and activated charcoal, but no improvement of precision and recovery was observed.

Mato et al. (25) developed an enzymatic specific micro-method for honey's citric acid, by using an UV-test with previous clarification of the sample with polyvinylpolypyrrolidone (PVPP). In this work, authors compared precision and accuracy of the proposed procedure (with clarification) in relation to those of the direct analysis, that is to say, without any clarification. Authors concluded that precision and accuracy of the method with PVPP clarification (precision between 0.26 and 1.60%, and recovery between 98.0 and 100.9%) were better than the results of the direct method (precision between 1.02 and 2.66%, and recovery between 84.0 and 115.6%).

Cossu and Alamanni (26) adapted to honey an enzymatic method widely used for the analysis of oxalate in urine, by determining oxalic acid in 40 Sardinian (Italy) honeys. Samples were previously clarified with activated charcoal. The detection limit for oxalic acid was 0.40 mg/L and the quantification limit was 1.33 mg/L. Average recovery percentage was 90.8 ± 1.0%. Alamanni et al. (27) analyzed oxalic acid in 49 floral honeys by applying the enzymatic method set up by Cossu and Alamanni (26). Results were compared with those obtained by using a HPLC method set up by these authors for the simultaneous determination of oxalic, formic and lactic acids with previous solid-phase extraction (SPE) of the samples' acids. Alamanni et al. (27) claimed that there was a good correlation between the results obtained by both methods, enzymatic and chromatographic. The authors specified that enzymatic quantification of oxalic acid in tree strawberry (*Arbutus unedo*) honeys was not possible due to the fact that there was a parallel uncontrollable reaction, which prevented absorbance stabilization.

Bogdanov et al. (28) determined formic and oxalic acid contents in honey by applying the enzymatic test used for oxalic acid analysis, which allows quantifying both acids in the same sample. Coefficients of variation (RSD %) ranged between 2.9 and 4.4% for formic acid, and between 13.4 and 18.4% for oxalic acid. Recovery for formic acid was of 93 ± 4%, whereas for oxalic acid was of 84 ± 24%. Specific detection limits were not shown for both determinations, because authors had pointed out that these limits ranged between 3 and 5 mg/kg. In this work, Bogdanov et al. (28) found a small, but significant, increase of formic acid in honey after a 7-day treatment of hives with formic acid. Increases were similar throughout the different years of analysis, so that the authors thought that formic acid remains in the sugar feed in the brood combs. According to these authors, these residues were not problematic, because they had no influence on the quality of honey. On the contrary, they found that oxalic acid content remained unchanged, even after two successive treatments during the same autumn. No rise of free acidity was found after combining a treatment with formic and oxalic acids throughout the 3 years of assays.

The main advantages of enzymatic methods include high specificity that even allows the determination of D/L isomers of some organic acids. In addition, the equipment and materials necessary for enzymatic analysis are very simple and available in the vast majority of honey quality control laboratories. Enzymatic procedures could also be considered excellent as reference methods, but in this case, more precision and accuracy studies would be necessary, because several researchers point out nothing about these studies. And, the precision and recovery results of other authors are not good enough as they would be expected with enzymatic methods. The main disadvantage of enzymatic tests is that they allow the determination of only one acid, so that if an organic acid profile of a given honey is necessary to characterize the sample botanically or geographically, the enzymatic analysis would be tedious and time consuming. Another disadvantage is the stability of these kits, which is not very long, plus the assays must be carried out in a few days.

CHROMATOGRAPHIC METHODS

The chromatographic methods developed for the determination of organic acids in honey samples have been divided into three groups: paper and on-column ion exchange chromatography, gas chromatography and liquid chromatography.

Paper and On-Column Ion Exchange Chromatography

Stinson et al. (29) first reported a separation and quantification of several organic acids simultaneously in honey samples (butyric, acetic, formic, lactic, succinic, pyroglutamic, malic, citric and gluconic acids) by using chromatographic methods as paper or on-column ion exchange chromatography.

Gas Chromatography (GC)

Speer and Montag (30, 31) quantified phenylacetic and benzoic acids in 32 honeys of different botanical origin by using GC-FID. These acids were extracted from honey with ethyl acetate. The method recoveries were 85.9% for phenylacetic acid and 97% for benzoic acid. Heather honeys contained the higher amounts of both phenylacetic and benzoic acids.

Steeg and Montag (32–34) determined the content of 24 aromatic organic acids as free acids in honeys from different floral sources. These acids as their trimethylsilyl derivatives were analyzed by a GC-MS method after extraction with ethylacetate. Benzoic, β-phenylactic, 4-hydroxybenzoic and 4-hydroxyphenylacetic acids were found in all samples. The authors found differences in the content of the aromatic organic acids according to the floral source of honey. For example, heather honeys could be identified by the presence of a high concentration of benzoic acid, phenylacetic acid, mandelic acid and β-phenylactic acids. The differentiation of honeydew and floral honeys were also possible because of the difference in the concentration of protocatechuic acid.

