Herramientas Analíticas en la Clasificación de Mieles en Base a Criterios de Calidad e Inocuidad

TESIS DOCTORAL

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Hace constar que:

La memoria titulada **"Herramientas analíticas en la clasificación de mieles en base a criterios de calidad e inocuidad"** que presenta **Dª Marisol Juan Borrás** para optar al grado de Doctor por la Universitat Politécnica de Valencia, ha sido realizada en el Instituto de Ingeniería de Alimentos para el Desarrollo (IuIAD – UPV) bajo su dirección y que reúne las condiciones para ser defendida por su autora.

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Fdo. Isabel Escriche Roberto

A mis padres

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Resumen

La miel, consumida por el hombre desde hace miles de años debido a sus propiedades organolépticas y terapéuticas, es el producto de la unión entre el mundo animal, la abeja (*Apis melífera*), y el vegetal, el néctar de las flores y/o secreciones azucaradas de las plantas o insectos. Hoy en día las empresas envasadoras y comercializadoras de miel, para cumplir las exigencias legales y comerciales, deben llevar a cabo una etapa previa al proceso industrial que consiste en clasificar las mieles que entran en la empresa como materia prima, tanto en lo que respecta a criterios de calidad como de inocuidad. Entre los primeros, además de los referentes al cumplimiento de los niveles de los parámetros fisicoquímicos exigidos por la normativa nacional e internacional, destaca la necesidad de clasificar las mieles atendiendo al origen botánico, por el consiguiente valor añadido que implica para las empresas. En este sentido, la búsqueda de nuevas herramientas analíticas que faciliten la diferenciación botánica de las mieles sería de gran utilidad para el sector apícola. Esto es debido a que la metodología tradicional basada en la cuantificación del contenido polínico presenta no solo el inconveniente de requerir personal experto, sino además el de estar sujeto a interferencias, especialmente cuando el contenido polínico esté infrarrepresentado, como sucede en algunos tipos de miel.

Otro aspecto esencial para el sector, es el relacionado con la posible presencia en la miel de residuos químicos (antibióticos o pesticidas) como consecuencia directa de los tratamientos veterinarios o indirecta de los tratamientos agrícolas. A este respecto, garantizar el cumplimiento de la legislación y la reducción de riesgos para el consumidor es un requisito fundamental en el campo de la Seguridad Alimentaria. Para ello, se debe controlar la materia prima en la recepción industrial, llevando a cabo los análisis adecuados mediante métodos validados y contrastados.

En este sentido, la presente tesis doctoral se plantea con dos objetivos claramente diferenciados: 1. Evaluar las técnicas que se vienen utilizando de forma rutinaria en el control de calidad de mieles, tanto a nivel industrial como comercial, y compararlas con otras alternativas no convencionales y 2. Evaluar la efectividad del control de la materia prima (llevado a cabo en la etapa de recepción industrial) para cumplir los límites legales establecidos en lo referente a la presencia de residuos químicos en miel. Además, valorar el riesgo para el consumidor como consecuencia de la exposición a dichos residuos cuando legalmente tenga establecido un LMR (Límite máximo de residuos).

De los resultados obtenidos se concluye que, en general, los parámetros fisicoquímicos, que se vienen utilizando de forma convencional en la clasificación de mieles, no permiten una buena diferenciación de las mismas en términos de monofloralidad. Si bien, el origen botánico de las mieles tiene un claro impacto en algunos de ellos, como es el color y la conductividad eléctrica, los niveles de ciertos parámetros fisicoquímicos pueden variar en función del año de recolección (especialmente el color) y de las prácticas apícolas. En este sentido, el apicultor tiene un importante papel en la variabilidad de algunos de estos

parámetros, especialmente en lo que respecta al HMF y la humedad, e incluso al color varietal característico que el mercado requiere. Por lo tanto, unas buenas prácticas apícolas son vitales para obtener el producto que el consumidor espera y la legislación exige.

Las técnicas alternativas ensayadas en el presente estudio, como es el caso de la identificación de compuestos volátiles característicos en la fracción volátil de las mieles, y la aplicación de un sistema de lengua electrónica construido con sensores metálicos, han proporcionado resultados útiles y esperanzadores en clasificación de mieles para complementar la información obtenida mediante el análisis polínico.

La utilización de marcadores químicos, como es el caso del antranilato de metilo en las mieles de cítrico, resulta especialmente útil cuando éstas presenten un bajo porcentaje de polen de la especie botánica de la que mayoritariamente procedan (por variedades híbridas estériles o producción de polen y néctar no simultánea). Sin embargo, inexplicablemente las transacciones comerciales son, en ocasiones más exigentes con esta variedad de miel que con otras, ya que no solo exigen la presencia de un porcentaje de polen mínimo de *Citrus* spp. (10-20%), sino que además requieren un nivel de antranilato de metilo no inferior a 2 mg/kg. En este sentido, la presente tesis doctoral propone reconsiderar la concentración requerida de este compuesto para la miel de cítricos españoles, a un valor mínimo de 1.2 mg/kg (superior al sugerido en otros estudios para mieles de cítrico italianas), y además sólo tener en cuenta este parámetro en el caso de las mieles con un sorprendente bajo porcentaje de polen de cítricos, y después de la evaluación de sus propiedades organolépticas y fisicoquímicas.

La presencia de determinados compuestos, en la fracción volátil de las mieles, resulta determinante en su diferenciación; siendo el origen botánico el que mayor influencia tiene en su discriminación y en menor medida el origen geográfico. Por ejemplo, carvacrol y α-terpineno son característicos de la miel de tilo; α-pineno y 3-methyl-2-butanol de la miel de girasol, y óxido de cis-linalool de la miel de acacia.

Con relación a la lengua electrónica, construida con sensores metálicos, la combinación de la información con ella generada junto con la aplicación de adecuadas técnicas estadísticas multivariantes (Análisis de Componentes Principales y Redes Neuronales) ha demostrado que este sistema permite la diferenciación de mieles según su origen botánico con un porcentaje de éxito del 100%. Además, se ha confirmado una buena correlación entre la lengua electrónica y la capacidad antioxidante de las mieles (0.9666).

En lo que respecta al control de residuos químicos, los resultados confirman que un control de calidad apropiado en la recepción de la materia prima, aplicando una metodología analítica adecuada y validada, resulta eficaz para reducir en la miel comercializada el riesgo de exposición por la presencia de sulfonamidas. En este sentido, se puede considerar que está garantizada la seguridad del consumidor de miel en lo que respecta no solo a la presencia de sulfamidas, sino también de otras sustancias químicas

como antibióticos y pesticidas, ya que el control que las empresas llevan a cabo de forma rutinaria los engloba a todos.

En relación al riesgo de exposición a pesticidas a través del consumo de miel, se concluye que, aunque en el estudio llevado a cabo en muestras comerciales, no se superó el Límite Máximo de Residuos (LMR) para ninguno de los pesticidas analizados, el consumidor está expuesto a muchos de ellos a concentraciones inferiores a dichos límites (especialmente para los acaricidas destinados al tratamiento de la varroa). Sin embargo, el "Indice de Peligro" (Hazard Index: HI) para la presencia de pesticidas en las mieles obtenido como sumatorio del peligro individual de cada pesticida (Hazard Quotient: HQ) presente en ellas, fue en el peor de los casos 500 veces inferior al valor de 1, considerado como límite de aceptabilidad. Aunque los consumidores no están expuestos a niveles tóxicos de pesticidas a través del consumo de miel, sin embargo, siguiendo el principio de que una exposición tiene que ser "tan baja como sea razonablemente posible", el sector primario, apicultores y agricultores, debe hacer un mayor esfuerzo ya que sus prácticas pueden influir directamente en el problema de la presencia de residuos en la miel.

Resum

La mel, consumida per l'home des de fa milers d'anys degut a les seues propietats organolèptiques i terapèutiques, és el producte de la unió entre el món animal, l'abella (Apis mel·lífera), i el vegetal, el nèctar de les flors y/o secrecions ensucrades de les plantes o insectes. Hui en dia les empreses envasadores i comercialitzadores de mel, per a complir les exigències legals i comercials, han de dur a terme una etapa prèvia al procés industrial que consistix a classificar les mels que entren en l'empresa com a matèria primera, tant pel que fa a criteris de qualitat com d'innocuïtat. Entre els primers, a més dels referents al compliment dels nivells dels paràmetres fisicoquímics exigits per la normativa nacional i internacional, destaca la necessitat de classificar les mels atenent a l'origen botànic, pel consegüent valor afegit que implica per a les empreses. En este sentit, la busca de noves ferramentes analítiques que faciliten la diferenciació botànica de les mels seria de gran utilitat per al sector apícola; ja que la metodologia tradicional basada en la quantificació del contingut pol·línic presenta no sols l'inconvenient de requerir personal expert, sinó a més el d'estar subjecte a interferències, especialment quan el contingut pol·línic estiga infra-representat, com succeïx en alguns tipus de mel.

Un altre aspecte essencial per al sector, és el relacionat amb la possible presència en la mel de residus químics (antibiòtics o pesticides) com a conseqüència directa dels tractaments veterinaris o indirecta dels tractaments agrícoles. A este respecte, garantir el compliment de la legislació i la reducció de riscos per al consumidor és un requisit fonamental en matèria de Seguretat Alimentària. Per a això, s'ha de controlar la matèria primera en la recepció industrial, duent a terme les anàlisis adequats per mitjà de mètodes validats i contrastats.

En este sentit, la present tesi doctoral es planteja amb dos objectius clarament diferenciats: 1. Avaluar les tècniques que s'utilitzen de forma rutinària en el control de qualitat de mels, tant a nivell industrial com comercial, i comparar-les amb altres alternatives no convencionals i 2. Avaluar l'efectivitat del control de la matèria primera (dut a terme en l'etapa de recepció industrial) per a complir els límits legals establits pel que fa a la presència de residus químics en mel. A més, valorar el risc per al consumidor com a conseqüència de l'exposició als anomenats residus quan legalment tinguen establit un LMR (Límit màxim de residus).

Dels resultats obtinguts es conclou que, en general, els paràmetres fisicoquímics, que s'utilitzen de forma convencional en la classificació de mels, no permeten una bona diferenciació de les mateixes en termes de monofloralidat. Si bé, l'origen botànic de les mels té un clar impacte en alguns d'ells, com és el color i la conductivitat elèctrica, els nivells de certs paràmetres fisicoquímics poden variar en funció de l'any de recol·lecció (especialment el color) i de les pràctiques apícoles.En este sentit, l'apicultor té un important paper en la variabilitat d'alguns d'estos paràmetres, especialment pel que fa al HMF i la humitat, i inclús al color varietal característic que el mercat requerix. Per tant,

unes bones pràctiques apícoles són vitals per a obtindre el producte que el consumidor espera i la legislació exigix.

Les tècniques alternatives assajades en el present estudi, com és el cas de la identificació de compostos volàtils característics en la fracció volàtil de les mels, i l'aplicació d'un sistema de llengua electrònica construït amb sensors metàl·lics, han proporcionat resultats útils i esperançadors en classificació de mels per a complementar la informació obtinguda per mitjà de l'anàlisi pol·línica.

La utilització de marcadors químics, com és el cas de l'antranilato de metil en les mels de cítric, resulta especialment útil quan estes presenten un baix percentatge de pol·len de l'espècie botànica de què majoritàriament procedisquen (per varietats híbrides estèrils o producció de pol·len i nèctar no simultània). No obstant això, inexplicablement les transaccions comercials són, de vegades més exigents amb esta varietat de mel que amb altres, ja que no sols exigixen la presència d'un percentatge de pol·len mínim de Citrus spp. (mínim 10-20%), sinó que a més requerixen un mínim contingut d'antranilato de metil (2 mg/kg) .En este sentit, la present tesi doctoral proposa reconsiderar el nivell de MA requerit per a la mel espanyola de cítrics, a un valor mínim de 1.2 mg/kg (superior al suggerit en altres estudis per a mels de cítric italianes), i a més només tindre en compte este paràmetre en el cas de les mels amb un sorprenent baix percentatge de pol·len de cítrics, i després de l'avaluació de les seues propietats organolèptiques i fisicoquímiques.

La presència de determinats compostos, en la fracció volàtil de les mels, resulta determinant en la seva diferenciació; sent l'origen botànic el que major influència té en la discriminació i en menor mesurada l'origen geogràfic. Per exemple, carvacrol i α-terpineno són característics de la mel de til·ler; α-pineno i 3-methyl-2-butanol de la mel de girasol, i òxid de cis-linalool de la mel d'acàcia.En relació amb la llengua electrònica, construïda amb sensors metàl·lics, la combinació de la informació amb ella generada juntament amb l'aplicació d'adequades tècniques estadístiques multivariant (Anàlisis de Components Principals i Xarxes Neuronals) ha demostrat que aquest sistema permet la diferenciació de mels segons el seu origen botànic amb un percentatge d'èxit del 100%. A més, s'ha confirmat una bona correlació entre la llengua electrònica i la capacitat antioxidant de les mels (0.9666).

Pel que fa al control de residus químics, els resultats confirmen que un control de qualitat apropiat en la recepció de la matèria primera, aplicant una metodologia analítica adequada i validada, resulta eficaç per reduir en la mel comercialitzada el risc d'exposició per la presència de sulfonamides. En aquest sentit, es pot considerar que està garantida la seguretat del consumidor de mel en el que respecta no tan sols a la presència de sulfamides, sinó també d'altres substàncies químiques com a antibiòtics i pesticides, ja que el control que les empreses duen a terme de forma rutinària els engloba a tots.

En relació al risc d'exposició a pesticides a través del consum de mel, es conclou que, encara que en l'estudi dut a terme en mostres comercials, no es va superar el Límit Màxim

de Residus (LMR) para cap dels pesticides analitzats, el consumidor està exposat a molts d'ells a concentracions inferiors a aquests límits (especialment per als acaricides destinats al tractament de la varroa). No obstant això, el "Indice de Perill" (Hazard Index: HI) per a la presència de pesticides en les mels, obtingut com a sumatori del perill individual de cada pesticida (Hazard Quotient: HQ) present en elles, va anar en el pitjor dels casos 500 vegades inferior al valor d'1, considerat com a límit d'acceptabilitat. Encara que els consumidors no estan exposats a nivells tòxics de pesticides a través del consum de mel, no obstant això, seguint el principi que una exposició ha de ser "tan baixa com sigui raonablement possible", el sector primari, apicultors i agricultors, ha de fer un esforç ja que les seves pràctiques poden influir directament en el problema de la presència de residus en la mel.

Abstract

Honey, consumed by people for thousands of years due to its organoleptic and therapeutic properties, is the product of the union of two worlds, the animal (the bee *Apis mellifera*), and the plant (nectar from flowers and/or sweet secretions from plants and insects). Today, the honey packaging and retail business must classify honey before the manufacturing process, taking into account quality and safety criteria in order to meet legal and commercial requirements. In addition to the requirements relating to compliance with the levels of physico-chemical parameters obligatory by national and international law, the quality criteria require classification according to botanical origin, which results in added value for the companies. In this regard, looking for new analytical tools that facilitate the botanical differentiation of honey would be useful for the beekeeping sector. This is because the traditional method based on the quantification of the pollen content not only has the disadvantage of requiring skilled technicians, but is also sometimes subject to interference, especially when the pollen content is underrepresented, as occurs in some types of honey.

Another essential aspect for the sector, is that related to the possible presence of chemical residues (antibiotics or pesticides) in honey, as a direct consequence of veterinary treatments or indirectly due to agricultural treatments. In this regard, guaranteeing compliance with the legislation and reducing risks to consumers is an essential requirement in the field of food safety. For this reason, on receiving batches of raw honey, the packaging industry must conduct proper analysis using proven and validated methods.

Taking this into account, the present PhD thesis has two distinct objectives: 1. To evaluate the techniques that have been used routinely in the quality control of honey, at both an industrial and commercial level, and to compare them with other unconventional alternatives, and 2. To evaluate the effectiveness of monitoring the raw material in the packaging industry (carried out on receiving batches of raw honey,) to meet the legal limits regarding the presence of chemical residues. Also, to assess the risk to the consumer as a result of exposure to such residues when there is a legally established maximum residue limit (MRLs).

Based on the results obtained it is concluded that, in general, the physicochemical parameters that have been used conventionally in the classification of honey do not permit good differentiation in terms of monoflorality. While the botanical origin of honey has a clear impact on some of them, such as the color and electrical conductivity, levels of certain physicochemical parameters may vary depending on the year of harvest (especially color) and beekeeping practices. In this line, the beekeeper has an important role in the variability of some of these parameters, especially in regard to HMF and moisture, and even in the characteristic varietal color that the market requires.

