INFORME FINAL

NOMBRE DEL INVESTIGADOR

Patricia Vit

NOMBRE DEL PROYECTO

"Valorización de mieles de pote producidas por Meliponini en Ecuador"

ÁREA DE DESARROLLO

Area Ciencias de la Salud

NOMBRE DE LA INSTITUCIÓN DE ACOGIDA

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Contenido

ANEXOS EN CD

1. **Referencias Bibliográficas**
2. **Listado de Abejas Meliponini de Ecuador**
3. **Fichas de abejas**
4. **Mieles recolectadas**
5. **Certificados**
6. **Publicaciones**
7. **Gestión de la queja**
8. **Evaluación sensorial**
9. **INEN**
10. **Convenio ULA-UTMACH**
11. **Notas Conceptuales SENESCYT**
12. **Ruta de Museos Vivientes de Abejas Meliponini en el Mundo**
13. **Informes de Visitas Científicas**
14. **Arbitraje manuscritos y evaluación proyectos**
15. **I Congreso de Apicultura y Meliponicultura en Ecuador**

INFORME FINAL DE ACTIVIDADES

INTRODUCCIÓN

Aproximadamente 500 especies de abejas sin aguijón pertenecen a la tribu Meliponini (Michener, 2007), y viven en las regiones tropicales y subtropicales del planeta La Tierra (Crane, 1992). Estas abejas –a diferencia de *Apis mellifera* y otras especies de *Apis*, almacenan su miel en potes de cerumen, por este motivo se creó el término "miel de pote" (Vit y col., 2013), para diferenciarla de la miel más comercial extraída de panales de cera. En América Latina la cría de abejas sin aguijón se conoce como meliponicultura; sin embargo el origen del término es incierto, quizás se derivó del género *Melipona* o de la tribu Meliponini. La meliponicultura necesita ser protegida para prevenir su extinción (Villanueva y col., 2005) como tradición precolombina considerada hoy día un saber ancestral; a su vez, paradójicamente, las abejas sin aguijón deben ser protegidas de los meliponicultores a fin de lograr una práctica sostenible y no depredadora de los recursos naturales. La disminución de bosques y selvas, de la diversidad de especies de plantas, aumenta la competencia por los alimentos en los grandes meliponarios con más de 100 colmenas (Villanueva-Gutiérrez y col., 2013), y reduce el rendimiento de los potes de miel. Por lo tanto, las prácticas tradicionales necesitan actualizarse con el conocimiento moderno de meliponicultura y protección ambiental, para apuntar hacia un filosofía de cosmovisión "cuidar abejas mansas para proteger selvas" (Vit, 2000). Como indicador de la gran biodiversidad de abejas sin aguijón en Ecuador, 89 especies de Meliponini han sido informadas para la región sur, correspondiente a la zona 7, la cual comprende las tres provincias limítrofes con Perú: El Oro, Loja y Zamora Chinchipe (Ramírez y col., 2013).

Si bien el fósil más antiguo de una abeja en nuestro planeta es precisamente el de una abeja sin aguijón (comunicación personal JMF Camargo†, 2008; Vit, 2010), y la miel precolombina fue producida sólo por abejas sin aguijón, la miel de pote aún no está incluída en las normas de miel porque hasta hoy sólo se reconoce la miel producida por *Apis mellifera* –especie introducida en el Nuevo Mundo luego del descubrimiento de América (Vit, 2008).

MARCO TEÓRICO

Los usos medicinales de la miel y el polen producidos en potes de cerumen por ocho taxones de abejas sin aguijón de Brasil, fueron investigados en el estudio de zooterapia de Costa-Neto (2004). Estas propiedades medicinales necesitan ser demostradas, y una aproximación es el estudio de sus compuestos bioactivos como los flavonoides (Truchado y col., 2011). Recientemente se revisó el uso medicinal de la miel sola o combinada con terapias convencionales, como un antioxidante novedoso (Erejuwa y col., 2012). En el estudio con mieles de pote peruanas, se encontró que la actividad antioxidante de la miel varía de acuerdo con su origen entomológico (Rodríguez-Malaver y col., 2007). El estudio biofarmacéutico realizado por la Universitá di Ferrara con mieles de pote ecuatorianas producidas por mezclas de *Scaptotrigona* sp. recolectadas por los indígenas Achuar en la provincia Morona Santiago, es un valioso referencial (Guerrini y col., 2009).

El primer borrador para una norma de miel producida por abejas sin aguijón en potes de cerumen, fue presentado durante la reunión anual de de la Comisión Internacional de la Miel en el Centro Europeo de las Ciencias del Gusto (del inglés *European Center of Taste Science*) en la ciudad de Dijon, Francia, con representantes científicos de 18 países (Vit, 1999). Desde los estándares sugeridos por Guatemala, Mexico y Venezuela en el año 2004 (Vit y col., 2004), y la revisión realizada para establecer los estándares de calidad dos años más tarde (Souza y col., 2006), la propuesta de una norma para las mieles de pote está ahora sustentada por datos nuevos, e.g. Argentina (Sgariglia y col., 2010), Australia (Persano Oddo y col., 2008), Bolivia (Ferrufino y Vit, 2013), Brasil (Almeida-Muradin y col., 2013), Colombia (Fuenmayor y col., 2013), Guatemala (Dardón y Enríquez, 2008; Gutiérrez y col., 2008) y Venezuela (Vit y col., 2012).

Además del problema de ausencia de nofrmas para las mieles de pote, existe otro problema con las mieles falsas por el cual se realizan estudios de autenticidad (Vit y col., 1998; Latorre y col., 2000; Baroni y col., 2002; Arvanitoyannis y col., 2005), ya que las imitaciones de mieles requieren la participación del gobierno para su control. La autenticidad de la miel genuina se basa en la producción de este producto de la colmena por las abejas, y también en sus denominaciones de procesamiento como miel cruda o miel orgánica, e informaciones según su origen geográfico o de las plantas visitadas (Arvanitoyannis y col., 2005), y el tipo de abejas que la produce (Vit y col., 1998). Algunos indicadores químicos usados para la quimiometría se basan en based en descriptores físicoquímicos como proteínas (Baroni y col., 2002; Ramón-Sierra, 2015), metales (Latorre y col., 2000) y evaluación sensorial (Deliza y Vit, 2013), para mencionar algunos de ellos. Estos métodos han sido usados solos o combinados.

La producción de miel en Ecuador es menor que la demanda local. Este hecho junto con

Honey production in Ecuador is lower than the local demand. This fact besides the lack of a sanitary control to remove fake honey from the market, keep genuine and false honeys on the shelves. Consumers are confused in front of honey and non-honey syrups, and the choice of a genuine product is hard. Chemical analyses are expensive and sensory detection needs specialized training. Therefore a kit was proposed to help honey consumer's choice with an authentication test based on the number of phases formed after shaking diethyl ether and a honey dilution in water (Vit, 1998).

On the other hand, a classic honey quality control is based on the content of hydroxymethylfurfural (HMF) (Figura 1) as an indicator of aging or heating of honey, with a maximum standard of 40 mg HMF/kg by the Codex Alimentarius Commission (Codex-Stan, 1981). Several methods have been used to measure HMF in honey, with different reagents and techniques (Subovsky y col, 2004; INN, 2006; Lemos y col.; Kukurová y col., 2006). NMR for HMF detection and quantification in honey (Rastrelli y col., 2009) is used in the present study to provide distinctive spectra besides the information of this parameter measuring degradation of fructose.

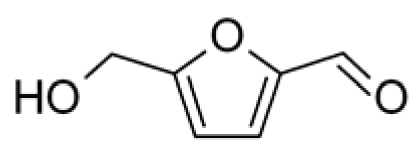

Figura 1. Hidroximetilfurfural

Los azúcares son los componentes mayoritarios de la miel, y también juegan un rol en los análisis de autenticidad. Las normas de miel ecuatorianas (INEN, 1988) y las venezolanas (COVENIN, 1984a) aun se refieren a los estándares cuprimétricos evaluados con el métodod de Lane & Eynon para medir azúcares reductores y sacarosa aparente (INEN, 1989, COVENIN 1984b). Sin embargo el HPLC con detector RI es el método aceptado para identificar y cuantificar azúcares en la miel por la Comisión del Codex Alimentarius (CODEX STAN, 2008). Los espectros de azúcares por HPLC han sido usados para describir los tipos de azúcares (fructosa, glucosa, sacarosa, turanosa, maltosa, trehalosa, erlose) (Bogdanov y col., 1996) y para agrupar los tipos de miel según su origen entomológico usando sólo el contenido de tres azúcares (fructosa, glucosa, maltosa) (Vit y col., 1997) o los factores de calidad de la miel (acidez libre, humedad, sacarosa, azúcares reductores, HMF, actividad de la diastasa) (Vit y col., 1998).

NMR spectroscopy is a powerful tool for sugar detection in honey, but the diverse forms of sugars in water solutions increase complexity for full assignments of shifts. Not identified ^{1}H NMR resonances were assigned to the keto and α-pyranose tautomers of D-fructose analysed by NMR spectroscopy, for a final resolution at tautomeric equilibrium (20°C in D_2O) of 68.23% β-pyranose, 22.35% β-furanose, 6.24% α -furanose, 2.67% α -pyranose and 0.50% keto tautomers (Figura 2) (Barclay y col., 2012). The botanical origin of Italian (Consonni y col., 2008, 2012) and Brazilian (Boffo y col., 2012) honeys were identified by NMR. Saccharides by NMR were useful predictors of botanical origin in Italian honeys, as an alternative to the chromatographic and melissopalynological methods (Consonni y col., 2012). Additionally, the entomological origin of pot-honey produced by stingless bees was also predicted by NMR (Schievano y col., 2013).

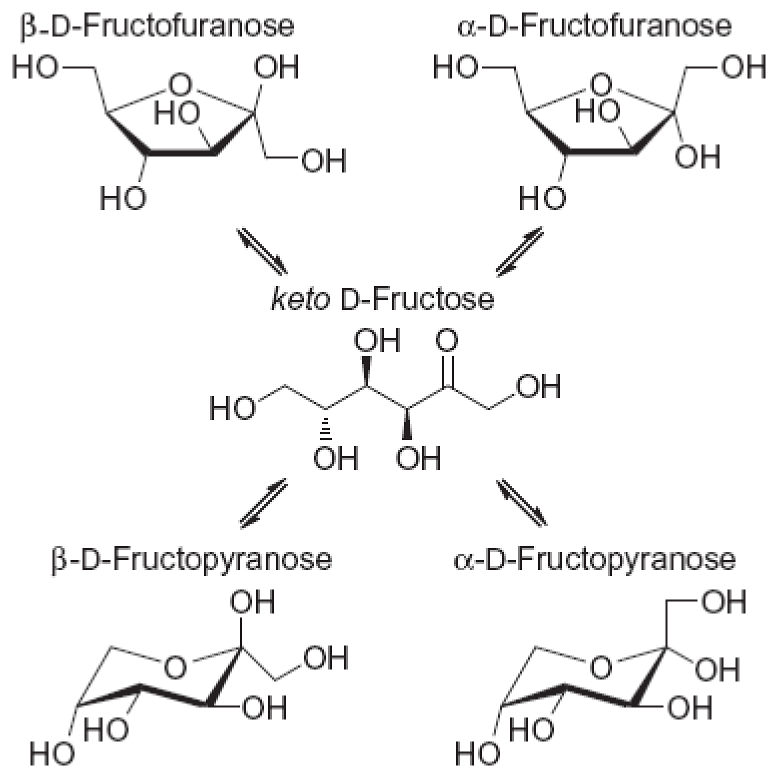

Figura 2. Formas tautoméricas de D-Fructose en diluciones de agua

Tomado de: Barclay y col., 2012

Se ha demostrado que el contenido de nitrógeno es un descriptor útil para discriminar mieles genuinas y falsas (Vit, 1987). After studies of aminoacid contents in honey, proline revealed to be the most abundant with more than 200 mg/kg Hermosin y col., 2003). Proline is originated from the honeybee (Ohe, 1991), and is added during the transformation of nectar into honey. Free aminoacid contents varied between 340 to 1322 mg/kg in Spanish honeys (Sancho y col., 1991), and 390.63 to 633.50 mg/kg in Polish honeys (Janiszewska, 2012). The variation of proline is not dependent on unifloral sources, therefore proline is not a good indicator of botanical origin (Janiszewska, 2012; Bogdanov y col., 2004) but still needs to be explored as a predictor of entomological origin. Selective 1D TOCSY is used to assign aminoacids by NMR (Sandusty, 2005). Ver las estructuras de la prolina, fenilalanina y tirosina en la Figura 3.

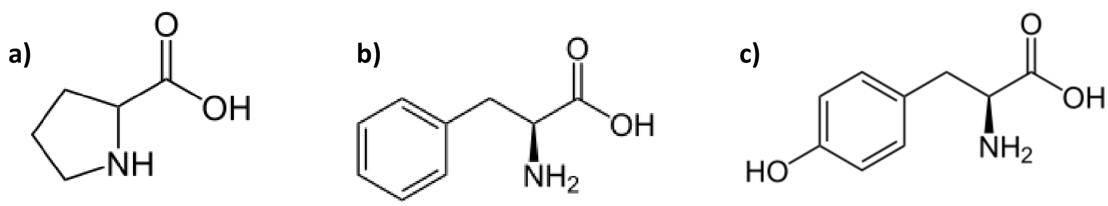

Figura 3. Aminoácidos visibles en espectros RMN de diluciones de miel en agua deuterada.
a) Prolina, el más abundante, **b)** Fenilalanina, **c)** Tirosina

La mayoría de los alimentos, incluyendo la miel, contienen diversos metabolitos y residuos químicos en matrices sólidas, semi-sólidas, o líquidas. En ciencias de alimentos, se usa la metabolómica para asesorar la calidad, la historia de procesamiento, y la inocuidad de materia prima y de los productos finales (Schievano y col., 2013). Esta investigación fue iniciada como proyecto colateral para tratar el problema de las mieles falsas encontradas durante los muestreos de miel de pote. Para su detección se observó el número de fases de diluciones de mieles mezcladas por agitación con éter etílico (Vit, 1998). Se seleccionaron indicadores de calidad clásicos (HMF, prolina, azúcares) y más recientes (ácido acético, ácido cítrico, etanol) como marcadores en los espectros RMN de miel diluída con agua fueron investigados para confirmar el diagnóstico de miel genuina o falsa. La ^{1}H-RMN en diluciones de agua deuterada y en extractos de cloroformo de la miel fueron usados para discriminar entre el origen entomológico de la miel producida por *Apis mellifera* y las mieles de pote producidas por los géneros *Geotrigona*, *Melipona* y *Scaptotrigona*.

PLANTEAMIENTO DEL PROBLEMA

- Los meliponinos de Ecuador han sido poco estudiados y su biodiversidad debe ser grande como en los países vecinos.
- Es necesario hacer un inventario de las especies de abejas sin aguijón (Meliponini) de Ecuador y caracterizar las mieles que producen en potes de cerumen, a fin de valorar este producto y sus usos tradicionales. Las mieles de Meliponini no están en la Norma Miel de Abeja. Requisitos NTE-INEN 1572.
- Estas mieles precolombinas elaboradas y almacenadas en botijas de cerumen representan un conocimiento muy valioso por la megabiodiversidad neotropical de Meliponini y sus potenciales aplicaciones medicinales, nutricionales, para la protección ambiental y el desarrollo socio-económico.
- Causas probables: Indiferencia ante los saberes ancestrales. Factores asociados: Desconocimiento, capacidad de análisis, recursos disponibles para el control de calidad de meile ecuatorianas..
- Los análisis de azúcares reductores y de sacarosa aparente son análisis de rutina que tuvieron problemas de cuantificación.
- Actores y/o instituciones involucradas: INEN, ARCSA, AGROCALIDAD, MAGAP, PROTAL- ESPOL, PROMETEO, UTMACH, UEA, SAE, comunidades de meliponicultores ecuatorianos.
- Soluciones que se han intentado: Gestión de la queja ante el SAE.
- Interrogantes fundamentales, que debieron ser resueltas a través de la propuesta de trabajo: Composición quImica de mieles de pote producidas por abejas Meliponini en Ecuador. Los valores de sacarosa aparente y de azúcares reductores no fueron confiables, por lo cual no se pudieron publicar y tampoco sugerir para los estándares de calidad en la noram reviada o en una nueva norma para miel de pote.
- Innovación: En vista de no haber logrado los análisis de composición, se elaboró una Guía para la Ruta de Museos Vivientes de Abejas Meliponini en el Mundo, con listas de meliponicultires ecuatorianos, recopilada durante las visita científicas.

DELIMITACIÓN DE LA INVESTIGACIÓN

- Se recolectaron abejas sin aguijón y mieles de pote en 20 provincias de Ecuador.
- Se entrevistaron meliponicultores ecuatorianos sobre usos medicinales de las mieles de pote.

- Se trabajó desde el 10.06.14 al 09.06.15
- Se analizaron las mieles según su contenido de humedad, acidez libre, azúcares reductores, sacarosa aparente (problemas analíticos indicados en resultados), evaluación sensorial (color, olor, sabor, aroma, análisis descriptivo, pruebas de aceptación, perfil de libre elección) y Resonancia Magnética Nuclear (RMN) por colaboración con la Dra. Elisabetta Schievano, del Departamento de Ciencias Químicas de la Universitá di Padova UNIPD, Italia (se realizaron tres visitas cientIficas a la UNIPD en septiembre 2014, diciembre 2014 y abril 2015). Se realizó una visita científica para iniciar mediciones de actividad cititóxica de tres mieles de pote en un modelo con células de cáncer de ovario con el Dr. Fazlul Huq, Ciencias Médicas, The Sydney University USYD, pero se necesitan muchas repeticiones. Todas las variables estudiadas fueron presentadas en resúmenes de congresos, incluídos en los Anexos del CD.

JUSTIFICACIÓN

En este apartado se refleja la importancia y la relevancia que tiene la propuesta del trabajo realizado>:

- Evidencias que demuestren la magnitud de la problemática o necesidad para profundizar en el análisis: Luego de un año de revisiín de la norma Miel de Abejas. Requisitos NTE INEN 1572, ninguna institución participante pudo ofrecer resultados de mieles ecuatorianas para ser considerados en los estándares de calidad, y los requisitos volvieron a estar basados en otras normas. Los análisis de sacarosa aparente y de azúcarea reductore no pudieron ser realizados satisfactoriamente por un laboratorio privado, financiados por mi persona (3 mieles), la UEA (16 mieles) y la UTMACH (32 mieles). Tampoco se pudieron corregir durante gestión de la queja ante el Servicio de Acreditación Ecuatoriano (SAE). La magnitud del problema inicia en la ausencia de un laboratorio especializado en miel de abejas, o la falta de capacidad analítica por los laboratorios del ARCSA.
- Se necesita una norma de calidad para mieles de pote producidas por abejas Meliponini en Ecuador, y para ello es necesario proponer estándares de calidad según el tipo de abeja puesto que la biodiversidad entomológica se refleja en las características sensoriales y químicas de las mieles producidas.

- Los análisis químicos y sensoriales de la miel de pote, junto con la identificación entomológica de la abeja productora de miel son necesarios para caracterizar las mieles de pote producida en Ecuador.
- Diversos beneficios futuros derivan de esta investigación apenas iniciada, la cual requiere el aporte científico de las nuevas generaciones de las universidades ubicadas en diferentes localidades geográficas de Ecuador. Las personas consumidoras de miel reciben una oferta de miel genuina y protección ante las mieles falsas. Los grupos sociales dedicados a la meliponicultura se benefician del estudio científico de sus mieles para valorar su calidad con una Norma Técnica Ecuatoriana y los usos medicinales conocidos con los aberes ancestrales, los cuales requieren demostraciones científicas. La comercia.ización de estas mieles autóctonas favorecerá conumidores, analistas, instituciones y grupos sociales dedicados a promover la biodiveridad y el naturimo,
- La aplicabilidad en áreas estratégicas del desarrollo de la salud puede fortalecer la meliponiterapia y la meliponicultura sustentable por grupos capaces de manejar meliponarios (niños, mujeres, ancianos) pero débiles ante el manejo de apiarios más pesados. El hecho que estas abejas no pican, facilita su manejo

Objetivo general

Caracterizar las mieles de pote ecuatorianas según su composición química, sensorial y origen entomológico

Objetivos específicos

Investigación

1. Mantener actualización bibliográfica.
2. Realizar inventario de abejas sin aguijón en las provincias de Ecuador (recolección de abejas sin aguijón y miel de pote, recopilaci'on de nombres étnicos). Enviar las abejas a Brasil para su identificación entomológica por la Dra. Silvia RM Pedro.

3. Conocer los usos tradicionales de las mieles de pote en Ecuador.

4. Caracterizar las mieles de pote según su composición química (humedad, acidez libre, cenizas, azúcares reductores, sacarosa aparente, nitrógeno) según disponibilidad en UTMACH o por colaboración con laboratorio bromatológico en ESPOL, Guayaquil

5. Obtener un espectro sensorial de mieles de pote (descripción y aceptación).

6. Participar en Congreso Científico Internacional 6. Participación en Congreso de la Sociedad Italolatinoamericana de Etnomedicina (SILAE) en Marsala, Sicilia, Italia 7-12 Septiembre 2014. 6.1 Presentación sobre caracterización de miel de pote y poster sobre abejas sin aguijón de Ecuador.

7. Aplicar de RMN en mieles de diverso origen entomológico y geográfico en Ecuador (obtención de espectros y análisis quimiométrico con la Dra. Elisabetta Schievano).

Capacitación científica en el área pertinente a su especialidad

1. Capacitar en trabajo de campo para recolectar sistemáticamente abejas, información de nidos, mieles de pote, cuestionarios para usos tradicionales.
2. Establecer vínculo con entomólogo para identificar las abejas.
3. Formar o actualizar en análisis químicos de rutina para el control de calidad de la miel de pote.
4. Formar en evaluación sensorial de las mieles de pote (descripción y aceptación).
5. Interpretar espectros RMN en mieles como herramienta discriminatoria de origen entomológico y geográfico.
6. Presentar el libro Pot-honey: A legacy of stingless bees, para actualizar el conocimiento sobre mieles producidas por abejas sin aguijón en potes de cerumen (dirigido a profesores, personal y estudianntes universitarios)

Gestión de recursos nacionales e internacionales (económicos, humanos)

Formular un proyecto para valorar las mieles de pote de Ecuador con un museo.

Formular un proyecto para caracterizar mieles ecuatorianas.

Formular un proyecto con propuesta sensorial en escuelas para diferenciar mieles genuinas y falsas, además de identificar las mieles de pote ecuatorianas.

Relacionamiento estratégico interinstitucional a nivel nacional e internacional

Proponer convenio con la Universidad de Los Andes, Mérida, Venezuela. Este convenio facilitará futuras interacciones e intercambio académico con profesionales requeridos para el proyecto.

Formar grupos multidisciplinario para agrupar meliponicultores del mundo desde un museo que valora sus mieles de pote, “Ruta de Museos Vivientes de Abejas Meliponini en el Mundo”.

RESULTADOS OBTENIDOS

Investigación

En la investigación realizada con este proyecto se estudiaron algunas mieles de pote ecuatorianas (producidas por *Melipona indecisa* “cananambo”, “bermejo” *Melipona mimetica*, "bunga negra" *Melipona grandis*, *Geotrigona* sp. y *Trigona* sp. “abeja de tierra”, *Nannotrigona* y *Scaptotrigona ederi* “catiana” o "catana") para conocer las propiedades medicinales atribuídas por los meliponicultores, y contribuir con algunos requisitos referenciales en la propuesta de su inclusión en las normas ecuatoianas para miel de abejas NTE INEN 1572 (INEN, Instituto Ecuatoriano de Normalización, 1988) durante su revisión iniciada en el año 2014, bien sea en la misma tabla de los estándares para *Apis mellifera* –como se sugirió para la miel de pote más abundante en Venezuela, producida por *Melipona favosa* y conocida como “erica” (Vit, 2013), o en un anexo como en las normas colombianas (ICONTEC, Instituto Colombiano de Normas Técnicas y Certificación, 2007). Se realizaron análisis de humedad y acidez libre; sin embargo, hubo problemas analíticos con las determinaciones de azúcares reductores y sacarosa aparente. Se inició un manuscrito con las primeras mieles (16) analizadas por la Universidad Estatal Amazónica, y las mieles (3) con financiamiento propio. En las segundas mieles (32) analizadas por la Universidad Técnica de Machala se detectaron errores analiticos por lo cual no se puede publicar así. El 6 de Diciembre de 2015 se solicitaron correciones. El 15 de Febrero de 2015 se introdujo una solicitud de gestión de la queja ante el Servicio de Acreditación Ecuatoriano. El Laboratorio privado repitió los análisis junto con un laboratorio externo de su elección, pero no se lograron las correcciones requeridas y se decidió no publicar esos resultados y tampoco se pudieron usar como requisitos de calidad para la norma ecuatoriana. Se recolectaron 108 mieles, se analizaron 51, y el resto ya no se pudo analizar en espera de las repeticiones. Se hicieron análisis en otros laboratorios para llegar a esa conclusión.