Echigo and Takenaka (35) used a gas-chromatographic (GC) method for the determination of 10 honey organic acids: oxalic, malonic, succinic, fumaric, malic, α-ketoglutaric, tartaric, cis-aconitic, citric and gluconic acids in about 45 minutes. Because of the fact that these acids are hardly volatile, it was necessary to obtain their trimethylsilyl derivatives.

Wilkins et al. (10) identified 32 honey dicarboxylic aliphatic acids as methyl esters, analyzing them by gas-chromatography and mass spectrometry detection (GC-MS). Then, 18 acids were quantified, among them malic and succinic acids. Methodology, setting up extraction, derivatization and chromatographic analysis had been previously described by Tan et al. (36) for the determination of different compounds, such as hydrocarbons (C_{21}-C_{33}), aromatic acids, diacids and linear chain fatty acids (C_8-C_{28}). Two aliphatic dicarboxylic acids (2-methoxybutanedioic and 4-hydroxy-3-methyl-trans-2-pentenedioic) were identified in *Knightea excelsa* honeys. After analyzing more than 200 New Zealand honeys from different botanical origins, these two acids were only found in honeys with an important contribution of pollen from Knightea excelsa, so that according to the researchers, 2-methoxybutanedioic and 4-hydroxy-3-methyl-trans-2-pentenedioic acids could be considered as markers of *Knightea excelsa* honeys.

Some methods, originally developed for the determination of carbohydrates in honeys, can be used for the analysis of some acids. Horváth and Molnár-Perl (37) developed a GC-MS procedure for the simultaneous determination, as trimethylsilyl derivatives, of major honey constituents (sugars) and minority compounds (such as organic acids).This method allowed quantifying 5 aliphatic carboxylic acids (malic, citric, isocitric, succinic and quinic acids) and 4 aromatic acids (benzoic, 4-hydroxibenzoic, 2,4-dihydroxibenzoic and 2,6-dihydroxibenzoic acids). Sanz et al. (38) determined quinic or gluconic acids by using a GC-FID and GC-MS methods. A derivatization process was also necessary for the detection of these acids which was analysed as trimethylsilyl derivatives.

Verzera et al. (39) set up a GC-MS method with previous solid phase microextraction (SPME) that allowed the identification of 113 volatile compounds in 15 honeys from different botanical sources coming from Sicily (Italy). Aliphatic organic acids, such as acetic acid, and aromatic acids, such as benzoic acid, were found among the volatile compounds. According to these researchers, their SPME-GC-MS method was simple, rapid, easily reproduced, and provided useful information for the determination of the floral source of honey. However, in this paper, no result about the quantification of these compounds was given.

Pilz-Güther and Speer (40) developed a GC-FID to analyze different organic acids like D,L-lactic, citric, succinic acid, and L-malic acids simultaneously in honey after clean-up by a SAX-cartridge. As occurs in others GC methods, it was necessary to carry out a derivatization process. Citric and malic acid concentrations obtained by this method are compared with the results obtained by the enzymatic method, and there were not statistically significant differences between both groups.

Gas chromatography is greatly adequate to determine volatile organic acids, such as aromatic acids, which are identified together with other compounds of honey flavor. Nevertheless, when the analysis of aliphatic organic acids is necessary, gas chromatography does not seem to be the most suitable technique, because the vast majority of these acids are not volatile, thus, making it necessary to derivatize them previously.

Liquid Chromatography (HPLC, IC)

Pérez-Cerrada et al. (41) developed a method to determine inorganic anions such as chloride, phosphate and sulphate by ionic chromatography (IC) with conductivity detection that also allowed determining malic, tartaric and oxalic acids. This technique was applied to food samples, such as cane sugar and dietetic vegetable sugar, honey, lemon syrup, condensed milk and liquid caramel, among others. No matrix interferences were observed and the reproducibility, according to these authors, was acceptable. Validation data for these organic acids determination were not given.

Jörg and Sontag (42) developed a HPLC method for qualitative and quantitative determination of phenolic acids (2-hydroxybenzoic, 3-hydroxybenzoic, 4-hydroxybenzoic, 3,4-hydroxybenzoic, 4-hydroxycinnamic and 4-hydroxy-3-methoxycinnamic acid) in 11 honeys from different botanical origin. The phenolic acids were previously extracted from honey using ethyl acetate. The chromatographic separation was performed in a reversed phase column using a mixture of methanol, glacial acetic acid and bidistilled water at pH of 3.0 as mobile phase. These acids were detected electrochemically using a coulometric dual electrode detector, so they were oxidized in the first cell and then reduced in the second one. Detection limits ranged between 0.3 to 1.5 ng for the oxidative mode and from 0.1 to 1.6 ng for the reductive detection mode. The recoveries ranged from 87.8 and 97.8%, with a relative standard deviation between 2.0 and 6.8%. No result about method precision was shown in this work. The authors concluded that distribution pattern of phenolic acids allows to differentiate between some honeys of different botanical origin, for example, 3,4-hydroxybenzoic was found in large amount in honeydew honeys. Therefore, further investigations would be necessary.