Therefore, good beekeeping practices are essential to obtain the product that the consumer expects and legislation requires.

The alternative techniques tested in this study, such as identifying characteristic volatile compounds in the volatile fraction of honey, and the application of an electronic tongue made with metals, have provided useful and promising results in the classification of honey to complement the information obtained by pollen analysis.

The use of chemical fingerprinting, such as for methyl anthranilate in citrus blossom honey, is particularly useful when the percentage of pollen is particularly low, as in the case of sterile hybrids or when pollen and nectar production is not simultaneous. However, inexplicably commercial transactions are sometimes more demanding with this type of honey than with others, as they not only require the presence of a minimum percentage of *Citrus* spp. pollen (at least 10-20%), but also require a minimum presence of methyl anthranilate (2 mg/kg). This PhD thesis suggests reconsidering the level of this compound required in Spanish citrus honey; proposing a minimum value of 1.2 mg/kg (greater than that recommended in other studies for Italian citrus honey). However, only taking this parameter into consideration in the case of honey with a surprising low percentage of citrus pollen, and after evaluating its organoleptic and physicochemical properties.

The presence of certain compounds, in the volatile fraction of the honey, is decisive in its differentiation; botanical origin having the greatest influence on discrimination and to a lesser extent the geographical origin. For example, carvacrol and α-terpinene are characteristic of tilia honey; α-pinene and 3-methyl-2-butanol of sunflower honey; and cis-linalool oxide of acacia honey.

The information obtained with an electronic tongue (made with metal sensors) in combination with appropriate multivariate statistical techniques (Principal Component Analysis and Neural Networks) has demonstrated that this system allows the differentiation of honey by botanical origin with a success rate of 100%. A good correlation between the electronic tongue and the antioxidant capacity of honey has also been confirmed (0.9666).

With regard to the control of chemical residues, the results confirm that proper quality control on receiving batches of raw honey, applying appropriate validated analytical methods, is effective in reducing the risk of exposure to sulfonamides in commercialized honey. In this respect, it can be considered that honey consumer safety, with regard not only to the presence of sulfonamides, but also to other chemical residues such as antibiotics and pesticides is guaranteed, as the control that companies carry out routinely covers all this aspects.

Regarding the risk of exposure to pesticides through consumption of honey, it is concluded that, although in this study, carried out with commercial samples, the

Maximum Residue Level (MRL) was not exceeded for any of the pesticides analyzed; the consumer is exposed to many of them at concentrations below these limits (especially acaricides used against varroa). However, the "hazard index" (HI) for the presence of pesticides in honey obtained as the addition of the individual risk of each pesticide (Hazard Quotient: HQ) present in them, was in the worst case 500 times lower than the value of 1, considered as the limit of acceptability. Although consumers are not exposed to toxic levels of pesticides through consumption of honey; the principle that exposure to residues has to be "as low as reasonably possible", means that the primary sector, beekeepers and farmers, has to strive to improve their practices as these directly influence the problem of the presence of residues in honey.

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1. INTRODUCCIÓN

1. Introducción

1.1.-La miel como producto

1.1.1. Formación de la miel

La miel, consumida por el hombre desde hace miles de años, es el producto de la unión entre el mundo animal, la abeja (*Apis melífera*), y el vegetal, el néctar de las flores y/o secreciones azucaradas de las plantas o insectos. Hoy en día sigue siendo el mismo alimento que nuestros antepasados consumían con importantes propiedades nutricionales y terapéuticas, así como con características sensoriales muy atractivas.

No se sabe a ciencia cierta desde cuando el hombre introdujo la miel en su dieta, sin embargo, sí que hay constancia de que ya la recolectaba hace más de 8000 años, como queda patente en las pinturas rupestres de la cueva de la Araña (Bicorp, Valencia). Hasta la aparición del azúcar cristalizado, hace unos 2600 años, la miel ha sido el alimento para endulzar por excelencia.

Las abejas producen la miel a partir de néctar o de secreciones azucaradas. El néctar es una disolución acuosa de azúcares (sacarosa, glucosa y fructosa) en diferentes proporciones y de otras sustancias como sales minerales, ácidos orgánicos, aromas, etc.). Es segregado por las plantas en los nectarios, situados habitualmente en la flor, aunque también en las hojas o estípulas. Estos nectarios se encuentran en lugares estratégicos con la finalidad de que los insectos “atrapen” los granos de polen en su cuerpo y garanticen así la polinización. Este hecho ocasiona que el polen, en mayor o menor cantidad, se encuentre presente en la miel. Además del néctar, las abejas recogen y transforman las secreciones azucaradas o mielatos de ciertas plantas, así como de ciertos insectos chupadores de savia, homópteros, que viven parásitos sobre algunas plantas y succionan de ellas la savia elaborada.

Las abejas recolectan las soluciones azucaradas que tienen a su disposición cerca de la colmena, si bien es cierto que tienen predilección por ciertas especies botánicas (Crane, 1975). En una primera etapa, las abejas pecoreadoras, absorben con su lengua el néctar de las flores que visitan o los mielatos, los introducen en su buche y vuelven a la colmena para regurgitarlos, posteriormente, los vuelven a absorber o los pasan a otras abejas,

incorporando así enzimas adicionales (diastasa y glucoxidasa) y continuando con la transformación iniciada en el buche de la abeja recolectora. En este proceso se incorpora también la enzima invertasa, que ayuda a transformar el néctar o las secreciones de las plantas en miel. Los líquidos azucarados depositados en el panal siguen deshidratándose y experimentando transformaciones bioquímicas. Las abejas ventiladoras con el movimiento de sus alas producen unas corrientes de aire, introduciendo del exterior aire seco y eliminando del interior el húmedo. De esta manera, consiguen rebajar el porcentaje de humedad hasta llegar a un 16-20%, grado de maduración que tiene la miel cuando las obreras operculan, es decir sellan la celda con una fina capa de cera. Es en este momento cuando la miel está lista para ser recogida del panal por los apicultores (Del Baño Breis, 2000). Este nivel de concentración de agua, hace a la miel un producto biológico bastante estable, impidiendo que los hongos y las levaduras encuentren un medio favorable para su desarrollo. Sin embargo, la miel sigue transformándose, por acción de las enzimas, una vez extraída de los panales y durante su almacenamiento posterior (Figura 1.1).

Figura 1.1.- Abejas depositando miel en el panal

1.1.2. Composición de la miel y su implicación en la calidad

La miel es básicamente una solución de azúcares en agua y aproximadamente un 1% de otros constituyentes menores (Tabla 1.1). Estos componentes le confieren unas características de color, sabor y aroma típico, así como ciertas propiedades físico-químicas, típicas del origen botánico de las plantas de las que procede. Las características intrínsecas de cada miel, además, pueden variar en función de la climatología, el estado de la colmena y las prácticas apícolas (Sáenz & Gómez, 2000).

Carbohidratos

Los carbohidratos son los principales componentes de la miel, siendo los mayoritarios los monosacáridos fructosa y glucosa, representado solo ellos casi el 80%. En menor proporción se encuentran los disacáridos sacarosa y maltosa, y por último otros disacáridos y oligosacáridos como erlosa, turanosa, melecitosa, etc. La composición en

azúcares de una miel puede estar relacionada con: la procedencia botánica, el estado de madurez, la adulteración, la capacidad de cristalizar, la capacidad de fermentación, el desarrollo de hidroximetilfurfural (HMF), etc.

Tabla 1.1- Rango de variabilidad de los componentes químicos de la miel

Componentes mayoritarios (99%)	g/100g
Fructosa	21.7-53.9
Glucosa	20.4-44.4
Sacarosa	0.0-7.6
Otros azucares	0.1-16.0
Componentes minoritarios (1%)	**g/100g**
Minerales	0.02-1.03
Nitrógeno (proteínas)	0.00-0.13
Enzimas	>0.1%
Aromas	>0.1%
Otros	>0.1%

Fuente: Codex alimentarius, 2001

La relación de ciertos monosacáridos, como son la fructosa y glucosa (F/G), puede ser útil para la caracterización botánica de las mieles. Así por ejemplo, altos ratios F/G son propios de la mieles de acacia y castaño, por el contrario bajos ratios F/G son típicos de mieles de girasol, colza y diente de león (Persano-Oddo & Piro, 2004).

El porcentaje de sacarosa de una miel puede depender no solo de su procedencia botánica sino también de su estado de maduración. Una miel extraída del panal antes de que esté madura, puede tener un excesivo contenido en sacarosa, pudiendo superar el nivel permitido por la legislación (máximo de 5% en general, o entre 10%-15% en ciertas excepciones) (Serra-Bonvehí & Ventura, 1995). El contenido del resto de azúcares minoritarios (producidos por la acción de la invertasa) es mayor en las mieles de mielada que en las de néctar, difiriendo poco entre ellas (Bogdanov, 2011).

El conocimiento de la composición de los diferentes azúcares presentes en la miel puede proporcionar información sobre su posible adulteración. La miel puede adulterarse por la adición directamente de jarabes azucarados (jarabes de azucares invertidos, jarabes de caña de azúcar o remolacha, jarabes de arce, etc.).

La relación de los dos principales azucares de la miel, fructosa y glucosa (F/G), condiciona tanto la capacidad de cristalizar como su velocidad. La miel es una solución sobresaturada de azúcares; por ello en su estado líquido es bastante inestable y tiene una tendencia natural a cristalizar. La fructosa al ser más soluble en agua permanece fluida, siendo la glucosa la que cristaliza debido a su menor solubilidad (Hamdan, 2010). Una miel con un ratio F/G >1.33 tardará en cristalizar, sin embargo, cuando éste sea <1.11 el fenómeno

se producirá más rápidamente (Smanalieva & Senge, 2009). White et al. (1962) demostraron que la concentración de glucosa y el contenido de humedad de una miel (G/H) ayudan a predecir la tendencia de una miel a la cristalización. Así, para valores de esta relación inferiores a 1.60 la capacidad de cristalización de una miel es prácticamente nula o muy lenta, en cambio para valores superiores a 2 es más rápida y completa. Además de estos factores, el fenómeno de la cristalización también estará condicionado por la presencia en la miel de "semillas o núcleos de cristalización", como son: granos de polen, partículas de cera, impureza, burbujas de aire, etc.

Un problema asociado a la cristalización es la fermentación, algunas mieles cristalizan uniformemente y otras parcialmente, formando en muchas ocasiones 2 fases; quedando los cristales en el fondo y la fase más liquida en la superficie. Cuando esto sucede, en la parte superior rica en agua se favorece el desarrollo de las levaduras (propias de un alimento de origen animal, principalmente genero *Bacillus*), comenzando entonces la transformación de los azúcares en alcohol y la producción de dióxido de carbono (Zamora & Chirife, 2006). Es necesario un contenido de 100 levaduras/g miel para que el proceso tenga lugar, así como un contenido en humedad superior a 20% y una temperatura ambiente de unos 25ºC (Díaz-Moreno, 2009).

La deshidratación espontanea en un medio ácido como es la miel, especialmente de la fructosa y de la glucosa, produce un aldehído cíclico llamado hidroximetilfurfural (HMF) ($C_6H_6O_3$) (Figura 1.2). Esta reacción se acelera por el calor y el tiempo. La miel recién extraída con buenas prácticas de manipulación contiene pequeños porcentajes de este aldehído (0 a 7 mg/kg), sin embargo, se incrementa con el sobrecalentamiento y/o envejecimiento de la miel, siendo más pronunciado cuanto más ácida es esta (Sancho et al., 1992). Por este motivo, para garantizar que la miel llegue al consumidor lo más fresca posible y sin alteración de sus características intrínsecas, la legislación establece que este parámetro no debe ser superior a 40 mg/kg durante toda la vida útil de la miel. La destinada a uso industrial y miel de origen procedente de regiones de clima tropical y mezclas de estas puede llegar a contener un máximo de 80 mg/kg (Tabla 1.3) (Real Decreto 1049/2003). Es por ello que en las transacciones comerciales, este parámetro se utiliza como indicador de la frescura de la miel (Fallico et al., 2004).

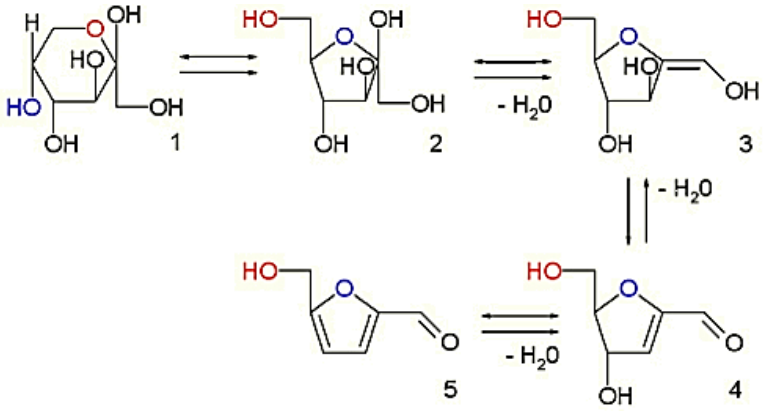

Figura 1.2.- Reacciones de equilibrio entre la fructosa y el HMF en medio ácido.1: Fructosa, 2: Fructofuranosa, 3–4: Etapas intermedias de deshidratación, 5HMF (Stahlberg et al., 2011).

Humedad

El nivel de agua de una miel contribuye en su peso específico, viscosidad, sabor, calidad y en definitiva en el valor comercial del producto (Sáenz & Gómez, 2000). Su contenido está comprendido entre 14 y 21%, con un promedio de 17%. El contenido de agua va a depender del origen botánico de la miel, las condiciones climáticas, la temporada de producción, la manipulación humana y las condiciones de almacenamiento, y por todo ello, va a influir en su calidad final (Gallina et al., 2010). Bajos porcentajes de agua dificultan la manejabilidad de la miel, por el contrario altos porcentajes pueden propiciar efectos negativos ya mencionados como es la fermentación. Si una miel llega a la industria con un alto contenido en agua, propiciado bien por la climatología anual o por una extracción prematura del panal, existe el riesgo de que posteriormente en el almacenamiento pueda fermentar por levaduras osmófilas. Aunque esto no ocasiona ningún peligro de salud para el consumidor, invalidará el producto para su comercialización, con la consecuente pérdida económica para la industria (tiempo y dinero). Con valores de humedad <17.1% desaparece prácticamente este peligro. Sin embargo, con valores por encima de este nivel la posibilidad de fermentación aumenta considerablemente en función de la carga microbiana (Belitz & Grosch, 1997).

Enzimas

Las enzimas son componentes minoritarios de la miel, cuya presencia diferencia claramente la miel de otros edulcorantes. Algunas de ellas son introducidas por las abejas procedentes de su tracto gastrointestinal y otras proceden del néctar o mielatos (Anclan, 1998). Las enzimas son las encargadas de transformar los azúcares del néctar o de los mielatos. Las diastasas (α-amilasas), son enzimas transformadoras de almidón, que proceden tanto de la abeja como del néctar. No está clara su función en el proceso de obtención de la miel, ya que el néctar no contiene almidón (White, 1978; Ropa, 2010), aunque al parecer estas enzimas participa en la digestión del polen (del Baño-Breis,

2000). La invertasa (α-glucosadas), de origen animal, es la encargada de desdoblarla sacarosa en fructosa y glucosa (E 1).

$$C_{12}H_{22}O_{11} + H_2O \text{ ------ } C_6H_{12}O_6 + C_6H_{12}O_6 \qquad \text{Eq. (1)}$$

Las mieles frescas no procesadas de diferentes procedencias florales presentan grandes variaciones en su actividad diastásica (Tabla 1.2). Esta variación es debida a las diferencias de pH entre mieles, a la cantidad de néctar que procesan las abejas en un momento dado y a las diferencias en la forma de recolección de cada abeja pecoreadora (Nacional Hoyen Borde, 2015). Aunque de manera general las enzimas son sensibles al calor, la diastasa es relativamente estable al calor y al almacenamiento, por el contrario la invertasa es mucho más susceptible. Estas dos enzimas juegan un papel muy importante en la calidad de la miel y son usadas como indicadoras de la pérdida de su frescura (Bogdanov, 2011), tanto por envejecimiento como por un excesivo calentamiento durante su procesado. Cabe destacar que la legislación solo contempla la actividad de la diastasa, aun siendo la más estable al calor. Esto unido al hecho de la gran variabilidad que presenta esta enzima en los diferentes tipos de miel, hace pensar que su nivel no está muy correlacionado con la calidad de la miel (Visquert, 2015). Aun así, la legislación, para preservar la naturaleza biológica de la miel, establece que el contenido enzimático de diastasas no deberá ser menor de 8 ID (escala Schade), salvo en el caso de mieles que por su origen de manera natural tienen bajo contenido enzimático (p.e. miel de cítricos), en cuyo caso este valor no debe ser menor de 3 ID.