1. Actualización bibliográfica.
   Se recopilaron y leyeron casi 300 artículos científicos. Ver **Anexo 1** Referencias Bibliográficas en CD.

2. Realizar inventario de abejas sin aguijón en las provincias de Ecuador (recolección de abejas sin aguijón y miel de pote). Se enviaron las abejas a Brasil para su identificación entomológica por la Dra. Silvia RM Pedro. Se encontraron otros taxones en los

especímenes enviados (Diptera, Halictidae, Coleoptera), lo cual sucede cando se recolectan especímenes sobre las flores y no en las colmenas de Meliponini. A continuación se presentan las identificaciones realizadas. Ver CD **Anexo 2** Listado de Abejas Recolectadas, **Anexo 3** Fichas de abejas (216) y **Anexo 4** Mieles Recolectadas (117).

3. Conocer los usos tradicionales de las mieles de pote en Ecuador. Se encuestaron los productores durante el muestreo, con encuestas sobre usos medicinales donde se solicitaron quince descriptores: Identificación Personal (1. Nombre, 2. Apellido, 3. Edad, 4. Dirección, 5. Teléfono, 6. Email); Meliponicultura (7. Años de experiencia, 8. Nombres étnicos de las especies de abejas que se cuidan, 9. Origen de los nidos, 10. Tipo de meliponario tradicional o racional, 11. Número de colmenas, 12. Productos extraídos, 13. Método de extracción de miel, 14. Conservación de la miel); 15. Usos medicinal de las mieles de pote recabados por enumeración y escuchando los casos.

   Este trabajo fue presentado como poster en el 23rd. Italo-Latinamerican, Asian & African Congress of Ethnomedicine. Marsala, Sicily, Italia, 7-12 Septiembre. A continuación se muestra el manuscrito realizado, el cual ya fue publicado: Vit P, Vargas O, López, T.V., Maza F. Meliponini biodiversity and medicinal uses of pot-honey from El Oro province in Ecuador. Emirates Journal of Food and Agriculture, Emir. J. Food Agric 27(6): 502-506. Online First: 13th April 2015. Ver **Anexo 5** Certificados y **Anexo 6** Publicaciones en el CD.

Meliponini biodiversity and medicinal uses of pot-honey from El Oro province in Ecuador

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Abstract
Ecuadorian stingless bees (Apidae, Meliponini) have ethnomedicinal interest because their products are used in healing. Diverse remedies consist on pot-honey alone or mixed with infusions. This set of medicinal uses were informed in El Oro province by Ecuadorian stingless bee keepers –known as meliponicultors– in Latin America: bruises, tumors, ocular cataracts, pterygium, inflammation, infections, varicose veins, cleaning blood after childbirth, kidney diseases, tumor, wound healing, and soothing balm before sleeping. *Scaptotrigona ederi* named “catiana” or “catana” is the most frequent bee in the visited cantons Las Lajas, Balsas, Piñas, and Zaruma. Other important stingless bees are *Melipona indecisa* “cananambo”, *Melipona mimetica* “bermejo”, *Nannotrigona* cf. *perilampoides* “pitón”, and *Paratrigona* aff. *eutaeniata* “pirunga”. A bioprospective research will follow to value this ancient tradition and the honey processed in cerumen pots, with sound inclusion in the Ecuadorian honey regulation NTE INEN 1572, currently under revision

Keywords Ecuador, *Geotrigona*, medicinal uses, *Melipona*, Meliponini, pot-honey, *Scaptotrigona*

Introduction

Ecuador has a surface of almost 300,000 km² divided into 24 provinces with the highest biodiversity in the planet. El Oro province is divided into 14 cantones, and belongs to the region 7 of Ecuador, located in the South West of the country, besides Loja and Zamora Chinchipe provinces (Figure 1). El Oro’s population is 559,846 inhabitants living in 5,850 km^2 –roughly 1/50 the country surface; the capital city Machala is internationally known as the banana capital of the world. Besides the ecologically controversial exploitation of shrimp farming and intensive banana plantations, El Oro province has protected areas: Arenillas Ecological Reserve, Buenaventura National Park and Isla Santa Clara Wildlife Sanctuary. This coastal province is home to the Mullopungo, Chilla and Tioloma foothills, Hummingbird Sanctuary, and stingless bees.

Las Lajas is a 300 Km^2 Southern West canton of El Oro province, capital La Victoria, known for the production of cocoa, corn, and livestock. The temperature of 19 to 30˚C, and altitudes between 80 and 900 m.a.s.l. are good for stingless bee life, indeed few species are currently kept.

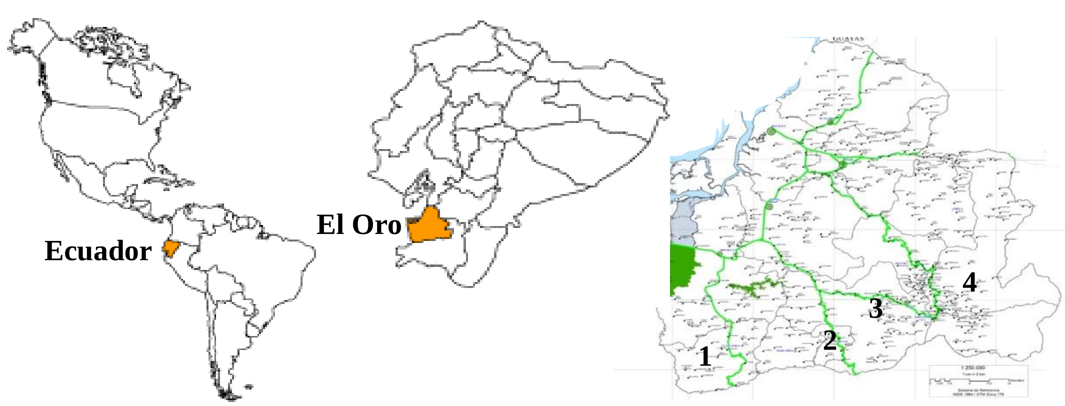

Figure 1. Location of El Oro province in the Southwest of Ecuador
1. Las Lajas, 2. Balsas, 3. Piñas, and 4. Zaruma cantons.
From: http://www.parks.it/world/EC/Eindex.html

Stingless bees (Hymenoptera; Apidae; Meliponini) are a tropical group with more than 500 known species, and perhaps 100 more to be named (Michener, 2013). This great biodiversity is mostly represented by Neotropical Meliponini with almost 400 species group (Camargo and Pedro, 2007; Camargo, 2013). Different species of stingless bees thrive in El Oro province, and some of them are used in traditional meliponiculture. The following species of stingless bees were previously reported in this province (Ramírez et al., 2012): *Cephalotrigina capitata* Smith,1854; *Geotrigona fumipennis*, Camargo & Moure, 2066; *Oxytrigona mellicolor*, Packard, 1869; *Scaptotrigona* sp. cf. *postica*, *Scaptotrigona* sp., *Trigona fulviventris, Trigona matera, Trigonisca* sp. 1 and sp. 2.

"Stingless bees process honey and pollen in cerumen pots" is the title of an e-book, to reflect on differences from and similarities to honey and pollen processed in beeswax combs (Vit and Roubik, 2013). See honey and pollen pots from a nest of "catana" *Scaptotrigona ederi* in Chiriboga, Libertad parrish, Las Lajas canton, El Oro province, Ecuador (Figure 2).

Photography: P. Vit

Figure 2. Honey and pollen pots from "catana" *Scaptotrigona ederi*

Knowledgeable medicinal properties of honey produced by stingless bees from Guatemala, Mexico and Venezuela were informed (Vit et al., 2004). Achuars from the Amazonian forest of Ecuador treat throat inflammation with pot-honey (Guerrini et al., 2009). Honey ‒as an effective curative product with religious and mythical powers‒ was pinpointed in a retrospective review on the medicinal uses of *Melipona beecheii* pot-honey by the ancient Maya to restore balance of ill patients (Ocampo Rosales, 2013). Pot-honey was a name suggested to group honey produced by Meliponini because stinglessness –intellectual term used by Michener (2013)‒ possibly gives no input to think on the process by which honey is made inside cerumen containers, a component that may explain bioactive properties (Vit et al., 2013).

Natives from South America, such as the Kayapós, from southern Pará, Brazil, use the products of stingless bees in food and medicine, and also as a model for social organization for their own communities (Posey and Camargo, 1985; Camargo and Posey, 1990). Local knowledge on medicinal use, management and ecological aspects were studied in a contribution of ethnoentomology for eight stingless bee species from Michoacan, Mexico (Reyes-González et al., 2014).

In this work we studied Ecuadorian meliponines from Las Lajas, Balsas, Piñas, and Zaruma cantons from El Oro province and the different medicinal uses of their honey produced in cerumen pots, to retrieve the relation man-bee-environment-health.

Materials and Methods

Stingless bee keepers –named meliponicultors in Latin America– were visited in four cantons (Las Lajas, Balsas, Piñas, and Zaruma) from Provincia El Oro in Ecuador. Questionnaires were used with seventeen meliponicultors, six female and eleven male, to inform medicinal uses of pot-honey by themselves and pot-honey consumers. The questionnaire consisted in 15 structured questions on Personal Identification (1. Name, 2. Surname, 3. Age, 4. Location, 5. Phone, 6. Email); Meliponiculture (7. Years of experience, 8. Ethnic names of species kept, 9. Origin of the Nests, 10. Rational or traditional meliponary, 11. Number of hives, 12. Products extracted, 13. Method of honey extraction, 14. Conservation of the honey); 15. Medicinal uses of pot-honey were elicited by enumeration and listening cases. Data on medicinal uses of pot-honey were analyzed and summarized by using Microsoft Excel to determine relative frequencies (%) of citations so as to identify the most common and popularly uses in the studied area.

The behavior of the bees was observed, and information on type of meliponary, and shape of the nest entrance was recorded. Ethnic names of the stingless bees were taken in each sampling, and used for preliminary identification (Ramírez et al., 2013). Stingless bees were collected in isopropyl alcohol, kept in boxes, and sent to Dr. S.R.M. Pedro, Camargo's Collection, Biology Department, Universidade de São Paulo, Ribeirão Preto, Brazil, for entomological identification. Another set was sent to Prof. J. Ramírez for subsequent mounting to be deposited in entomological boxes at Universidad Nacional de Loja, Loja, Ecuador. Further duplicates were sent to Professor Charles D. Michener at the University of Kansas, Lawrence, USA; to Dr. Clifford Keil, Director of the Invertebrates Museum, Pontificia Universidad Católica de Ecuador, Quito, Ecuador; and Dr. David W. Roubik, Smithsonian Tropical Research Institute; Panama.

Results and discussion

Stingless bees kept by seventeen visited Ecuadorian meliponicultors, aged 12 to 76-y-o in El Oro province are "bermeja" *Melipona mimetica* Cockerell, 1914; "cananambo" *Melipona indecisa*, Cockerel, 1919; "catiana" or "catana" *Scaptotrigona ederi* Schwarz, unpublished; "pirunga" *Paratrigona* aff. *eutaeniata* Camargo & Moure, 1994, "piton" *Nannotrigona* cf. *perilampoides* (Cresson, 1878). All of them commented on decrease of "bermejo" and "cananambo" nests as a more sensitive bee in frank decrease.

The dark *Scaptotrigona ederi* has variable defensive behavior, generally entangles in the hair and bites, therefore the use of the veil is advised for harvesting. The other species mentioned here are gentle bees, *Nannotrigona* sp. hides easily inside the nest. Two of the interviewed meliponicultors kept more than one type of stingless bee species, namely *Melipona indecisa* and *Scaptotrigna ederi*, *Melipona mimetica* and *Scaptotrigna ederi*; whereas a female meliponicultor kept *Nannotrigona* cf. *perilampoides*, *Paratrigona* aff. *eutaeniata*. and *Scaptotrigna ederi*. In Table 1 we show the ethnomedicinal uses of pot-honey produced by these five species of stingless bees, with healing properties known by producers and consumers in the locality. In this work the simple expressions were chosen to group the way honey is used, instead of systemic categorizations of medicinal interventions. The use of mixtures with plants would deserve another work by its own, therefore only the pot-honey is considered here.

Table 1. Ethnomedicinal uses of pot-honey produced by five types of stingless bees in El Oro province

Stingless bee names		n	Canton Location	Healing uses (%)							
Ethnic	Scientific			balm	blood	kidney	eyes	inflammation	sour throat	tumor	wound healing
"bermeja"	*Melipona mimetica*	1	Las Lajas	-	2.7	2.7	2.7	2.7	2.7	-	-
"cananambo"	*Melipona indecisa*	1	Piñas	-	-	-	-	-	2.7	-	-
"catiana" "catana"	*Scaptotrigona ederi*	13	Balsas, Las Lajas, Piñas, Zaruma	5.4	27.1	5.4	8.1	13.5	10.8	5.4	2.7
"pirunga"	*Paratrigona* aff. *eutaeniata*	1	Las Lajas	-	-	-	2.7	-	-	-	-
"piton"	*Nannotrigona* cf. *perilampoides*	1	Las Lajas	-	-	-	2.7	-	-	-	-

Pot-honey is widely used alone or mixed with medicinal plants to treat tumors, eyes (ocular cataracts, pterygium), inflammation, sour throat infections, blood (bruises, varicose veins, purifying blood, cleaning blood after childbirth), kidney diseases, wound healing, and soothing balm before sleeping. The most frequent medicinal use was related to blood in 27% of the reported uses.

Their sensory qualities are appreciated by meliponicultors, who perceive floral smell and enticing sour flavor. In Table 1, the ethnic and scientific names of five stingless bee species from El Oro province are given with the medicinal uses of the pot-honey attributed by meliponicultors investigated here. Eight healing effects were registered, and besides being the most frequent, "catiana" or "catana" pot-honey has all these putative medicinal properties. Generally meliponicultors do not keep *Apis mellifera*, but the uses of honey are similar as a sweetener in plant infusions with antiinflammatory, emollient, against colds, and invigorative properties, as reported in the Granada study (Benítez, 2011). In the review on edible insects of Ecuador (Onore, 2005) and in our study, medicinal uses of stingless bee bodies were not informed by stingless bee keepers. However, whole body extracts of bees are used as anticancer and antibacterial agents, namely for their antimicrobial peptides (AMPs) (Ratcliffe et al., 2011).

Insects represented the major animal group (23%) of folk medicinal bio-resources in the study of Bahia, Brazil (Costa-Neto, 2004). Therefore entomotherapy has implications for public health and biological conservation (Alves and Alves, 2011); in their review 18 species of stingless bees were retrieved in studies on medicinal properties. However, compared to fitotherapy, the number of studies on zootherapy is very limited. Antibacterial and antioxidant activity of honey vary according to the botanical and entomological (Rodríguez-Malavaer et al., 2007) origin. The bioactive properties of honey are ascribed to specific factors such as the synergistic action of sugar and hydrogen peroxide for wound healing (Kwakman et al., 2010).

Further ongoing studies are of interest to identify the megabiodiversity of stingless bees in Ecuador, the traditional meliponiculture, and medicinal uses of pot-honey as ancestral knowledge. Although these pot-honeys were produced and used before Columbus, they are not yet considered in the honey regulations (Vit, 2008). This joint effort besides the characterization of pot-honeys, and its inclusion in the honey standards of the INEN 1572 regulation (Vit et al., unpublished), using the *Melipona favosa* pot-honey model (Vit, 2013), would increase its current value in the market up to USD 27/kg, promote the study of its medicinal properties and praise the activity of meliponicultors. The role of honey is perceived therapeutic in 90% of multispecies medicinal recipes.

Traditional medicine (TM) remains a simple therapy for health care in low income countries because it is the most available and affordable form of healing, as well as for the naturism philosophy, and combined with alopatic medicine as complementary alternative medicine (CAM) (WHO, 2000). It is readily available in ethnic groups such as Pankararé from Brazil who use *Frieseomelitta* sp., *Cephalotrigona* cf *capitata*, *Melipona* sp., *Melipona scutellaris*, *Partamona* cf. *cupira*, *Tetragonisca* sp., *Trigona* (*Trigona*) *spinipes*, *Plebeia* sp., pot-honey or pollen eaten or mixed with plants (Costa-Neto, 2002). The fact that pot-honey has cultural value connecting with ancient curative skills, does not exclude its use as raw material in the preparation of industrial drugs.

The ecological contribution of stingless bees as organisms is encapsulated in their pollinating service to about 50% of flowering plant species in the Neotropics (Biesmeijer, 1997) and Australia (Heard, 1999). The role of honey is perceived as therapeutic in 90% of multispecies medicinal recipes from Misiones, Argentina (Kujawsca, 2012).

Stingless bee keepers from Zona Maya in Mexico experienced colony losses of *Melipona beecheii* due to competition for food (Villanueva-Gutiérrez et al., 2013), especially in meliponaries with more than 100 nests. Besides the rescue of tradition, environmental protection is needed to achieve sustainable meliponiculture. To protect wild populations of stingless bees, the Ecuadorian Ministry of Environment started to fine extractions of nests from wild logs five years ago in Las Lajas canton from El Oro province, as informed by a meliponicultor from Amarillos, parrish La Libertad (M. Estrada, personal communication).

Besides the nutritional, organoleptic and sanitary values of a medicinal food like honey, an enterprising concept on the quality of the agri-food systems –as reviewed by Monastra and Crisponi (2013), considers animal welfare and defence of the ecosystem, as practiced by stingless bee keepers in modern days.

Conclusions

Honey is a medicinal food product of plant-animal-based pharmacopeia used in entomotherapy. Seventeen meliponicultors provided information on the honey produced in cerumen pots by five types of stingless bees from el Oro province from Ecuador. Pot-honey is mostly harvested from "catana" or "catiana" because the other bees –"bermeja", "cananambo", "pirunga" anf "piton"− are less frequent. This described knowledge has ecological and sanitary implications, and deserves careful considerations.

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Caracterizar las mieles de pote según su composición química (humedad, acidez libre, cenizas, azúcares reductores, sacarosa aparente, nitrógeno) según disponibilidad en UTMACH. No se recibió colaboración con laboratorio bromatológico en PROTAL-ESPOL, Guayaquil. Yo financié los análisis de humedad, acidez libre, azúcares reductores y sacarosa aparente de 3 mieles; la Universidad Estatal Amazónica (UEA) financió 16 mieles y posteriormente la Universidad Técnica de Machala (UTMACH) financió 32 mieles. A continuación se presenta el manuscrito enviado a la revista indexada Molecules para las primeras mieles, el cual no fue aceptado y se solicitó un mayor muestreo. Los resultados siguientes presentaron problemas analíticos que no se pudieron resolver. Esta situación fue presentada oralmente en el congreso From Honey to Table. International Congress on Safety and Authenticity of Bee Products. Istanbul, Turkey, 21-22 Mayo, como “Assessment of honey analysis in an Ecuadorian laboratory during a scientific research service”, luego de lo cual INTERTEK, Bremen, Alemania, ofreció colaboración analítica y se enviaron 90 de las 117 mieles muestreadas para realizar análisis de parámetros de calidad (humedad, sustancias insolubles en agua, acidez libre, conductividad eléctrica, pH, actividad diastasa, cenizas, actividad de agua, HMF, espectro de azúcares, y si alcanza muestra, glicerina, actividad de invertasa, prolina) hasta donde se pueda con la cantidad de muestra de miel enviada. A continuación se inserta el manuscrito realizado para 15 mieles (no se pudo continuar) por Vit P, Vargas JC, Andino M, Maza F. Physico-chemical and sensory characterization of Ecuadorian (Hymenoptera: Apidae: Meliponini) commercial pot-honeys: *Geotrigona* sp. “abeja de tierra”, *Melipona* spp. “cananambo” and “bermejo”, and *Scaptotrigona* sp. “catiana”. Ver **Anexo 7** Gestión de la queja.

(Article)

Physico-chemical and sensory characterization of Ecuadorian (Hymenoptera: Apidae: Meliponini) commercial pot-honeys: *Geotrigona* sp. "abeja de tierra", *Melipona* spp. "cananambo" and "bermejo", and *Scaptotrigona* sp. "catiana"

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Abstract: The quality of fifteen commercial pot-honeys produced by "abeja de tierra" *Geotrigona*, "bermejo" and "cananambo" *Melipona*, and "catiana" *Scaptotrigona* stingless bees species in Ecuador was evaluated for free acidity, reducing sugars, sucrose and water contents. These pot-honeys were also described for their visual viscosity, color, smell, aroma, dominant taste and other physiological sensations in the mouth. Pot-honey produced by *Geotrigona* is the most different from *Apis mellifera* standards, with free acidity some 12-20 times higher than the maximum of 40 meq/kg, double water content of the maximum 20 g/100 g, and a third of the minimum 65 g/100g of reducing sugars. Pot-honey produced by *Melipona* and *Scaptotrigona* may fulfill *Apis mellifera* standards, with a slightly higher moisture up to 27.88 g/100 g and free acidity up to 49.01 g/100 g, but lower contents of reducing sugars [36.33-51.82] g/100 g. Sucrose content of pot-honey produced by *Geotrigona*, *Melipona* and *Scaptotrigona* is lower than 5 g/100g as in the *Apis mellifera* honey standards. Compositional and sensory data on pot-honey produced by Meliponini is a contribution to the database of the revised Ecuadorian honey standards NTE INEN 1572, and will eventually support the inclusion of pot-honey standards in the norm.

Keywords: Ecuador; entomological origin; Meliponini; physic-chemical analysis; pot-honey, quality standards; sensory descriptors.

1. Introduction

Aproximately 500 species of stingless bees belong to the Meliponini sub-family [1], and live in tropical and subtropical regions [2]. These bees store honey in cerumen pots, therefore the term "pot-honey" was coined [3] to differentiate them from honey produced in beeswax combs by *Apis mellifera* and other *Apis* spp. In Latin America stingless bee keeping is known as meliponiculture, the origin of the term is uncertain, and could be linked to the *Melipona* genus or to the subfamily Meliponini. The traditional stingless bee keeping or meliponiculture should be protected to prevent its extinction [4], and paradoxically, stingless bees should be protected from stingless bee keepers for a sustainable instead of predatory practice. The decline of forest and plant species diversity, increase competition for food in large meliponaries [5], and reduce pot-honey yields. Therefore, the traditional practice needs input from current knowledge on stingless bee keeping and environmental protection, to pinpoint an ultimate philosophy "caring gentle bees to protect forests" [6]. As an indicator of the great biodiversity of stingles bees, 89 species of Meliponini are reported in the Southern region of Ecuador [7].

The medicinal use of honey or pollen produced in cerumen pots by eight taxa of Brazilian stingless bees was investigated in the zootherapy study of Costa-Neto [8]. These medicinal properties need to be demonstrated, and one approach is to study bioactive compounds such as flavonoids [9]. Honey alone or combined with conventional therapy was recently reviewed as a novel antioxidant [10]. The antioxidant activity of honey varies according to the entomological source of honey [11]. In the research on Ecuadorian pot-honey, a comprehensive biopharmaceutical approach was done in a *Scaptotrigona* mixture collected by Achuars in Morona Santiago province [12].

Although the oldest fossil of a bee in our planet is a stingless bee [13], and Precolumbian honey was produced only by stingless bees; pot-honey is not included in the honey regulations because they are currently devoted to *Apis mellifera* which was a species introduced after the discovery of America [14].

The first draft for a norm of honey produced by stingless bees was presented by Vit during the 1999 Annual Meeting of the International Honey Commission held in the European Center of Taste Science in Dijon, France, with scientific representatives of 18 countries [15]. Since the standards suggested by Guatemala, Mexico and Venezuela in 2004 [16], and the review done to set quality standards in 2006 [17], the proposal of a norm for pot-honeys of the world is now supported by new data, e.g. from Argentina [18], Australia [19], Bolivia [20], Brazil [21], Colombia [22], Guatemala [23] and Venezuela [24].