Cherchi et al. (9) identified several organic acids in honey and quantified gluconic, pyruvic, malic, citric, succinic and fumaric acids in 48 floral honeys by using a HPLC method with UV detection. Samples were previously purified by solid phase extraction (SPE) with an ionic exchange cartridge. Chromatographic separation was carried out in 60 minutes by employing two reversed-phase C_{18} columns connected in series, with sulphuric acid ($pH = 2.45$), at a flow rate of 0.7 mL/min as mobile phase. The detection limits ranged between 0.002 to 3 ppm (w/w). Average recovery percentages ranged from 89 and 104%, depending on the organic acid. In this paper, precision data were not given. According to the authors, the described method would allow the simultaneous determination of a number of organic acids, assisted by the fact that the problem of overlapping peaks could be overcome by varying the mobile phase pH. Polycarboxylic acids such as citric and fumaric acids might be more sensitive to any change regarding the mobile phase. Cherchi et al. (43) quantified gluconic, pyruvic, malic, citric, succinic and fumaric acid contents of 37 unifloral Sardinian (Italy) honeys, purifying the samples by solid phase extraction (SPE) and analyzing them by HPLC. They followed the method of Cherchi et al. (9). Furthermore, in order to characterize those Sardinian samples, these authors determined other physical, chemical, organoleptic, microbiological and palynological parameters. All these parameters were subdivided into two groups: "Characterization parameters" and "Quality

Control parameters." Organic acids and gluconic acid in particular, were included in characterization parameters by Cherchi et al. (43). Serra-Bonheví et al. (44) applied, with some modifications, the Cherchi et al. (9) method to determine 12 organic acids (as gluconic, oxalic, pyruvic, malic, citric, succinic, fumaric, propionic, 2-oxopentanoic, glutaric, isobutyric, butyric acids) in broom honey (*Spartocytisus supranubius* L.) produced in The Canary Islands (Spain). In this work, the samples were determined directly without a clean-up procedure with cartridges or other extraction procedure. Two columns connected in series are used for performing the acid separation. Besides, data of method validation (detection and quantification limits, recoveries and precision) is shown. In the summary, the authors suggested that malic acid can be used as a marker for the differentiation of broom honey, but any statistical treatment was apply to confirm this idea.

Defilippi et al. (45) determined formic acid by ionic chromatography (IC) with conductivity detector, using as mobile phase carbonate/bicarbonate buffer 0.25:2.75 mM ($pH = 10.1$), at a flow rate of 1 mL/min. Sample preparation was a simple dilution, because neither purification nor honey extraction were necessary. Analysis time was shorter than 5 minutes. The detection limit was 1.4 mg/kg. According to the authors this procedure is simple, accurate and very sensitive for honey, which allows its use as a honey quality control method. However, in the paper neither precision nor recovery results were given. The authors concluded that natural formic acid content in honey is very variable and is influenced by honey botanical origin.

Ferreres et al. (11) isolated, identified, and quantified cis, trans-abscisic acid and trans, trans-abscisic acid in Portuguese bell heather (*Erica* sp.) honey by using reverse phase HPLC with diode array detection. To confirm the structures, EIMS, ^{1}H NMR and ^{13}C NMR techniques were used. These authors did not find these two acids in any other type of unifloral honey analysed, so they could be useful markers of *Erica* sp. honeys, after the analysis of a higher number of samples from different geographical origins. In a recent study, Gheldof et al. (5) found cis, trans-abscisic acid in honeys from different botanical origin (buckwheat, soy, tupelo, clover, fireweed, acacia), in quantities approximately 10 times less than the quantities found by Ferreres et al. (11). Later, abscisic acids (cis, trans-abscisic acid and trans, trans-abscisic acid) were found in large amounts in both Australian and New Zealand *Leptospermun* honeys (46). In this work, the level of trans-trans abscisic acid in New Zealand manuka honey were much higher than that in Australian jelly bush honey. Yao et al. (47) also investigated the presence of these acids in Australian eucalyptus honeys. In studies, cis, trans-abscisic acid and trans, trans-abscisic acid could be used individually and/or jointly for the authentication of the botanical origins of these kind of honeys.

Del Nozal et al. (12) quantified citric, pyruvic, galacturonic, gluconic, malic, citramalic, quinic, succinic, fumaric and formic acids in 57 honeys from different botanical sources. Samples were previously purified by solid phase extraction (SPE) with anionic exchange cartridges. These authors employed two systems of HPLC with UV detection. One of the procedures was based on the use of an anionic exchange column (specific for organic acids) with sulphuric acid 5 mM as mobile phase, at a flow rate of 0.5 mL/min. The other procedure used two reversed phase C_{18} columns connected in series, by employing ammonium dihydrogen phosphate $pH = 2.2$ as mobile phase, at a flow rate of 0.7 mL/min. With both methods the approximate analysis time was 30 minutes. In this paper, no result of precision and recovery in honey samples is shown. Data of both precision and recovery were given on the basis of organic acids standards or syrups that tried to imitate honey composition.