La glucosa-oxidasa, de origen animal, actúa sobre la glucosa para producir ácido glucónico y peróxido de hidrogeno. Este compuesto actúa como antibacteriano protegiendo a la miel hasta que esté madura (Eq 2). Es una enzima sensible a la luz, que está activa en el néctar, pero inactiva en la miel, aunque puede volver a activarse si la miel es diluida. Otras enzimas como la fosfatasa acida y la catalasa se encuentran también presentes en las mieles (White, 1978; Crane, 1990; Sáenz & Gómez, 2000).

$$\textit{D-glucosa} + O_2 \text{-----} \textit{D-gluconolactona} \text{-----} \textit{ácido glucónico} + H_2O_2 \qquad \text{Eq. (2)}$$

La fosfatasa ácida, está presente principalmente en el polen, aunque también en el néctar. Esta enzima está relacionada con la fermentación de la miel, de manera que aquellas presenten mayores valores de actividad de esta enzima tendrán una mayor tendencia a fermentar. Dicha actividad está fuertemente influenciada por el pH de la miel, por lo que a mayores valores de pH, mayor es su actividad.

El estudio de la actividad enzimática de una miel puede ayudar a detectar cierto tipo de adulteraciones. En ocasiones se adiciona fraudulentamente a la miel jarabes de azúcares invertidos (HFCS) (producidos enzimáticamente a partir de β-amilasa y γ-amilasa), o bien

se adicionan directamente enzimas (β-fructofuranosidasa) para compensar la disminución de actividad enzimática por la adición de estos jarabes.

Tabla 1.2.- Contenido diastásico en mieles de diferentes orígenes. Unidades expresadas como valores de la escala Schade.

Origen floral	Índice diastasico (ID)
Azahar	4.25
Trébol	5.73
Pimienta	13.2
Milflores	22.0
Eucalipto	24.0
Trigo sarraceno	36.8

Un método que posibilita la detección de algunos de estos enzimas ajenos a la miel, se basa en la comparación de la actividad enzimática (diastasa) antes y después de someter la miel a un tratamiento térmico; éste destruye completamente la diastasa y no a las enzimas adicionadas. Si después del tratamiento se detecta actividad enzimática, ésta es debida a la presencia de las enzimas resistentes adicionadas en la adulteración. Para poner de manifiesto la adulteración de la miel por adición de siropes de azúcar invertido tipo C3, se evalúa la presencia de la enzima β- fructofuranosidasa, mediante la detección cromatográfica de los metabolitos que ésta produce a partir de la adición de un sustrato específico.

Compuestos aromáticos

Los compuestos volátiles (ácidos alifáticos y aromáticos, aldehídos, cetonas y alcoholes, etc.), junto con los azúcares, los ácidos de la miel, así como ciertos componentes del néctar contribuyen definitivamente en su aroma y sabor. Muchos estudios se han llevado a cabo en las últimas décadas en relación a los componentes volátiles de las diferentes variedades de mieles y distintas procedencias (Radovic et al., 2001; Soria et al., 2003; De la Fuente, 2005; Castro-Vázquez et al., 2009). En algunos de ellos la caracterización de la fracción volátil ha ayudado en la autentificación de su origen botánico (Kadar et al, 2011). Un ejemplo claro de este hecho lo encontramos en la miel de azahar (*Citrus* spp.), que se caracteriza por la presencia en ella del metilantranilato (MA). Se trata de un compuesto volátil específico del néctar de la miel de cítricos, y característico de aroma propio de su flor (ISO 5496,2006). Su presencia en la miel indica que las abejas han recogido el néctar de las flores de cítricos (White & Bryant, 1996; Castro-Vázquez et al., 2007). Por esta razón el MA ha sido y sigue siendo usado como marcador de este tipo de mieles y puede ser una buena herramienta para la clasificación de la miel de azahar cuando el nivel de contenido en polen es muy bajo, como suele suceder en este tipo de mieles (Sesta et al., 2008).

Antioxidantes en la miel

La miel fresca posee una actividad antioxidante significativa, similar a la de muchas frutas y verduras (Gheldof et al., 2002). Las plantas contienen una variedad de derivados polifenólicos, y por lo tanto, cuando las abejas recogen el néctar o los mielatos, estos compuestos bioactivos son transferidos de las plantas a la miel (Silici et al., 2010). Los compuestos fenólicos, antioxidantes naturales, (especialmente flavonoides) constituyen un grupo importante por sus propiedades funcionales y su importancia terapéutica (Yao et al., 2004). Mieles con altos niveles de antioxidantes, pueden contribuir a mejorar la salud humana (Oroian & Escriche, 2015).
Varios estudios han demostrado una fuerte correlación entre el contenido de compuestos fenólicos en mieles de diversas fuentes florales, sus capacidades antioxidantes y actividades antibacterianas (Gheldof et al., 2002; Meda et al., 2005; Escriche et al., 2011; Álvarez -Suárez et al., 2012; Escuredo et al., 2012).

Otros componentes

Además de los anteriormente citados, la miel contiene otros componentes entre los que destacan: aminoácidos, ácidos, proteínas y minerales. Los aminoácidos libres están presentes en aproximadamente 100 mg/100 g materia seca. Entre éstos destaca la prolina (procedente de las abejas) que constituye entre el 50 y 85% de esta fracción aminoacídica. El ácido más abundante es el ácido glucónico, aunque también están presentes otros como: acético, butírico, cítrico, fórmico, láctico, málico, piroglutámico, y succínico. La presencia de estos ácidos le confiere el pH ácido a la miel, comprendido en un rango de 3.4 a 6.1. Este medio ácido inhibe la presencia y crecimiento de microorganismos. Las proteínas en la miel representan una cantidad muy pequeña y su presencia está ligada, mayoritariamente a los granos de polen que se encuentran en ella; su contenido aproximado es de 0.26 g/100 g de proteínas. El contenido en sales minerales varía notablemente con relación al origen botánico, a las condiciones edáfico-climáticas, así como a las técnicas de extracción; oscila entre 0.1 y 0.2 %, siendo el elemento dominante el potasio, seguido de: cloro, azufre, sodio, calcio, fósforo, magnesio, manganeso, silicio, hierro y cobre (Belitz & Grosch, 1997). Algunos autores han observado correlación entre el contenido en minerales y el color de las mieles (González-Miret, 2005).

1.2.-Marco legislativo

Los criterios de calidad para la miel están definidos tanto por el Codex Alimentarius (2001) para la miel como por la Comisión Europea (Directiva 2001/110/CE), con escasas diferencias entre ellos. El Codex es un referente internacional que ha servido de base para elaborar normas más específicas a nivel de cada país (Oyarzun et al., 2005). En ambos estándares se describen los parámetros de calidad de la miel, definiendo sus mínimos y máximos. En España, el Real Decreto 1049/2003, transposición de la citada Directiva, establece la norma relativa a la calidad de la miel y define los criterios de diferentes parámetros físico-químicos (comentados en el apartado 1.1.2) (Tabla 1.3), así como otras condiciones que deben de cumplir las mieles vendidas en el territorio español. Se recoge que la miel vendida como tal no debe tener ningún ingrediente (incluyendo aditivos alimentarios), no debe contener nada que no sea miel. Asimismo, no debe presentar ningún aroma o sabor que provenga de su procesado o almacenamiento. Tampoco debe presentar principios de fermentación o estar fermentada. La miel no debe ser sometida a calentamiento hasta tal punto que éste le pueda inducir cambios esenciales en su composición o daños en su calidad.

Los métodos analíticos para el análisis de los parámetros fisicoquímicos, son recogidos por la legislación española en el BOE 145 (1986). Además, también se han publicado métodos revisados y recopilados por la Comisión Internacional de la Miel (IHC) (Bogdanov et al., 2002). La IHC se constituyó en el año 1990 con la finalidad de desarrollar y mejorar las normas relativas a los estándares de calidad de la miel.

Los criterios de calidad recogidos en las diversas normativas se centran en tres conceptos: autenticidad, frescura y seguridad.

Tabla 1.3.- Características composicionales de la miel según los estándares europeos (Directiva 2001/110/CE).

Contenido de azúcares	
-Contenido de fructosa y glucosa (suma de ambas)	
Miel de flores	≥60 g/100 g
Miel de mielada, mezclas de miel de mielada con miel de flores	≥ 45 g/100 g
-Contenido de sacarosa	
En general	≤5 g/100 g
Falsa acacia (Robinia pseudoacacia), alfalfa (Medicago sativa), Banksia de Menzies (Banksia menziesii), Sulla (Hedysarum), Eucalipto rojo (Eucalyptus camaldulensis), Eucryphia lucida, Eucryphia milliganii, Citrus spp.	≤10 g/100 g
Espliego «Lavandula spp.», borraja «Borago officinalis»	≤15 g/100 g
Contenido de agua	
En general	≤20%
Miel de brezo «Calluna» y miel para uso industrial en general	≤23%
Miel de brezo «Calluna vulgaris » para uso industrial	≤25%
Contenido de sólidos insolubles en agua	
En general	≤0,1 g/100 g
Miel prensada	≤0,5 g/100 g
Conductividad eléctrica	
Miel no incluida en la enumeración de los dos párrafos más abajo indicados, y mezclas de estas mieles	<0,8 mS/cm
Miel de mielada y miel de castaño, y mezclas de éstas, excepto con las mieles que se enumeran a continuación	≥0,8 mS/cm
Excepciones: madroño «Arbutus unedo», argaña «Erica», eucalipto, tilo «Tilia spp», brezo «Calluna vulgaris», manuka o jelly bush «Leptospermum», árbol del té «Melaleuca spp.»	
Ácidos libres	
En general	≤50 miliequivalentes de ácidos/ kg
Miel para uso industrial	≤80 miliequivalentes de ácidos/ kg
Índice diastásico (escala de Schade)	
En general, excepto miel para uso industrial	no menos de 8
Mieles con un bajo contenido natural de enzimas (por ejemplo, mieles de cítricos) y un contenido de HMF no superior a 15 mg/kg	no menos de 3
HMF	
En general, excepto miel para uso industrial	≤40 mg/kg
Miel de origen declarado procedente de regiones de clima tropical y mezclas de estas mieles	≤80 mg/kg.

1.2.1. Autenticidad y frescura

La miel al ser un edulcorante con precio elevado ha sido y es hoy día muy susceptible de adulteraciones y fraudes. La miel adulterada apareció en el mercado mundial en la década de 1970, coincidiendo con el desarrollo industrial de los jarabes provenientes de materias primas vegetales como los de maíz de alta fructosa (JMAF) (Cordella et al., 2002). Datos recientes publicados por la Comisión Europea demuestran que la miel sigue siendo objeto de estos fraudes (Parlamento Europeo, 2013). Según este estudio, la miel ocupa la sexta posición entre los diez alimentos con mayor riesgo de sufrir fraude alimentario (aceite de oliva, pescado, alimentos ecológicos, leche, cereales, miel y jarabe de arce, café y té, especias, vino y zumos de frutas). En la última década la miel adulterada, procedente mayoritariamente de países terceros como China, están causando un importante problema para el sector, por la competencia desleal que esto implica (White, 2000; Bogdanov & Martin, 2002; Chen et al., 2011). La Tabla 1.4 muestra como ejemplo algunas de las alertas alimentarias que al respecto han tenido lugar en los últimos años.

Tabla 1.4.- Alertas relativas a mieles adulteradas de venta en comercios de distintos países.

Origen	Fecha publicación
Malasia	2 octubre 2012
Nueva Zelanda	12 junio 2012
Nueva Zelanda	21 mayo 2012
Arabia Saudí	6 febrero 2013
Turquía	30 marzo 2013
Turquía	3 octubre 2011
Vietnam	12 septiembre 2012

Fuente: FDA (USA)

La extracción prematura de la miel del panal también implica un fraude ya que la legislación define a la miel como "*la sustancia natural….., que las abejas recolectan, transforman combinándolas con sustancias específicas propias, depositan, deshidratan, almacenan y dejan en colmenas para que madure*" (Directiva 2001/100/CE; Real Decreto 1049/2003). La miel debe de madurar dentro del panal, y solo debe ser extraída por el apicultor en el momento adecuado de maduración. Si se recolecta antes de tiempo, la sustancia azucarada resultante tiene un elevado contenido en agua, llegando fácilmente a sobrepasar el 25%. Este tipo de fraude, se ha observado también en mieles procedentes de China y probablemente en otros países (Bogdanov & Martin, 2002).

El ultrafiltrado de la miel, tiene por objeto quitar su polen para impedir identificar la fuente botánica y/o geográfica de la misma. Esta práctica dificúltala detección de posibles fraudes, bien sea en la declaración del país o países de origen o bien en la variedad

botánica expresada en la etiqueta; impidiendo por consiguiente su trazabilidad. Es un procedimiento que requiere de alta tecnología, en el que la miel se calienta generalmente diluida con agua, y se fuerza a pasar, a alta presión, a través de unos filtros de malla muy pequeña, finalmente el exceso de humedad es eliminada. En el mercado de Estados Unidos durante años se han encontrado mieles filtradas (Schneider, 2011).

Por ello, para proteger a la miel recientemente la unión europea modificó una parte de la Directiva 2001/110/CE, estableciendo en la nueva Directiva 2014/63/UE que la eliminación parcial o total del polen de la miel está específicamente prohibida y aduce que "*el polen entra en la colmena como resultado de la actividad de las abejas y está presente en la miel de forma natural, con independencia de que los explotadores de empresas alimentarias extraigan o no esa miel*".

Métodos analíticos para la detección de adulteraciones en miel

La Comisión Europea 2001/110/EC, con la finalidad de asegurar un negocio justo, proteger a los consumidores del fraude y mantener un alto nivel de capacidad de su identificación, está fomentando la investigación y el desarrollo de métodos analíticos que faciliten la detección de dichas prácticas. Una parte importante del trabajo de la Comisión Internacional de la Miel (IHC) en los últimos años se ha centrado en cuestiones relativas a la autenticidad de la miel.

Son muchos los trabajos publicados por diferentes autores en relación a la aplicación de técnicas analíticas que permiten detectar las adulteraciones de la miel; sin embargo, ninguna de dichas técnicas resulta concluyente por si misma ya que, tal y como se ha comentado, son muy diferentes los procedimientos llevados a cabo para realizar tales adulteraciones: incorporación de jarabes (jarabes de maíz, de caña de azúcar, de agave, etc.), adición de enzimas, incorporación de colorantes, extracción prematura de la miel del panal, etc. Además, como sucede siempre, "la técnica de la adulteración" va siempre un paso por delante de la de la "técnica de la detección".

Entre las diferentes técnicas que actualmente existen para la detección de adulteraciones en la miel cabe mencionar:

- Estudio del residuo microscópico. Esta técnica se emplea como un primer cribado para identificar la adición de jarabes de caña de azúcar o de maíz, evaluando en la miel el residuo microscópico presente (Kerkvliet & Meijer, 2000) y posteriormente verificando con la medida de su relación isotópica 13C/12C de miel y su proteína (Simsek et al., 2012; Tosun, 2013).
- Métodos isotópicos a través de la resonancia magnética nuclear (SNIF-NMR) que permiten obtener los espectros característicos de la miel sin adulterar (Cote et al., 2003). Posteriormente, se comparan éstos con las mieles sospechosas de estar adulteradas.

- Evaluación del perfil de azúcares por métodos cromatográficos. Esta técnica es especialmente recomendada para la detección de jarabes de remolacha (Elflein & Raezke, 2005; Cabañero et al., 2006).
- Espectroscopía de infrarrojos, por transformada de Fourier, que combinada con análisis multivariantes son capaces de determinar el nivel de adición de azúcar a la miel (fructosa, sacarosa y jarabe de maíz) (Sivakesava & Irudayaraj, 2001; Kelly et al, 2006).
- Cromatografía capilar de gases (GC) para la detección de oligosacáridos ajenos a la miel. Especialmente útil para adición de jarabes de maíz ricos en fructosa (HFCS) (Low & South, 1995).
- Determinación de glicerol, butanediol y etanol mediante test enzimáticos que permitirá determinar adulteración por "miel inmadura". Se admite un máximo de 300 mg/kg de glicerol para mieles de néctar y un máximo de 150 mg/kg de etanol para mieles de néctar españolas e italianas Además estas técnicas se complementan mediante la detección de altos niveles de levaduras muertas por microscopía óptica. (Huidobro et al., 1994; Flores et al., 2002).