In this work, Ecuadorian pot-honeys produced by *Melipona* spp. named "cananambo" and "bermejo", *Geotrigona* sp. named "abeja de tierra" and *Scaptotrigona* sp. named "catiana" are studied to contribute for the proposal of its inclusion in the Ecuadorian honey norm [25] either in the same table of standards for *Apis mellifera* –as suggested for the most abundant pot-honey in Venezuela produced by *Melipona favosa* known as "erica" [26], or in the annex like in the Colombian regulations [27].

2. Results

2.1 Physico-chemical composition of Ecuadorian pot-honeys

Fifteen commercial pot-honeys were collected during field work in El Oro, Loja and Manabí Ecuadorian provinces, and analyzed for four physicochemical parameters currently done in routine analysis by the Ecuadorian sanitary authority ARCSA, namely free acidity, moisture, reducing sugars and apparent sucrose. Raw data, averages and *Apis mellifera* Ecuadorian standards for three honey types are given in Table 1.

Table 1. Physico-chemical analysis of *Melipona*, *Geotrigona* and *Scaptotrigona* pot-honey, and *Apis mellifera* honey standards

Bee taxa	Pot-honey sample	Free acidity (meq/kg)	Moisture (g/100 g)	Reducing sugars (g/100 g)	Apparent sucrose (g/100 g)
***Geotrigona* sp.**	**1**	626.66	38.39	33.74	3.73
	2	502.28	36.84	17.44	2.70
	3	659.10	34.86	31.64	2.49
	4	497.09	36.78	27.31	2.12
	5	563.49	34.62	20.79	4.11
	6	807.25	37.04	16.24	1.44
***Melipona* spp.**	**7**	31.81	21.74	52.36	1.64
	8	39.74	18.85	38.70	1.09
	9	48.97	22.80	44.38	4.01
***Scaptotrigona* sp.**	**10**	46.25	22.09	51.75	2.37
	11	48.87	27.82	38.05	4.29
	12	35.30	31.29	43.45	3.77

	13	36.29	19.51	44.28	2.03
	14	48.04	20.32	39.50	3.24
	15	25.30	20.09	30.49	1.35
Averages & standards					
Geotrigona	**n=6**	609.33^{b} (111.30) [497.09-807.25]	37.06^{b} (1.38) [34.62-38.39]	24.53^{a} (7.09) [16.24-33.74]	2.76^{a} (0.95) [1.44-4.11]
Melipona	**n=3**	44.36^{a} (5.33) [31.81-49.97]	20.82^{a} (2.28) [18.85-22.80]	41.54^{b} (3.28) [38.70-52.36]	2.24^{a} (1.39) [1.09-4.01]
Scaptotrigona	**n=6**	40.01^{a} (8.89) [25.30-48.87]	21.79^{a} (2.94) [19.51-31.29]	42.25^{b} (5.30) [30.49-51.75]	2.84^{a} (1.07) [1.35-4.29]
Apis mellifera	**NTE INEN 1572**	**Max 40**	**Max 20** **Max 23**	**Min 65** **Min 60**	**Max 5** **Max 7**

Individual values are given in the upper table where minimum and maximum are highlighted. Averages ± (SD), [minimum-maximum] values are given in the lower table. Different superscripts indicate significant difference in honey composition between the two groups. $P < 0.05$. The NTE INEN 1572 standards have two honey types, Class I (upper value) and Class II (lower value). Class II possibly will be deleted in the revised norm.

2.2 Sensory features of Ecuadorian pot-honeys

The sensory perceptions of the three types of Ecuadorian honey are given in Table 2. All the pot-honeys analyzed here were liquid, and few of them developed tiny crystals after freezing, causing a visual milky viscosity in three *Geotrigona* honeys. The color varied from light to dark amber. The smell varied along the bee, candy, caramel, menthol, fermented, floral, fruity notes. The aromas were similar with bee, citrusy, floral, lemon zest, fermented, fruity, menthol, pollen, and resinous. A *Geotrigona* honey had book glue off-odor and stable off-aroma. Dominat flavors are sweet for *Melipona* honeys and sour for *Geotrigona* honeys, *Scaptotrigona* honeys are more variable sweet, sour sweet, sour astringent and even bitter. Four honey samples −two of *Geotrigona* and two of *Scaptotrigona*− caused salivation while tasting. Floral for *Melipona* and citrusy for *Geotrigona* were the most frequent descriptors perceived in the smell and aroma; pollen was frequently perceived in the smell and aroma of *Scaptotrigona*.

Table 2. Sensory analysis of *Melipona*, *Geotrigona* and *Scaptotrigona* pot-honey

Bee genus	Pot-honey sample	Visual viscosity	Color	Smell	Aroma	Dominant taste	Other physiological sensations
Geotrigona	1	liquid very thin	dark amber	citrusy, bee	citrusy, resinous	sour, astringent	salivation
	2	milky liquid	creamy	citrusy	citrusy	sour	-
	3	liquid	dark amber	citrusy, bee	citrusy	sour	-
	4	liquid	light amber	book glue	citrusy stable	sour	salivation
	5	liquid	creamy	floral	citrusy	sour	-
	6	milky liquid	dark amber	citrusy floral	citrusy candy lemon zest	sour	-
Melipona	7	liquid	creamy	menthol	menthol	sweet	-
	8	liquid	light amber	floral	floral	sweet	-
	9	liquid	light amber	floral	floral	sweet	-
Scaptotrigona	10	liquid	amber	pollen	floral	sweet	-
	11	liquid	light amber	fruity "sarrapia"	fruity	sour-sweet	-
	12	liquid	amber	fermente d	fermented	sour, astringent	salivation
	13	liquid	amber	tobacco	bee	bitter	salivation
	14	liquid	light amber	citrusy	pollen, bee	bitter	-
	15	liquid	dark	pollen	pollen	sweet	-

amber

The acceptance of the honeys varied as follows: *Apis mellifera* light amber 6.5 ± 2.8, *Apis mellifera* amber 5.1 ± 2.8, *Geotrigona* amber 3.9 ± 3.0, *Melipona* light amber 6.7 ± 2.5, *Scaptotrigona* light amber 5.8 ± 2.8. There were no significant differences between the acceptances in the five honey types tested here $P < 0.05$. The highest acceptances were assessed for the light amber honeys, both *Melipona* and *Apis mellifera*, and slightly lower *Scaptotrigona*. For a group of 16 over 40 assessors, the light amber *A. mellifera* was the best honey, while the *Melipona* honey was the best for 10 assessors, both rated with values from 5.9 to 10.0; the *Scaptotrigona* honey was chosen as the best honey by 9/40 assessors who rated it with acceptances from 8.2 to 10.0.

3. Discussion

Tropical honeys have great diversity for new research and discoveries compared to the greatly studied and characterized unifloral European honeys, such as the distinctive 15 honey sheets for *Brassica napus, Calluna vulgaris, Castanea sativa, Citrus* spp., *Eucalyptus* spp., *Helianthus annuus, Lavandula* spp., *Rhododendron* spp., *Robinia pseudacacia, Rosmarinus officinalis, Taraxacum officinale, Thymus* spp., *Tilia* spp. floral honeys and two honeydew types [28] that continuously improve analytical methods. Besides the diverse botanical origin, the entomological origin of the honey is also important in the tropics and subtropics to explain bioactive properties: 1. The antioxidant activity, phenolics and flavonoids were studied in Argentinian and Paraguayan *Tetragonisca fiebrigi* "yateí" pot-honey [29], 2. Composition, antioxidant activity [19] and antimicrobial activity [30] of *Tetragonula carbonaria* honey from Australia that was of briad spectrum similar to medicinal honey, 3. Antibacterial, antiyeast and antifungal activities of the Thai *Tetragonula laeviceps* raw pot-honey showed that yeasts are more sensitive to diluted honey, and antimicrobial activity is higher in extracts with solvents of mild polarity [31], 4. Antioxidant capacity was higher in Malaysian Gelam *Melaleuca spp.* than Pineapple *Ananas comosus* honey [32], 5. Selective harvest was proposed for the dark amber true acacia honey, *Acacia mangium* from Malaysia [33].

Although pot-honeys are major honeys in the forests −as stated by Dr. D.W. Roubik, they are still minor honeys in the market. Therefore, they need promotion, protection and development of their infant industry [34]. Educational initiatives from Australia [35] and pot-honey shows [36] do expand the knowledge of stingless bees, meliponiculture, pot-honey, sensory appeal, composition, and understanding of medicinal uses by the public. Traditional medicinal properties attributed to pot-honeys need scientific demonstrations and the honeys need to be characterized for fair trading. The physico-chemical composition is discussed below for each of the three pot-honey types studied here: *Melipona*, *Scaptotrigona* and *Geotrigona*, before a forthcoming proposal of pot-honey standards valid at this early stage of the research.

3.1 Melipona pot-honey type

Melipona stingless bee species build bigger cerumen pots to process their honey [37] and have higher honey yields. Therefore their honeys have been studied more frequently. The physico-chemical composition of *Melipona* honeys varies according to the species. Average water contents (g/100 g) are 24.9 for *M. brachychaeta* and 24.1 for *M. grandis* from Bolivia [20], 28.02 *M. quinquefasciata* [38], 28.84 *M. scutellaris* [39] and 24.8 *M. subnitida* [40] from Brazil, 25.8 *M. compressipes*, 27.6 *M. eburnea*, and 24.8 *M. favosa* from Colombia [22], 17.32 for *M. beecheii*, 19.66 for *M. solani* and 20.37 for *M. aff. yucatanica* from Guatemala [41], and 28.0 *M. favosa* from Venezuela [42]; with a range of 17.32 to 28.84 g water/100 g. Moisture varied from 18.85 to 22.80 g/100 g for the Ecuadorian *Melipona* honeys in Table 1, these values are within the moisture range of honey from eleven *Melipona* species from Bolivia, Brazil, Colombia, Guatemala, and Venezuela [20, 22, 38, 40, 41, 42].

Average free acidities (meq/kg) are 10.4 for *M. brachychaeta* and 16.0 for *M. grandis* from Bolivia [20], 28.02 for *M. quinquefasciata* [38], and 32.49 for *M. subnitida* [40] from Brazil, 23.23 for *M. beecheii*, 4.95 for *M. solani* and 10.59 for *M. aff. yucatanica* from Guatemala [41], and 51.7 for *M. favosa* from Venezuela [42]; with a range of 4.95 to 51.7 meq/kg. In our study with three *Melipona* honeys, the variation of free acidity from 31.81 to 49.97 meq/kg in Table 1 is within the free acidity range of honey produced by eight *Melipona* species from Bolivia, Brazil, Guatemala, and Venezuela [20, 38, 40, 41, 42].

3.2 Scaptotrigona pot-honey type

Scaptotrigona mexicana honey from Guatemala has a free acidity of 12.68 meq/kg, 18.74 g/100g water content, 57.22 g reducing sugars/100 g, and 0.06 g apparent sucrose/100 g [41]; the water content for the Ecuadorian *Scaptotrigona* honey in Table 1 is also 18.74 g/100g, but the free acidity 40.1 meq/kg is higher than that found in Guatemala, and consequently the content of 42.25 g/100 g reducing sugars is lower than the minimum 65% of the *Apis mellifera* standard. The low sucrose has no problem because the standard establishes a maximum of 5%, perhaps a more refined limit could be suggested with a lower maximum value for sucrose of pot-honeys.

3.3 Geotrigona pot-honey type

Dardón and Enríquez [41] reported a free acidity of 85.53 meq/kg and a water content of 32.09 g/100g for the honey produced by *Geotrigona acapulconis*; these were the highest acidity and moisture between the honeys produced by nine species of Meliponini in Guatemala. Also in Table 1, the Ecuadorian *Geotrigona* honey shows the highest free acidity and moisture. The fact that average of free acidity in the Ecuadorian *Geotrigona* is 609.33 meq/kg should be explained by the species and the interactions of the underground nest with the soil. Behavioral observations of the underground bees and their nests are needed to understand such a different pot-honey [43], and hypothesize the origin of such chemical array. This is a very thin honey with 37.06 g/100 g water content, almost double of the 20% maximum permitted in the Ecuadorian honey norm [25]. These elevated compositional values of water and acids, explain a decrease on reducing sugars to a 24.53 g/100 g average which is lower than the minimum of 65% established in the norm [25].

3.4 Approaching a proposal of pot-honey standards

Honey bees and stingless honey bees produce honey with similarities and differences that vary with the bee species [44]. The statistical analysis from Table 1 shows that honey produced by *Melipona* and *Scaptotrigona* are more similar between each other in free acidity, moisture, and reducing sugar contents than honey produced by *Geotrigona* in Ecuador. However apparent sucrose is not different in the three honey types. Similarly, these observations were done with *Melipona*, *Scaptotrigona* and *Trigona* honeys from Venezuela in 1998 [45], although pot-honey produced by *Geotrigona* and *Trigona* are different. Quality standards needed by pot-honey were substantiated in 1999 [15] during the Annual Meeting of the International Honey Commission, and later proposed for Guatemala, Mexico, and Venezuela in 2004 [16], and also for Brazil in 2006 [46] and 2013 [39]. The first official insert of pot-honey produced by native bees was done with the Annex of the Colombian honey norm ICONTEC in 2007 [27] derived from the comprehensive review done in 2006 [17]. The proposal to expand the Ecuadorian honey norm NTE INEN 1572 [25] to other honey bee species, namely stingless honey bees was one reason for the current revision according to his President Dr. Hugo Rosero; during the third meeting of the Technical Committee of the revision of the NTE INEN 1572, the *M. favosa* model [26] was presented as an option for the crucial decisions to be taken, and the best option for further development of meliponiculture.

A provisional idea for pot-honey standards in Ecuador given in Table 3, shows similarities and differences with *Apis mellifera* standards, and between the thre genera studied here, with a recommendation that 30-40 honey samples of each group should be analyzed for a sensible and solid database. Averages and [minimum-maximum] values from Table 1 are retained here to visualize the proposal of quality standard for each parameter (free acidity, moisture, reducing sugars and apparent sucrose) for the three pot-honey groups based on their entomological origin *Geotrigona*, *Melipona* and *Scaptotrigona*. The column of apparent sucrose is highlighted in grey because the standard of a permitted maximum of 5 g/100 g remains the same for the honey produced by the three genera. Compared to the free acidity maximum value 40 meq/kg in the *Apis mellifera* honey standards, reference values should be increased up to 800 for *Geotrigona* and 50 for *Melipona* and *Scaptotrigona* honey. The maximum moisture of 20% also needs to be increased up to 25% for *Melipona*, 30% for *Scaptotrigona* and 40% for the *Geotrigona*. On the other hand, the 65% minimum of reducing sugars requires a reduction to 16% for *Geotrigona*, and 35% both for *Melipona* and *Scaptotrigona*.

Table 4. Suggested pot-honey standards for *Geotrigona*, *Melipona*, and *Scaptotrigona*

Bee	Number of pot-honeys & honey norm	Free acidity (meq/kg)	Moisture (g/100 g)	Reducing sugars (g/100 g)	Apparent sucrose (g/100 g)
Geotrigona	**n=6**	609.33[b] [497.09-807.25]	37.06[b] [34.62-38.39]	24.53[a] [16.24-33.74]	2.76[a] [1.44-4.11]

		Max. 800	**Max. 40**	**Min. 16**	**Max. 5**
Melipona	**n=3**	44.36[a] [31.81-49.97] **Max. 50**	20.82[a] [18.85-22.80] **Max. 25**	41.54[b] [38.70-52.36] **Min. 35**	2.24[a] [0.99-4.01] **Max. 5**
Scaptotrigona	**n=6**	40.01[a] [25.30-48.87] **Max. 50**	21.79[a] [19.51-31.29] **Max. 30**	42.25[b] [30.49-51.75] **Min. 35**	2.84[a] [1.35-4.29] **Max. 5**
Apis mellifera **Honey Type 1**	**NTE INEN 1572**	**Max 40**	**Max 20**	**Min 65**	**Max 5**

This proposal is a trend that needs to be validated with more pot-honeys from Ecuador. This is not the first proposal, and therefore it is supported by previous research done in Brazil [39, 46], Colombia [27], Guatemala [16], Mexico [16], and Venezuela [16, 26]. Ecuador is about to start a contribution in regulatory promotion of pot-honey. The final outcome would have consensual value by the Technical Committee TC-NTE INEN 1572 revising and updating the honey norm.

3.5 Sensory analysis

Sensory characteristics and defects of honey from *A. mellifera* were based on the wine sensory experience adapted to the perception of honey bee keepers and consumers by Gonnet and Vache (1984) [47]. A leading research with *Melipona quadrifasciata* by French and Brazilian scientists made the observations of her delicious thin and sour honeys [48] –always re-discovered by young melittologists and endlessly communicated in seminars, workshops and papers of pot-honey produced by stingless bees− as already stated by Schwarz since 1948 [49] for the widely relished honeys in tropical America before Columbus. An ayurvedic observation on dominant flavor of food to prepare balanced meals was incorporated in early sensory approach of commercial honeys from Venezuela, where the sugary matrix does not always convey to the sweet perception collectively assigned to honey, even in botanical types with more than 65% reducing sugars, such as the bitter *Castanea* and *Arbutus unedo* European honeys. Comparing honey extracted from beeswax combs and cerumen pots, needs adaptations to keep the similarities and to insert differences. For instance, the fermentive off-odor is a defect for unmatured *Apis mellifera* honey [47], but it is a distinctive feature in some types of pot-honeys [50]. Therefore fermentation is a valid descriptor because Meliponini process their honey diversely.

The overall acceptability of honey is a result of the sensory qualities and the attitude of the consumer towards that particular honey, as for any other food [51]. In this study the higher scores for light amber honeys such as those of *Melipona* and *Scaptotrigona*, indicated the consumers were keen for delicate aromas, instead of the strongly sour *Geotrigona* honey. However, when consumers explain their choices, *Geotrigona* honey is perceived as more medicinal, definitely not as a sweetener.

Bee products have healing properties known to improve circulation, decrease inflammation and boost immune protection [52, 53]. Somehow the consumer has a curative relationship with bee products. Pot-honey has been studied for the antibacterial [54], antioxidant [11] and anticancer activity [55]. A vast research is needed to demonstrate putative medicinal properties and the active components derived from stingless honey bees, their diet or perhaps originated with their interactive microbiota [56]. Discoveries such as the presence of C-glycosides in pot-honeys [9] are possible due to the great biodiversity of Meliponini [57], and sustained research of specializaed groups.

3.6 Presence of pot-honey in the Ecuadorian market and prospective development

In the Ecuadorian coast *Geotrigona* and *Scaptotrigona* honeys are more abundant. According to meliponicultors the two *Melipona* species known as “bermejo” and “cananambo” were easily kept in the past, some 10-20 years ago, but now feral nests of *Melipona* are scarce and these bees abandon the hive more easily than *Scaptotrigona* named “catiana”, as if the bees are more sensitive to management. In the Ecuadorian Amazon rainy forest, *Melipona* honey is harvested by pot-honey hunters and sold by Kichwa and Shuar nationalities in Native Indian fairs from Puyo, Pastaza province, Tena, Napo province and El Coca, Orellana province with a top price of almost 10 $/125 g, whereas in El Oro province the cost is 15-20 $/750 g. Ecuadorian Kichwas and Shuars, like Brazilian Enawene-Nawe [58] do not breed bees, but other Brazilian indigenous societies do, like the Guarani [59] and the Pankararé [60], as well as Huottuja from Venezuela [61]. Native knowledge about stingless bees connects social groups with nature, in a sort of ecological cosmovision of ancestral awareness.

Government enterprises are needed for producers, possibly to subsidize the quality control procedures, to generate scientific research needed to demonstrate putative medicinal properties, and to open marketing channels. Ministries of Health, and of Agriculture and Livestock could support a strategical planning for ancestral knowledge embracing traditional stingless beekeeping and pot-honey-hunting in Ecuador, among other agricultural practices to participate in the Change of Productive Matrix as envisaged by the Knowledge Sharing Program (KSP) from South Korea [62] and the Good Living (originally from Kichwa language *Sumak Kawsay*, translated into Spanish as *Buen Vivir*). Ecuador will join the seminal initiative Route of Living Museums of Meliponini in the World, launched by Costa Rica and Venezuela in 2013 [37], with a leading role for the regulatory concepts, with the current systematic Prometeo Project location of stations for meliponaries and stingless bee nests. Besides their great biodiversity, for a number of reasons, the identified stingless bees keep changing their names [63], therefore a specialized entomologist is mandatory in any scientific team investigating pot-honey. The unifloral Italian honeys were carefully filed in a comprehensive book [64] that could be imitated to illustrate the tropical meliponine honeys for a better knowledge of the public and the melittologists.

4. Experimental Section

4.1 Pot-honey samples

Fifteen pot-honeys were purchased from local stingless bee keepers or in markets from El Oro, Loja and Manabí Ecuadorian provinces, and kept frozen until analysis. Stingless bees were collected from the entrances of nests in logs or hives using isopropyl alcohol, dried and kept in plastic boxes before sending them to Dr. SRM Pedro for identification at Universidade de São Paulo, Ribeirão Preto, Brasil; and using the book on stingless bees from the South of Ecuador [7] for "abeja de tierra" that is referred to few species of *Geotrigona* spp. Bees were also deposited in the Laboratory of Entomology with Professor José Ramírez, at Universidad Nacional de Loja, Loja province, Ecuador. The entomological and geographical origin of the pot-honey samples is given in Table 4.

Table 4. Entomological and geographic origin of the 15 pot-honey samples

Ethnic name of stingless bees	Scientific name of stingless bees	n	Locations
"abeja de tierra"	*Geotrigona* spp.	6	Zapotillo, Loja province; Quimís, Manabí province.
"bermejo" and "cananambo"	*Melipona* spp.	3	Pindal, Loja province; La Moquillada, Piedras Blancas, El Oro province.
"catiana" or "catana"	*Scaptotrigona* spp.	6	La Moquillada, Moromoro, Piedras Blancas, Piñas, El Oro province; El Trapiche, Loja province.

4.2 Physico-chemical analysis

Only *Apis mellifera* is considered for the ten quality standards in the Ecuadorian honey norm NTE INEN 1572 [25] and corresponding analytical methods: relative density and moisture, reducing sugars, sucrose, ratio fructose: glucose, free acidity, insoluble solids, ash, hydroxymethylfurfural (HMF), and diastase number. ARCSA (Spanish acronym for *Agencia Nacional de Regulación, Control y Vigilancia Sanitaria* (in English National Agency of Regulation, Control and Sanitary Vigilance) complies with five of these parameters for the sanitary registration of honey: Acidity, moisture, relative density, reducing sugars and sucrose. Therefore these analysis, except relative density that will be removed from the Ecuadorian standards, were done in duplicates by service ordered to the PROTAL-ESPOL Laboratory in Guayaquil, Ecuador, due to the importance to provide data generated in an Ecuadorian Accredited Laboratory to be considered in a proposal of new standards for pot-honey to be considered in the Ecuadorian Honey Norm NTE INEN 1572 [25]. The methods used by this laboratory are gravimetric for moisture NTE INEN 1632 [65], Lane & Eynon for reducing sugars, and apparent sucrose NTE INEN 1633 [66], titrimetric for free acidity NTE INEN 1634 [67].

4.3 Sensory analysis

Sensory analysis was done to describe the dominant taste [68] –as learned from the ayurvedic technique to equilibrate food according to the taste [69], visual appearance, smell, and aroma, using the odor-aroma table for pot-honey [70]. Other physiological sensations were also observed. An acceptance test [71, 72] was done with 40 assessors for a *Geotrigona*, *Melipona*, *Scaptotrigona*, two commercial *Apis mellifera* honeys, one amber and the other light amber using a 10-cm unstructured line scale anchored with the expressions 'like it a little' at 1 cm from the left end, and 'like it a lot', at 9 cm from the left end.

3.4 Statistical analysis

Physico-chemical results were statistically processed with SPSS [73] to compare means of *Geotrigona*, *Melipona* and *Scaptotrigona* pot-honeys with ANOVA, *post-hoc* Tukey test. The acceptance scores were measured and the data were analyzed, by ANOVA, followed by Tukey test to check differences between means.