Alamanni et al. (27) analyzed oxalic, lactic and formic acids contents in 49 unifloral Sardinian (Italy) samples. Samples were previously purified by solid phase extraction (SPE) with anionic exchange cartridges and subsequently analyzed by HPLC with UV detection. Chromatographic separation took place in 20 minutes. These authors used a specific column for organic acids; mobile phase was sulphuric acid 0.025 N, at a flow rate of 0.6 mL/min. Repeatability coefficients of variation were 2.8% for oxalic acid, 1.3% for lactic acid and 4.3% for formic acid. Average recovery percentages were 91.3% (oxalic acid), 94.5% (lactic acid) and 93.7% (formic acid). Detection limits ranged from 0.96 mg/L (formic acid) and 6.37 mg/L (lactic acid) and quantification limits ranged from 3.19 mg/L (formic acid) and 21.22 mg/L (lactic acid). As it has been already commented, oxalic acid results were compared with those obtained by the enzymatic analysis, showing a good correlation between both procedures. In this work, the average natural oxalic acid content was evaluated in honeys from different botanical sources to observe if eventual treatment in beehives with oxalic acid determined substancial or abnormal increases of the acid in honey. No increases were found when oxalic acid was used for the treatment of Varroa infestation.

Del Nozal et al. (48) determined oxalic acid (as oxalate), together with other inorganic anions such as sulphate and nitrate, in 99 floral and honeydew honeys by ionic chromatography (IC) with conductivity detection. To avoid matrix interferences, samples were purified by solid phase extraction (SPE) with ionic exchange cartridges, procedure already set up by these authors. Chromatographic separation was carried out, in about 50 minutes with an anionic exchange column and a mixture of lithium borate-gluconate (1.4%), *n*-butanol (2.0%), acetonitrile (12.0%) and water (84.6%) as mobile phase at a flow-rate of 2 mL/min. Repeatability for oxalic acid was 4.05%, whereas average recovery percentage was 92.72 ± 3.34%. The content of oxalic acid was evaluated in honey samples before and after a treatment with oxalic acid for varroasis control. The conclusion was that this therapeutical treatment of the beehives did not influence the initial value of oxalic acid in honey.

Casella and Gatta (49) determined organic acids such as gallic, ascorbic, gluconic, lactobionic, galacturonic, and glucuronic acids by an anionic exchange chromatographic. The detection was amperometric with constant voltage. Chromatographic separation took place in less than 20 minutes with an anionic exchange column, using an alkaline mobile phase composed by NaOH 0.1 M and sodium acetate 0.08 M. This method was applied to different samples such as tea, vinegar or honey. For honey, only gluconic acid was quantified, with a coefficient of variation between 6% and 9% for the precision and 98% for the recovery. According to these authors, their procedure allowed determining various organic acids in real matrices avoiding complicate extraction and derivatization procedures and providing with good sensitivity, reproducibility and recovery.

Suárez-Luque et al. (50, 51) set up a HPLC method with UV detection that allowed determining malic, maleic, citric, succinic and fumaric acids in 15 minutes. Chromatographic separation was carried out on a single C_{18} reversed phase column, using as mobile phase Milli-Q water acidified with m-phosphoric acid 4.5% (p/v) at pH 2.20 and using a flow rate of 0.7 mL/min. In order to avoid matrix interferences, sample was previously purified by solid phase extraction (SPE) with ionic exchange cartridges. These authors studied repeatabilities (%RSD $\leq$ 3.20% for the different acids), reproducibilities (%RSD $\leq$ 4.86% for the different acids) and recoveries (percentages that ranged between 62.9% for malic acid and 99.4% for citric acid).

Del Nozal et al. (52, 53) separated oxalic, D-glucuronic, citric, galacturonic, propionic, pyruvic, malic, citramalic, quinic, D-gluconic, lactic, formic, glutaric, fumaric, succinic and butyric acids in 39 honeys (52) and 58 honeys (53), all of them from the same geographical origin and different botanical sources. These researchers set up a HPLC, with UV (210 nm) detection procedure, by using four ion-exclusion columns connected in series, employing water with an 0.1% (v/v) of *o*-phosphoric acid as a mobile phase. The separation time was almost 60 minutes. To determine organic acids previous purification of the honey samples was not necessary, because only dilution and later filtration were needed. With these conditions oxalic, D-glucuronic, citric, galacturonic, propionic, quinic, D-gluconic, formic, and glutaric acids were quantified. Injection repeatability studies as well as complete repeatability studies of the method with both standards and honey were carried out. As regards honey, authors only gave results of oxalic, D-glucuronic, citric, galacturonic, pyruvic, D-gluconic, formic and glutaric acids. RSD% was $\leq$5.5% for the repeatability of the injection and $\leq$7.2% for the repeatability of all the procedure. In the paper, no result of accuracy was given. To evaluate only the oxalic acid, two columns coupled in series were enough. In this case, analysis time was reduced to 10–15 minutes. Concentrations of the organic acids studied were very variable, and even although they could be influenced by the botanical origin of the samples, within each group, an important variability of concentrations was observed.