1.2.2. Seguridad

En materia de seguridad la miel, al igual que cualquier otro alimento, no debe contener microorganismos o residuos químicos que puedan alterar la salud del consumidor. Debido a la baja a_w de la miel, desde el punto de vista microbiológico es muy estable y no contiene microrganismos tóxicos. La única excepción puede ser la presencia de esporas de *Clostridium botulinum*, motivo por el cual las empresas envasadoras suelen indicar en la etiqueta que el producto no es apto para niños menores de 1 año.

Residuos químicos y su determinación

En lo que respecta a la presencia de residuos, la miel puede tener sustancias químicas procedentes tanto de los tratamientos veterinarios (contaminación directa) como de la contaminación agrícola (contaminación indirecta). Las abejas padecen enfermedades infecciosas, y en ciertas ocasiones los apicultores se ven obligados a aplicar medicamentos veterinarios. Las que más problemas causan a la apicultura a nivel mundial, por afectar a larvas y pupas, son la Loque americana (AFB) producida por el bacilo *Paenibacillus larvae* y la Loque europea (EFB) producida por la bacteria no esporulante *Melissococcus pluton* (Hammel et al., 2008). Para el tratamiento de AFB se utilizan compuestos de la familia de las sulfamidas y para la EFB se usan las tetraciclinas, ya que las sulfamidas no tienen acción curativa para esta última (Mundo Apícola, 2012).Actualmente en la Unión Europea no se han fijado límite máximos de residuos (LMR) para medicamentos veterinarios en los productos de la colmena, lo que significa que éstos compuestos nunca deben estar presentes por encima del límite de cuantificación del método analítico utilizado (Maudens et al., 2004). A pesar de su

prohibición, no es infrecuente la utilización de sulfonamidas y otros antibióticos con fines sanitarios apícolas.

Por otro lado, la abeja melífera se ve afectada por la presencia de parásitos que disminuyen su producción, y en mayor medida provocan su muerte. El más importante es el ácaro *Varroa destructor* (Anderson & Trueman, 2000), considerado como la más problemática plaga de Apis mellifera en el mundo, la varroasis (Sammataro & Finley, 2004). Este ácaro provoca pérdidas directas de producción e indirectas en la polinización de cultivos (Ledoux et al., 2000). El ácaro parasita las larvas de abeja en la propia celdilla y, desde ese momento, la abeja llevará el parásito que, además de alimentarse de su hemolinfa, le producirá un tremendo desgaste energético durante el pecoreo, llevándola hasta la extenuación y por consiguiente a su muerte (Willians, 2000). La varroasis se ha expandido con el tiempo y hoy se encuentra presente en todas las regiones de importancia apícola. Para luchar contra este parásito se utilizan diferentes pesticidas con acción acaricida. Entre los principios activos más usados actualmente en España encontramos el coumafos (organofosforado), el amitraz (amidina) y el tau-fluvalinato (piretroide). Los organofosforados y piretroides inhiben la transmisión de estímulos en el sistema nervioso de los ácaros (Simon-Delso, 2015; Soderlund, 2012). Las amidinas son antagonistas de los receptores de la octopamina en el cerebro de los parásitos, provocando hiperexcitabilidad, seguidamente parálisis y muerte. Al aplicar estos tratamientos en la colmena, si no se llevan a cabo de manera adecuada, pueden aparecer posteriormente sus residuos en la miel.

La vía indirecta que ocasiona la presencia de residuos en la miel es la debida a los tratamientos agrícolas llevados a cabo porros agricultores para proteger a los cultivos de plagas y enfermedades (Kujawski & Namiesnik, 2008).Las abejas entran en contacto con estos plaguicidas agrícolas durante la actividad de recogida del néctar (en un radio de 3-6 km de la colmena) (Beekman & Ratnieks, 2000; Rial-Otero et al., 2007). Alrededor del 80% de los insecticidas agrícolas que se usan actualmente en Europa son organofosforados y carbamatos (Blasco et al., 2011). En los últimos años han aparecido otros insecticidas llamados neocotinoides, que vienen siendo muy usados, por presentar una serie de ventajas en la agricultura: son altamente eficaces, selectivos, de efecto prolongado y no presentan resistencia cruzada como los carbamatos, organofosforados o piretroides sintéticos. Sin embargo cuando la abeja entra en contacto con los insecticidas neonicotinoides su sistema nervioso central se ve afectado, provocando su desorientación y muerte por su incapacidad de volver a la colmena, ya que son agonistas en los receptores postsinapticos del receptor de la acetilcolina nicotínica del sistema central de los insectos (Taner & Czerwenka, 2011).

La combinación de la exposición a dosis subletales de pesticidas, y la presencia de la varroasis, junto con otros problemas que pueda tener la colmena, a menudo producen un efecto sinérgico sobre el comportamiento de las abejas provocando su muerte, originando lo que se viene denominando como síndrome de despoblamiento o

disminución de las abejas en la colmena (Colony Collapse Disorder, CCD) (Williamson & Wright, 2013.; Van Engelsdorp et al., 2009). Los dos últimos años han sido dramáticos en relación al CCD, ya que un elevado número de colmenas lo mostraron provocando una disminución de su rendimiento entre el 30 y el 50%. Este hecho ha determinado que este episodio, en muchos casos, haya sido el más grave desde el primer embate de la varroa a finales de los años 80 (Calatayud & Simó, 2015).

Control de los residuos

Actualmente, a este respecto, se exige el cumplimiento del Plan de Control de Residuos acordado por la Unión Europea y llevado a cabo por la Autoridad Europea de Seguridad Alimentaria (EFSA). Con ello, se persiguen los siguientes objetivos: garantizar la protección de la salud humana, controlar y vigilar las transacciones de alimentos de acuerdo a la ley, y facilitar el comercio mundial de piensos y alimentos seguros y saludables; todo ello teniendo en cuenta las normas y acuerdos internacionales. Cuando los pesticidas son usados de la manera recomendada, no deberían implicar un riesgo para el consumidor. Sin embargo, una mala aplicación de los mismos (dosis, procedimiento, período, etc.) podría causar la contaminación del aire, agua, suelos además de las especies animales (García-Chao et al., 2010) y de sus productos. En la UE, desde septiembre de 2008, ha entrado en vigor el Reglamento que establece las normas revisadas sobre los residuos de plaguicidas (Reglamento CE 396/2005). En la base de datos de la página web de la Comisión Europea (EU Pesticides database, 2015) se encuentran los límites máximos de residuos (LMR) relativos a todas las matrices alimentarias y a todos los plaguicidas. Los LMR establecidos de los diferentes pesticidas para la miel abarcan un rango de concentraciones que van desde 10 hasta 50 μg/kg. Por otro lado el Reglamento 37/2010 *sobre tratamientos farmacológicos en animales productores de alimentos*, en relación a las abejas (*tejido diana: la mie*l) fija los límites máximos de 2 productos acaricidas: el amitraz (puede estar presente hasta un máximo de 200 μg/kg) y el coumafos (puede estar presente hasta un máximo de 100 μg/kg).

Métodos analíticos de control de residuos químicos. Validación

Para evitar introducir estos residuos en la cadena alimentaria y reducir riesgos para la salud humana, el control de su presencia en la miel es un requisito fundamental de seguridad alimentaria (Bargańska et al., 2013). Entre las diferentes técnicas analíticas que se utilizan con este propósito, destacan el test Elisa y el Charm como técnicas cualitativas (screening), ambas ampliamente establecidas para el análisis de sulfamidas y antibióticos. Asimismo, existen diversos métodos cuantitativos tanto para el análisis de antibióticos, sulfamidas y pesticidas. Entre estos métodos cabe destacar la cromatografía líquida y de gases, unidas a diferentes técnicas de detección, como son de ultravioleta (Viñas et al., 2004; Carrasco-Pancorbo et al., 2008), de fluorescencia (Pang et al., 2005; Tsai et al., 2010) o de espectrometría de masas (MS/MS) (Kaufman et al., 2002; Khong et al., 2005; Pang et al., 2006). Actualmente la técnica de elección es LC-MS/MS junto con GC-MS/MS,

ya que permiten la cuantificación de residuos (a niveles de concentraciones de μg/kg) con una alta sensibilidad y selectividad en matrices biológicas.

Los laboratorios analíticos que se dedican al análisis de residuos químicos tienen la obligación de validar los métodos analíticos antes de su aplicación. En este sentido, hay que realizar una serie de comprobaciones que aseguren que el método es científicamente correcto en las condiciones en que va a ser aplicado. En el proceso de validación se deben comprobar sus características técnicas en cuanto a selectividad y especificidad, sensibilidad, linealidad, límite de detección, límite de cuantificación, exactitud y precisión. El proceso para determinar la exactitud de los métodos se lleva a cabo con materiales de referencia certificados, si es posible, o con muestras enriquecidas artificialmente en el caso de no disponer de los materiales de referencia adecuados. La precisión del método se calcula mediante el análisis repetido de una misma muestra. Respecto al control de calidad externo, el laboratorio debe participar en ejercicios de intercomparación y ensayos de aptitud que se organizan a nivel nacional e internacional en el contexto de los métodos a validar considerando tanto el tipo de matriz como de analito.

Con respecto a la validación del método, existen guías, proporcionadas por diferentes organizaciones: la Directiva 2002/657/EC, la guía Sanco, la International Conference for Harmonization (ICH, 1996), la guía Eurachem (2014). Todas ellas presentan ligeras variaciones, sin embargo, tienen un objetivo común: establecer un sistema armonizado de aseguramiento de la calidad para asegurar la exactitud y la comparabilidad de los resultados analíticos.

La Directiva 2002/657/EC, *relativa al funcionamiento de los métodos analíticos y la interpretación de los resultados*, establece una serie de requisitos para llevar a cabo la validación de métodos analíticos tanto cualitativos como cuantitativos. En ella se definen los criterios de funcionamiento y otros requisitos que son función de la técnica usada, para la detección y cuantificación (LC-UV-Vis, LC-fluorimetría, ICP-MS, LC-MS/MS,…). Para demostrar que el método analítico cumple los criterios de funcionamiento la citada directiva establece los parámetros que hay que estudiar e indica que la validación puede realizarse a través de un estudio interlaboratorios, o de acuerdo con métodos alternativos en un solo laboratorio, o mediante la validación interna (Tabla 1.5).

El Comité sobre Residuos de Plaguicidas (CCPR, 2013) de la Comisión del Codex Alimentarius (FAO y OMS) en relación al Programa conjunto sobre Residuos de Plaguicidas para su control en los alimentos, considera que la guía SANCO/2007/3131 ofrece una cobertura en profundidad de la validación de métodos para residuos de plaguicidas. El documento SANCO *Validación de métodos y procedimientos del control de la calidad para el análisis de residuos de plaguicidas en los alimentos y los piensos* contiene directrices sobre criterios de rendimiento, incluyendo límites de recuperación y precisión. Abarca las exigencias y evaluaciones a aplicar a los datos obtenidos de los

métodos de análisis. Además la guía, en respecto a la utilización de técnicas de espectrometría de masas, especifica los requisitos necesarios, en relación a las intensidades relativas de los iones obtenidos, para la identificación de compuestos y sus tolerancias máximas.

Tabla 1.5.-Criterios de validación de métodos analíticos según la Directica 2002/657/EC.

Parámetros a evaluar independientes del método	Parámetros a evaluar dependientes del método
Especificidad	La recuperación
Veracidad	Respetabilidad
Robustez: cambios menores	Reproducibilidad intralaboratorio
Estabilidad	Reproducibilidad
	El límite de decisión (CCα)
	Capacidad de detección (CCβ)
	Curvas de calibración
	Robustez: cambios importantes

1.3.-Clasificación industrial de la miel

1.3.1. Métodos tradicionales en la clasificación de mieles

El origen botánico del néctar o de los mielatos empleados por las abejas para producir miel será determinante en sus características y propiedades organolépticas. La miel de flores, se puede clasificar en: a) miel unifloral o monofloral, cuando el néctar procede principalmente de un especie botánica, de la que toma el nombre (miel de colza, miel de romero, miel de azahar, miel de girasol, miel de cantueso, miel de castaño, etc.) y b) miel multifloral, polifloral o milflores, que es aquella procedente del néctar de diversas especies botánicas, sin que predomine ninguna de ellas. Por otro lado cuando la materia prima no ha sido el néctar, la miel se puede denominar como “miel de mielada”. Estas mieles contienen menor número de pólenes de plantas entomófilas y una cantidad mayor de pólenes de plantas anemófilas, restos de hifas, esporas de hongos y algas verdes indicadoras de mielada. Se suelen considerar como bioindicadores de mielada determinadas clorofíceas (algas verdes) del género Pleurococcus y, en menor medida, especies de los géneros Chlorococcus y Cystococcus y que normalmente aparecen agrupas en estructuras “coloniales” denominadas cenobios (Garcia-Pérez, 2003).

Las mieles monoflorales son importantes en el mercado de la miel, por tener un mayor valor añadido. Su producción depende tanto del apicultor, de la gestión que este hace a través de la selección del sitio y de la recolección selectiva, como del industrial que mezcla diferentes mieles para conformar lotes homogéneos. El aumento de los niveles de

exigencia por parte del consumidor y la apreciación que este tiene por la mieles monoflorales, están abriendo nuevos nichos de mercado, que presentan una oportunidad para el sector de la miel. En España y otros países como Italia y Francia casi la mitad de la miel comercializada tiene una denominación botánica.

Las diversas denominaciones botánicas deben ser verificables, a fin de proteger al consumidor, y de preservar la genuinidad de las mismas. Tanto el estándar para la miel Codex Alimentarius (Codex Alimentarius, 2001) como el europeo (Comisión Europea, 2002) permiten usar denominaciones específicas para las mieles producidas a partir de fuentes botánicas concretas (miel de romero, miel de eucalipto, miel de girasol,…), siempre que éstas procedan esencialmente del origen indicado y tengan las correspondientes características fisicoquímicas, organolépticas y microscópicas (Persano-Oddo & Bogdanov, 2004). Sin embargo, aunque estas normas especifican algunos límites para la denominación de miel de flores y mieles de mielada, no establecen ningún criterio legal para mieles monoflorales, por lo tanto no garantizan un control eficaz de la monofloralidad para estas denominaciones. Algunos autores (Gómez-Pajuelo, 2004; Sáenz & Gómez, 2000) así como ciertos laboratorios europeos han establecido límites para las mieles monoflorales, que pueden servir como control de calidad a nivel nacional, pero presentan el inconveniente de que estos límites pueden variar entre países, lo que implica una dificultad para el comercio internacional de este tipo de mieles.

Existen D.O. que especifican que para que una miel pueda ser considerada como monofloral de una especie botánica, ésta debe tener un mínimo porcentaje de polen de dicha variedad de miel. Por ejemplo, están reconocidas entre otras, con una calidad diferenciada mediante D.O.P. las mieles españolas de Granada, La Alcarria, Tenerife y Villuercas-Ibores; con I.G.P. las mieles de Galicia; con Marcas de Calidad de comunidades autónomas la miel de azahar y la miel de romero con Marca C.V., etc. Cabe mencionar que a menudo, la importación de ciertas mieles monoflorales ha sido rechazada debido al incumplimiento de los límites locales (Persano-Oddo & Bogdanov, 2004).

No es fácil definir una miel como monofloral, ya que como se ha dicho no hay referencia de mieles puras monoflorales, puesto que las abejas pecorean siempre en diferentes especies botánicas incluso cuando hay una especie predominante. Ciertos tipos de mieles pueden ser producidos en varios países con diferentes niveles de 'unifloralidad', a raíz de la mayor o menor presencia de la planta correspondiente, y esto puede conducir a una percepción ligeramente diferente, de un país a otro. El enfoque clásico para verificar la denominación botánica de la miel tiene en cuenta tres metodologías complementarias, el análisis sensorial, el físico-químico y el melisopalinológico. La decisión de clasificar una miel como monofloral se debe basar en una interpretación global delos resultados obtenidos por estas tres metodologías. Este enfoque clásico para la definición de mieles monoflorales conlleva el uso de técnicas laboriosas que deben ser llevadas a cabo por personal experto. Este hecho es especialmente evidente en el análisis del polen, ya que los técnicos encargados de esta tarea deben tener amplia experiencia en el

reconocimiento de la morfología y otros aspectos de los pólenes de las diferentes especies botánicas. El técnico también debe ser conocedor de los aspectos sensoriales característicos de las diferentes variedades de miel. A pesar de la problemática relacionada con estas dos metodologías, en la actualidad la industria sigue utilizándolos para la clasificación botánica de las mieles como parte del control rutinario realizado en la etapa de recepción.