5. Conclusions

After the analysis of fifteen Ecuadorian pot-honeys, the following tendencies of pot-honey contrasted with the NTE INEN 1572 *Apis mellifera* honey were observed: 1. Moisture is generally higher in pot-honey [18.85-38.39] g/100g, compared to the *Apis mellifera* standard, maximum 20 g/100g. 2. Free acidity is variable, *Melipona* and *Scaptotrigona* pot-honeys contents of 25.30-49.97 meq/kg are more similar to the *Apis mellifera* standard, maximum 40 mg/kg; whereas *Geotrigona* has contents 12-20 times higher with a range of 497.09-807.25 meq/kg. 3. Reducing sugars are lower in pot-honey (16.24-52.36 g/100 g) than the *Apis mellifera* standards, minimum 65 g/100g. 4. Sucrose content of *Geotrigona*, *Melipona* and *Scaptotrigona* pot-honeys studied here is lower than the maximum 5 g/100 g permitted for *Apis mellifera* honey. The sensory perception of *Melipona* and *Scaptotrigona* honeys was high by the acceptance test, with frequent floral and citrusy notes for *Melipona*, and pollen and fruity notes for *Scaptotrigona*. A proposal of pot-honey standards needs no changes for sucrose content, but the 20% moisture and 40 meq/kg free acidity standards for *Apis mellifera* need higher maximum values, whereas the minimum 65% reducing sugars needs a standard with lower value.

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Author Contributions

Conceived and designed the analytical needs: PV, FMV. JVC coordinated the analytical service. Performed the sensory acceptance tests: PV, MA, JCV. Discussed data: PV, MA, JCV, FMV. Wrote the paper and statistical analysis: PV. All authors have drafted the outline, read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Access to stingless bee samples: The stingless bees are deposited in the Entomology Laboratory of Universidad Nacional de Loja, with Professor José Ramírez.

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Obtener un espectro sensorial de mieles de pote (descripción y aceptación). La descripción sensorial de las mieles fue incluida en el primer manuscrito de composición química, indicado arriba en el manuscrito enviado a Molecules. Ver registros sensoriales de aceptación en **Anexo 8** Evaluación sensorial. Además, se hicieron otras evaluaciones sensoriales de aceptación de miel ecuatoriana, inclusive en un ceviche de concha, aprovechando la realización del FIRMA en Machala, magistralmente presidido por el Dr. William Senior Prometeo-UTMACH, quien culminó con la publicación de las Memorias *in extenso*. A continuación, se presenta el trabajo en la Memoria *in extenso* y los dos resúmenes: 1. Presentado en el Congreso Internacional sobre Seguridad y Autenticidad de Productos de la Colmena en Istanbu, Turquía (21-22 Mayo 2015), sobre aceptación de 18 mieles ecuatorianas degustadas en el I Congreso de Apicultura y Meliponicultura en Ecuador, Machala 21-22 Febfrero 2015, y 2. A ser presentado en el Pangborn Sensory Science Symposium, en Gothenborg, Suecia, 23-27 Agosto 2015, sobre el perfil de libre elección realizado con asesores kichwas en la comunidad de Rio Chico provincia de Pastaza. Ver **Anexo 5** Certificados y **Anexo 6** Publicaciones en el CD.

Vit P (2014) Evaluación sensorial y emocional de ceviche de "concha prieta" *Anadara tuberculosa* con miel de "abeja de tierra" *Geotrigona* sp. y "catiana" *Scaptotrigona* sp. de la provincia El Oro, Ecuador. Memorias **VIII FIRMA 2014, Foro Iberoamericano de Recursos Marinos y Acuicultura**, Machala, Ecuador, 18-21 Noviembre.

Evaluación sensorial y emocional de ceviche de "concha prieta" Anadara tuberculosa con miel de "abeja de tierra" Geotrigona sp. y "catiana" Scaptotrigona sp. de la provincia El Oro, Ecuador

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RESUMEN
La acuicultura y la meliponicultura son más recientes en Ecuador, comparadas con la domesticación de cuyes y chanchos. Tanto la "concha prieta" Anadara tuberculosa como la miel de pote elaborada por Meliponini, se producen en la provincia El Oro en Ecuador. El consumo de concha prieta en ceviche es frecuente en la ciudad de Machala, y en el Restaurante Al Brujo llaman "pantera negra" al ceviche de concha prieta servido en copa con mezcla de choclo, camote, cebolla morada y cilantro. En este trabajo se evaluó el efecto sensorial y emocional de combinar miel de "abeja de tierra" Geotrigona sp. o miel de "catiana" Scaptotrigona sp. con ceviche de concha prieta en proporción 1:5, contrastadas

con un control sin miel, en una planilla con dos secciones. Arriba se colocaron tres líneas no estructuradas de 10 cm, ancladas con las palabras poco y mucho, para evaluar la aceptación del asesor Abajo se utilizaron 39 emociones validadas por EsSense® Profile. Los ceviches con miel evocaron más emociones del grupo energético y el ceviche solo evocó más emociones del grupo feliz.

PALABRAS CLAVE
Aceptación sensorial, Anadara tuberculosa, ceviche, concha prieta, evaluación emocional, Geotrigona, Meliponini, miel de pote. Scaptotrigona.

INTRODUCCIÓN

Comparadas con la domesticación de cuyes y de chanchos, la acuicultura y la meliponicultura son más recientes en Ecuador. La vasta biodiversidad neotropical alcanza casi 400 especies de abejas sin aguijón –pertenecientes a la tribu Meliponini (Camargo y Pedro, 2007), de las cuales 89 se encuentran en la zona 7, conformada por las provincias de El Oro, Loja y Zamora Chinchipe del sur de Ecuador (Ramírez et al., 2013).

Tanto la “concha prieta” Anadara tuberculosa como la miel de pote elaborada por Meliponini, se producen en la provincia El Oro en Ecuador. El consumo de concha prieta en ceviche es frecuente en la ciudad de Machala, y en el Restaurante Al Brujo llaman “pantera negra” al ceviche de concha prieta servido en copa con mezcla de choclo, camote, cebolla morada y cilantro.

En este trabajo se evaluó el efecto sensorial y emocional de combinar miel de “abeja de tierra” Geotrigona sp. o miel de “catiana” Scaptotrigona sp. con ceviche de concha prieta en proporción 1:5, contrastadas con un control sin miel.

MATERIALES Y MÉTODOS

Asesores La aceptación y la emoción evocada por cada tipo de ceviche de concha prieta, fue medida en 18 asesores de 18 a 65 años, 8 mujeres y 10 hombres. Aceptación Se usaron tres líneas no estructuradas de 10 cm, ancladas con las palabras “poco” y “mucho”, para evaluar la aceptación del asesor. Emociones Se utilizaron 39 emociones validadas por EsSense® Profile (King y Meiselman, 2010a,b) y traducidas al español (Vit, 2013) presentadas en siete grupos (Ver Tabla 1) para facilitar su elección, cinco con emociones positivas: 1. Energético, 2. Feliz, 3. Amoroso, 4. Seguro, 5. Calmado, y dos con emociones negativas: 6. Triste, 7. Asqueado. Cada emoción evocada se valoró con su intensidad (1. Baja, 2. Media, 3. Alta).

Tabla 1. Emociones presentadas en siete grupos.

Grupos de emociones						
‘Energético'	**‘Feliz'**	**‘Amoroso'**	**‘Seguro'**	**‘Calmado'**	**‘Triste'**	**‘Asqueado'**
Activo	Contento	Cariñoso	Satisfecho	Calmado	Triste	Agresivo
Aventurero	Bien	Amigable	Seguro	Saludable	Aburrido	Asqueado
Atrevido	Buen carácter	Amoroso	Firme	Liberado	Nostálgico	Ávido
Energético	Feliz	Tierno		Apacible		Culpable
Entusiasta	Alegre	Comprensivo		Pacífico		Descontrolado
Interesado	Dichoso	Acogido		Tranquilo		Preocupado
Creativo	Agradable			Dócil		
Estimulado	Complacido					
Sorprendido	Amable					
	Divino					
	Completo					
☺☺☺	☺☺	☺	☺😐	😐	☹	☹☹

RESULTADOS Y DISCUSIÓN

Los ceviches con miel evocaron más emociones positivas del grupo energético y el ceviche solo sin miel evocó más emociones del grupo feliz. Tanto el ceviche sin miel como el ceviche con miel de Scaptotrigona, evocaron una emoción negativa; mientras que en cuatro asesores el ceviche con miel de Geotrigona evocó emociones negativas de los dos grupos (Ver Tabla 2).

Tabla 2. Emociones positivas y negativas evocadas por el ceviche

TIPO de MIEL	Emociones positivas					Emocioes negativas	
	Energético	**Feliz**	**Amoroso**	**Seguro**	**Calmado**	**Triste**	**Asqueado**
Sin miel	4	**9**	**2**	**1**	**1**	**0**	**1**
***Geotrigona* "abeja de tierra"**	**7**	**3**	**2**	**0**	**2**	**2**	**2**
***Scaptotrigona* "catiana"**	**8**	**5**	**1**	**1**	**2**	**1**	**0**

En la Tabla 3 se aprecia una tendencia de preferir el ceviche control sin miel, y el motivo fue porque es más ácido. Entre las dos mieles, la de Scaptotrigona fue percibida como mejor combinación que la de Geotrigona, con el ceviche de "concha prieta". Las aceptaciones en los tres grupos fueron: 6,4 ± 2,6 (ceviche solo), 6,2 ± 2,3 (ceviche con miel de Scaptotrigona), y 5,4 ± 1,8 (ceviche con miel de Geotrigona). Además de tener mayor aceptación, aunque esta diferencia no es estadísticamente significativa con ANOVA ($P<0.05$), el ceviche control fue el mejor de los tres para 12/18 asesores, el ceviche con miel de Scaptotrigona fue el mejor para 5/18 y sólo 1/18 prefirió la mezcla con miel de Geotrigona.

Tabla 3. Aceptación (media ± DE) y Emociones - intensidad (frecuencia)

Variables	402 ceviche solo	551 ceviche con Miel *Geotrigona*	686 ceviche con *Miel Scaptotrigona*
Aceptación	6,4 ± 2,6	5,4 ± 1,8	6,2 ± 2,3
Emociones	Intensidades		
Activo	2 (1)		3 (1)
Aventurero			1 (1)
Energético		2 (1), 3 (2)	3 (1)
Entusiasmado	1 (1)	2 (1)	2 (2), 3 (1)
Interesado			1 (1)
Estimulado			2 (1)
Sorprendido	1 (1)	2 (1)	
Bien	3 (1)	2 (1)	2 (1)
Feliz	3 (1), 2 (1)	2 (1)	3 (1)
Alegre	3 (1)	2 (1)	3 (1)
Agradable	3 (4)		3 (1)
Complacido	2 (1)		
Completo			2 (1)
Amigable	3 (1)	1 (1)	
Amoroso	3 (1)	2 (1)	
Acogido			1 (1)
Satisfecho			3 (1)
Seguro	3 (1)		
Calmado		1 (1)	
Saludable			2 (1)
Liberado	2 (1)		
Apacible			2 (1)
Pacífico		2 (1)	
Aburrido		2 (1)	
Nostálgico		2 (1)	3 (1)
Agresivo		2 (1)	
Asqueado	2 (1)	3 (1)	

Este trabajo permitió valorar el uso de un ingrediente nuevo en la gastronomía a base de concha prieta ecuatoriana –la miel de pote, producto de los saberes ancestrales de la meliponicultura.

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Vit P, Isitua C, Deliza R (2015) Sensory evaluation of Ecuadorian honeys in a bee congress. **From Honey to Table. International Congress on Safety and Authenticity of Bee Products. Istanbul, Turkey**, 21-22 Mayo. p. 33.

Sensory evaluation of Ecuadorian honeys in a bee congress

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Ecuador has a great biodiversity of pot-honeys produced by stingless bees (Apidae: Meliponini) and Apis mellifera honeys produced in combs. However, imitations if these honeys are also present in the market. The acceptance of honey is a good indicator of the familiarity of consumers with genuine and false honey.
Objective: Honey consumers attending a bee congress in Ecuador discriminated 18 honey types by sensory analysis with the acceptance test.

Materials-Methods: The acceptance test used a 10 cm unstructured line scale by 58 participants of the First Congress of Apiculture and Meliponiculture in Ecuador, held in Machala 21-22 February 2015. Honey was served in crystal goblets and tasted with plastic spoons. Seventeen genuine honeys with diverse entomological origin (Apis mellifera, Geotrigona fumipennis, Melipona indecisa, Scaptotrigona ederi, Tetragonisca angustula) and botanical origin (multifloral, eucaliptus Myrtaceae, ceiba Bombacaceae) and one false. ANOVA and Duncan post-hoc were applied for statistical analysis

Results: The extreme acceptance values consisted on the honeys more and less accepted by the Ecuadorian assessors. One Apis mellifera honey was the least accepted lower than 4/10. Two Eucaliptus Apis mellifera honeys scored over 6/10, as well as the unique false honey.

Conclusion: Pot-honeys produced by local stingless bees obtained intermediate scores, and the extreme positions were given to Apis mellifera honey, lower than 4/10 and higher than 6/10. One fact that illustrates the need of sensory education of Ecuadorian honey consumers is that the unique false honey also scored over 6/10, and was not recognized as a manufactured syrup.

Vit P, Deliza R (2015) Sensory discrimination of genuine and false honeys by native Kichwa assesors from Rio Chico, Pastaza province, Ecuador". **11th Pangborn Sensory Science Symposium, Gothenborg, Suecia**, 23-27 Agosto 2015; sección Cross Cultural Sensory and Consumer Research (aceptado).

Sensory discrimination of genuine and false honeys by native Kichwa assessors from Rio Chico, Pastaza province, Ecuador

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Pastaza is the largest and least populated province in Ecuador, with seven native indigenous nationalities. The Kichwas from the Rio Chico community live near to the capital city Puyo, and were chosen to take part in this study for their knowledge on stingless honey bees. From the 400 species of Neotropical Meliponini that make honey in cerumen pots, almost 100 thrive in Southern Ecuador, and confer such biodiversity to pot-honey. In this research sensory characteristics of Ecuadorian false and genuine honeys with diverse entomological orign (*Apis mellifera* –light amber and amber, *Geotrigona* sp., *Melipona grandis* and *Scaptotrigona ederi*) were investigated with Kichwa assessors (four female and four male, aged from 18 to 62-y-o). The panel was asked to taste and to identify sensory attributes of honey (appearance, taste, smell, aroma, mouthfeel, other tactile sensations), and to score their intensities in 10 cm unstructured line scales anchored with the words weak and strong, using the Free-Choice Profile (FCP) methodology The Generalized Procrustes Analysis (GPA) was used on the FCP data. The first and second dimensions accounted for by 61.1% of the variance. In the descriptive sensory evaluation, darker honeys (amber *Apis mellifera*, false and *Geotrigona*) were separated from lighter colored (*Apis mellifera*, *Melipona* and *Scaptotrigona*) by the first dimension; whereas thicker honeys (*Apis mellifera* and false) were discriminated from thinner honeys (*Geotrigona*, *Melipona* and *Scaptotrigona*) by the second dimension. The assessors were able to evaluate and differentiate honey types without previous sensory training. Remarkably, two Kichwa ladies immediately spit out the false honey, in contrast to another acceptance study on 18-honeys, where the unique false honey was scored among the best three by 58 participants of a bee congress. Therefore, results suggest that Ecuadorian native Kichwas keep a sensory legacy of ancestral knowledge, and are more familiar with natural products.

Keywords: Kichwa, Ecuador, honey, FCP

4. <u>Aplicación de RMN en mieles de diverso origen entomológico y geográfico en Ecuador</u> (obtención de espectros y análisis quimiométrico con la Dra. Elisabetta Schievano). Se presentaron avances en tres eventos científicos. Ver **Anexo 5** Certificados y **Anexo 6** Publicaciones en el CD.

Schievano E, Zuccato V, Finotello C, <u>Vit P</u> (2015) NMR spectroscopy as a tool for honey analysis: Adulteration and entomological discrimination of Ecuadorian honeys. **I Congreso de Apicultura y Meliponicultura en Ecuador. Machala**, El Oro, Ecuador, 21-22 Febrero.

NMR spectroscopy as a tool for honey analysis:
Adulteration and entomological discrimination of Ecuadorian honeys

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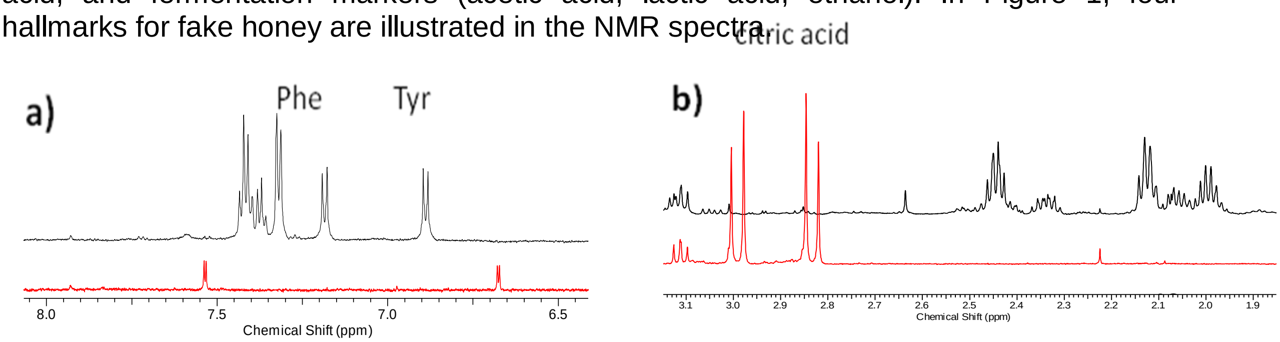
Honey is produced in combs by *Apis* species and in pots by Meliponini species of bees in the world. Meliponini are known as stingless bees. In Ecuador, honeys are produced in combs and pots. We studied honey produced by *Apis mellifera*, *Geotrigona*, *Scaptotrigona* and *Melipona* bees. Honey production in Ecuador is lower than the local demand. This fact besides the lack of a sanitary control to remove fake honey from the market, keep genuine and fake honeys on the shelves. Consumers are confused in front of honey and non-honey syrups, and the choice of a genuine product is hard. We performed a NMR study on aqueous honey dilution with D_2O and on chloroform extracts with the aim to identify the fake or adulterated honeys and to obtain an entomological discrimination of genuine honeys. The 1D spectra were acquired at 298 K, with a 600 MHz NMR Bruker instrument, signals of ^1H NMR spectra were integrated and used as inputs for PCA, PLS-DA analysis. Honey adulteration and entomological discrimination were successfully obtained. This study allowed the characterization and discrimination of the different honeys in terms of sugars, hydroxymethylfurfural, aminoacids and other organic compounds such as citric acid, and fermentation markers (acetic acid, lactic acid, ethanol). In Figure 1, four hallmarks for fake honey are illustrated in the NMR spectra.



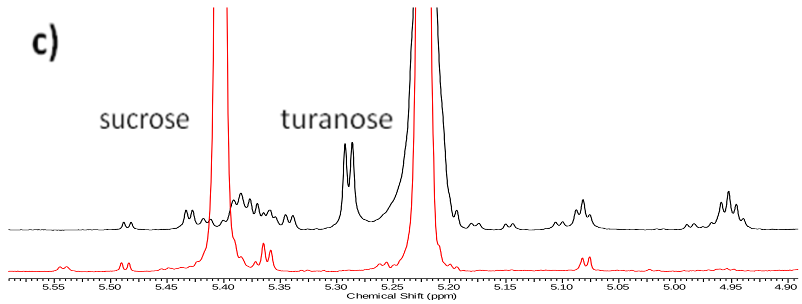


Figure 1. Hallmarks of fake honey in NMR spectra of honey diluted in D_2O (genuine honey in black, fake honey in red) a) Lack of aminoacids and high content of HMF, **b)** Presence of citric acid, **c)** Lack of natural sugars, but high sucrose concentration.

Keywords: honey, adulteration, *Apis mellifera*, aqueous dilutions, chloroform extracts, Ecuador, entomological origin, Meliponini, NMR, PCA, PLS-DA.

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Authenticity of Ecuadorian Commercial Honeys

Elisabetta Schievano, Valentina Zuccato, Claudia Finotello, Patricia Vit

Abstract—**Control of honey frauds is needed in Ecuador to protect bee keepers and consumers because simple syrups and new syrups with eucalyptus are sold as genuine honeys. Authenticity of Ecuadorian commercial honeys was tested with a vortex emulsion consisting on one volume of honey:water (1:1) dilution, and two volumes of diethyl ether. This method allows a separation of phases in one minute to discriminate genuine honeys that form three phase and fake honeys that form two phases; 34 of the 42 honeys analyzed from five provinces of Ecuador were genuine. This was confirmed with ^1H NMR spectra of honey dilutions in deuterated water with an enhanced aminoacid region with signals for proline, phenylalanine and tyrosine. Classic quality indicators were also tested with this method (sugars, HMF), indicators of fermentation (ethanol, acetic acid), and residues of citric acid used in the syrup manufacture. One of the**

honeys gave a false positive for genuine, being an admixture of genuine honey with added syrup, evident for the high sucrose. Sensory analysis was the final confirmation to recognize the honey groups studied here, namely honey produced in combs by *Apis mellifera*, fake honey, and honey produced in cerumen pots by *Geotrigona*, *Melipona*, and *Scaptotrigona*. Chloroform extractions of honey were also done to search lipophilic additives in NMR spectra. This is a valuable contribution to protect honey consumers, and to develop the beekeeping industry in Ecuador.

Keywords— fake, genuine, honey, ^{1}H NMR, Ecuador

I. INTRODUCTION

IF honey is not genuine, it is fake honey. Tropical markets are places of great biodiversity, but also of imitations of natural products such as honey. Genuine honey is sotred either in beeswax combs by *Apis* species and in cerumen pots by Meliponini species of bees in the world [1].

Diverse chemometric studies on honey authentication are based on chemical indicators based on physicochemical descriptors [2] proteins [3], metals [4] and sensory [5], to mention some of them –used alone or combined.

A honey authentication test with diethyl ether, discriminates genuine honeys with three phases and fake honeys with two phases. This test and ^{1}H NMR on deuterated water honey dilutions were used to standardize key components of genuine honey produced by diverse entomological sources such as *Apis mellifera* and pot-honey produced by the genera *Geotrigona*, *Melipona* and *Scaptotrigona*, contrasted with the abundant fake honeys in the Ecuadorian market.

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II. METHODS

A. Honey Samples

Forty two Ecuadorian commercial honeys from four entomological origins *Apis mellifera*, *Geotrigona*, *Melipona* and *Scaptotrigon*, plus fake honey were collected during field work in El Oro, Loja and Pastaza provinces, and kept frozen until analysis.

A. Authentication Test

Honey dilution was prepared with a fixed volume of liquid or cristallyzed honey, e.g. 1.0 mL plus the same volume of water, later 2.0 mL of diethyl ether were added and vigourosly shaked in the tube, let stand for one minute before observing the number of phases [6].

B. Deuterated Water Dilutions of Honey

For each honey sample, 200 mg of honey were dissolved in 600 µL of D_2O (Sigma-Aldrich, 99.96 atom % D, Milan, Italy), up to 1 ± 0.025 mL, 450 µL of the solution were transferred to 5 mm precision glass NMR tubes (Wilmad 535-pp). This extractive procedure yields a solution adequate for fast NMR analysis.

C. Chloroform Extraction of Honey

For each honey sample a chloroform extraction was done following a previous protocol [7] to find lipophilic additives.

D. ^{1}H NMR Spectra Acquisition

The ^{1}H NMR spectra were acquired at 298 K, with a 600 MHz NMR Bruker instrument.

E. NMR Data Processing Before Statistics

A data matrix was built, for statistical analysis if NMR spectra by considering only the sugar signals form to 3.6 ppm to 5.9 ppm excluding the segments containing water, to eliminate the variation in the water signals suppression, and the segments containing HMF resonances at 4.5 ppm. Data reduction was done by segmenting the spectra in 0.03 ppm intelligent buckets and the integral value was normalized by Total Sum Normalization. The calculations were performed using the program ACD. The obtained dataset (X matrix) was exported to Microsoft Excel and transferred into SIMCA-P+ software (v 13.0 Umetrics, Umea, Sweden).

F. Multivariate Data Analysis

The multivariate analysis was carried out onto mean-centered and unit variance (UV) scaled data through Projection to Latent Structures-Discriminant Analysis (PLS-DA). The supervised pattern recognition models Partial Projection to Latent Structures Discriminant Analysis (PLS-DA) has been chosen in order to attain classification rules for predicting the correct class. The quality of the models was described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the models and indicates the goodness of fit. Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability.

III. RESULTS

The authentication test was positive for 81% genuine honeys with three phases, of the 42 tested honeys. See Fig.1.