Liquid chromatography is a technique widely used by lots of researchers to determine organic acids in many foodstuffs due to its sensitivity, versatility and reproducibility. Nevertheless the quantities of honey's organic acids are very low and thereby, there are many compounds that interfere in the quantification of organic acids, as for example, sugars. Interferences must be removed by means of a pretreatment of the sample or by employing columns in series, thus making these methods tedious and time consuming.

ELECTROPHORETIC METHODS

Organic acids separation and quantification is a very important application of capillary electrophoresis (54). There are lots of papers in which organic acids of several foodstuffs have been quantified by this method, many of these papers being review articles (55–59). Surprisingly, there are few papers of honey's organic acids determination by capillary electrophoresis.

Boden et al. (60) only identified honey's citric acid and the inorganic ions chloride and phosphate. Separation was carried out in 8 minutes at 22°C, with indirect-UV detection at 232 nm, using as electrolyte salicylic acid 7.5 mM, TRIS (tris[hydroximethyl]aminomethane) 15 mM, $Ca(OH)_2$ 1 mM and DOTAOH (dodecyltrimethylammonium hydroxide) 0.35 mM.

Navarrete et al. (61) applied a CZE method for the determination of oxalic, fumaric, maleic and malic acids in honey. The separation has been carried out in 14 minutes. The composition of running buffer was 50 mM sodium phosphate, 25% 2-propanol and 0.001% HDB (hexadimethrine bromide) adjusted to pH 8 with 1.0 NaOH. The instrumental parameters have been also optimized (voltage of -20 kV, hydrodynamic injection for 15 seconds and UV detection at 210 nm). Repeatability and reproducibility studies were not carried out and the recovery percentages ranged between 97.62% and 101.81%. Therefore, several important organic acids, as for example gluconic acid which is the predominant acid in honey or others, as citric, acetic, formic or lactic acids were not determined.

Mato et al. (62) developed a simple capillary electrophoresis method with direct-UV detection for the determination of oxalic, formic, malic, succinic, pyruvic, acetic, lactic, citric and gluconic acids in less than 4 minutes. The electrolyte composition was phosphate (7.5 mM NaH_2PO_4 and 2.5 mM Na_2HPO_4) as the carrier buffer, 2.5 mM TTAOH (tetradecyltrimethylammonium hydroxide, commercial name OFM-OH) as electroosmotic flow modifier and 0.24 mM $CaCl_2$ as selectivity modifier, adjusting the pH at 6.40 constant value. The running voltage was -25 kV at controlled temperature of 25°C. The injection was carried out in hydrodynamic mode and the detection mode was UV direct at 185 nm. Only a simple treatment of dilution and filtration was necessary for the determination of organic acids in honey by this electrophoretic method. Repeatability and reproducibility studies were carried out and showed RSD (%) $\leq$4.6% and $\leq$10.0 %, respectively. Recovery percentages ranged between 95% and 103%.

Despite capillary electrophoresis has been hardly applied to honey's organic acids determination, seems to show lots of advantages if compared with other procedures. In general, this technique is simple, rapid and low cost, because it needs neither laborious treatment of the samples nor long times of analysis. Furthermore, capillary electrophoresis avoids the most problematic interferences found by applying HPLC, such as sugars, which are the main honey constituents (which represent 99% total solids). Capillary electrophoresis main disadvantage is its lower reproducibility, if compared with other methods, and its little lower sensitivity as well, because limits of detection and quantification are in general, higher than those obtained by enzymatic and chromatographic procedures. Nevertheless, capillary electrophoresis is one of the techniques with better prospects for honey's organic acids determination, because it has been hardly developed for this purpose, unlike its development regarding other foods.

CONCLUSION

Firstly, it is necessary to highlight that there are few literature references as regards honey's organic acids determination. Nevertheless, due to the importance of these compounds, both traditional and new analytical procedures are very likely to be widely studied and developed within future years.

Enzymatic analysis should be used when only one organic acid must be determined, because this method is very specific and provides excellent precision and accuracy.

Gas chromatography should be the chosen technique for volatile or semi-volatile organic acids. This technique, together with liquid chromatography and capillary electrophoresis, provides the advantage of determining numerous organic acids. Despite being a versatile, sensitive and reproducible technique, HPLC is tedious and time consuming because it requires previous purification of the samples and/or the use of several columns in series to improve the separation of organic acids.