Análisis melisopalinológico

La miel contiene células de polen como consecuencia del arrastre por adhesión al cuerpo de la abeja. Este hecho es precisamente el fundamento del análisis polínico, que asume que una determinada cantidad del polen de cada especie, permanece en la miel, y por lo tanto a partir de su estudio es posible conocer su origen floral. En definitiva, el polen proporciona una buena huella digital de la procedencia botánica, así como del medio ambiente donde esa miel ha sido cosechada. El estudio y reconocimiento microscópico de la morfología de los granos de polen de las diferentes especies botánicas, y la asignación de las frecuencias relativas de aparición de las mismas, con la finalidad de asignar una variedad a la miel, es lo que se denomina melisopalinología. Por lo tanto, el análisis de polen es útil tanto para determinar el origen botánico de las mieles como de la zona geográfica de procedencia (Persano Oddo & Bogdanov, 2004).

No existe un método oficial para llevar a cabo este análisis. La International Commission for Bee Botany en 1978 elaboró y publicó un método melisopalinológico (Louveaux et al., 1978), y aunque posteriormente diversos autores propusieron otras metodologías, hoy en día sigue siendo un método establecido en la mayoría de los laboratorios europeos que realizan controles de calidad rutinarios a la miel. En 2004 Von Der Ohe et al. implementaron y validaron esta metodología, la consistencia del método fue probada en un ensayo interlaboratorio en el que participaron 9 países europeos.

El principal punto crítico del análisis melisopalinológico sigue siendo la correcta identificación del polen y la interpretación posterior de los resultados, así como de la discriminación de las especies melíferas y/o nectaríferas. Es importante destacar que la presencia por ejemplo de un 20% de polen de una determinada especie presente en la miel no indica, sin más, que la miel se ha formado con un 20% de néctar procedente de dicha planta, sino que esta proporción puede ser mayor o menor. De manera general para poder definir una miel como monofloral, el contenido de polen de la especie vegetal dominante deberá ser ≥ 45% (polen predominante). Se establecen excepciones para especies en las que el polen puede estar supra-representado (castaño, eucalipto o myosotis, etc.) o infra-representado (azahar, romero, tilo) (Celis & Díez, 1995; Sáenz & Gómez, 2000).

Aunque no está claramente determinada la relación entre la cantidad de néctar usada para producir una miel y la proporción de pólenes en el sedimento polínico de ésta, se

sabe que depende de una serie de factores que deben ser tenidos en cuenta, son los llamados factores primarios, secundarios y terciarios.

El aporte primario es el procedente de la flor en la que pecorea la abeja. Los granos de polen caen en el néctar de la propia flor, por las acciones mecánicas bien del aire o bien de ciertos animales. La succión de néctar por parte de la abeja arrastra estos granos en suspensión. Este fenómeno está influenciado directamente por distintos factores:

- la arquitectura floral (flores abiertas, tubulares, cerradas, etc.). Por ejemplo, en el caso de lavandas y cítricos, el polen se deposita en polinias o en los estambres curvados hacia fuera, lo que conlleva a que el néctar contenga poco polen.
- coincidencia de la secreción nectarífera con la dehiscencia de la antera.
- localización cercana de los estambres a los nectarios.
- tamaño y ornamentación de los granos: influyen en la cantidad susceptible de ser filtrada por el proventrículo en el buche melario.
- distancia entre la fuente nectarífera y la colmena: a mayor distancia, más tiempo dura la filtración y más se reduce el contenido polínico en la materia prima.

El aporte secundario al sedimento de la miel, se debe a granos de polen presente en el microambiente que hay en el interior de la colmena. Estos entran a través de la piquera por corrientes de aire o son traídos en el cuerpo de la abeja al rozar con las anteras.

El aporte terciario, lo constituye el modo de extracción de la miel del panal. El uso de instrumentos para desopercular los cuadros provoca la apertura de las celdillas con polen, incorporándose éste a la miel. Este aporte es relevante, sobre todo en aquellos casos en que la extracción se realiza por prensado de los panales o tipo de colmena, debido a que las celdillas que contienen polen (si las hay) son también procesadas y su contenido pasa a formar parte del producto final. A partir de extracciones por centrifugación, el aporte polínico terciario puede ser también considerable (Del Baño-Breis, 2000).

Análisis sensorial

La calidad sensorial de un alimento determina la aceptación del mismo por parte del consumidor y su decisión de volver a comprarlo, por ello debe de ser muy tenida en cuenta por productores y envasadores al establecer la diferencia entre sus productos (Díaz-Moreno, 2009). El consumidor de miel define sus preferencias en base a la vista, el olfato y la boca. En el caso concreto de la miel, la evaluación sensorial va a permitir definir su origen botánico, así como identificar y detectar algunos defectos tales como: fermentación, impurezas, olores y sabores extraños, etc.

Queda claro que la miel de una determinada procedencia botánica está definida por ciertas características organolépticas. Para una correcta clasificación de la miel en base a su origen botánico, el dictamen organoléptico es fundamental. No solo es una ayuda para completar la información obtenida en el estudio polínico y fisicoquímico; sino que en ocasiones resulta determinante para sacar conclusiones (ej. miel de lavanda). Así, por

ejemplo: la miel de romero tiene un aroma poco intenso, con notas alcanforadas y tonos florales aumentando la intensidad de los aromas en boca; la miel de eucalipto se define por su aroma intenso y persistente a madera mojada, con gusto dulce y notas ligeramente ácidas y la miel de espliego por su aroma muy intenso, siendo al gusto dulce con tonos ácidos y ligeramente salado (Gómez-Pajuelo, 2004). En la Figura 1.3 se muestra la rueda resultado de la estandarización de la terminología para definir sensorialmente a una miel (odour and aroma wheel for honey) realizada por un grupo de trabajo de la IHC (Piana et al., 2004).

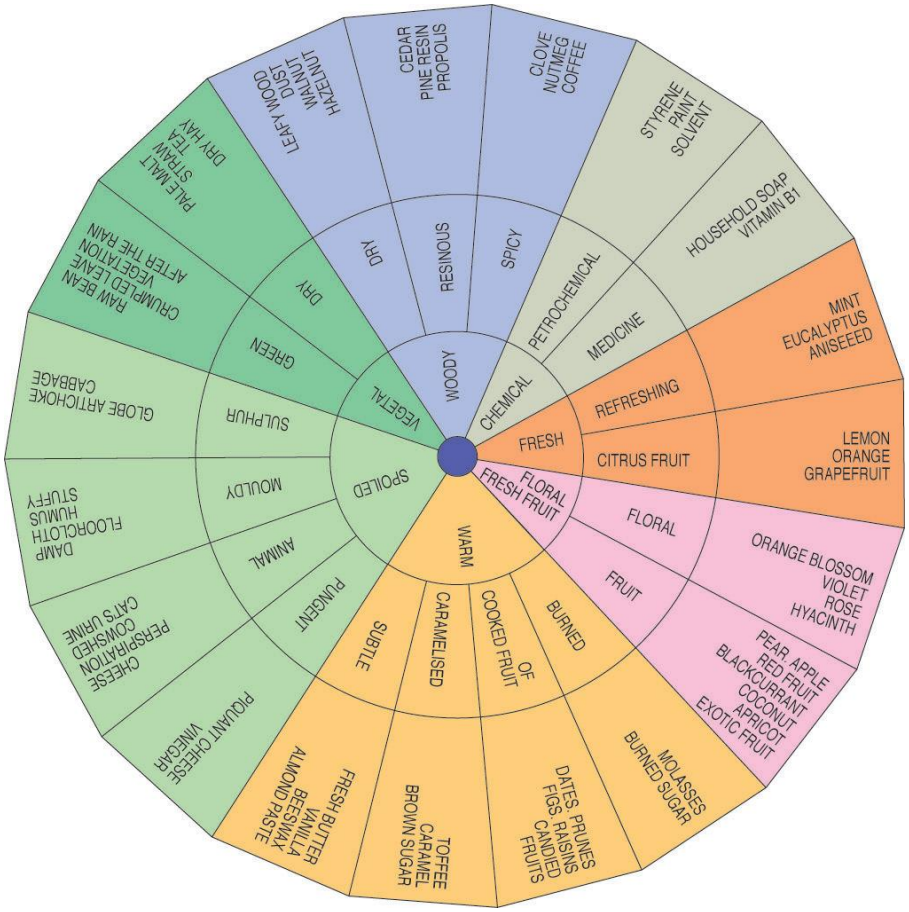

Figura 1.3.-Rueda de olor y aroma de la miel (IHC, 2001)

Análisis físico-químico y color

Los métodos físico-químicos utilizados para el control rutinario de la calidad de la miel han sido validados y armonizadas por la Comisión Internacional de la Miel (IHC) y pueden ser utilizados dentro del alcance tanto del Codex Alimentarius Honey Standard (Codex Alimentarius, 2001) como de la Directiva de la Unión Europea (European Commission, 2002).

La conductividad eléctrica es un parámetro de calidad de gran utilidad para la clasificación de mieles (Persano-Oddo & Piro, 2004). El hecho de que la conductividad eléctrica se

correlacione bien con el contenido mineral de la miel, permite distinguir perfectamente la miel de mielada de la de néctar. La conductividad se determina en un conductímetro a 20°C, considerando el peso de la muestra sin humedad; expresando el resultado en mS/cm (Accorti et al., 1987).

La actividad enzimática difiere entre tipos de mieles, pudiéndose utilizar para su clasificación siempre y cuando se trate de mieles frescas y crudas ya que como se ha comentado anteriormente, algunas enzimas como la diastasa desciende con el calentamiento y el almacenamiento (Visquert, 2015). La medida de la actividad diastásica se puede llevar a cabo por 2 procedimientos: 1. Definido por el BOE 145/1986, basado en la determinación de la velocidad de hidrólisis del almidón en una disolución de miel y medición del punto final de la reacción por espectrofotometría a una absorbancia de 660nm; 2. El método Phadebas descrito por la IHC, se basa en la medida de la hidrolización de un sustrato estandarizado (pastillas) por la acción de la enzima diastasa. En ambos casos ocurre que esta reacción produce una solución azulada, cuya intensidad es proporcional a la cantidad de enzima presente. Este método presenta la ventaja de ser más rápido y fiable que el anterior (variabilidad del almidón usado), ya que usa un sustrato estandarizado. En ambos casos, la absorbancia de la solución es directamente proporcional a la actividad diastásica de la muestra.

Los azúcares de una miel; se pueden determinar por diferentes métodos, siendo la cromatografía liquida (HPLC) el más extensamente utilizado, en diferentes modalidades: detección de índice de refracción (IR), detección de la dispersión de luz (ELSD) o iónico (PAD). La cromatografía de gases con ionización de llama (FID) es también útil en el análisis de azúcares. Presenta la ventaja frente a la cromatografía líquida de permitir mayor sensibilidad en la detección de azúcares minoritarios. Por el contrario, su mayor inconveniente es que se requiere una etapa previa de derivatización de la muestra, lo que lo convierte en un método muy tedioso. Otro método ampliamente extendido, por su rapidez y facilidad en el análisis de azúcares, son los kits enzimáticos, especialmente en lo que se refiere a la determinación de fructosa, glucosa y sacarosa. Este procedimiento aunque no es especialmente precisos, sirve como método de rutina en el control de calidad de la miel.

El colores la propiedad física percibida de manera más inmediata por el consumidor. Es un criterio muy útil para la clasificación de mieles monoflorales, variando desde blanco agua, a través de tonos ámbar, hasta casi negro. Algunas mieles presentan tonalidades típicas, como son el color amarillo brillante (miel de girasol), verdoso o rojizo (miel de tomillo, brezo). Para medir el color el método más extensamente usado es el colorímetro PFund, que se basa en la comparación óptica de la miel con una escala de color. También se puede medir con técnicas que miden la reflectancia o transmitancia de la muestra, para ello se usan equipos conocidos como espectrofotómetros o colorímetros triestimulos (espacios cromáticos CIELAB). En el año 2004 apareció en el mercado un espectrofotómetro (Hanna) específico para medir el color de la miel, cuyos resultados se

expresan en la misma escala de color Pfund (mm), y por su gran versatilidad es hoy en día ampliamente usado en los laboratorios de control de calidad de mieles.

1.3.2. Métodos alternativos en la clasificación de mieles

En las últimas décadas se vienen desarrollando métodos alternativos para la clasificación de mieles, que aunque están dando resultados prometedores a nivel científico, en su mayoría no están validados ni armonizados para ser empleados como análisis de rutina. En general se trata de técnicas analíticas que generan una enorme cantidad de datos, por lo que para dar resultados concluyentes, se requiere del apoyo de técnicas estadísticas multivariantes. En la Tabla 1.6 se resume la utilidad de los métodos tradicionales, así como de los nuevos que actualmente se están desarrollando y aplicando.

Entre los nuevos métodos cabría mencionar:

La espectroscopia de infrarrojos (IR). Se fundamenta en la absorción de la radiación IR por parte de las moléculas en vibración. En principio, cada molécula presenta un espectro IR característico (huella dactilar), debido a que la mayoría de las moléculas tienen algunas vibraciones que, al activarse, provocan la absorción de una determinada longitud de onda en la zona del espectro electromagnético correspondiente al infrarrojo. De esta forma, analizando cuales son las longitudes de onda que absorbe un compuesto en la zona del infrarrojo, podemos obtener información acerca de las moléculas que componen dicho compuesto. Así, en la miel se determinan los espectros significativos en las regiones de absorción de diferentes grupos funcionales de los componentes de la miel, por ejemplo los C–O y C–OH de la glucosa y fructosa. De esta manera se estudia el ratio F/G característico de los diferentes orígenes botánicos y se obtienen sus F/G tipificados, que posteriormente se compararan con los de la miel a estudiar.

Compuestos fenólicos (flavonoides). Son metabolitos secundarios de los vegetales, necesarios para su desarrollo y defensa frente a infecciones por microorganismos que las atacan. La miel al proceder de las plantas (néctares y mieladas) contiene pequeñas partes de estos compuestos. Desde que se descubrió la existencia de compuestos fenólicos en la miel ha resultado ser un importante tema de estudio. Por una parte ha resultado de gran interés para conocer mejor sus propiedades beneficiosas en el organismo humano, y por otra permite explorar un campo en la caracterización geográfica/botánica de este producto (Tomás-Barberán et al., 1994). En un estudio llevado a cabo por estos autores sobre miel de La Alcarria concluyen que al analizar mieles de distinto origen geográfico, las mieles tropicales carecen de ciertos compuestos característicos hallados en La Alcarria; y por otra parte analizando mieles monoflorales del mismo origen demostraron que el análisis discriminante clasificó el 85,7% de las mieles monoflorales correctamente. Estos autores concluyeron que el análisis de flavonoides puede ser de gran utilidad en estudios de origen y caracterización de mieles.

En un reciente estudio llevado a cabo por Escriche et al. (2014) sobre diferentes mieles españolas, deduce que el origen botánico afecta al perfil de flavonoides y compuestos fenólicos lo suficientemente como para permitir la discriminación entre ellas. En la miel de cítricos encontraron predominio de hesperetina, en la de romero de kaempferol, crisina, pinocembrina, ácido cafeico y naringenina y en la de mielada, miricetina, quercetina, galangina, y en particular el ácido p- cumárico.

Análisis de los componentes volátiles por GC-MS, como una medida indirecta de la percepción aromática de las mieles, es uno de los métodos más prometedores para la diferenciación de mieles (Bogdanov et al., 2004). Son numerosos los compuestos que han sido identificados como característicos de algunas fuentes florales, lo que se refleja en las recientes publicaciones mencionadas en el punto 1.1.2. Los componentes volátiles contribuyen significativamente al flavour de la miel y está claro que varían con el origen floral. Aunque cabe señalar que los resultados del análisis, sobre los compuestos del aroma de la miel, dependen de las técnicas de aislamiento, así como de los métodos de detección. Un análisis cuidadoso de los componentes de la fracción volátil en la miel podría ser una herramienta útil para la caracterización de la fuente botánica (Anklam, 1998).

Análisis de aminoácidos. Las relaciones entre las concentraciones de diversos aminoácidos pueden ser útiles para determinar el origen geográfico de una miel. De igual manera, los perfiles de aminoácidos podrían dar una indicación de la fuente botánica de muestras de miel. En un estudio llevado a cabo sobre la composición de aminoácidos, analizados por GC-MS, en mieles de acacia, cítrico, castaño, rododendro, romero y tilo se concluyó que ciertos aminoácidos como arginina, triptófano y cistina son característicos de algunos tipos de mieles florales. Sin embargo, es el perfil general de todos ellos el que puede permitir la diferenciación entre tipos de miel e incluso en función del origen geográfico (Pirini et al., 1992).