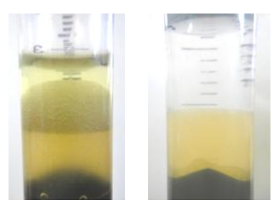

Fig. 1 Authentication test: Three phases for genuine honey (right) and two phases for fake honey (left)

In Fig. 2 we show two expanded regions of the NMR spectra of the chloroform phase in eight false Ecuadorian honeys: a) Region [5.45 – 4.85 ppm] has visible genuine honey signals (e.g. wax) indicating admixtures of syrups and honey in the lower spectra, while the upper spectra are flat as syrups; b) Region [8.5 – 5.0] with characteristic signals of manufactured honeys with residues of benzoic acid, hydroxymethylfurfural (HMF), 2-hydroxyacetyl-furan, sorbic acid and vanillin signals.

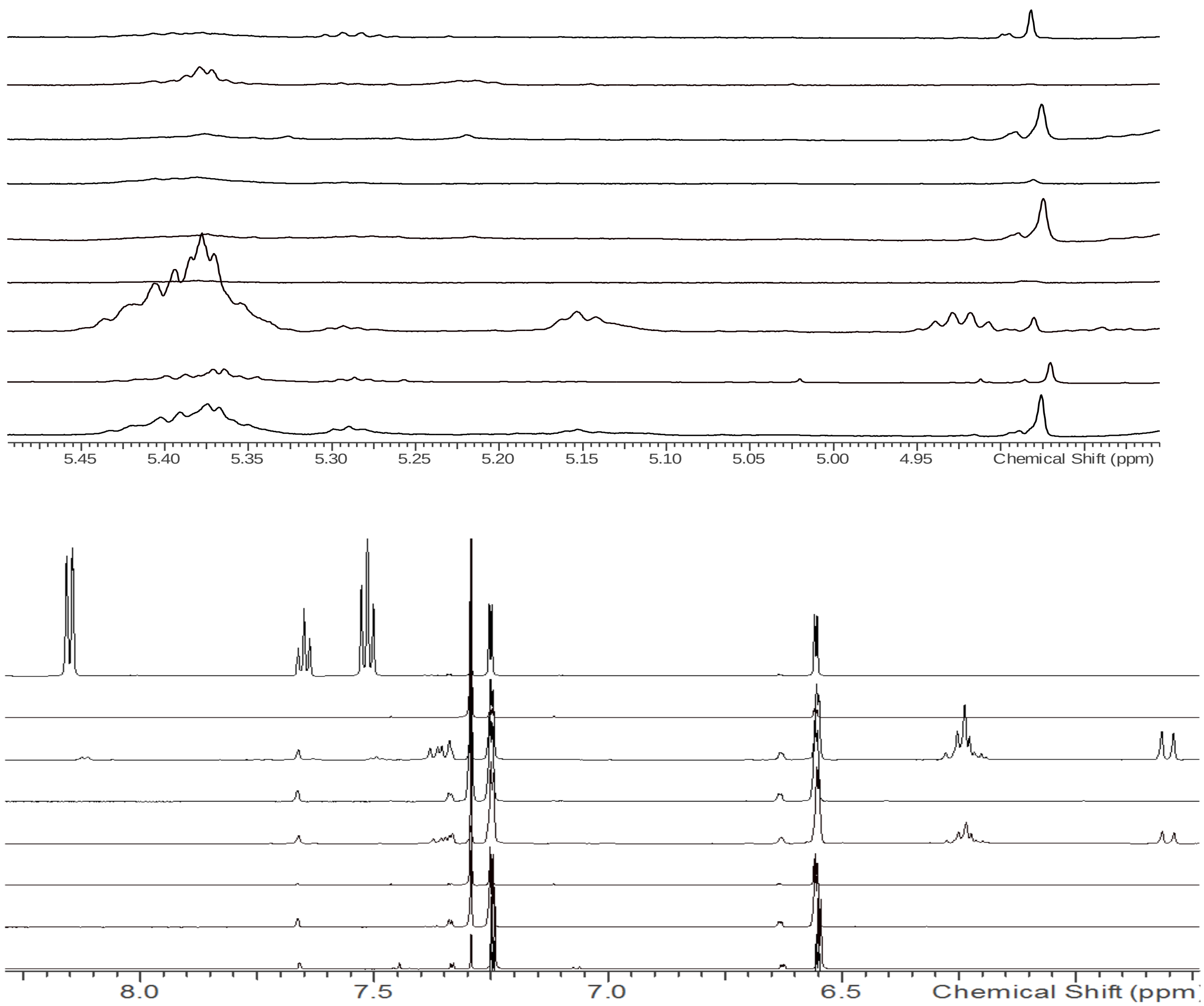

Fig. 2 Expansion of the NMR spectra of the chloroform phase of the eight false or adulterated Ecuadorian honeys: a) Region [5.45 – 4.85 ppm], b) Region [8.5 – 5.0]

NMR spectra of the eight false or adulterated honeys studied here (*Apis mellifera*, *Geotrigona*, *Melipona* and *Scaptotrigona*) were compared.

Besides the contrast of complex spectra profiles in genuine honey compared to poorer profiles in false honeys, the admixture of genuine honey and syrup is also confirmed with the NMR spectra, because of the simultaneous presence of natural sugars –like those of genuine honey– but a signal of excessive sucrose like in syrup.

Five chemical indicators are suggested to detect the presence of fake honey, or admixtures of fake honey with genuine honey, in Table 1. First of all, compared to genuine honey, fake honey lacks aminoacids. Second, fake honeys show high HMF signals that are not seen in genuine honey. Third, Ecuadorian fake honeys use citric acid and sorbic acid as additives in their manufacturing process, and therefore these acids are detected in the NMR spectra. The sugar composition is the fourth component to differentiate genuine from fake honeys, reported as the fourth indicator [8] but sugars really represent two hallmarks: A wide spectra of natural sugars is present in genuine honey (fructose, glucose, kojibiose, maltose, melibiose, nigerose, turanose, etc.) is the fourth indicator, whereas fake honey has important signals on sucrose as a fifth indicator. A sucrose content lower than 5 g/100 g is advised for genuine honey in the Ecuadorian honey standards [9] as well as by the Codex Alimentarius Commission [10].

Table 1. Chemicals markers of honey diluted in deuterated water.

Chemical indicators	**Honey Type**	
	Genuine	**Fake**
1. Aminoacids	Present	Absent
2. HMF	Low	High
3. Additives	Absent	Present
4. Natural sugars	Present	Absent
5. Sucrose	Low	High

The ^{1}H NMR spectra of honey dilutions in deuterated water with an enhanced aminoacid region showed signals for proline, phenylalanine and tyrosine in genuine honey, but these aminoacids were absent in false honey. The classic honey quality indicators (sugars, HMF) tested with ^{1}H NMR, confirmed that HMF content is very high in fake honeys derived from heated sucrose and syrups, compared to the low concentration up to 40 mg HMF/kg in the genuine *Apis mellifera* honey regulations [9, 10]. Sugars are also regulated compounds in honey norms, where sucrose has an upper limit of 5 g/100 g. Therefore, besides HMF, sucrose is also a target compound to detect false honey, in contrast to fructose, glucose major sugars [11, 12] and distinctive maltose in pot-honey produced by non*Melipona* stingless bees [13]. High concentrations of sucrose indicated manufactured honey. However some honeys had signals of genuine origin, with a diversity of natural minor sugars such as raffinose, turanose, nigerose, palatinose, kojibiose, others recently informed in a worldwide honey collection (erlose, isomaltose, maltose, melezitose, trehalose) of more than 800 honeys [11], and arabinose informed in a method to discriminate botanical origin in 328 honeys (manuka, multifloral, sunflower, honeydew, chestnut, acacia,orange, rape, eucalyptus) [12]. Indicators of fermentation (ethanol, acetic acid), and residues of citric acid used in the syrup manufacture, as declared in one label, in contrast to the natural citric acid reported as typical honey components [11, 12]. One of the honeys gave a false positive for genuine, being an admixture of genuine honey with added syrup, evident for the high sucrose. Sensory analysis was the final confirmation to recognize the honey groups studied here, namely honey produced in combs by *Apis mellifera*, with chatracteristic floral descriptors. Fake honeys have a major candy like odor-aroma [14]. Whereas honey produced in cerumen pots by *Geotrigona*, *Melipona*, and *Scaptotrigona*, have the entomological sensory descriptors more distinctive than the botanical origin, as previously observed for the perceptions of pot-honeys harvested in forests with the Huottuja assessors in the Venezuelan Amazon [15] and Kichwas in the Pastaza province of Ecuador.

IV. Conclusion

A simple method based on the number of phases after vigorous shaking of honey water dilution and diethyl ether, was complemented with NMR approach to dilucidate what are the key components of genuine and fake honeys useful in authentication routine. Five chemical indicators were suggested for that purpose because fake honeys: 1. Lack of aminoacids, 2. Show high HMF contents, 3. Keep citric acid as a marker of their human manufacture, 4. Lack of natural honey sugars, and 5. exceed the sucrose limits allowed for genuine honey.

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Entomological Origin of Honey Discriminated by NMR Chloroform Extracts in Ecuadorian Honey

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***Abstract*— Honeys are produced by *Apis mellifera* and stingless bees (Meliponini) in Ecuador. We studied honey produced in beeswax combs by *Apis mellifera*, and honey produced in pots by *Geotrigona* and *Scaptotrigona* bees. Chloroform extracts of honey were obtained for fast NMR spectra. The 1D spectra were acquired at 298 K, with a 600 MHz NMR Bruker instrument, using a modified double pulsed field gradient spin echoes (DPFGSE) sequence. Signals of ^{1}H NMR spectra were integrated and used as inputs for PCA, PLS-DA analysis, and labelled sets of classes were successfully identified, enhancing the separation between the three groups of honey according to the entomological origin: *A. mellifera*, *Geotrigona* and *Scaptotrigona*. This procedure is therefore recommended for authenticity test of honey in Ecuador.**

***Keywords*—** *Apis mellifera*, honey, ^{1}H NMR, entomological origin, Meliponini

Introduction

HONEY is the sweet product extracted from combs by *Apis* species and from pots by Meliponini species of bees in the world [1]. Meliponiculture is the art to keep stingless bees. "Stingleness seems rather sensational" although it does not refer to a lack of sting, but to a modified non-functional sting [2]. These bees of the Meliponini Tribe live in the tropics, and their honeys processed in cerumen pots were enjoyed before Columbus as *Apis mellifera* was introduced later [3].

In a human/animal interface study Aboriginal honey hunting in Australia is reviewed [4]. Domesticating stingless bee feral colonies in hives still needs strategies that facilitate the location of nests, and that also facilitate honey harvest [4,5].

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Networking between stingless bee keepers is an indicator of success to multiply colonies and marketing of pot-honey [5]. In a Mexican survey with Mayan stingless bees, *Melipona beecheii* showed colony loss caused by abundance of Africanized honey bee, or beekeeping experience was lower than that caused by competition for food [6]. The Brazilian large-scale survey to support and improve meliponiculture by a quantitative approach and analysis, offers a solid basis needed to increase economic profits of stingless bee products, especially pot-honey [5]. Four key roles are highlighted here to explain why Brazilian Government officers in the tropics are interested to develop the stingless bee industry by promoting: 1. Their local biodiversity, 2. Their economic importance for the role as pollinators in natural flora and commercial crops, 3. The fact that lack of stings facilitates management, 4. Easier manipulation of smaller hives than *Apis mellifera*, demanding technical skills instead of physical strength, accessible to children, women, elderly unable to care for an apiary by themselves.

Standards of pot-honey have not been created [7]. The *Codex Alimentarius Commission* international honey standards for honey are designed for *Apis mellifera* only [8]. Only the Colombian Honey Norm has included an annex with quality chemical factors of pot-honey [9]. Needs of chemical markers to authenticate the entomological origin have been approached by multivariate analysis of fructose, glucose and maltose [10], physic-chemical quality factors [11], sensory Free Choice Profile (FCP) [12], NMR and chemometrics [13], flavonoid C-glycosides [14] and O-glycosyl flavones [15], and more recently by electrophoretic patterns because different species of stingless bees produce honey with distinctive protein bands [16].

Pot-honey is known to be consumed either for family use or to reach up to 1,100% the price of *Apis mellifera* honey [17]. This great difference is a motivation to declare a false entomological origins, causing an Economically Motivated Adulteration (EMA), to be added to the four types of EMAs causing vulnerabilities in the US honey market: 1. Dilution with syrups, 2. Feeding sugar to bees during nectar offer, 3. Masking countries of origin, and 4. Antibiotic residues after excessive veterinary doses [18]. Therefore the importance to certify the entomological origin of honey.

In this work, ^{1}H NMR on chloroform extracts of honey were used to discriminate between the entomological origin of honey produced by *Apis mellifera* and pot-honey produced by the genera *Geotrigona* and *Scaptotrigona*.

METHODS

Honey samples

Ecuadorian commercial honeys from three entomological origins *Apis mellifera*, *Geotrigona* and *Scaptotrigona* were collected during field work, and kept frozen until analysis.

Chloroform extract of honey

A water:chloroform mixture (1:1) was used as extractive solvent to discard sugars −the major compounds of honey, retained in the water phase. The aroma compounds and other hydrophobic substances are separated in the organic phase. This extractive procedure yields a concentrated solution adequate for fast NMR analysis. For that purpose, portions of 6.0 ± 0.1 g honey were weighed in a 20 mL capped centrifuge tube and dissolved with 15 mL of deionized water, 15 mL of $CHCl_3$ were added and the mixture was stirred for 10 min. The biphasic mixture was then centrifuged at 10,000 rpm for 15 min at 4 °C. The lower chloroform phase was collected in a glass vial and the solvent was evaporated under a gentle stream of nitrogen. The solid residue was dissolved in 600 µL of $CDCl_3$ and placed in an NMR tube, following the scheme of NMR-based metabolomic approach previously published. Chloroform is a convenient solvent compared to dimethyl sulfoxide (DMSO) and methanol (MeOH) previously used in NMR studies of honey, because the residual chloroform signal is very sharp, in a very small region at 7.26 ppm, which does not overlap with important regions of the spectra [19].

C. ¹H NMR spectra acquisition

The 1D spectra were acquired at 298 K, with a 600 MHz NMR Bruker instrument, using a modified double pulsed field gradient spin echoes (DPFGSE) sequence [20]. This pulse in the DPFGSE sequence allows the removal of signals present in the (0-2 ppm) region, required to improve digitization of the weaker peaks, lower integration errors, and also better quantification of the number of resonant spins. The spectra collection, processing and analysis required thirty minutes.

Statistical analysis

Signals of ^{1}H NMR spectra were integrated and used as inputs for PCA (Principal Component Analysis) and PLS-DA (Partial Least Squares – Discriminant Analysis) analysis [6]. PCA discriminates how one sample is different from another, and identifies the variables contributing to the variation. PLS-DA are based on similar principles as PCA, but additionally labelled sets of classes are identified, enhancing the separation between groups of observations.

RESULTS

The spectra of the three entomological origins of honeys studied here (*Apis mellifera*, *Geotrigona*, and *Scaptotrigona*) were compared. The honey produced by these groups of bees share common features and have distinctive regions that merit further analysis. Expanded ^{1}H-NMR spectra of chloroform extracts of honey produced by these three types of bees, are compared in Fig. 1. A central region (3.5-4.5) ppm is characteristic for each bee type, because it contains the endogenous lipophilic markers, independent of floral and geographical origin. Other substances vary their concentrations.

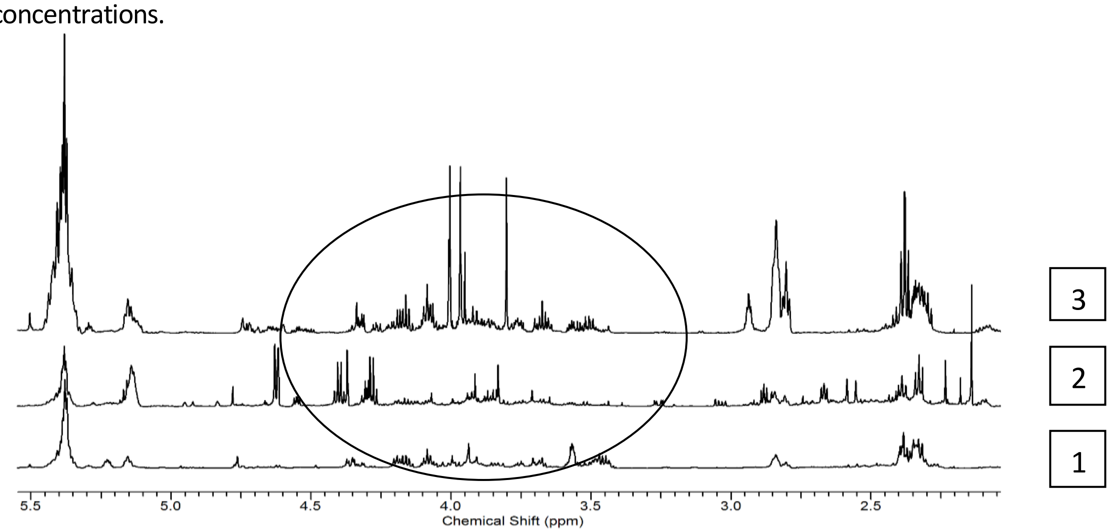

Fig. 1 Comparison in the expanded region (2.0-5.5 ppm) of ^{1}H-NMR spectra of chloroform extracts of honeys produced by: 1. *Apis*, 2. *Scaptotrigona*, and 3. *Geotrigona* bees in Ecuador.

In Fig. 2 the PLS-DA discriminates the three Ecuadorian honey groups according to the entomological origin.

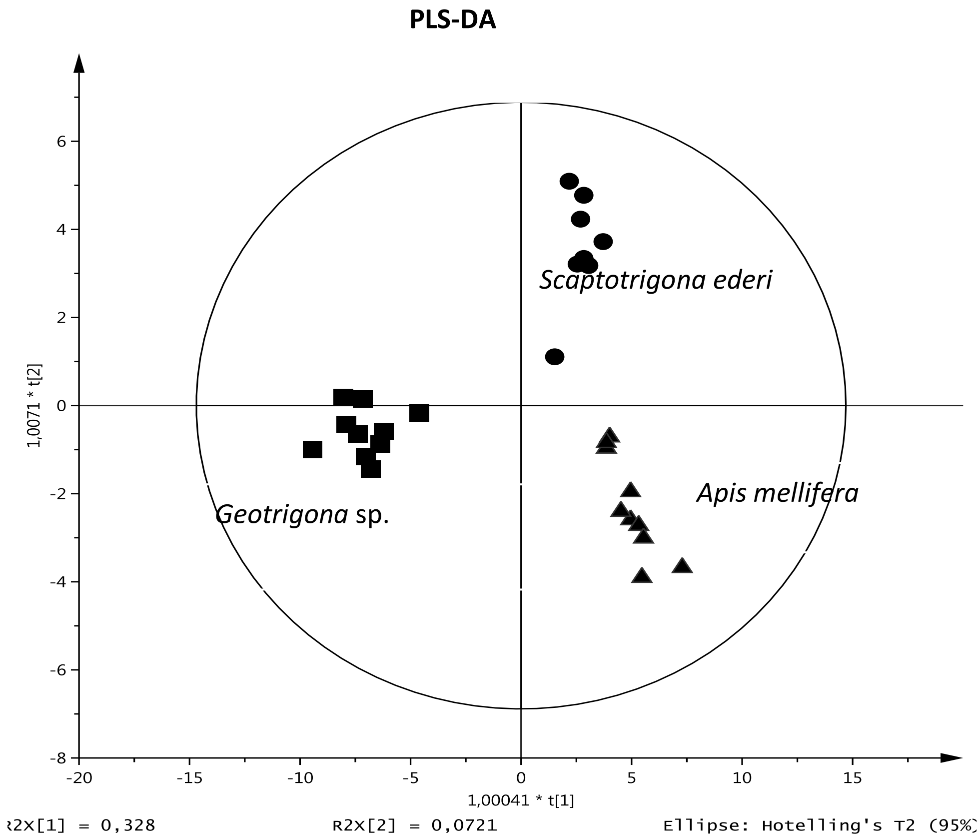

Fig. 2 Principal Component Analysis and PLS-DA on 1H NMR spectra of chloroform extracts of Ecuadorian honey produced by *Apis mellifera*, and pot-honeys produced by *Geotrigona* sp. and *Scaptotrigona ederi*.

This graphic separation shows more similarities between *Scaptotrigona* and *Apis mellifera* honey, separated only by the second component, because *Geotrigona* honeys are separated from them by the first component.

Besides the recent NMR profiling investigation with 800 honeys originated worldwide [21], the unifloral honeys from Italy [22] and Germany [23], and the entomological and geographical origin prediction [13], this research confirms a new element for denominations of protected origins in honey: The entomological origin, so important in tropical and subtropical ecosystems with stingless bees.

The Australian cosmovision of "Sugarbag Dreaming" connects honey to the knowledge of the surrounding environment and accepted hunting practices in the Northern Territory [4]. Stingless bees, sugarbag structures and honey are conceived in their relationship to humans and other beings as an "indigenous philosophical ecology" [24]. In this way, honey is a complex matrix, and ^{1}H-NMR one complex method to perceive its chemical composition, adequate to uncover the type of bee originating each honey. Direct observation of the spectra and clusters after integrating NMR signals of honey chloroform extracts by PCA will do that. Therefore, this method is a useful reference for the official agencies and becomes an alternative method for the Ecuadorian Honey Norm [25] to test the entomological origin of Ecuadorian honey, with a biodiversity of 89 stingless bee species in the El Oro, Loja and Zamora Chinchipe provinces in the South of Ecuador [26].

Conclusion

The entomological origin of the *Apis mellifera*, and the two major pot-honeys in the Ecuadorian market –*Geotrigona* and *Scaptotrigona ederi*, were successfully discriminated after PLSD-DA on ^{1}H NMR spectra of chloroform honey extracts.

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También se iniciaron varios manuscritos pero nunca se enviaron y aun se está decidiendo a cuál revista, posiblemente sean dos manuscritos en dos revistas diferentes. El compromiso con los objetivos del proyecto Prometeo quizás aumentó, por ello solicité que en sus publicaciones incluyan en Materiales y Métodos que las mieles ecuatorianas fueron recibidas del Proyecto Prometeo-UTMACH "Valorización de Mieles de Pote producidas por Meliponini en Ecuador" y ya no seré coautora.

A continuación el manuscrito enviado en Mayo, siguiendo las instrucciones de autor para Food Chemistry como decidió la Dra. Elisabetta Schievano, lo cual puede cambiar sin ningún problema ya que el trabajo fue realizado con el equipo RMN 600 MHz, los reactivos y el personal especializado del Departamento de Ciencias de la Universitá di

Padova, y que ellos decidan lo que ya no se pudo hacer para enviar este trabajo antes de concluir mi vinculación Prometeo-UTMACH.

Authenticity and biodiversity approach to Ecuadorian commercial *Apis mellifera* and Meliponini honeys

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ABSTRACT
The Ecuadorian biodiversity of stingless honey bees, defies false honeys in the market. Proton Nuclear Magnetic Resonance ^{1}H-NMR 600 MHz and multivariate statistical models were used to confirm false honey diethyl ether authentication test of 85 Ecuadorian commercial honeys. Genuine honey produced by *Apis mellifera* and Meliponini, was successfully discriminated from false honey, derived from inverted sugars. Clusters of entomological origin of honey were obtained. A set of adulteration and fermentive indicators was created for NMR spectra of D_2O honey dilutions and $CDCl_3$ honey extracts. Beeswax was identified as a component of the authentication test interface present in genuine honey and absent in false honey. Regulatory quality factors (HMF, glucose, fructose, and sucrose) were quantified by ^{1}H-NMR.

Keywhords: *Apis mellifera*, authentication test, Ecuador, honey, hydroxymethylfurfural, Meliponini, Nuclear Magnetic Resonance, quality control

1. Introduction

Bees of the world produce and store honey in beeswax combs ─*Apis* species, and in cerumen pots ─Meliponini species. Stingless honey bees belong to the Meliponini Tribe (Michener, 2007). The colony size varies with the species (Roubik, 1989). These bees thrive in the tropics, and their honeys stored in pots were delighted in Tropical America before Columbus, as *Apis mellifera* was introduced later (Schwarz, 1948). The oldest fossil of a bee is *Cretotrigona prisca*, dating back to the late Cretaceous (Michener & Grimaldi, 1988). Therefore the first honey in the planet was produced in pots by a stingless honey bee, until an oldest fossil of *Apis* may be found (Camargo, Personal Communication). Besides the fact of being the oldest bees in Earth planet, and their pantropical distribution in changing tropical zones (Engel & Michener, 2013), diverse evolutionary factors caused a great biodiversity up to 400 species group living in the Neotropics (Camargo & Pedro, 2007). The 89 species of Meliponini reported for three provinces in the South of Ecuador (Ramirez, Ureña & Camacho, 2013) provide a dimension on the complexity caused by entomological biodiversity to characterize genuine Ecuadorian honey.