Capillary electrophoresis is a technique that takes very short time. In addition, sample preparation is simple. On the contrary, capillary electrophoresis is less sensitive and more difficult to reproduce than other techniques. Nevertheless, the development of capillary electrophoresis procedures to determine organic acids in honey is extremely interesting, in particular due to the fact that presently there are few papers published on this topic.

REFERENCES

1. J. W. Jr. White, Composición y propiedades de la miel, in *La Apicultura en los Estados Unidos*, ed. S. E. McGregor (México D. F.: Limusa, 1979), 57–66.
2. E. Crane, The traditional hive products: honey and beeswax, in *Bees and Beekeeping. Science, Practice and World Resources*, ed. (Oxford: Heinemann Newnes, 1990), 388–451.
3. S. Bogdanov, Nature and origin of the antibacterial substances in honey. *Lebensm.-Wis. u.-Technol.* 30 (1997):748–753.
4. R. J. Weston, K. R. Mitchell, and K. L. Allen, Antibacterial phenolic components of New Zealand manuka honey. *Food Chemistry* 64 (1998):295–301.
5. N. Gheldof, X. -H. Wang, and N. J. Engeseth, Identification and quantification of antioxidant components of honeys from various floral sources. *Journal of Agricultural and Food Chemistry* 50 (2002):5870–5877.
6. H. M. Eshbah and F. F. Ahmed, Screening certain natural products and organic acids for controlling Varroa mite (Varroa jacobsoni Oudemans). *Shashpa* 1 (1994):53–62.
7. F. Mutinelli, A. Baggio, F. Capolongo, R. Piro, L. Prandin, and L. Biasion, A scientific note on oxalic acid by topical application for the control of varroosis. *Apidologie* 28 (1997):461–462.
8. N. W. Calderone, Effective fall treatment of Varroa jacobsoni (Acari: Varroidae) with a new formulation of formic acid in colonies of Apis mellifera (Hymenoptera: Apidae) in the Northeastern United States. *Journal of Economic Entomology* 93 (2000):1065–1075.
9. A. Cherchi, L. Spanedda, C. Tuberoso, and P. Cabras, Solid-phase extraction and high-performance liquid chromatographic determination of organic acids in honey. *Journal of Chromatography A* 669 (1994):59–64.
10. A. L. Wilkins, Y. Lu, and S.-T. Tan, Extractives from New Zealand honeys. 5. Aliphatic dicarboxylic acids in New Zealand Rewerewa (Knightea excelsa) honey. *Journal of Agricultural and Food Chemistry* 43 (1995):3021–3025.
11. F. Ferreres, P. Andrade, and F. A. Tomás-Barberán, Natural occurrence of abscisic acid in heather honey and floral nectar. *Journal of Agricultural and Food Chemistry* 44 (1996):2053–2056.
12. M. J. Del Nozal, J. L. Bernal, P. Marinero, J. C. Diego, J. I. Frechilla, M. Higes, and J. Llorente, High performance liquid chromatographic determination of organic acids in honey from different botanical origin. *Journal of Liquid Chromatography and Related Technologies* 21 (20) (1998):3197–3214.
13. I. Mato, J. F. Huidobro, J. Simal-Lozano, and M. T. Sancho, Significance of nonaromatic organic acids in honey. *Journal of Food Protection* 66 (2003):2371–2376.
14. *Methods of Enzymatic BioAnalysis and Food Analysis Using Test-Combinations* (Mannheim, Germany: Boehringer Mannheim GmbH, 1995).
15. M. L. Tourn, A. Lombard, F. Belliardo, and M. Buffa, Quantitative analysis of carbohydrates and organic acids in honeydew, honey and royal jelly by enzymic methods. *Journal of Apicultural Research* 19 (1980):144–146.
16. W. Stoya, G. Wachendoerfer, I. Kary, P. Siebentritt, and E. Kaiser, Formic acid as a therapeutic against varroatose and its effect on honey. *Deutsche Lebensmittel-Rundschau* 82 (1986):217–221.
17. W. Stoya, G. Wachendoerfer, I. Kary, P. Siebentritt, and E. Kaiser, Milchsäure als Therapeutikum gegen Varroatose und ihre Auswirkung auf den Honig. *Deutsche Lebensmittel-Rundschau* 83 (1987):283–286.
18. H. Hansen and M. Guldborg, Residues in honey and wax after treatment of bee colonies with formic acid. *Tidsskr. Planteavl.* 92 (1988):7–10.
19. B. Talpay, Inhaltsstoffe des Honigs-Citronensäure (Citrat). *Deutsche Lebensmittel-Rundschau* 84 (1988):41–44.
20. B. Talpay, Inhaltsstoffe des Honigs-Ameisensäure (Formiat). *Deutsche Lebensmittel-Rundschau* 85 (1989):143–147.
21. A. G. Sabatini, G. L. Marcazzan, R. Colombo, and M. Garagnani, Applicazione di un metodo enzimatico per la determinazione

dell'acido formico e dell'acido lattico presenti nel miele. *Apicoltura* 9 (1994):135–145.