Análisis de proteínas. El análisis de proteínas mediante electroforesis de gel de poliacrilamidas junto con la aplicación de un análisis discriminante, permitió una cierta clasificación de mieles gallegas (Rodríguez Otero et al., 1990). Sin embargo, estos autores llegaron a la conclusión de que el análisis de los perfiles de aminoácidos parece ser más adecuado, para la detección del origen botánico y geográfico, que el de la composición de proteínas.

La determinación de minerales y oligoelementos en la miel podrían ser adecuados para la detección de origen geográfico, debido al hecho de que éstos se ven afectados en gran medida por la contaminación ambiental. La investigación de estos perfiles de elementos traza en combinación con técnicas de evaluación de datos estadísticos podría ser un enfoque prometedor (Sevlimli et al., 1992; Rodríguez -Otero et al., 1995).

Tabla 1.6.- Métodos para la determinación del análisis botánico de mieles (Bogdanov et al., 2004)

Métodos y Parámetros	Estado
Métodos clásicos	
Determinación de parámetros fisicoquímicos rutinarios: conductividad eléctrica, azucares, ratio F/G, actividad enzimática, prolina, color, rotación óptica, pH, acidez	Junto con el análisis sensorial y el polínico la conductividad y el ratio F/G son muy útiles Parámetros rutinarios pueden ser determinados por espectroscopia IR
Otros métodos	
Determinación de polifenoles por HPLC	Presenta buenos resultados, aunque son métodos laboriosos para ser de rutina
Determinación de componentes volátiles por espacio de cabeza o SPME seguido de GC-MS o narices electrónicas	Métodos prometedores, que debería ser investigados y desarrollados para análisis cuantitativo de volátiles
Lenguas electrónicas	Son también prometedoras, pero no se encuentran disponibles en laboratorios de alimentos de manera habitual
Aminoácidos	Tienen algún poder discriminante pero dependen del origen geográfico
Análisis de inmunotransferencia de las proteínas de la miel, originadas por el polen	Método complementario al clásico melisopalinológico
Elementos traza	Muestra alguna discriminación, pero puede depender del origen geográfico y de la climatología
Ácidos carboxílicos alifáticos	Limitado poder discriminante ya que muchos ácidos son aporte de la abeja
Espectroscopia de infrarrojos (IR)	Prometedor nuevo y rápido método, que debería ser desarrollado
Espectrometría de masas por pirolisis	Prometedor nuevo método, aunque necesita cara instrumentación

La composición de ácidos orgánicos en la miel. El perfil de ácidos orgánicos y su evaluación mediante métodos estadísticos puede resultar útil para proporcionar información adicional sobre mieles de diversas fuentes. En miel de rewarewa de Nueva Zelanda (*Knightea excelsa*), se han identificado treinta y dos ácidos dicarboxílicos alifáticos (analizados por GC), tres de ellos se han propuesto como marcadores (Wilkins et al.,

1995). Cherchi et al. (1994) en un estudio de ácidos orgánicos alifáticos de mieles italianas describieron un método de extracción en fase solida (SPE) y posterior análisis por HPLC.

Las lenguas electrónicas. Entre las técnicas actualmente más prometedoras, destacan las lenguas electrónicas, ya que se trata de equipos de medida rápidos y de bajo coste (García-Breijo, 2005). Según la IUPAC, una lengua electrónica es "un sistema multisensor, que poseen una baja selectividad y que utilizan procedimientos matemáticos avanzados para el procesamiento de las señales obtenidas. Estos procedimientos matemáticos están basados en la formación de Patrones de Reconocimiento y/o Análisis de datos Multivariados, como son las Redes Neuronales Artificiales (RNA), los Análisis por Componentes Principales (PCA), etc. (Campos et al., 2013). Las lenguas electrónicas están siendo aplicadas en el control de calidad de diferentes alimentos: aguas minerales (Martínez-Máñez et al., 2005), (Zhuiykov, 2012), vinos (Campos et al., 2013), leche (Dias et al., 2009), miel (Dias et al., 2008; Kadar, 2011), etc.

La lengua electrónica consta de: un conjunto de sensores de distinta especificidad, una instrumentación para adquirir la señal y un sistema de procesado de datos (ver Figura 1.4) (Jiménez et al., 2002). Los sensores electroquímicos transforman el efecto de la interacción electroquímica analito-electrodo en una señal. El instrumento para adquirir la señal (sistema de medida), se encarga de captar las señales eléctricas generadas por los sensores, y, posteriormente, digitalizarlas y enviarlas al sistema de procesado de datos. Sistema de procesado de datos (Software), creado con los algoritmos apropiados, permite procesar las señales obtenidas por el sistema de medida (Jiménez et al., 2002).

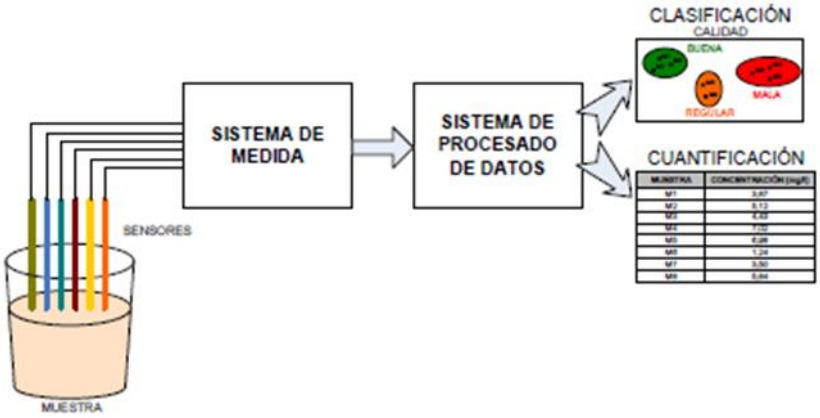

Figura 1.4.- Esquema de los componentes que forman la lengua electrónica (Alcañiz, 2011).

1.4.-El sector

1.4.1. Producción primaria

El proceso de producción de la miel comienza cuando el apicultor decide poner las colmenas en una determinada zona geográfica, con la finalidad de cosechar un tipo de miel determinado. Una vez la miel ha sido producida por las abeja y está ya madura el apicultor extrae la miel de los panales, bien in situ en el campo o bien transportando los cuadros para extraer la miel posteriormente. El paso siguiente es extraerla miel (normalmente por centrifugación), eliminando la capa de cera que han depositado las abejas para tapar las celdillas (desoperculado) (Figura 1.5). La miel se recoge en bidones metálicos (aprox. 300 kg) que se cierran y serán la materia prima que entra en las industrias de procesado y envasado de miel.

Figura 1.5.- Desoperculado de las celdillas y extracción de miel por centrifugación

La calidad con la que entra la miel en la industria es responsabilidad del apicultor. De él depende cosechar en el momento adecuado de maduración (humedad) y conservar los bidones en un lugar fresco y seco. Pero además, hay otro aspecto importante relacionado con los tratamientos veterinarios. Las abejas desarrollan infecciones y se requiere la aplicación de tratamientos químicos con antibióticos, sulfamidas, acaricidas, etc. Estos tratamientos de deben aplicar en el momento adecuado y a dosis correctas. De no ser así, el resultado es una miel contaminada por dichas sustancias.

En los últimos años los problemas de presencia de residuos han aumentado, por ello las administraciones y los compradores están cada vez más alerta. Otra fuente de residuos químicos en la miel es la debida a los pesticidas utilizados en los tratamientos agrícolas, que llegan a la miel por la ubicación de los panales próximos a zonas de cultivo. Este problema es usual en mieles de cultivo como el azahar, el girasol, el almendro, etc.

1.4.2. Procesado de la miel y controles

En la industria actual el control de la calidad del proceso es fundamental para conseguir un producto adecuado que cumpla los requisitos y exigencias establecidos, y esto tiene especial relevancia en la industria alimentaria, ya que el producto final va a tener incidencia en la salud humana. Por parte de las administraciones y de los consumidores cada vez es mayor la exigencia de alimentos con mayores índices de calidad y de seguridad. La administración debe asegurar un nivel elevado de protección de la salud de las personas, y el consumidor tiene derecho a disponer de un producto adecuado a sus exigencias y expectativas.

La industria de la miel, como parte del sector agroalimentario, debe controlar la calidad tanto en la recepción de su materia prima, como en el proceso, hasta la expedición del producto terminado. Entre los parámetros más frecuentemente controlados por las empresas envasadoras los residuos químicos ocupan un lugar destacado, además de las características organolépticas (sabor y aroma), color, contenido de humedad, frescura de la miel (medida por la diastasa y el contenido de HMF), composición de azúcares mayoritarios y el examen microscópico para la determinación de origen botánico y geográfico. Este control de calidad de la miel tiene dos propósitos principales, por un lado el de verificar su autenticidad, es decir, para revelar posibles fraudes tales como mieles adulteradas etc. y por otro el determinar su calidad con respecto a las necesidades del procesador y del mercado.

La mayoría de las empresas envasadoras de miel tienen establecidos ciertos parámetros de calidad internos tanto para la materia prima que adquieren, bien directamente de los apicultores, bien de terceros, así como para el producto terminado listo para la venta. De esta manera controlan la producción, ajustando los costes a los requisitos del producto y a sus diferentes niveles de calidad. Dichos niveles de calidad pueden ser los requeridos por el mercado, o bien por otra empresa bajo cuyo sello se comercializará el producto. El proceso que se lleva a cabo en la industria de manera general sigue el diagrama de flujo de la Figura 1.6.

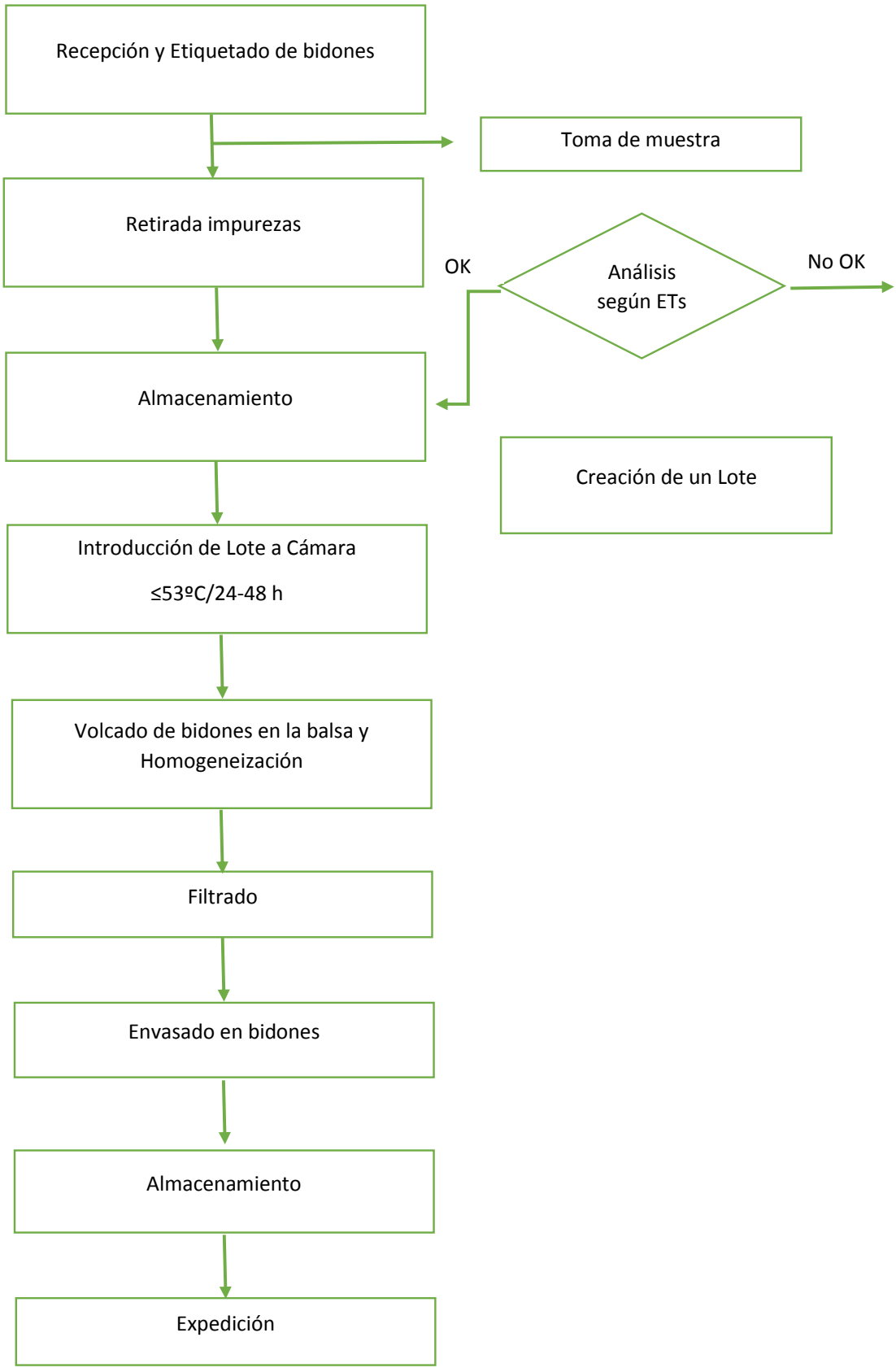

Figura 1.6.- Diagrama de flujo del procesado de miel.

1.4.3. España en el contexto de producción y comercialización de miel

Según datos extraídos de la FAO, casi la mitad de la producción mundial de miel del año 2012 se concentró en Asia (737.000 toneladas), correspondiendo a China 436.000 toneladas, lo que representa el 60%. El segundo continente productor fue Europa seguido de América y finalmente África y Oceanía (Figura1.7).

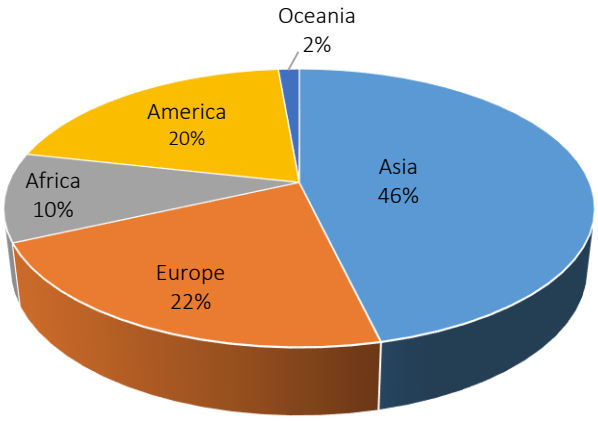

Figura 1.7.- Producción mundial de miel en 2012 (en Toneladas)

Dentro de Europa los principales productores de miel en 2012 fueron Ucrania, España y Rumania (Figura 1.8). España cuenta con el mayor número de colmenas y tasa de profesionalización, dentro de la Unión Europea. El censo total de colmenas, verificado sobre la base del Registro de explotaciones apícolas en el estado español, ascendió a 2.533.270 (abril de 2012). La mayor parte de dicho censo se encuentra repartido en Andalucía (22.25%), Extremadura (18.64%), Comunidad Valenciana (15.55%) y Castilla y León (14.94%). Un 78.7 % del total de las colmenas de España pertenecen a apicultores profesionales (dato de diciembre de 2012). Esto significa que pertenecen a explotaciones apícolas con más de 150 colmenas (dato superior al del conjunto de la UE, que sólo cuenta con un 32.8% de las colmenas en manos de apicultores profesionales).

Según DataComex, en 2012 el 89.8% de la miel importada por España procedía de terceros países, especialmente China (14.232 toneladas), seguido por Argentina (587 toneladas), lo que supone 3.7% del total. Según la misma fuente, México habría exportado a nuestro país en 2012 un total de 309 toneladas, lo que constituiría únicamente un 2% del total.

Los principales destinos en la UE de las exportaciones de miel desde España en 2012 fueron Francia con 5.734 toneladas (34.8%), Alemania con 4.201 toneladas (25.5%), Italia con 1.495 toneladas (9.1%) y Portugal con 1.114 toneladas (6.8%) (COAG, 2015).