Honey production in Ecuador is lower than the local demand, and some private initiatives from Carchi province export unifloral avocado, eucalyptus, "chaparro", tangerine and coffee honeys to Belgium, Canada, France, Spain, and United States (El Comercio, 2012). In the central market of Tulcan, capital of Carchi provic=nce, only false honey is available (P.V. Personal Observation). These facts besides the lack of a sanitary control to remove fake honey from the market, keep genuine and false honeys on the shelves in local markets. False honeys or honeys adulterated with various cheaper sweeteners, such as refined cane sugar, high fructose corn syrup and maltose syrup, result in higher commercial profits. Consumers are confused in front of honey, non-honey syrups or admixtures, and the choice of a genuine product is hard. This is an example of economically motivated adulteration (EMA), term created for "fraudulent, intentional substitution or addition of a substance for the purpose of increasing the apparent value of the product or reducing the cost of its production, i.e., for economic gain" (US Food and Drug Administration FDA). The high value of pot-honey reaching up to 1,100% to that of *Apis mellifera* honey (Alves, 2013) puts at risk the declared entomological origin. In Ecuador the cost of pot-honey sold in markets without labels reaches up to 40 USD/kg. The fact that United States of America imports 60% of honey, causes four types of EMAs causing vulnerabilities in the honey market: 1. Dilution with less expensive and always available syrups, 2. Feeding sugary supplements to bees to increase honey productiron, 3. Masking the country of origin to pretend internal production at higher price, and 4. Presence of antibiotic residues in a medicinal food (Easter Strayer, Everstine & Kennedy, 2014).

As major components of honey, sugars also play a key role in honey authentication. HPLC sugar spectra were used to describe (fructose, glucose, sucrose, turanose, maltose, trehalose, erlose) (Bogdanov, Vit & Kilchenman, 1996) and to cluster honey types from Venezuela according to the entomological origin using only sugars fructose, glucose, and maltose contents (Vit, Fernández-Maeso, & Ortiz-Valbuena, 1997) or in a multifactorial approach with six quality factors of honey: free acidity, moisture, sucrose, reducing sugars, HMF, and diastase activity (Vit, Persano Oddo, Marano, & Salas de Mejías, 1998).

NMR spectroscopy is a powerful tool for sugar detection in honey, but the diverse forms of sugars in water solutions increase complexity for full assignments of shifts. The botanical origin of Italian (Consonni & Cagliani, 2008; Consonni, Cagliani, Cogliati, 2012) and Brazilian (Boffo, Tavares, Tobias & Ferreira, 2012) honeys were identified by NMR. Saccharides by NMR were useful predictors of botanical origin in Italian honeys, as an alternative to the chromatographic and melissopalynological methods (Consonni, Cagliani & Cogliati, 2012). Mexican honey adulterations with sugar solutions (D-glucose, D-fructose and sucrose), and syrups (corn, inverted and cane sugar) were studied by Fourier transform infrared (FTIR) spectroscopy as non-destructive alternative to chemical measurement techniques for qualitative characterization, coupled to multivariate analysis (Rios-Corripio, Rojas-Lopez & Delgado-Macuil, 2012). Arabinose and L-rhamnose were also used besides major honey sugars D-fructose and D-glucose to discriminate the botanical origin in 328 honeys (manuka, multifloral, sunflower, honeydew, chestnut, acacia, orange, rape, eucalyptus) (Ohmenhaeuser, Monakhova, Kuballa, Lachenmeier, 2013). Additionally, the entomological origin of pot-honey produced by stingless honey bees was also predicted by NMR coupled to multivariate analysis (Schievano, Mammi, & Menegazzo, 2013). More recently, natural minor sugars such as erlose, isomaltose, maltose, melezitose, raffinose, trehalose, turanose, nigerose, palatinose, kojibiose, were reported after proton NMR profiling in a worldwide honey collection of more than 800 honeys (Spiteri, Jamin, Thomas, Rebours, Lees, Rogers, Rutledge, 2014). Both detections of foreign sugars and natural honey sugars are useful to control honey authenticity.

Pot-honey from Ecuador is used in traditional remedies to treat bruises, tumors, ocular cataracts, pterigium, inflammation, infections, varicose veins, cleaning blood after childbirth, kidney diseases, wound healing, and soothing balm before sleeping (Vit, Vargas, López & Maza, 2015). Pharmacological studies of pot-honey from the Chankuap Cooperative in Morona Santiago initiated the scientific basis to explain these medicinal properties (Guerrini, Bruni, Maietti, Poli, Rossi, Paganetto, Muzzoli, Scalvenzi & Sacchetti, 2009). In this work the authenticity of honey is attributed after observing a physical behavior of honey dilutions shaked with diethyl ether (Vit, 1998). A selection of classic (HMF, proline, sugars) and innovative (acetic acid, citric acid, ethanol) markers in the ^{1}H NMR spectra on honey diluted with deuterated water and honey extracted with deuterated chloroform were investigated to confirm the genuine or fake outcome of 85 commercial Ecuadorian honey, and a further discrimination of entomological origin.

2. Materials and methods

2.1. Chemicals and materials

Deuterated water (D_2O, ≥99.96%-d, Eurisotop); deuterated dimethyl sulfoxide-d_6 (DMSO-d_6, 99.9%-d, Sigma-Aldrich); isobutyraldehyde (≥99%, Sigma-Aldrich); N,N-dimethylformamide (DMF, ≥99.99% (GC), Fluka-Sigma-Aldrich); Calcium formate (standard for quantitative NMR, TraceCERT®, Fluka-Sigma-Aldrich). 5 mm precision glass NMR tubes (535-pp, Wilmad), coaxial insert (wgs-5bl, Wilmad). Pure saccharides were purchased from Sigma Aldrich (Milan, Italy).

2.2. Honey samples

Eighty-five commercial honeys from Ecuador were purchased or received from local bee keepers and stingless bee keepers, or in markets from Azuay, Carchi, Cochabamba, El Oro, Esmeraldas, Galápagos, Guayas, Imbabura, Loja, Los Ríos, Manabí, Morona Santiago, Napo, Orellana, Pastaza, Pichincha and Zamora Chinchipe provinces Ecuadorian provinces, identified as genuine or false by sensory analysis, and kept frozen until analysis. Stingless honey bees were collected in isopropyl alcohol when possible, and sent dried for entomological identification to Dr. S.R.M. Pedro, Biology Department, Universidade de São Paulo, Ribeirão Preto, Brasil. Ethnic names were given to one or more species of stingless honey bees, with commercial pot-honeys available in Ecuador: “abeja de tierra” *Geotrigona* and *Trigona*, “catiana” or “catana” *Scaptotrigona ederi* and *Nannotrigona*, “bermejo” *Melipona mimetica*, “bunga negra” *Melipona grandis*, “cananambo” *Melipona indecisa*. The vast biodiversity of Meliponini causes lack of ethnic names for bees ─called “mosco” besides the color, as observed during field work and reported in a thesis on ethnomeliponiculture (Chieruzzi Löwenstein, 1989).

From the 85 Ecuadorian honeys, 66 genuine honeys had the following entomological and geographical origin: 16 *Apis mellifera* (Agua Blanca, Manabí province; Cuenca, Azuay province; Chemico, Zamora Chinchipe province; Loja, Loja province; Portoviejo, Manabí province; Mombaiza, Morona Santiago province; Quevedo, Los Ríos province; Tablazo, Esmeraldas province), 13 *Geotrigona-Trigona* (Agua Blanca, Portoviejo and Quimís, Manabí province; Balsas and Los Ceibitos, Santa Elena province; Camarones, Esmeraldas province; Alamor, Cochas, Macará and Zapotillo, Loja province), 16 *Melipona* (Chontayacu, Puyo, Pastaza province; La Chiquita, Esmeraldas; Pindal, Loja province; La Moquillada, El Oro province; Tamiahurco, Napo province; via Los Zorros, Orellana province), 21 *Nannotrigona-Scaptotrigona* (Calera Chica, Chiriboga, Juan XXIII, Machala, Moromoro, Piedras Blancas, El Oro province; 12 de Diciembre, Denavip, El Trapiche, Potrerillos, San Pedro, Loja province; Tamiahurco, Napo province; via Los Zorros, Orellana province), and 19 were false honeys (Babahoyos, Los Ríos province; Ibarra, Imbabura province; Patate, Pastaza province; Quito, Pichincha province; Riobamba, Cochabamba province; San Joaquín, Guayas province; Santa Cruz, Galápagos province; Tulcán, Carchi province; Zamora, Zamora Chinchipe province; Zapotillo, Loja province).

2.3. Honey preparations for NMR

2.3.1. Aqueous samples

For each honey sample, about 200 mg of honey were precisely weighted and dissolved in D_2O, to reach a total volume of 1.00 ± 0.025 mL, 450 µL of the solution were transferred to 5 mm precision glass NMR tubes.

2.3.2 Chloroform extracts

Portions of 6.0 ± 0.1 g of honey were weighed in Teflon tubes and dissolved in 15 mL of deionized water; 15 mL of $CDCl_3$ were added and the mixture was mechanically stirred for 10 min and then centrifuged at 7200 rpm for 15 min at 4 °C. The lower chloroform phase was collected in a glass vial and the solvent was evaporated under a gentle stream of nitrogen. The solid residue was dissolved in 600 µL of $CDCl_3$ and placed in an NMR tube for the analysis.

2.4. Observation of the interface formed in honey/water/organic admixtures

2.4.1. Chloroform extraction

The insoluble material formed during extraction at the interface between the organic and the aqueous layer, was washed several times with water and chloroform to purify it from sugar residues and cold chloroform-soluble organic compounds. Once dried in an oven, the insoluble material was dissolved in chloroform at 40°C.

2.4.2. Diethyl ether test

A honey dilution was prepared with a fixed volume of liquid or crystallized honey, e.g.

0.5 ml plus the same volume of water, then 2 mL of diethyl ether were added and vigorously shaked in the tube, let stand for 1 minute to observe the number of phases (Vit, 1998).

2.5. ^{1}H NMR profiling

2.5.1. Spectra acquisition and processing

NMR experiments were recorded at 298 K (338 K in the case of acquisition of waxes extracts in $CDCl_3$) using a Bruker Avance DMX600 operating at 599.90 MHz for ^{1}H and equipped with a 5 mm TXI xyz-triple gradient probe.

All the spectra of the honey samples dissolved in D_2O and the spectra of chloroform extracts were first acquired using a common 1D-sequence, 256 transients were collected as 64K points with a spectral width of 14 ppm and a relaxation time of 2 s. For the HMF quantification a double pulsed field gradient spin echo (DPFGSE) sequence was used, modified by the addition of an inversion hard pulse after the first gradient in the G-S-G cluster, to obtain a G-π-S-G block (G is a pulsed field gradient and S is a soft π pulse). As a soft pulse, we used an inversion Reburp sequence of 1.2 kHz sweep width and 3867 ms duration centered at 4.00 ppm (Schievano, Finotello, Mammi, Illy Belci, Colomban & Navarini 2015) All gradient pulses were followed by a 100 μs recovery delay. The use of this sequence was necessary for quantifications in honey, while the NMR spectra are dominated by the intense signals of sugar protons, which limit the value of the Receiver Gain (RG) that can be used and consequently the digitalization of smaller signals, lengthening the acquisition time needed to reach a good signal to noise ratio and increasing the LoD and LoQ values. Line broadenings of 0.3 Hz were applied before Fourier transformation. Phase and baseline corrections were performed manually and as calibration we considered the signal of the αH1 proton of glucose at 5.22 ppm for aqueous samples, and of chloroform at 7.27 ppm for the extracts.

Every sample was analyzed in duplicate, the spectra were processed four times and data are reported as mean ± standard deviation expressed as mg/kg of honey.

Metabolites assignments are based on literature data (Spiteri, Jamin, Thomas, Rebours, Lees, Rogers & Rutledge, 2014) and spiking.

2.5.2 Quantifications

For the quantification of the absolute concentrations of HMF, a solution of an external standard was placed in a coaxial tube inserted in the NMR tube. The standard chosen is DMF dissolved in D_2O: its aldehydic proton resonate in the same spectral region of the analyte, not overlapping with any interfering signal. To have two final concentrations close to that expected in honey samples and in fake honeys, two standard solutions were prepared by accurately weighing the DMF and the solvent. 0.00203 M/0.000315 M .The method to calculate the concentration of the analytes has been previously described (Schievano, Finotello, Mammi, Illy Belci, Colomban & Navarini 2015).

To produce quantitative data, the relaxation delay was at least 5 times the longest measured T_1, corresponding to the aldehydic signal of DMF in D_2O, determining a relaxation delay of 51 s. (Schievano, Finotello, Navarini & Mammi, 2015). Depending on HMF content, the number of scans collected varied between 32-256, resulting in acquisition times between 30 min and 4 h.

2.5.3 The sugar resonances were identified by the addition of standard compounds

The content of some sugars in the honey aqueous solutions was measured considering in each case a non-overlapped proton signal where it was possible and the percentage of the corresponding sugar isoform. For fructose the signals at 4.10 ppm were considered, corresponding to protons H3, H4 of β-furanose (22.35%) and H3 of α-furanose (6.24%) (Barclay, 2011). In the case of glucose, the multiplet at 3.23 ppm was integrated corresponding to the H2 of β-glucose (62.8 %). In the case of sucrose and maltose the signals integrated were partially overlapped so the respective sugar content is an estimation, always higher than the actual content. The sucrose resonance at 4.21 ppm was considered, corresponding to proton H3, for maltose the resonance at 3.26 ppm corresponding to H2 of β-maltose (53% (o 61.1%) (Jamroz, Paradowska, Zawada, Makarova, Sławomir Kazmierskia & Wawera, 2013).

2.6 Statistical analysis

2.6.1 Pre-statistical processing of NMR data.

For statistical analysis of NMR spectra, a data matrix was built, excluding the segments containing water [4.77-4.90] ppm, to eliminate the variation in the water signals suppression, and the segments containing HMF resonances at [4.69, 6.68], [7.54, 9.45] ppm. Data reduction was conducted by segmenting the spectra in 0.03 ppm intelligent buckets obtaining 253 bins and the integrals value was normalized by Total Sum Normalization. The calculations were performed using the program ACD. The obtained dataset (X matrix) was exported to Microsoft Excel and transferred into Soft Independent Modeling of Class Analogy SIMCA-P^+ software (v 13.0 Umetrics, Umea, Sweden).

2.6.2 Multivariate data analysis

The multivariate analysis was carried out onto mean-centered and unit variance (UV) scaled data through Partial Least Squares-Discriminant Analysis (PLS-DA). The supervised pattern recognition modeling Partial Projection to PLS-DA has been chosen in order to attain classification rules for predicting the correct class. The quality of the models was described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the models and indicates the goodness of fit. Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability (Jung, Lee, Kwon, Lee, Ryu & Hwang, 2010).

3. Results and Discussion

Genuine commercial honeys available in Ecuador are produced by *Apis mellifera* and by several species of Meliponini, most exploited to collect honey from underground nests, logs, or stingless bee hives in traditional meliponaries. Different ethnic names are given to stingless honey bees from the same genus *Melipona*. This is the case for “bermejo” *Melipona mimetica*, “bunga negra” *Melipona grandis*, and “cananambo” *Melipona indecisa*. However, the same ethnic name is given to stingless honey bees from different genera producing pot-honey called “abeja de tierra” *Geotrigona* and *Trigona*, and “catiana” or “catana” *Nannotrigona* and *Scaptotrigona*. False honeys imitate both groups of genuine honeys.

3.1 Authentication test, hydroxymethylfurfural, sugar contents and ratios

According to the diethyl ether authentication test, three phases are produced in genuine honeys and two phases in the fake honeys (Vit, 1998). This authentication test was useful to discriminate genuine and fake honeys. Results of the test on the eighty-five Ecuadorian commercial honeys (from Azuay, Carchi, Cochabamba, El Oro, Esmeraldas, Galápagos, Guayas, Imbabura, Loja, Los Ríos, Manabí, Morona Santiago, Napo, Orellana, Pastaza, Pichincha and Zamora Chinchipe provinces) are given in Table 1. Besides the number of phases formed after shaking aqueous dilutions of honey with diethyl ether, or during the chloroform extraction, the concentration ranges of HMF, sucrose content and the fructose/glucose ratios are given. The *Apis mellifera*, *Geotrigona-Trigona*, *Melipona* and *Nannotrigona-Scaptotrigona* honeys produced the characteristic three phases for genuine honey. The commercial honeys with only two phases as response to the test, were classified as false.

Table 1. Authentication test, HMF, sucrose contents and fructose/glucose ratios of Ecuadorian commercial honeys

Honey type	Number of honeys	Number of phases		HMF content			Fructose/ Glucose ratio
		Diethyl ether test	Chloroform extraction	ND	NQ	mg/kg	
Geotrigona-Trigona	13	3	3	7	2	[51-409]	[1.00-1.13]
***Melipona* spp.**	16	3	3	3	1	515	[0.91-1.27]
Nannotrigona-Scaptotrigona	21	3	3	3	1	169	[0.98-1.41]
Apis mellifera	16	2	3	3	4	[2.5-836]	[1.00-1.13]
False	19			2	-	[93-2757]	[0.85-0.94]

ND= not detectable NQ= not quantifiable HMF values > 40 mg/kg and sucrose < 5 g/100 g does not comply with the *Apis mellifera* honey standard

3.1.1 Interface of the chloroform/water honey extraction

In Table 1, the number of phases observed in the authentication test (Vit, 1998) is the same number obtained with the chloroform/water procedure of honey extraction (Schievano, Pasini, Cozzi & Mammi, 2008). The intermediate phase between the organic chloroform and the aqueous layer was collected at refrigerated centrifuge temperature (at 4 °C) to investigate its nature. Then, the insoluble material of this interface was dissolved in deuterated chloroform at 40 °C. The NMR spectra of these solutions reveal that they contain waxes. For example, the spectrum coming from the insoluble material of *Apis* *mellifera* honey is very similar to the spectrum of *Apis mellifera* beeswax received from a beekeeper. The resonances of long chain esters of fatty acids are present in both honey and beeswax spectra. important NMR signals of beeswax present in the intermediate phase. Clearly, fake honeys produced by acidic hydrolysis of sucrose or starch, do not contain any beeswax, explaining why they originate only two phases in the authentication test, without the intermediate phase.

3.1.2 HMF in Ecuadorian commercial honeys

The HMF content is the classic quality indicator of freshness for honey. The Ecuadorian norm for honey does not comprise honey produced by other bees than *Apis mellifera* (Instituto Ecuatoriano de Normalización, 1988). Here we will consider the *Apis mellifera* HMF limit of 40 mg/kg honey and 80 mg/kg for tropical honey (Codex Alimentarius Comission, 1981, Revised Codex Standard 2001) as a reference for pot-honey, too.

As expected, the HMF values in fake honeys are high. The HMF content of 66 genuine honeys ─according to the authentication test─ was mostly non-detectable (ND), non-quantifiable (NQ) but seven of them have HMF values exceeding the accepted limits. Two *Geotrigona* honeys ranged 51-94 mg/kg whereas other two honeys ranged higher HMF values of 346-408 mg/kg. High HMF values of one *Apis mellifera* (836 mg/kg), one *Melipona* (515 mg/kg) and one *Nannotrigona-Scaptotrigona* (169 mg/kg) honeys exceeded many times the standard limits. These values may indicate a non-adequate processing, possibly due to heating, inadequate conservation, a too long shelf-life, or on the other hand, may suggest honey adulteration by syrup addition. The HMF formation was explained in four Italian unifloral honeys of *Citrus aurantium*, *Eucalyptus camaldulensis*, *Hedysarium coronarium* and *Castanea sativa*, and correlated with pH, free acidity, lactones and total acidity (Fallico, Zappala, Arena & Verzera, 2004). The particular chemical environment of unifloral honeys substantiated a consideration to adapt HMF limits according to the characteristic pH of each honey type. This could be also valid for the chemical environment caused by the entomological origins, especially for the sour and thin honeys produced by *Geotrigona-Trigona* bees.

3.2 Ecuadorian false honeys are inverted sugar

A syrup can derive from starch hydrolyzed with acids or with enzymes. Hydrolyzed starch syrup treated with isomerase produces high fructose corn syrup (HFCS). The other type of syrup derives from inverted sugar. It is possible to distinguish the different types of syrups by considering the fructose/glucose (F/G) ratio, the maltose and the sucrose contents. Therefore, the sugar composition was analyzed to distinguish these three types of commercial syrups in false honey. In general, the F/G ratio of syrups is always lower than the medium value found for genuine honeys. Ratios of F/G were calculated for starch syrup (0.05), HFCS (0.78-1.30), and for inverted sugar (1-1.18) from reported fructose and glucose data in the literature (Cordella, C., Militao, J. S. L. T., Clement, M. Drajnudel, P. & Cabrol-Bass, 2005; White, 2008; Bertelli, Lolli, Papotti, Bortolotti, Serra, & Plessi, 2010). In the Ecuadorian honeys, all the fake honeys have a F/G ratio ranging from 0.87 to 0.9 (see Table 1). These F/G ratios exclude that they are produced by starch hydrolysis, but this observation is not enough to distinguish the inverted sucrose from the HFCS origin of the syrup. However, sucrose was present in low (traces) and high concentrations (31- 300/100

g) in the false honeys. The negligible quantity of maltose ─in contrast to high maltose content in hydrolyzed starch and HFCS syrup─ and the high concentrations of citric acid, point out that Ecuadorian false honeys is manufactured from hydrolyzed sucrose with citric acid to obtain inverted sugar. The heating process to obtain inverted sugar dramatically increases the HMF content. Interestingly, false honeys with the highest sucrose contents also have the lowest HMF contents, indicating shorter heating time or lower temperature.

3.3 Comparison of false and genuine honey spectra

The ^{1}H NMR spectra of honey D_2O dilutions are a chemical fingerprint containing signals from sugars, amino acids, organic acids, HMF and other hydrophilic compounds. The ^{1}H NMR spectra of honey chloroform extracts are needed to analyze the hydrophobic fraction. Thus, a combined analysis of NMR spectra of these two honey preparations complement each other, providing an overview of the honey composition.

A set of NMR spectra was chosen to illustrate differences and similiarities of Ecuadorian honey types. The spectra of aqueous dilutions of genuine and fake honey are shown in Fig. 1. The natural sugars (e.g. raffinose, turanose, nigerose, palatinose, kojibiose) and the aminoacids (e.g. glutamine, phenylalanine, proline, tyrosine) are completely absent in false honeys (Fig.1).