22. I. Mato, J. F. Huidobro, M. P. Sánchez, S. Muniategui, M. A. Fernández-Muiño, and M. T. Sancho, Enzymatic determination of total D-gluconic acid in honey. *Journal of Agricultural and Food Chemistry* 45 (1997):3550–3553.
23. P. Pulcini, M. G. Piazza, and F. Allegrini, Total gluconic acid (AGT) content in Italian unifloral honeys. *Industrie Alimentari* XLIII (2004):263–268.
24. I. Mato, J. F. Huidobro, M. P. Sánchez, S. Muniategui, M. A. Fernández-Muiño, and M. T. Sancho, Enzymatic determination of L-malic acid in honey. *Food Chemistry* 62 (1998):503–508.
25. I. Mato, J. F. Huidobro, V. Cendón, S. Muniategui, M. A. Fernández-Muiño, and M. T. Sancho, Enzymatic determination of citric acid in honey by using polyvinylpolypyrrolidone-clarification. *Journal of Agricultural and Food Chemistry* 46 (1998):141–144.
26. M. Cossu and Alamanni, Possibilità di impiego di una metodica enzimatica per la determinazione dell'acido ossalico in campioni di miele della Sardegna. *La Rivista di Scienza dell'Alimentazione* 28 (3) (1999):315–319.
27. M. C. Alamanni, M. Cossu, and F. Sanna, Un metodo HPLC per il dosaggio degli acidi ossalico, lattico e formico quali acaricidi e componenti naturali nel miele. *La Rivista di Scienza dell'Alimentazione* 29 (2000):139–147.
28. S. Bogdanov, J.-D. Charrière, A. Imdorf, V. Kilchenmann, and P. Fluri, Determination of residues in honey after treatments with formic and oxalic acid under field conditions. *Apidologie* 33 (2002):399–409.
29. E. E. Stinson, M. H. Subers, J. Petty, and J. W. J. White, The composition of honey. V. Separation and identification of the organic acids. *Archives of Biochemistry and Biophysics* 89 (1960): 6–12.
30. V. K. Speer and A. Montag, Beitrag zum Vorkommen von Benzoesäure und Phenylessigsäure in Honig. *Deutsche Lebensmittel-Rundschau* 80 (1984):103–105.
31. V. K. Speer and A. Montag, Phenylacetic acid and benzoic acid contents in honey. *Lebensmittelchem. Gerichtl. Chem.* 39 (1985):11–12.
32. E. Steeg and A. Montag, Nachweis aromatischer carbonsäuren in honig. Aromatic carbonic acids of honey. *Z. Lebensm. Unters. Forsch.* 184 (1987):17–19.
33. E. Steeg and A. Montag, Quantitative Bestimmung aromatischer Carbonsäuren in Honig (Quantitative determination of aromatic carbonic acids in honey). *Z. Lebensm. Unters. Forsch.* 187 (1988):115–120.
34. E. Steeg and A. Montag, Minorbestandteile des Honigs mit Aroma-Relevanz. I. Aromatische Carbonsäuren und Ester aromatischer Carbonsäuren. *Deutsche Lebensmittel-Rundschau* 84 (1988):103–108.
35. T. Echigo and T. Takenaka, Production of organic acids in honey by honeybees. *Nippon Nogei Kagaku Kaishi* 48 (1974):225–230.
36. S.-T. Tan, P. T. Holland, A. L. Wilkins, and P. C. Molan, Extractives from New Zealand honeys. 1. White Clover, Manuka, and Kanuka Unifloral honeys. *Journal of Agricultural and Food Chemistry* 36 (1988):453–460.
37. K. Horváth and I. Molnár-Perl, Simultaneous GC-MS quantitation of *o*-phosphoric, aliphatic and aromatic carboxylic acids, proline, hydroxymethylfurfurol and sugars as their TMS derivatives: in honeys. *Chromatographia* 48 (1998):120–126.
38. M. L. Sanz, M. González, C. de Lorenzo, J. Sanz, and I. Martínez-Castro, A contribution to the differentiation between nectar honey and honeydew honey. *Food Chemistry* 91 (2005):313–317.
39. A. Verzera, S. Campisi, M. Zappalà and I. Bonaccorsi, SPME-GC-MS analysis of honey volatile components for the characterization of different floral origin. *American Laboratory* 33 (2001):18–21.
40. D. Pilz-Güther and K. Speer, Development of a GC method for the simultaneous determination of organic acids in honey. *Deutsche Lebensmittel-Rundschau* 100 (2004):84–87.
41. M. Pérez-Cerrada, M. A. Herrero-Villen, and A. Maquieira, Sugar-rich food: Determination of inorganic anions by ionic chromatography. *Food Chemistry* 34 (1989):285–294.
42. E. Jörg and G. Sontag, Determination of phenolic acids in honey by HPLC using coulometric dual electrode detection. *Deutsche Lebensmittel-Rundschau* 88 (1992):179–183.
43. A. Cherchi, M. Porcu, L. Spanedda, C. I. G. Tuberoso, S. Cosentino, and F. Palmas, Individuazione di parametri utili per la caratterizzazione e la valorizzazione di mieli tipici della Sardegna: asfodelo, cardo e corbezzolo. *La Rivista di Scienza dell'Alimentazione* 24 (1995):523–534.
44. J. Serra-Bonvehí, A. Bentabol-Manzanares, and J. M. Santos-Vilar, Quality evaluation of broom honey (*Spartocytisus supranubius* L) produced in Tenerife (The Canary Islands). *Journal of the Science of Food and Agriculture* 84 (2004):1097–1104.
45. A. Defilippi, G. Piancone, A. Prandtatter, and G. P. Tibaldi, Honey quality: Ion chromatographyc determination of formic acid. *Industrie Alimentari* 34 (33) (1995):495–497, 503.
46. L. Yao, N. Datta, F. A. Tomás-Barberán, F. Ferreres, I. Martos, and R. Singanusong, Flavonoids, phenolic acids and abscisic acid in Australian and New Zealand *Leptospermum* honeys. *Food Chemistry* 81 (2003):159–168.
47. L. Yao, Y. Jiang, R. Singanusong, N. Datta, and K. Raymont, Phenolic acids and abscisic acid in Australian Eucalyptus honeys and their potential for floral authentication. *Food Chemistry* 86 (2004):169–177.
48. M. J. Del Nozal, J. L. Bernal, J. C. Diego, L. A. Gómez, J. M. Ruiz, and M. Higes, Determination of oxalate, sulfate and nitrate in honey and honeydew by ion-chromatography. *Journal of Chromatography A* 881 (2000):629–638.
49. I. G. Casella and M. Gattta, Determination of electroactive organic acids by anion-exchange chromatography using a copper modified electrode. *Journal of Chromatography A* 912 (2001):223–233.
50. S. Suárez-Luque, I. Mato, J. F. Huidobro, J. Simal-Lozano, and M. T. Sancho, Rapid determination of minoritary organic acids in honey by high performance liquid chromatography. *Journal of Chromatography A* 955 (2002):207–214.
51. S. Suárez-Luque, I. Mato, J. F. Huidobro, and J. Simal-Lozano, Solid-phase extraction procedure to remove organic acids from honey. *Journal of Chromatography B* 770 (2002):77–82.
52. M. J. Del Nozal, J. L. Bernal, J. C. Diego, L. A. Gómez, and M. Higes, HPLC determination of low molecular weight organic acids in honey with series-coupled ion-exclusion columns.