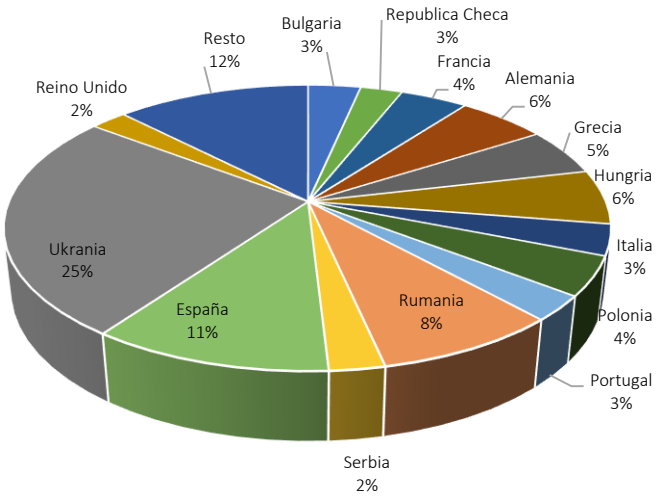

Figura1.8.- Producción de miel en Europa en 2012 (en Toneladas). Datos según FAO

1.5.-Bibliografía

Accorti M., Piazza M.G. & Persano-Oddo L. (1987). La conductivité électrique et le contenu en cendre du miel, Apiacta 22, 19–20.

Alcañiz M. (2011). Diseño de un sistema de lengua electrónica basado en técnicas electroquímicas voltamétricas y su aplicación en el ámbito agroalimentario. Tesis doctoral. Universidad Politécnica de Valencia. Departamento de Ingeniería Electrónica.

Álvarez-Suárez J.M., Giampieria F., González-Paramás A.M., Damianic E., Astolfid P. & Martínez-Sanchez G. (2012) Phenolics from monofloral honeys protect human erythrocyte membranes against oxidative damage. Food and Chemical Toxicology, 50 (5), 1508–1516.

Anderson D. L. & Trueman J. W. H. (2000). Varroa jacobsoni (Acari: Varroidae) is more than one species. Experimental & applied acarology, 24(3), 165-189.

Anklam E. (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. Food chemistry, 63(4), 549-562.

Bargańska Ż., Ślebioda M. & Namieśnik J. (2013). Pesticide residues levels in honey from apiaries located of Northern Poland. Food Control, 31(1), 196-201.Beekman M., & Ratnieks F. L. W. (2000). Long-range foraging by the honey-bee, Apis mellifera L. Functional Ecology, 14(4), 490-496.

Beekman M. & Ratnieks F. L. W. (2000). Long-range foraging by the honey-bee, Apis mellifera L. Functional Ecology, 14: 490-496.

Belitz H. D. & Grosch W. (1997). Química de los Alimentos. Editorial Acribia. Zaragoza, España.

Blasco C., Vázquez-Roig P., Onghena M., Masia A. & Picó Y. (2011). Analysis of insecticides in honey by liquid chromatography-ion trap-mass spectrometry: Comparison of different extraction procedures. Journal of Chromatography A, 1218, 4892-4901.

BOE 145.Orden de 12 de junio de 1986 por la que se aprueban los métodos oficiales de análisis para la miel.

Bogdanov S. & Martin P. (2002). Honey Authenticity: a Review. Swiss Bee Research Centre.

Bogdanov S., Martin P. & Lullmann C. (2002). Harmonised methods of the international honey commission. Swiss Bee Research Centre, FAM, Liebefeld.

Bogdanov S., Ruoff K. & Persano Oddo L. (2004). Physico-chemical methods for the characterisation of unifloral honeys: a review. Apidologie, 35(Suppl 1), 4-17.

Bogdanov S. (2011). The Honey Book. Bee Product Science. www.bee-hexagon.net.

Brookes S.T., Barrie A. & Davies J.E. (1991). A rapid 13C/12C test for determination of corn syrups in honey, J. Assoc. Off. Anal. Chem. 74, 627-629.

Cabañero A.I., Recio J.L. & Ruperez M. (2006). Liquid chromatography coupled to isotope ratio mass spectrometry: A new perspective on honey adulteration detection, Journal of agricultural and food chemistry 54, 9719-9727.

Calatayud F. & Simó E. Una Varroa cada vez más virulenta. Apitemas -apipatologia y sanidad.http://www.apiads.es/index.php/apitemas, Consultada en septiembre de 2015.

Campos I., Bataller R., Armero R., Gandía J. M., Soto J., Martinez-Mañez R. & Gil-Sánchez L. (2013).Monitoring grape ripeness using a voltammetric electronic tongue. Food Research International 54, 1369–1375.

Carrasco-Pancorbo A., Casado-TerronesS., Segura-Carretero A. & Fernández-Gutiérrez A. (2008). Reversed-phase high-performance liquid chromatography coupled to ultraviolet and electrospray time-of-flight mass spectrometry on-line detection for the separation of eight tetracyclines in honey samples. Journal of Chromatography A, 1195(1), 107-116.

Castro-Vazquez L., Diaz-Maroto M.C. & Pérez-Coello M.S. (2007). Aroma composition and new chemical markers of Spanish citrus honeys. Food Chemistry, 103, 601-606.

Castro-Vazquez L., Diaz-Maroto M.C., González-Viñas M.A. & Pérez-Coello M.S. (2009). Differentiation on monofloral citrus, rosemary, eucalyptus, lavender, thyme and heather honeys based on volatile composition and sensory descriptive analysis. Food Chemistry, 112, 1022-1030.

CCPR- Comité sobre Residuos de Plaguicidas del Codex Alimentarius, (2013). Consultada en noviembre de 2015.
ftp://ftp.fao.org/Codex/Meetings/ccpr/ccpr45/pr45_12s.pdf

Celis M. & Díez M. J. (1995). Análisis polínico de mieles en la cabrera baja (montes de León, España). Acta Botanica Malacitana, 20: 91-96.

Chen L., Xue X., Ye Z., Zhou J., Chen F. & Zhao J. (2011). Determination of Chinese honey adulterated with high fructose corn syrup by near infrared spectroscopy. Food Chemistry 128, 1110–1114.

Cherchi A., Spanedda L., Tuberoso C. & Cabras P. (1994). Solid-phase extraction and high-performance liquid chromatographic determination of organic acids in honey. Journal of Chromatography A, 669(1), 59-64.

COAG. Coordinadora de Organizacines de Agricultores y Ganaderos. Informes-Apicultura. Anuario Agrario 2015. Consultada en septiembre de 2015. http://www.coag.org/rep_ficheros_web/e7a475275366fdbc0bed13f9f2b9d198.pdf

Codex Alimentarius. (2001). Draft revised standard for honey. Council Directive 2001/110/EC of 20 December 2001 relating to honey. Official Journal of the European Communities L10, 47-52.

Commission Regulation (EU) N º 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.

Cordella C., Antinelli J. F., Aurieres C., Faucon J. P., Cabrol-Bass D. & Sbirrazzuoli N. (2002). Use of differential scanning calorimetry (DSC) as a new technique for detection of adulteration in honeys. 1. Study of adulteration effect on honey thermal behavior. Journal of Agriculture Food Chemistry, 50, 203–208.

Cote J., Casabianca H., Chardon S., Lheritier J. &Grenier-Loustalot M. F. (2003). Application of carbohydrate analysis to verify honey authenticity. Journal of Chromatography A, 1021, 145–155.

Crane E. (1975). Honey: a comprehensive survey /edited by Eva Crane. London: Heinemann for the Bee Research Association.

Crane E. (1990). Bees and Beekeeping. Scientific, Practice and World Resources. 1st ed., Heinemann Newnes, Halley Court, Jordan Hill, Oxford OX2 8EJ, London, UK.

DataComex -Estadísticas del Comercio Exterior–Ministerio de Economía y Competitividad. datacomex.comercio.es. Consultada en noviembre de 2015.

Dias L.A., Peres A.M., Vilas-Boas M., Rocha M.A., Estevinho L. & Machado A.A.S.C. (2008). An electronic tongue for honey classification, Microchim Acta, 163, 97–102.

Dias L.A., Peres A.M., Veloso A.C.A., Reis F.S., Vilas-Boas M. & Machado A.A.S.C. (2009). An electronic tongue taste evaluation: Identification of goat milk adulteration. Sensors and Actuators B: Chemical, 136 (1), 209–217.

Directiva 2001/110/CE del Consejo de 20 de diciembre de 2001 relativa a la miel. Comunidad Europea.

Directiva 2002/657/EC. Decisión de la comisión, por la que se aplica la Directiva 96/23/CE del Consejo en cuanto al funcionamiento de los métodos analíticos y la interpretación de los resultados.

Directiva 2014/63/UE del Parlamento Europeo y del Consejo, de 15 de mayo de 2014, por la que se modifica la Directiva 2001/110/CE relativa a la miel

Del Baño Breis F. (2000). Mieles y Pólenes: palinoteca de la Región de Murcia.

De la Fuente E., Martinez-Castro I. & Sanz J. (2005). Characterization of Spanish unifloral honeys by solid phase microextraction and gas chromatography-mass spectrometry. Journal of Separation Science, 28, 1093–1100.

Diaz-Moreno A. (2009). La influencia de las condiciones de almacenamiento sobre la calidad físico-química y biológica de la miel. Tesis doctoral. Universidad de Zaragoza.

Elflein L. & Raezke K.P. (2005). Coupling of liquid chromatography with carbon stable isotope ratio mass spectrometry (LC-IRMS) – a promising new technique for detection of sugar adulteration of honey, Apimondia abstracts Ireland 2005, Apimondia International Apicultural Congress, Dublin, Ireland, 35-36.

Escriche I., Kadar M., Juan-Borrás M. & Domenech E. (2014). Suitability of antioxidant capacity, flavonoids and phenolic acids for floral authentication of honey. Impact of industrial thermal treatment. Food chemistry, 142, 135-143.

Escriche I., Kadar M., Juan-Borrás M. & Domenech E. (2011). Using flavonoids, phenolic compounds and headspace volatile profile for botanical authentication of lemon and orange honeys. Food Research International, 44, 1504-1513.

Escuredo O., Silva L., Valentao P., Seijo M.C. & Andrade P. (2012). Assessing Rubus honey value: pollen and phenolic compounds content and antibacterial capacity. Food Chemistry, 130 (3), pp. 671–678.

EU Pesticides database: http://ec.europa.eu/food/plant/pesticides. Consultada en octubre 2015.

Eurachem (2014). The Fitness for Purpose of Analytical Methods ALaboratory Guide to Method Validation and Related Topics.

Fallico B., Zappala M., Arena E. & Verzera A. (2004). Effects of conditioning on HMF content in unifloral honeys. Food Chemistry, 85, 305–313.

Fattori S. (2004). La Miel: Propiedades, Composición Y Análisis Físico-Químico. Apimondia.

Floris I., Satta A., Carpana E. & Sabatini A.G. (2002). The ethanol content as a useful parameter for the identification of honey. Symposio II Ruol della Recerca in Apicoltura, Bologna.

Gallina A., Stocco N. & Mutinelli F. (2010). Karl Fischer Titration to determine moisture in honey: A new simplified approach. Food control, 21(6), 942-944.

García-Breijo E. (2005). Diseño de un sistema multisensor en tecnología thick-film; fabricación, caracterización y estudio de su aplicabilidad a la medida de parámetros físico-químicos. Tesis doctoral. Universidad Politécnica de Valencia. Departamento de Ingeniería Electrónica.

García-Chao M., Agruña M. J., Flores-Calvete G., Sakkas V., Llompart M. & Dagnac T. (2010). Validation of an off line solid phase extraction liquid chromatography-tandem mass spectrometry method for the determination of systemic insecticide residues in honey and pollen samples collected in apiaries from NW Spain. Analytica Chimica Acta, 672, 107-113.

Garcia-Pérez R. (2003). Estudio palinologico y colorimétrico de mieles monoflorales de la Región de Murcia.Proyecto final de carrera. Uniniversidad Politécnica de Cartagena.

Gheldof N., Wang X.H. & Engeseth N. (2002). Identification and quantification of antioxidant components of honeys from various floral sources. Journal of Agricultural and Food Chemistry, 50 (21), 5870–5877.

Gómez-Pajuelo A. (2004). Mieles de España y Portugal. Montagud editores. Barcelona,

González-Miret M. L., Terrab A., Hernanz D., Fernández-Recamales M. A. & Heredia F. J. (2005). Multivariate correlation between color and mineral composition of honeys and by their botanical origin. Journal of agricultural and food chemistry, 53(7), 2574-2580.

Hamdan K. (2010). Crystallization of Honey. Bee World, 87 (4), 71-74.

Hammel Y. A., Mohamed R., Gremaud E., LeBreton M. H. & Guy P. A. (2008). Multi-screening approach to monitor and quantify 42 antibiotic residues in honey by liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1177(1), 58-76.

Huidobro J.F., Rea M.E, de Andrade P.C.B., Sanchez M.P., Sancho M.T., Muniategui S. & Simal-Lozano J. (1994). Enzymatic determination of primary alcohols as apparent ethanol content in honey. J. Agr. Food Chem. 42, 1975-1978.

ICH (2005).Validation of Analytical Procedures: Methodology, adoptedin 1996, Geneva Q2B, in 2005 incorporated in Q2(R1).

ISO 5496 (2006). Sensory Analysis. Methodology. Initiation and training of assessors in the detection and recognition of odours.

Jiménez C., Muñoz F. J., Bratov A. & Domínguez C. (2002). Lenguas electrónicas: sensores químicos aplicados a la medida del sabor y al control de calidad de los alimentos, Percepnet, 13, 1697-4131, Octubre.

Kadar M., Juan Borrás M., Carot J. M., Doménech E. & Escriche I. (2011).Volatile fraction composition and physicochemical parameters as tools for the differentiation of lemon blosson honey and orange blosson honey. Journal of the Science of Food and Agriculture, 91, 2768 – 2776.

Kaufmann A., Roth S., Ryser B. & Widmer M. (2002). Quantitative LC/MS-MS determination of sulfonamides and some other antibiotics in honey. Journal of AOAC International, 85 (4*),* 853-860.

Kelly J. D., Petisco C. & Downey G. (2006). Application of Fourier transform midinfrared spectroscopy to the discrimination between Irish artisanal honey and such honey

adulterated with various sugar syrups. Journal of agricultural and food chemistry, 54(17), 6166-6171.

Kerkvliet J.D. & Meijer H.A.J. (2000). Adulteration of honey: relation between microscopic analysis and deltaC-13 measurements, Apidologie 31, 717-726.

Khong S. P., Hammel Y. A. & Guy P. A. (2005). Analysis of tetracyclines in honey by high-performance liquid chromatography/tandem mass spectrometry. Rapid communications in mass spectrometry, 19(4), 493-502.

Kujawski M. W. & Namiesnik J. (2008). Challenges in preparing honey samples for chromatographic determination of contaminants and trace residues. Trends in Analytical Chemistry, Vol. 27 (9), 785-793.

Ledoux M. N., Pernal S. F., Higo H. A. & Winston M. L. (2000). Development of a bioassay to test the orientation behaviour of the honey bee ectoparasite, Varroa jacobsoni. Journal of Apicultural Research, 39(1-2), 47-54.

Louveaux J., Maurizio A. &Vorwohl G. (1978). Methods of Melissopalynology. Bee World, 59(4), 139-157.

Low N.H. & South W. (1995). Determination of honey authenticity by capillary gas chromatography, J. Assoc. Off. Anal. Chem. 78, 1106-1113.

Martínez Máñez R., Soto J., García-Breijo E., Gil L., Ibáñez J. & Llobet E. (2005). An "electronic tongue" design for the qualitative analysis of natural waters. Sensors and Actuators B: Chemical, 104(2), 302–307.

Maudens K. E., Zhang G. F. & Lambert W. E. (2004). Quantitative analysis of twelve sulfonamides in honey after acidic hydrolysis by high-performance liquid chromatography with post-column derivatization and fluorescence detection. Journal of Chromatography A, 1047(1), 85-92.

Meda A., Lamien C.E., Romito M., Millogo J. & Nacoulma O.G. (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well their radical scavenging activity. Food chemistry, 91(3), 571-577.

Mundo Apícola. Loque Europea, http://www.mundoapicola.com/pdf/loqueeuropea.pdf. Consultada en Mayo 2012.

National Honey Board. (www.honey.com). Consultada en septiembre de 2015.

Oroian M. & Escriche I. (2015). Antioxidants: Characterization, natural sources, extraction and analysis. Food Research International, 74, 10-36.

Oyarzun M.T., Figueroa A. & Tartanac F. (2005). Oportunidades de mejoramiento en la calidad e inocuidad de la cadena productiva de la miel en Chile. Organización de las Naciones Unidas para la Agricultura y la Alimentación, Santiago de Chile.