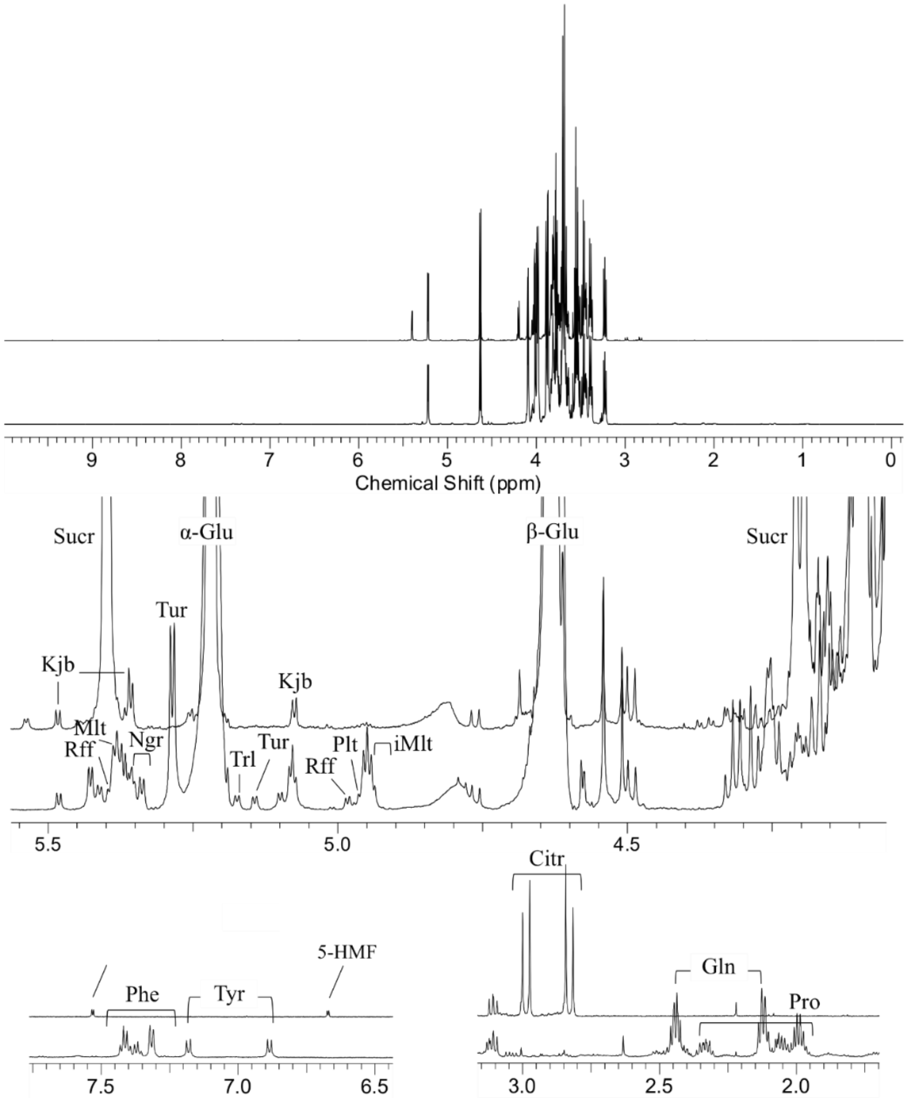

Fig. 1. NMR Spectra of Ecuadorian genuine and false honey diluted in D_2O.

However, it is possible to identify non aromatic organic acids (e.g. acetic, citric, lactic, formic), and ethanol as NMR fermentive indicators reported in Table 2 for 19 false honeys. Citric acid has a dual interpretation; although it is a component of genuine honey (Spiteri, Jamin, Thomas, Rebours, Lees, Rogers & Rutledge, 2014), its presence in high concentrations, is considered here as a residue of inverted sugar production. Propylene glycol is an additive not reported before in honey.

Table 2
NMR adulteration and fermentive indicators from D_2O and $CDCl_3$ honey spectra

NMR solvent	Adulteration indicator	NMR signals	Number of false honeys with adulteration

			indicators
$CDCl_3$	5-HMF	4,76(s); 6,56(d); 7,25(d); 9,64(s)	19
	2-Hydroxyacetyl-furan	4,77(s); 6,63(dd); 7,34(d); 7,66 (d)	19
	Sorbic acid	1,90 (d); 5,81 (d); 6,24 (m); 7,36 (dd)	2
	Benzoic acid	7,52 (t); 7,65 (t); 8,15 (d)	1
	Vanillin	4,00 (s); 7,07 (d); 7,46 (m); 9,86 (s)	2
D_2O	Lactic acid*	1,39 (d); 4,10(q)	3
	Ethanol*	1,18 (t); 3,62 (q)	6
	Citric acid	2,83 (d); 2,99 (d)	19
	Formic acid	8,26 (s)	9
	Acetic acid	2,08 (s)	13
	Propylene glycol*	1,13 (d); 3,41(dd); 3,51(dd); 3,84 (m)	4

*After spiking. (s) singlet, (d) doublet, (dd) double doublet, (m) multiplet, (q) quadruplet

Flavouring (vanillin), preservative additives (benzoic acid, sorbic acid), processing residues (citric acid), fermentive indicators (ethanol, acetic acid, lactic acid), as well as Maillard reaction products (5-HMF, 2-Hydroxyacetyl-furan) are chemical indicators of false honeys (Table 2). However pot-honey produced by Meliponini are naturally fermented. The presence of fermentive indicators in pot-honey has a different interpretation considering the limits if $CDCl_3$ adulterants. The fact that only 5/19 false honeys have sucrose contents above the permitted maximum 5 g/ g honey value in the honey standards, shows the low protection of this parameter to detect false honey in the Ecuadorian honey collection.

Representative expanded spectra of chloroform extracts for three fake honeys are reported in Fig. 2.

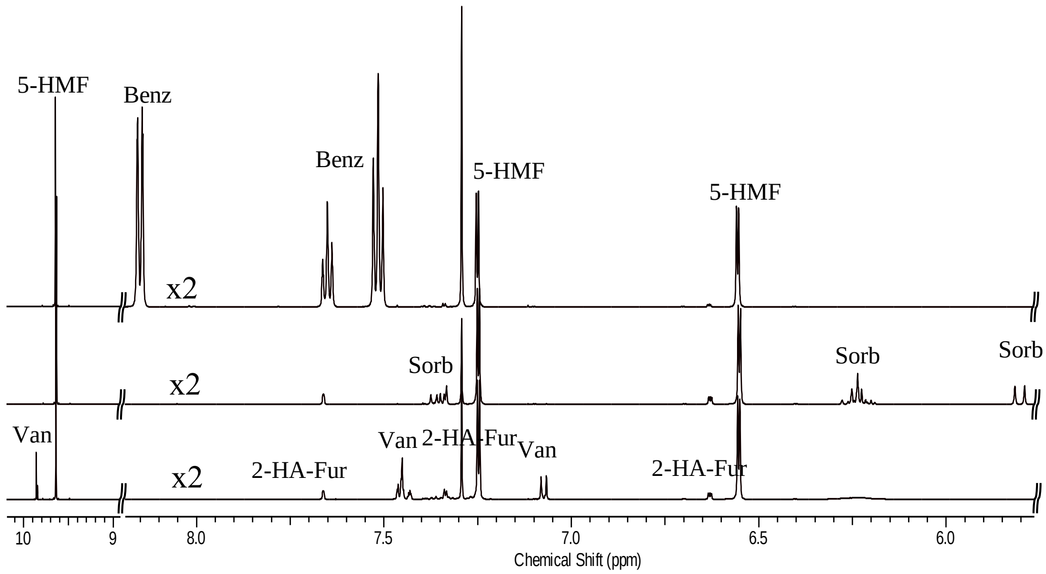

Fig. 2. Expanded NMR spectra of three representative false honeys to show adulteration indicators (see table 2 for compound abbreviations). 5-HMF is present in three honeys. Additional compounds in each spectra are: 1. Van, 2Ha-Fur; 2. Sorb; 3. Benz.

Four spectra for genuine honeys produced by *Apis mellifera*, *Geotrigona-Trigona*, *Melipona* and *Nannotrigona-Scaptotrigona* bees are shown in Fig. 3. A comparison of NMR spectra of the chloroform extracts provides key patterns to differentiate genuine and false honeys (Fig. 3). The presence of different types of compounds (e.g. amino acids, aromatics, fatty acids, phenolics, terpenes) originated from the biodiversity of plants (Schievano, Peggion & Mammi, 2010) and bees (Schievano, Peggion & Mammi, 2013) that make honey, confer complexity to the spectra of genuine honeys. The false honey spectra lack of floral and entomological signals.

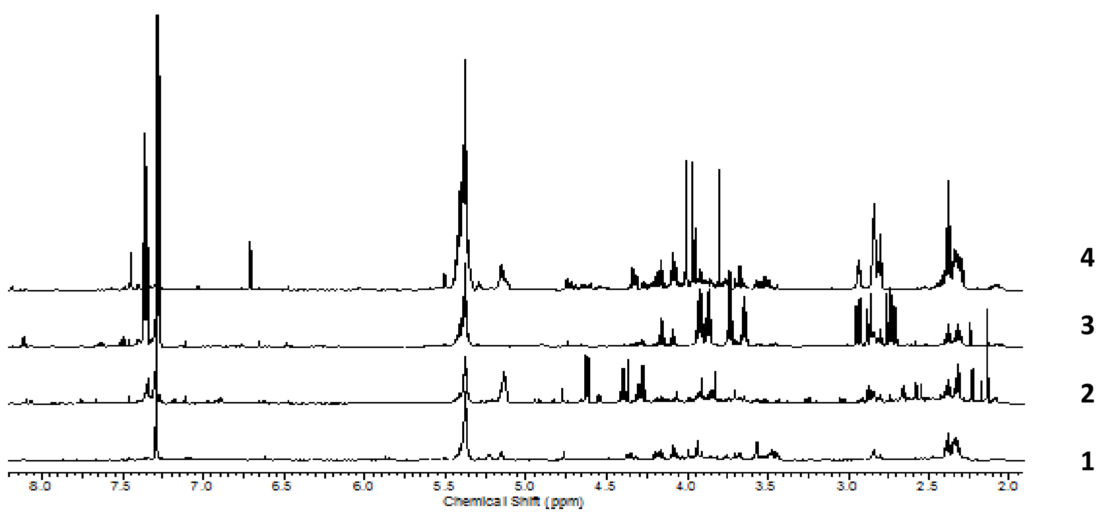

Fig. 3. Expansion of NMR spectra in representative genuine honeys produced by four bee groups: 1. *Apis*, 2. *Geotrigona-Trigona*, 3. *Melipona*, 4. *Nannotrigona-Scaptotrigona*.

3.4 Statistical analysis

An untargeted approach method was also used for the identification of adulterated samples, by using PLS-DA calculation. The PLS-DA was applied only to the sugar regions of the ^{1}H NMR spectra [4.0-5.5] ppm. The model was built by considering as unique class all the honeys classified as genuine by the authentication test vs all the false honeys. Graphical representations of the PLS-DA scores and loading plots of the 85 Ecuadorian honeys are shown in Fig. 4a, with subsequent eliminations of false honey (Fig. 4b) and "abeja de tierra" honeys produced by *Geotrigona* or *Trigona* bees are well separated from other genuine honeys and show large differences in the saccharide composition, because these samples cluster in a smaller region of the score plot.

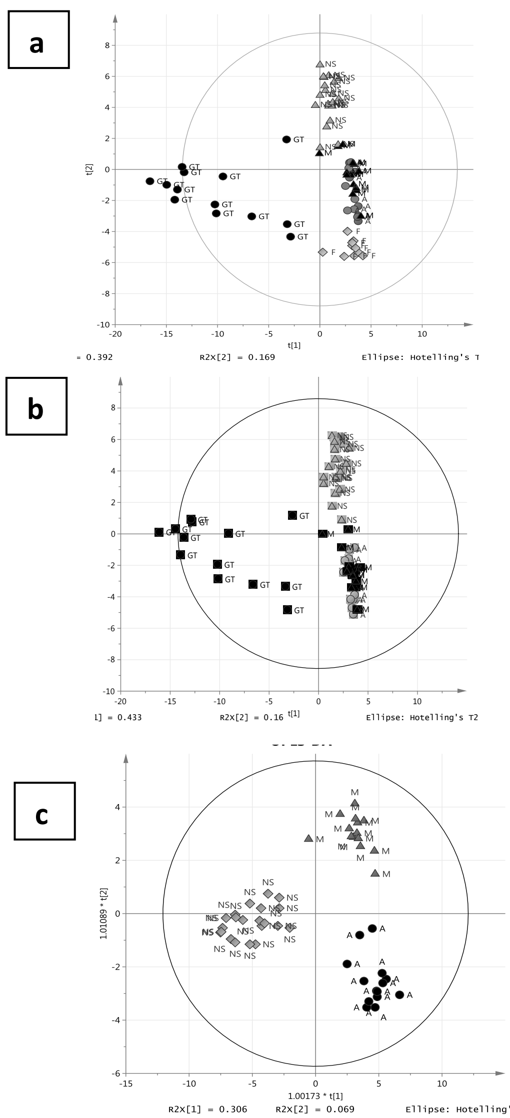

Fig. 4 Model score plots of genuine and false honey derived from ^{1}H NMR D_2O spectra. Honey classes: A *Apis mellifera*, GT *Geotrigona-Trigona*, NS *Nannotrigona-Scaptotrigona*, M *Melipona*, F False. Progressive groupings of honey types: **a)** Model with all the five honey classes: A-GT-NS-M-F, **b)** Model with four classes: A-GT-NS-M, without F, **c)** Model with three classes A-GT-NS, GT and F were removed for best fitting.

The chemometric analysis resulted in a very good entomological separation (Fig. 4c), after removal of false and GT to distinguish the A-M-NT classes. The corresponding loading plot indicated that glucose and sucrose are the characteristic variables for false honeys, while ─as expected, the natural saccharides, mainly kojibiose, nigerose, palatinose, raffinose, turanose, are the characteristics variables for genuine honey. In agreement with the fast and global authenticity screening of honey using ^{1}H-NMR profiling (Spiteri, Jamin, Thomas, Rebours, Lees, Rogers & Rutledge, 2014), also in this study of Ecuadorian honey, NMR is a remarkable option to quantify regulated parameters, and to check the onset of fermentation, as well as the entomological origin of Ecuadorian honey. In future, it is needed a melissopalynological database as done for Venezuela (Vit & Ricciardelli D'Albore, 1995; Vit, 2005), to check NMR markers for monofloral origin by NMR, as previously done for Italy (Schievano, Stocchero, Morelato, Facchin & Mammi, 2012) and Germany (Ohmenhaeuser, Monakhova, Kuballa & Lachenmeier, 2013). The botanical origin by itself is an authenticity test because fake honeys either lack of floral markers or will have a very low concentration of them in adulterations with syrups.

The model is able to classify correctly all the false and genuine honeys, with the exclusion of one sample initially labelled as genuine identified as adulterated by accurate analysis of NMR spectra of a "catiana" *Scaptotrigona* honey sold in a Pharmacy in canton Piñas, El Oro province, suspected of admixture. In conclusion the metabolomic approach allowed us to immediately identify the adulterated honey. The four *Geotrigona* samples with high HMF content, were correctly classified as genuine, confirming their authenticity. The high HMF content may be caused by the nature of such sour honey, as the HMF formation is enhanced by the low pH, as informed for Italian unifloral honey (Fallico, Zappala, Arena & Verzera, 2004).

Looking at pot-honeys, the diverse morphology and biology of stingless honey bees and their ancient natural history (Michener, 2013) predict new revelations in paleomelittology (Engel & Michener, 2013) to support the evolutionary success of the honey processed and stored in cerumen pots they still produce. The impressive underground honey named "abeja de tierra" produced by *Geotrigona* and *Trigona* species in Ecuador, is one of the most astonishing results of evolution in the honey world ─sour and thin as a tamarind juice, with distinctive bee and/or associated microbiota sensory remarks. Answering the course of this solution to store energy from nectars by a 2-3 m underground bee colony is beyond our understanding. Changing climates may have been a driving force, among others.

In Fig. 4, honeys produced by *Geotrigona-Trigona* (GT) are clearly clustered apart from *Apis mellifera* (A), *Melipona* (M) and *Nanotrigona-Scaptotrigona* (NT), as well as false honeys (F). The leitmotif for the prevalence of fake honey in the market is the economically motivated adulteration with thick syrups, finding room in a society with scant urban exposure to genuine honey types, limited honey availability, access to specialized honey laboratories and regulations without adapted standards. More similarities are visible between A-M-NT compared to GT, with chemical highest moisture and free acidity, perceived with thin visual consistency and sour taste (Unpublished Data). The PLS-DA model obtained with signals in the ^{1}H NMR spectra of honey D_2O dilutions, considers both the presence of chemical compounds and their concentrations in whole honeys as it is produced. We disagree with dehydrated honey preparations to have a dry basis database as previously investigated (Ohmenhaeuser, Monakhova, Kuballa & Lachenmeier, 2013) although it is useful for other purposes. More diluted GT honeys (right lower quadrant) may be separated from more concentrated F honeys (left lower quadrant), and the intermediate A-M-NT group (upper quadrants) with water contents near to 20 g/100 g honey. Chemical substances have a further meaning to identify species of bees, even by indirect metabolic processing of plant secondary metabolites such as flavonoids. The presence of their C- and O-glycosides in *Melipona favosa* pot-honey from Venezuela (Truchado, Vit, Tomás-Barberán & Ferreres, 2011), and O-glycosil flavones in *Tetragonula carbonaria* pot-honey from Australia (Truchado, Vit, Heard, Tomás-Barberán & Ferreres, 2015) is a new avenue to investigate the bee dependent fate of nectar chemical markers. Protein electrophoretic patterns of Mexican *Apis*, *Melipona* and *Trigona* honeys by SDS-PAGE have distinctive bands; therefore electrophoresis becomes an alternative method to detect entomological origin (Ramón-Sierra, Ruiz-Ruiz, Ortiz-Vázquez, 2015).

Alternative analytical methods are always useful to confirm official methods in food quality control, as needed for the honey industry. Several factors support the presence of false honey in the Ecuadorian market: 1. Decreased domestic production caused by climate change, 2. Regulatory efforts lack of Ecuadorian standards for honey and antibiotic control, 3. Insufficient analytical methods, 4. Limited food laboratories, 5. Absence of accredited laboratory for honey, 6. An official laboratory was suspended, 7. Investment on honey quality control is not planned, 8. Scanty knowledge of honey types by the public is a good ground for false honey industry growth and expansion, 9. Apiculture and meliponiculture need government protection to develop a solid genuine Ecuadorian honey prestige and availability to consumers, 10. Ecuadorian honey needs to be visible in a context of biodiversity, medicinal and economic opportunities to support upgrading of treasured ancestral knowledge with modern science.

Conclusions

Authentication test confirmed false honeys identified by sensory analysis, and was ratified by NMR spectroscopy. HMF content further characterized false honey and highlighted suspicious genuine honeys mixed with syrups. The PLS-DA analysis on the NMR sugar spectral region [3.0-4.5] ppm, revealed to be a rapid and reliable method to identify the adulterated honeys classified as false. Therefore a metabolomic approach together with the HMF determination, on aqueous dilutions, can represents a good procedure to identify adulterated honey. The usefulness of ^{1}H NMR is to detect false honey by adulteration indicators in D_2O honey dilutions (acetic acid, citric acid, formic acid, lactic acid, ethanol, propylene glycol, sucrose > 5 g/100 g, absence of natural sugars and aminoacids) and $CDCl_3$ extracts (benzoic acid, 5-HMF, 2-hydroxyacetyl-furan, sorbic acid, vanillin). It is necessary to enlarge the database of Ecuadorian commercial honey, and to analyze syrups of all types. Protection of genuine honey is an old problem, with updated technical resources such as the NMR spectroscopy presented here as a powerful tool coupled to chemometrics, or as an alternative method for some regulatory honey quality factors (fructose, glucose, HMF, sucrose).

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Conflicts of Interest

The authors declare no conflict of interest.

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Capacitación científica en el área pertinente a su especialidad

1. Capacitación en trabajo de campo para recolectar sistemáticamente abejas, información de nidos, mieles de pote, cuestionarios para usos tradicionales.Los

estudiantes Triny López y Cristian Trujillo asistieron a las primeras visitas científicas en Piñas y Las Lajas, para iniciar dos proyectos de investigación sobre inventario de abejas sin aguijón y caracterización de polen de pote producido por abejas Meliponini. A ellos se les mostraron los métodos de recolección de abejas como muestras entomol del polen para iniciar estudios de composición química. También se tuvieron varias runiones de búsqueda bibliográfica y revisín de la literatura científica. Hasta se creó un Grupo de investigación adscrito al Centro de Investigaciones Agropecuarias de la UTMACH. No hubo continuación por falta de tiempo de los estudiantes. Ellos fueron excelentes estudiantes y tuvieron mucho interés. Con Triny López se logró incirporar la información que obtuvimos en Las Lajas, en el poster presentado en el Congreso SILAE en Marsala, Sicilia, Italia Vit P, Vargas O, López T, Maza Valle F (2014) Traditional medicinal uses of pot-honey by meliponicultors from El Oro province in Ecuador. 23rd Italo-Latinamerican, Asian & African Congress of Ethnomedicine. Marsala, Sicily, Italia, 7-12 Septiembre. Esta articipación con poster se logró publicar en revista indexada en SCOPUS. Ver **Anexo 6** Publicaciones en CD. Vit P, Vargas O, López, T.V., Maza F. Meliponini biodiversity and medicinal uses of pot-honey from El Oro province in Ecuador. Emirates Journal of Food and Agriculture, Emir. J. Food Agric 27(6): 302-306. Online First: 13th April 2015. http://www.scopemed.org/?mno=184920 (Ver manuscrito en resultados de investigación).

2. Vínculo intitucional silviarmp@ffclrp.usp.br con la entomóloga Dra. Silvia RM Pedro, Departamento de Biología, Facultad de Filosofía, Ciencias y Letras, Universidad de São Paulo, Ribeirao Preto, Brasil, para identificar las abejas sin aguijón ecuatorianas, Meliponini. Ver **Anexo 2** Listado de Abejas Meliponini en Ecuador y **Anexo 3** Fichas de Abejas Meliponini en CD.

3. Formación y/o actualización en análisis químicos de rutina para el control de calidad de la miel de pote. No se realizaron análisis en la UTMACH, sino por servicio en laboratorio privado PROTAL ESPOL. Hubo una interacción teórica en la discusión de métodos oficiales durante la revisión de la norma Miel de Abejas. Requisitos NTE INEN 1572 y una demostración práctica del kit para detectar adulteraciones de miel, basado en el número de fases producidas al agitar una solución de miel con éter etílico y dejar en reposo (Vit, 1998), en la reunión No. 6. Ver **Anexo 9** INEN en CD.

4. Formación en evaluación sensorial de las mieles de pote (descripción y aceptación). Se realizaron evaluaciones de aceptació sensorial en Machala (provincia de El Oro), Puyo (provincia de Pastaza), Italia y Puerto Bolívar (provincia de El Oro), con más de 100 asesores (Ver Bases de datos del **Anexo 8** Evaluación sensorial en CD).

5. Interpretación de Resonacia Magnética Nuclear (RMN) en mieles como herramienta discriminatoria de origen entomológico y geográfico. Se realizaron tres visitas científicas al Departamento de Ciencias Químicas de la Universitá di Padova, con la Dra. Elisabetta Schievano, y sus asistentes Jalal Uddin, Valentina Zuccato y Claudia Finotello. Además de la discriminaci'on entomológica, se realizó un estudio de autenticidad de las mieles donde surgieron indicadores de RMN para diferenciar mieles genuinas de mieles falsas, junto con el método de Vit (1998) a base de agitación de solucion acuosa de miel con éter etílico. Estas interpretaciones fueron presentadas en: 1. I Congreso de Apicultura y Meliponicultura en Ecuador, I CAME 2015, Machala 21-22 Febrero. 2. International Conference of Nutritional and Nutraceutical Sciences, ICNNS 2015, Singapore 29-30 Marzo, 3. International Conference on Food Process Engineering, ICFPE 2015, Montreal 11-12 Mayo (Ver resúmenes y Memorias *in extenso* en los resultados de la investigación). Las interpretaciones realizadas en Italia fueron estudiadas junto con el Dr. Favian Maza, Contraparte Oficial de Prometeo UTMACH, con introduccion del Curso Básico RMN de la University of Calgary, Canadá y las clases gentilmente ofrecida con su usuario y palabra clave del Dr. Massimo Bellanda de la Universitá di Padova, Italia.

6. Se presentó el libro Pot-honey: A legacy of stingless bees, para actualizar el conocimiento sobre mieles producidas por abejas sin aguijón en potes de cerumen (dirigido a profesores, personal y estudiantes universitarios). Se presentó el libro durante el I Congreso de Apicultura y Meliponicultura en Ecuador, y una de las resoluciones fue apoyar la traducción del libro al español para que pueda llevarse la Academia al Campo, a fin de aumentar el conocimiento en meliponicultura en las comunidades.

Gestión de recursos nacionales

Se participó en la convocatoria SENESCYT 2014 con dos notas conceptuales avaladas por el Rector de la Universidad Estatal Amazónica, Puyo, Provincia de Pastaza, Dr. Julio César Vargas (Ver Notas conceptuales en CD **Anexo 11**):

1. Se formuló una nota conceptual para valorar las mieles de pote de Ecuador con un museo: "Museo Gota de Miel, para valorar las mieles de pote precolombinas y los cultores de las abejas sin aguijón (Meliponini) en Ecuador" (no pasó a la siguiente etapa).
2. Se formuló una nota conceptual para caracterizar mieles ecuatorianas: "Caracterización de mieles ecuatorianas falsas y genuinas de *A. mellifera* y Meliponini" (se encuentra en evaluación).