Journal of Liquid Chromatography and Related Technologies 26 (2003):1231–1253.

53. M. J. Del Nozal, J. L. Bernal, L. A. Gómez, M. Higes, and A. Meana, Determination of oxalic acid and other organic acids in honey and in some anatomic structures of bees. *Apidologie* 34 (2003):181–188.
54. F. S. Stover, Organic acids and organic ions, in *Handbook of Capillary Electrophoresis Applications* ed. H. Shintani and J. Polonský (London: Chapman and Hall, 1997), 550–579.
55. J. Lindeberg, Capillary electrophoresis in food analysis. *Food Chemistry* 55 (1996):73–94.
56. J. Lindeberg, Addendum to "Capillary electrphoresis in food analysis." *Food Chemistry* 55 (1996):95–101.
57. C. W. Klampfl, W. Buchberger, and P. R. Haddad, Determination of organic acids in food samples by capillary zone electrophoresis. *Journal of Chromatography A* 881 (2000):357–364.
58. R. A. Frazier, Recent advances in capillary electrophoresis methods for food analysis. *Electrophoresis* 22 (2001):4197–4206.
59. V. Galli, A. García, L. Saavedra, and C. Barbas, Capillary electrophoresis for short-chain organic acids and inorganic anions in different samples. *Electrophoresis* 24 (2003):1951–1981.
60. J. Boden, I. Haumann, and A. Mainka, Anwendung der Kapillarelektrophorese in der Lebensmittelanalytik. *GIT Labor-Fachzeitschrift.* 44 (2000):924–927.
61. M. Navarrete, S. Casado, M. Minelli, A. Segura, A. Bonetti, G. Dinelli, and A. Fernández, Direct determination of aliphatic acids in honey by coelectroosmotic capillary zone electrophoresis. Journal of Apicultural Research 44 (2005):65–70.
62. I. Mato, J. F. Huidobro, J. Simal-Lozano, and M. T. Sancho, Simultaneous determination of nonaromatic organic acids in honey by capillary zone electrophoresis with direct UV detection. *Journal of Agricultural and Food Chemistry* (Submitted for publication).