Además se elaboró otra nota conceptual que no ha sido avalada por ninguna universidad aún y se está gestionando con la Universidad Técnica de Quevedo, por la Dra. Janne Rojas, quien es Prometeo-UTEQ.

3. Se formuló una nota conceptual para formar niños y maestras en unidades educativas, con entrenamiento en evaluación sensorial de mieles para reconocer la biodiversidad de mieles ecuatorianas producidas en panal por *Apis mellifera* y en potes de cerumen por Meliponini, y también para detectar las mieles falsas: Defender la miel ecuatoriana conociéndola mejor con el kit educativo "Ama la Miel".

Relacionamiento estratégico interinstitucional a nivel nacional e internacional

Se elaboró y se firmó un convenio marco entre la Universidad de Los Andes, Mérida, Venezuela y la Universidad Técnica de Machala en el mes de Agosto de 2014. Este convenio ha facilitado interacciones y colaboraciones académicas con profesionales requeridos para el proyecto (Ver pdf de **Convenio ULA-UTMACH firmado** en CD **Anexo 10**)

Se asistió a las reuniones INEN para revisar la norma de miel. Además el 7 de Abril se propuso la elaboración de una norma para Miel de Pote, ya que no se incluyeron sus requisitos en la Revisión de la norma Miel de Abejas. Requisitos. NTE INEN 1572. Ver **Anexo 9** INEN en CD.

PAPER INDEXADO O ARTÍCULO CIENTÍFICO PUBLICADO

Se iniciaron varios manuscritos que no se publicarán por razones diversas, incluyendo el asunto relacionado con gestión de la queja ante el Servicio de Acreditación Ecuatoriano (SAE). Se continuaron manuscritos iniciados antes de la vinculación. Los manuscritos y/o publicaciones se colocaron en la sección de resultados y están en el CD **Anexo 6** Publicaciones. A continuación la lista de artículos publicados, Memorias de congreso, Memorias *in extenso*, manuscritos enviados y en elaboración:

Artículos publicados

Artículo iniciado antes de la vinculación

Truchado P, Vit P, Heard T, Tomás-Barberán FA, Ferreres F. Determination of interglycosidic linkages in O-glycosil flavones by high-performance liquid chromatography/photodiode-array detection coupled to electrospray ionization ion trap mass. Its application to *Tetragonula carbonaria* honey from Australia. Rapid Communications in Mass Spectrometry, **Rapid Commun Mass Spectrom 29: 948-954**. SCOPUS

Artículos del Proyecto Prometeo

Vit P, Vargas O, López, T.V., Maza F. Meliponini biodiversity and medicinal uses of pot-honey from El Oro province in Ecuador. Emirates Journal of Food and Agriculture, **EJFA 27(6): 502-506**. Online First: 13th April 2015. http://www.scopemed.org/?mno=184920 SCOPUS.

Vit P, Uddin J, Zuccato V, Maza F, Schievano E. Entomological origin of honey discriminated by NMR chloroform extracts in Ecuadorian honey. World Academy of Science, Engineering and Technology *International Journal of Biological, Food, Veterinary and Agricultural Engineering* 9 (5): 437-440. http://waset.org/publications/10001263/entomological-origin-of-honey-discriminated-by-nmr-chloroform-extracts-in-ecuadorian-honey INDEX COPERNICUS

Schievano E, Zuccato V, Finotello C, Vit P. Authenticity of Ecuadorian commercial honeys by NMR. World Academy of Science, Engineering and Technology *International Journal of Biological, Food, Veterinary and Agricultural Engineering* 9 (3): 303-306. http://waset.org/publications/10001258/authenticity-of-ecuadorian-commercial-honeys INDEX COPERNICUS

Revisión iniciada antes de la vinculación

Vit P, Huq F, Barth OM, Campo M, Pérez-Pérez EM,Tomás-Barberán FA, Santo EL. Use of propolis in cancer research. British Journal of Medicine and Medical Research **BJMMR 8 (2): 88-109**. Online First 18th. April 2015.

Pre-Print del Proyecto Prometeo

Vit P, Brito E, Maza Valle F. Reto científico de investigadores Prometeo en la Universidad Técnica de Machala, provincia El Oro, Ecuador. pp. 1-17 Online first Agosto 2014. http://www.saber.ula.ve/dspace/bitstream/123456789/38938/1/UTMACH.pdf

Manuscritos enviados

Manuscrito iniciado antes de la vinculación, enviado a EJFA 13.05.15

Vit P, Santiago B, Pedro SRM, Peña-Vera M, Pérez-Pérez E. Chemical and bioactive characterization of pot-pollen produced by Melipona and Scaptotrigona stingless bees from Paria Grande, Amazonas State, Venezuela.

Manuscrito del Proyecto Prometeo elaborado para Fuerza Farmacética (divulgación, no indexada)

Patricia Vit, Luis Xavier Freire, Ernesto Fuentes, Jorge Espinoza, Héctor Baque, Eduardo Vilao, Roberto Del Peso, Carlos Vergara, Antonio Mora, Favian Maza, Raul Casanova, Elizabeth Pérez-Pérez, Viviana Frisone. Una iniciativa con apoyo de la Unión Europea para el desarrollo apícola en el bosque seco de la Comuna Las Balsas, provincia de Santa Elena, Ecuador.

Memoria de Congreso

I Congreso de Apicultura y Meliponicultura en Ecuador, I CAME 2015. Machala, Ecuador, 21-22 Febrero 2015.

Vit P (editora) 2015. 2ª Edición. Memorias de Resúmenes I Congreso de Apicultura y Meliponicultura en Ecuador. Universidad Técnica de Machala; Machala, Ecuador. xxvii + 31 pp. http://www.saber.ula.ve/handle/123456789/40285

Memorias *in extenso*

Foro Iberolatinoamericano de Recursos Marinos y Acuicultura, VII FIRMA 2014. Machala, Ecuador, 18-21 Noviembre 2014.

Vit P. Evaluación sensorial y emocional de ceviche de "concha prieta" *Anadara tuberculosa* con miel de "abeja de tierra" *Geotrigona* sp. y "catiana" *Scaptotrigona* sp. de la provincia El Oro, Ecuador. Foro Iberoam. Rec. Mar. Acui. VII: 465-469.

XIII International Conference on Nutritional and Nutraceutiacal Sciences, ICNNS 2015. Singapore 29-30 Marzo 2015.

Schievano E, Zuccato V, Finotello C, Vit P. Authenticity of Ecuadorian commercial honeys. (2 pages) **13 (3) Part XV pp. 1488-1489.**

17th International Conference on Food Processing Engineering, ICFPE 2015. Montreal, Canadá, 11-12 Mayo 2015.

P. Vit, J. Uddin, V. Zuccato, F. Maza, E. Schievano. Entomological origin of honey discriminated by NMR chloroform extracts in Ecuadorian honey. (2 pages) **17 (5) Part XI pp. 1805-1806.**

Manuscritos elaborados

Manuscrito del Proyecto Prometeo iniciado para Food Chemistry SCOPUS

Schievano E, Vit P, Finotello C, Zuccato V, Pedro SRM, Maza M, Mammi S. Authenticity and biodiversity approach to Ecuadorian commercial *Apis mellifera* and Meliponini honeys

Manuscrito del Proyecto Prometeo iniciado para Anticancer Research SCOPUS

Fazlul Huq, Jun Qing Yu, Patricia Vit. Cytotoxicity of *Melipona*, *Scaptotrigona* and *Trigona* Ecuadorian pot-honeys in ovarian cancer cell model.

CONTRIBUCIÓN AL PLAN DEL BUEN VIVIR

Las mieles de pote de Ecuador obtuvieron visibilidad local, nacional e internacional con la iniciativa "Ruta de Museos Vivientes de Abejas Meliponini en el Mundo", la cual no recibió apoyo de la Prefectura de El Oro, pero si ha interesado a la Prefectura de Santa Elena. Prometeo no dispone de recursos para tal fin y la UTMACH tampoco.

Su caracterización química y sensorial es necesaria para incluirlas en las normas de Miel de Abejas de Pote, ya que actualmente sólo se refieren a la miel producida por *Apis mellifera*.

Los meliponicultores recibieron capacitación en talleres técnicos coordinados por la UTPL, en un proyecto de revitalización de la meliponicultura. Las asociaciones de meliponicultores permiten agruparse para resolver problemas compartir avances y progresos. La Prefectura de Loja y la Prefectura de Santa Elena tienen Asociaciones de Meliponicultores.

La producción de miel de pote ha motivado la formación de grupos de aprendizaje y práctica de meliponicultura por programas de prefecturas y por ONGs como Altrópico. Los análisis de sus mieles, permitirán dar un salto cualotativo para valorizar un producto sin normas de calidad. El grado de dificultad es mayor que el estudio de mieles de *Apis mellifera*, ya que presenta además el componenete de origen entomológico. La presente investigación ha aportado un método alternativo de análisis por RMN (por colaboración de la UNIPD) para identificar mieles según su origen entomológico: 1. *Apis mellifera*, 2. “abeja de tierra” *Geotrigona-Trigona*, 3. “bermejo” “bunga negra” “cananambo” *Melipona*, 4*.* “catana” “catiana” *Nannotrigona-Scaptotrigona.* Una denominación de origen entomológico protegido sería un gran valor para la gran biodiversidad de mieles ecuatorianas producidas en potes. La valorización de las mieles de pote en Ecuador podría incentivar microempresas de los saberes ancestrales de la meliponicultura ecuatoriana.

Este proyecto se enmarca preferencialmente en los objetivos 3, 7 y 8 del Plan Nacional del Buen Vivir (2013-2017).

Objetivo 3: Mejorar la calidad de vida de la población.

Objetivo 7: Garantizar los derechos de la naturaleza y promover la sostenibilidad ambiental territorial y global.

Objetivo 8: Consolidar el sistema económico social y solidario, de forma sostenible.

El fortalecimiento de la meliponicultura ecuatoriana aporta miele genuinas medicinales a la población y aumenta el ingreso de los productores. La protección de mieles genuinas por análisis de autenticidad, y propuesta de norma de calidad, aporta beneficos a la cadena de mercadeo (objetivo 3). La sostenibilidad ambiental para proteger la naturaleza y la biodversidad de abejas sin aguijón, aprendiendo a multiplicar nidos y realizando cosechas no destructivas de las colonias, se ha promovido con los contactos con meliponicultores durante las recolecciones de miel de pote en las visitas científicas (objetivo 7). Consolidar el manejo de colmenas adaptadas a las diferentes abejas sin aguijón, la cosecha, el envaado y la conservaciín de las mieles, junto con su denominación de origen entomológico para aportarvalor agregado de la miel de pote (objetivo 8).

El proyecto ha permitido visualizar los meliponicultores ecuatorianos que protegen el arte de criar abejas sin aguijón como saberes ancestrales
La producción artesanal se ha beneficiado de incursiones tecnológicas por ONGs y proyectos de universidades para el manejo de las abejas sin aguijón a fin de optimizar la meliponicultura tradicional.

Contribuciones con objetivos macro:

Las mieles de pote de Ecuador obtuvieron visibilidad local, nacional e internacional.

La caracterización química y sensorial fue útil para incluirlas en la propuesta de normas de miel de abejas, ya que actualmente sólo se refieren a la miel producida por *Apis mellifera*. Se detectó deficienicas analíticas en laboratorios, las cuales deben solventarse con asesoría de laboratorios internacionales acreditados en análisis de miel.

La valorización de las mieles de pote en Ecuador ha incentivado microempresas coordinadas por ONGs y la meliponicultura ecuatoriana.

Los meliponicultores recibieron capacitación familiar o en talleres técnicos. Las asociaciones de meliponicultores permiten agruparse para resolver problemas compartir avances y progresos, como en Loja (GADPL, UTPL), Esmeraldas (ALTROPICO) y Santa Elena (GADPSE).

La producción de miel de pote podría motivar la formación de grupos de aprendizaje y práctica de meliponicultura. La educación es lo más lento y la promoción debería ser centralizada por el MAGAP.

Contribuciones con objetivos micro

Los individuos que participaron en esta investigación conocieron las especies de abejas sin aguijón en Ecuador por sus nombres étnicos y científicos. La divulgación de los nombres científicos estará más arraigada con la promoción de placas para cada abeja de los Guías de Estación de la Ruta de Museos Vivientes de Abejas Meliponini en el Mundo (Ruta Meli) y su posible presentación web. También con la elaboración de un capítulo de abejas ecuatorianas para la traducción al español del libro “Pot-honey. A legacy of stingless bees” editado por Springer 2013 NY, Vit, Pedro, Roubik (editores).

Los investigadores encontraron similitudes y diferencias entre mieles producidas por diversas especies de abejas sin aguijón, desde las percepciones sensoriales hasta su composición química. Las publicaciones científicas de los resultados permitirán mayor comprensión del objeto de estudio, en este caso de las mieles de pote ecuatorianas silvestres y producidas en meliponarios. Ya se han iniciado. Son pequeñas contribuciones pero ya se han hecho visibles y se ha divulgado el nombre del Programa Prometeo, la Universidad Técnica de Machala y Ecuador en el ámbito nacional e internacional. Me parece injusto haber tenido que asumir los gastos de esas participaciones sin apoyo institucional. Espero que para los más jóvenes se generen oportunidades más estimulantes y de agradecimiento por la labor científica en Ecuador, ellos lo necesitan para avanzar y desarrollar la investigación científica en ciencias de la vida.

Sólo dos estudiantes de diversas carreras de ciencias de la vida pudieron aprender y practicar el estudio sistemático de la miel producida por abejas sin aguijón, y luego ya no tuvieron tiempo.

Además, deseo colocar en este informe la inclusión del Buen Vivir en las dos notas conceptuales enviadas a la Convocatoria 204 SENESCYT:

Museo Gota de Miel

1. Auspiciar la igualdad, la cohesión, la inclusión y la equidad social y territorial, en la diversidad. Una miel producida por abejas precolombinas actualmente ignorada en la normas de miel, pero apreciada en las comunidades rurales, merece ser incluida con los aportes de la ciencia para justificar y promover su producción y comercialización. Tanto

las mieles uniflorales y/o de mielada producidas por Apis mellifera como por Meliponini, deben ser diversificadas para entender la gran biodiversidad territorial en materia apícola. Evaluar residuos veteriarios y tóxicos permitirá adelantos en materia de seguridad alimentaria.

2. Favorecer el cambio de la matriz productiva en componentes de ingreso familiar de productores de miel, luego de valorizar su producto con información sobre composición e indicadores de calidad.
3. Fortalecer las capacidades y potencialidades de los apicultores y meliponicultores, empoderándolos de los indicadores de calidad de la miel que producen en Ecuador, para su toma de decisiones en asuntos sobre análisis de control y para registro sanitario.

6. Garantizar el trabajo digno en todas sus formas. El reconocimiento de las mieles genuinas y las mieles falsas aumentará el apoyo interinstitucional con los entes gubernamentales actuales (AGROCALIDAD y ARCSA) de gran impacto social cuando participen en esta labor preventiva y de detección y las nuevas generaciones, enmarcado en el Plan Nacional del Buen Vivir 2013-2017 (SENPLADES, 2013).

7. Según el artículo 74 de la Constitución Nacional de Ecuador, “Entre las instituciones de educación superior, la sociedad y el Estado, existirá una interacción que les permita contribuir de manera efectiva y actualizada a mejorar la producción de bienes y servicios y el desarrollo sustentable del país, en armonía con los planes nacionales, regionales y locales”. Así, las comunidades pueden beneficiarse sabiamente de las riquezas del medio ambiente.

Kit Ama la Miel

Este programa está concebido para contribuir con los siguientes objetivos del Plan Nacional del Buen Vivir:

1. Auspiciar la igualdad, la cohesión, la inclusión y la equidad social y territorial, en la diversidad. Una miel producida por abejas precolombinas actualmente ignorada en la normas de miel, pero apreciada en las comunidades rurales, merece ser incluida con los aportes de la ciencia para justificar y promover su producción y comercialización, junto

con las mieles uniflorales producidas por *Apis mellifera*, las cuales deben ser diversificadas para entender la gran biodiversidad territorial en materia apícola.

2. Promover la presentación comercial de diferentes tipos de miel y ofrecer vinculación universidad-comuidad mediante la capacitación, los servicios y la investigación científica para mejorar la calidad de vida, en la medida que se pueda aumentar el ingreso familiar gracias al reconocimiento de las mieles genuinas. Contribuye al cambio de la matriz productiva.

3. Fortalecer las capacidades y potencialidades de la ciudadanía, mediante la participación en eventos para actualizar y asesorar a maestros, estudiantes y todo público interesado en el uso medicinal de la miel genuina.

4. Construir espacios de encuentro común y fortalecer la identidad nacional, las identidades diversas, la plurinacionalidad y la interculturalidad. Este programa vincula directamente el lugar de producción de conocimiento innovador en base a una larga experiencia de investigación en la universidad, para hacerlo disponible a la comunidad escolar y consumidora de miel de abejas, a fin de empoderarla para reconocer las mieles falsas por evaluación sensorial, así las puede rechazar y evitar su consumo en recetas medicinales tradicionales.

5. Garantizar los derechos de la naturaleza y promover la sostenibilidad ambiental territorial y global. Este programa está orientado a múltiples facetas de las abejas y su entorno natural, la sostenibilidad ecológica porque hay que protegerlas en el tiempo y la interacción del hombre con los recursos nutricionales y medicinales que obtiene de la naturaleza para su consumo y supervivencia.

6. Garantizar el trabajo digno en todas sus formas. El programa propuesto será fuente de trabajo para diversas profesiones y una oportunidad de pasantías y becas para experiencias profesionales relacionadas. Las capacitaciones para compartir conocimientos en el reconocimiento de las mieles genuinas y las mieles falsas aumentará el apoyo interinstitucional con los entes gubernamentales actuales (AGROCALIDAD y ARCSA) aun no incorporados, pero de gran impacto social cuando participen en esta labor preventiva y de detección y las nuevas generaciones.

7. Garantizar la soberanía y la paz, profundizar la inserción estratégica en el mundo y la integración latinoamericana. La cata de mieles es un arte que requiere paz, contemplación y concentración para reconocer los indicadores sensoriales que la abeja recolecta en la naturaleza y compone en su obra de arte llamada "miel". Las personas que se entrenan en degustaciones de la miel aprenden a percibir el mundo en una gota de miel en su paladar. La inserción latinoamericana para proteger la apicultura beneficia la producción agropecuaria.

Ecuador es un país consagrado al Sagrado Corazón de Jesús y el mes de junio se celebra el Sagrado Corazón de Jesús. Junto con el Buen Vivir, el lema "Ecuador Ama la Vida" y otros lemas bien nombrados, esta tierra de gracia debe florecer. Algunos moradores son más organizados que otros, y pueden ayudar a quien necesitan orientación para lograr mejor calidad de vida con su conocimiento ancestral del campo. Hay tantos esperando la transferencia y el intercambio requeridos para el progreso.

DESCRIPCIÓN DE PRODUCTOS ALCANZADOS

Los productos de la propuesta realizada están enumerados y descritos en las secciones de RESULTADOS OBTENIDOS y PAPER INDEXADO O ARTÍCULO CIENTÍFICO PUBLICADO, con los respectivos respaldos en el CD, y en los anexos (participaciones en eventos científicos, organización de congreso I CAME, convenio ULA-UTMACH, colección de mieles, fichas entomológicas, referencias bibliográficas, resultados de análiisis PROTAL-ESPOL, gestión de la queja ante el SAE, nota conceptuales, Guía de la Ruta de Museos Vivientes de Abejas Meliponini en el Mundo. En el Cd se incluye el **Anexo 12** Informes de Visitas Científicas

Se presentan otros tres anexos de productos alcanzados en el CD:

Anexo 13 Ruta de Museos Vivientes de Abejas Meliponini en el Mundo, **Anexo 14** Arbitraje manuscritos y evaluación proyectos, y **Anexo** 15 I Congreso de Apicultura y Meliponicultura en Ecuador.

CONCLUSIONES Y RECOMENDACIONES

Conclusiones

1. Se pudo iniciar la investigación planteada "Valorización de mieles de pote producidas por Meliponini en Ecuador".

2. La oportunidad de aproximación a la gran biodiversidad de abejas Meliponini en Ecuador significó un mayor trabajo de visitas científicas, identificaciones entomológicas y análisis.

3. Se publicó un artículo científico derivado del poster presentado en el Congreso de la Sociedad Italolatinoamericana de Etnomedicina, SILAE 2013, Marsala, Italia, sobre las propiedades medicinales atribuidas a las abejas Meliponini en Ecuador, en revista indexada en SCOPUS.

4. La valiosa colaboración de la Dra. Silvia R.M. Pedro de la Universidade de São Paulo en Ribeirão Preto, Brasil permitió identificar las abejas recolectadas que pudieron llegar a Brasil (hubo muchas devoluciones por correo).

5. Los resultados de sacarosa aparente y de azúcare reductores de 52 mieles no se pueden usar por problemas analíticos. Esto demoró la realización de análisis para el total de las mieles recolectadas (117). Algunas mieles se agotaron pero 90 mieles fueron enviadas a INTERTEK, Bremen, Alemania para una colaboración analítica en laboratorio certificado.

6. La obtención de espectros de RMN por una inestimable colaboración con la Dra. Elisabetta Schievano de la Universitá di Padova, Italia, ha permitido detectar adulteraciones y separar mieles según su origen entomológico, los cuales se presentaron en tres congresos internacionales y se publicaron en dos memorias *in extenso*. Y también dos publicaciones indexadas en INDEX COPERNICUS, derivadas de las ponencias en ICNNS Singapore, 29-30 Marzo 2015, sobre autenticidad de la miel ecuatoriana, y la ICFPE en Montreal, 11-12 Mayo 2015, sobre origen entomológico de la miel ecuatoriana.

7. La organización del I Congreso de Apicultura Meliponicultura en Ecuador (I CAME 2015), reunió ocho Prometeo-UTMACH en el comité organizador. Fue un honor haber presidido este congreso con 130 inscritos y haber recibido solicitud de la sede para el II CAME 2016 por el MAGAP en Tungurahua.

Recomendaciones

1. Se recomienda crear una agencia para gestionar visitas científicas para estancias y/o congresos. Yo debí financiar personalmente mis participaciones, recibí ayuda de empresa privada para participar en congreso (medio boleto aéreo) y estancia (medio boleto aéreo).

2. Aunque se solicitó varias veces, no hay acceso institucional a las bases de datos científicas internacionales Web of Science, SCOPUS, etc.

3. Crear un laboratorio analítico especializado en mieles, puede ayudar al control de calidad requerido para promover la industria de miel ecuatoriana y proteger al consumidor.
4. Revisar la Agenda propuesta para el Desarrollo de la Apicultura y Meliponicultura en Ecuador.
5. Considerar las Resoluciones del I Congreso de Apicultura y Meliponicultura en Ecuador.
6. No olvidar a los meliponicultores ecuatorianos, quienes necesitan capacitación, canales de mercadeo y una norma de calidad para las mieles de pote que ellos producen. La traducción y distribución del libro Pot-honey. A legacy of stingless bees sería un gesto de llevar la academia al campo.
7. Seguir luchando con los recursos disponibldes e integrar más estudiantes en la investigación científica de mieles en Ecuador.
8. Seguir haciendo las cosas que están bien con la misma dedicación y entendimiento.
9. Seguir evolucionando como dice el Pabellón de Ecuador en la EXPO 2015 Milano "Discover ouR EVOLUTION". Allí deberían estar las mieles de pote.

LIMITACIONES

Falta de recursos para hacer análisis y movilización (estancias, congresos), competencia analítica limitada en laboratorio privado para análisis de sacarosa aparente y azúcares reductores, ausencia de un laboratorio especializado en mieles, falta de tiempo de estudiantes y profesores para aprender. Los saberes ancestrales son un objetivo del Buen Vivir, pero difícilmente reciben recursos. La meliponicultura no tiene un espacio en los planes de desarrollo y menos la valorización de sus productos. La capacidad de respuesta es lenta, lo cual afecta interacciones fluidas a todo nivel. Las abejas entregadas personalmente a la UNL jamás fueron montadas, supongo que deben darse por perdidas porque tampoco fueron devueltas. La cooperación entre instituciones académicas tiene procedimientos administrativos complejos quizás, motivo por el cual en lugar de hacer análisis en colaboración con Ecuador, se debe buscar colaboración internacional. Es necesario generar actividades nuevas para suplir las que no se pueden realizar, o tienen demoras. La demora en los pagos mensuales de la beca Prometeo.

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A N E X O 1

Memorias y Asistencia I Congreso de Apicultura y Meliponicultura en Ecuador