Pang G. F., Cao Y. Z., Zhang J. J., Jia G. Q., Fan C. L. & Li X. M. (2005). Simultaneous Determination of Oxytetracycline, Tetracycline, Chlortetracycline and Doxycycline

in Poultry Meat by High Performance Liquid Chromatography. Journal of Instrumental Analysis, 24(4), 61.

Pang G. F., Fan C. L., Liu Y. M., Cao Y. Z., Zhang J. J., Fu B. L. & Wu Y. P. (2006). Multi-residue method for the determination of 450 pesticide residues in honey, fruit juice and wine by double-cartridge solid-phase extraction/gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry. Food additives and contaminants, 23(8), 777-810.

Parlamento Europeo (2013). Report on the food crisis, fraud in the food chain and the control thereof [2013/2091(INI)].

Persano-Oddo L. & Piro R. (2004). Main European unifloral honeys: descriptive sheets, Apidologie 35 (Suppl. 1), S38–S81.

Persano-Oddo L. & Bogdanov S. (2004). Determination of honey botanical origin: problems and issues. Apidologie 35, S2–S3.

Piana L., Persano-Oddo L., Bentabol A., Bruneau E., Bogdanov S. &Guyot S. (2004). Sensory analysis applied to honey: state of the art. Apidologie, 35, S26–S37.

Pirini A, Conte L.S, Francioso O. & Lercker G. (1992). Capillary gas chromatographic determination of free amino acids in honey as a means of discrimination between different botanical sources. Journal of High Resolution Chromatography, 15,165–170.

Raude-Roberg L. (1994). Analyse der Saccharide spaltenden Enzyme des Bienenhonigs, Ph.D. thesis, Niedersächsisches Institut für Bienenkunde, Celle, Germany.

Radovic B.S., Careri M., Mangia A, Musci M., Gerboles M. & Anklam E. (2001). Contribution of dynamic headspace GC–MS analysis of aroma compounds to authenticity testing of honey. Food Chemistry, 72 (4), 511–520.

Real Decreto 1049/2003, de 1 de agosto, por el que se aprueba la Norma de calidad relativa a la miel.

Reglamento (CE) 396/2005 del parlamento europeo y del consejo de 23 de febrero de 2005 relativo a los límites máximos de residuos de plaguicidas en alimentos y piensos de origen vegetal y animal.

Reglamento 37/2010 (UE). Relativo a las sustancias farmacológicamente activas y su clasificación por lo que se refiere a los límites máximos de residuos en los productos alimenticios de origen animal. Diario Oficial de la Comunidad Europea, L15, 1-72.

Rial-Otero R., Gaspar E. M., Moura I. & Capelo J. L. (2007). Chromatrographic-based methods for pesticide determination in honey: An overview. Talanta, 71, 503-514.

Rodriguez-Otero J. L., Paseiro P., Simal J. & Cepeda A. (1990). Intento de caracterización de las mieles naturales de Galicia mediante las fracciones proteicas separadas por electroforesis. In Anales de Bromatologia (Vol. 42, No. 1, pp. 83-98). Sociedad Española de Bromatologia.

Rodriguez-Otero J. L., Paseiro P., Simal J., Terradillos L. & Cepeda A. (1995). Silicon, phosphorus, sulphur, chlorine and ash contents of Spanish commercial honeys.Zeitschrift für Lebensmittel-Untersuchung und Forschung, 200(3), 233-234.

Ropa D. (2010). Comparison of Mineral and Enzyme Levels in Raw and Processed Honey. Wisconsin: Ropa Science Research.

Rossmann A., Lüllmann C. & Schmidt H.L. (1992). Massenspektrometrische Kohlenstoff- und Wasserstoff-Isotopen-Verhältnismessung zur Authentizitätsprüfung bei Honigen, Zeitschrift für Lebensmittel-Untersuchung und -Forschung 195, 307-311.

Sáenz C. & Gómez C. (2000). Mieles españolas. Características e identificación mediante el análisis del polen. Ediciones Mundi-Prensa. Madrid (España).

Sammataro D. & Finley J. (2004).Obervations of the ectoparasitic bee mite Varroa destructor in honey bee (Apis mellifera) cells infected with chalkbrood (Ascosphaera apis). Journal of Apicultural Research, 43(1), 28-30.

Sancho M. T., Muniategui S., Huidobro J. F. & Simal Lozano J. (1992). Aging of honey. Journal of Agricultural and Food Chemistry, 40(1), 134-138.

Schneider A. (2011). Ultra-filtering Removes Pollen, Hides Honey Origins. http://www.foodsafetynews.com/2011/11/tests-show-most-store-honey-isnt-honey/#.Vky44XYve01. Consultada en noviembre de 2015.

Serra-Bonvehí J. & Ventura F. (1995). Characterization of Citrus honey produced in Spain. Journal of Agricultural and Food Chemistry, 43, 2053–2057.

Sesta G., Piana L, Persano-Oddo L., Lusco L. & Belligoli P. (2008). Methyl anthranilate in Citrus honey. Analytical method and suitability as a chemical marker. Apidologie, 39 (3), 334-342.

Sevimli H., Bayulgen N. & Varinlioglu A. (1992). Determination of trace elements in honey by INAA in Turkey. Journal of radioanalytical and nuclear chemistry, 165(5), 319-325.

Silici S., Sagdic O. & Ekici L. (2010). Total phenolic content, antiradical, antioxidant, andantimicrobial activities of Rhododendron honeys. Food Chem., 121, 238-243.

Sivakesava S. & Irudayaraj J. (2001). A rapid spectroscopic technique for determining honey adulteration with corn syrup. Journal of Food Science, 66(6), 787-791.

Simon-Delso N., Amaral-Rogers V. & Belzunces L.P. (2015). Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environmental Science and Pollution Research International 22, 5-34.

Simsek A., Bilsel M. & Goren A. C. (2012). 12C/ 13C pattern of honey from Turkey and determination of adulteration in commercially available honey samples using EA-IRMS. Food Chemistry, 130, 1115-1121.

Smanalieva J. & Senge B. (2009). Analytical and rheological investigations into selected unifloral German honey. Eur Food Res Technol, 229,107–113.

Soderlund D.M. (2012). Molecular Mechanisms of Pyrethroid Insecticide Neurotoxicity: Recent Advances. Archives of Toxicology, 86 (2), 165-181.

Soria A. C., Martínez-Castro I. & Sanz J. (2003). Analysis of volatile composition of honey by solid phase microextraction and gas chromatography-mass spectrometry. Journal of Separation Science, 26(9-10), 793-801.

Ståhlberg T., Fu W., Woodley J. M. & Riisager A. (2011). Synthesis of 5-(Hydroxymethyl) furfural in Ionic Liquids: Paving the Way to Renewable Chemicals. ChemSusChem, 4(4), 451-458.

Taner G. & Czerwenka C. (2011). LC-MS/MS Analysis of Neonicotinoid Insecticides in Honey: Methodology and Residue Findings in Austrian Honeys J. Agric. Food Chem., 59, 12271–12277.

Tomás-Barberán F.A, Ferreres F., Ortiz A. & Fernández M.C. (1994). Estudio sobre el contenido en flavonoides de las Mieles de La Alcarria: su aplicación a la caracterización Geográfico-Botánica. Convenio de colaboración entre el Consejo Superior de Investigaciones Científicas y la Junta de Comunidades de Castilla-La Mancha.Ed, CSIC. ISBN: 8460618323.

Tosun M. (2013). Detection of adulteration in honey samples added various sugar syrups with 12C/13C isotope ratio analysis method. Food Chemistry, 138, 1629-1632.

Tsai W. H., Chuang H. Y., Chen H. H., Wu Y. W., Cheng S. H., & Huang T. C. (2010). Application of sugaring-out extraction for the determination of sulfonamides in honey by high-performance liquid chromatography with fluorescence detection. Journal of Chromatography A, 1217(49), 7812-7815.

Van Engelsdorp D., Evans J., Saegerman C., Mullin C., Haubruge E, Nguyen B.K., Frazier M., Frazier J., Cox-Foster D., Chen Y., Underwood R., Tarpy D.R. & Pettis J.S. (2009). Colony Collapse Disorder: A Descriptive Study. Available at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0006481.

Visquert M. (2015).Tesis Doctoral. Influencia de las condiciones térmicas en la calidad de la miel. Universidad Politécnica de Valencia.

Viñas P., Balsalobre N., López-Erroz C. & Hernández-Córdoba M. (2004). Liquid chromatography with ultraviolet absorbance detection for the analysis of tetracycline residues in honey. Journal of Chromatography A, 1022(1), 125-129.

Von Der Ohe W, Persano-Oddo P., Piana L, Morlot M. & Martin P. (2004). Harmonized methods of melissopalynology. Apidologie, 35, S18–S25.

White Jr. (1978). Advances in Food Research Volume 24, Pag iii-vii, 1-378. Capitulo: Honey Original Research Article, 287-374, ISBN: 978-0-12-016424-0. Edited by C.O. Chicheste.

White J. W., Riethof M. L., Subers M. H. & Kushnir I. (1962). Composition of American honeys. Tech Bull. U.S. Dep. Agric., 1261.

White J.W. (1992). Internal standard stable carbon isotope ratio method for determination of C-4 plant sugars in honey: Collaborative study, and evaluation of improved protein preparation procedure, J. AOAC Int, 75, 543-548.

White J.W. & Bryant V.M. (1996). Assessing Citrus honey quality: pollen and methyl anthranilate content. Journal of Agricultural and Food Chemistry, 44, 3423–3425.

White J.W., Winters K., Martin P. & Rossmann A. (1998). Stable carbon isotope ratio analysis of honey: Validation of internal standard procedure for worldwide application. Journal of AOAC International, 81, 610-619.

White J.W. (2000). Isotope ratio testing of honey: Demystifying the internal standard test. Am. Bee J. 140, 318-321

Wilkins A. L. & Lu Y. (1995). Extractives from New Zealand honeys. 5. Aliphatic dicarboxylic acids in New Zealand rewarewa (Knightea excelsa) honey. Journal of Agricultural and Food Chemistry, 43(12), 3021-3025.

Williams D. L. (2000). A veterinary approach to the European honey bee (Apis mellifera). The Veterinary Journal, 160(1), 61-73.

Williamson S. M. & Wright G. A. (2013). Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. The Journal of experimental biology, 216(10), 1799-1807.

Yao L., Jiang Y., Singanusong R., Datta, N. & Raymont K. (2004). Phenolic acids and abscisic acid in Australian Eucalyptus honeys and their potential for floral authentication. Food Chemistry, 86(2), 169-177.

Zamora M.C. & Chirife J. (2006). Determination of water activity change due to crystallization in honeys from Argentina. Food Control, 17 (1), 59–64.

Zhuiykov S. (2012). Solid-state sensors monitoring parameters of water quality for the next generation of wireless sensor networks. Sensors and Actuators B: Chemical, 161 (1), 1-20.

2. OBJETIVOS

2. Objetivos

2.1.-Objetivo general

La presente tesis doctoral plantea dos objetivos generales:

2.1.1. Evaluar las técnicas que se vienen utilizando de forma rutinaria en el control de calidad de mieles, tanto a nivel industrial como comercial, y compararlas con otras alternativas no convencionales.

2.1.2. Evaluar la efectividad del control de la materia prima (llevado a cabo en la etapa de recepción industrial) para cumplir los límites legales establecidos en lo referente a la presencia de residuos químicos en miel. Además, valorar el riesgo para el consumidor como consecuencia de la exposición a dichos residuos cuando legalmente tenga establecido un LMR (Límite máximo de residuos).

2.2.-Objetivos específicos

2.2.1. Analizar en la etapa de recepción de las industrias de envasado de miel, la materia prima mediante las técnicas de rutina llevadas a cabo por la industria para la clasificación y control de calidad de las mismas.

2.2.2. Evaluar la influencia del tipo de miel, año de cosecha y prácticas apícolas en la variabilidad de los valores obtenidos mediante las técnicas de rutina.

2.2.3. Evaluar la efectividad del análisis de metilantranilato como técnica complementaria en la clasificación botánica de mieles españolas de cítrico.

2.2.4. Estudiar la capacidad discriminatoria de una lengua potenciométrica, desarrollada a partir de una combinación de electrodos metálicos, para la diferenciación de mieles.

2.2.5. Estudiar la capacidad discriminatoria de las técnicas convencionales (polínico, color, químicas y fisicoquímicas) para la clasificación de mieles de diferentes procedencias botánicas y geográficas.

2.2.6. Estudiar la capacidad discriminatoria del análisis de la fracción volátil para la clasificación de mieles de diferentes procedencias botánicas y geográficas.

2.2.7. Evaluar la presencia de sulfamidas en muestras de miel procedentes de la etapa de recepción de diferentes industrias envasadoras de miel, así como en muestras comerciales procedentes de las mismas industrias.

2.2.8. Cuantificar la presencia de diferentes pesticidas provenientes de tratamientos veterinarios o agrícolas, en mieles milflores comerciales (marcas blancas y líderes del mercado).

2.2.9. Evaluar la influencia del origen geográfico en la presencia de pesticidas en mieles milflores comerciales.

2.2.10. Evaluar el riesgo total para el consumidor en relación a la presencia de pesticidas en mieles.

3. RESULTADOS

3.1. Physicochemical quality parameters at the reception stage of the honey packaging process: Influence of type of honey, year of harvest and beekeeper

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Abstract

The aim of this paper was to evaluate the influence of the type of honey, year of collection and the beekeeper on the main physicochemical quality parameters (hydroxymethylfurfural "HMF", moisture and colour), measured on reception of the raw honey. 1593 samples (11 types of honey categorized by means of pollinic analysis), provided by 98 beekeepers, from 2009 to 2013, were analyzed. Colour was the parameter most affected by the type of honey and year, whereas HMF was the least affected in both cases. The clearest honeys were found to have the greatest moisture (orange, rosemary and lemon) and the darkest had the least moisture (lavender stoechas, eucalyptus, sunflower, honeydew and retama). Lavender, polyfloral and thyme had intermediate values of these parameters. For moisture, most samples were in accordance with international requirements (less than 20 g/100 g). All values were below the required limit for HMF (40mg/kg), although a few of them were abnormally high as they were raw honeys (i.e. 2% of the samples had values higher than 20 mg/kg). The fact that all the inadequate samples came from specific beekeepers, highlights the importance of their role, suggesting that training in good practices is the key to guarantee honey quality before it reaches the industry.

Keywords: honey quality, HMF, moisture, colour, year of harvest, beekeeper

1. Introduction

Food products have to satisfy numerous quality criteria before commercialization, especially in industrialized countries, where there is a need for high quality products with well-defined characteristics. Honey is not an exception and must be delivered to the consumer with its essential composition and quality minimally altered with respect to freshly harvested honey (Codex Alimentarius, 2001). There are international and sometimes local regulations that specify honey quality (Council Directive 2001/110; DOGV No. 4167/2002). Therefore, on receiving batches of raw honey the packaging industry has to carry out a wide range of analyses, such as quantification of pollen and physicochemical parameters (hydroxymethylfurfural "HMF", moisture and colour, among others). There are two main reasons for this: 1)to facilitate the classification of honeys according to their botanical origin (considering the pollinic percentage and colour) and 2)

to meet the legislated mandatory requirements during commercialization (e.g. HMF content less than 40 mg/kg or moisture content less than 20g/100g)(Council Directive 2001/110).

Honey classified as unifloral always has a higher commercial value than polyfloral. For this reason, the industry realizes this task prior to packaging, when according to information provided by the beekeeper there is a reasonable probability that a honey can be classified as unifloral. The identification and quantification of the percentage of pollen by microscopic examination is used to authenticate the botanical origin of honey (Von Der Ohe et al., 2004; Escriche et al., 2012; Panseri et al., 2013). The colour of honey is directly related to the botanical source of the nectar, and therefore can help in the classification of unifloral honeys. In addition, this parameter has commercial value as it is used as a criterion of acceptance or rejection by the consumers; however, it is only regulated by some Quality Marks (DOGV No. 4167/2002).

HMF is the most consistent indicator of honey freshness as it is practically absent in freshly harvested honey. However, it increases during handling, extraction, conditioning or storage operations, and also, as a consequence of the liquefaction and pasteurization carried out to improve manageability and destroy the crystallization nuclei (Visquert et al., 2014). Honey packaging plants must be very demanding about the HMF content of raw honey, as they are obliged by law to comply with the requirement established for this parameter during the best-before-date printed on the label. The moisture content of honey depends on the season in which it is harvested, the climatic conditions and the good practices carried out by the beekeepers (Persano-Oddo & Piro, 2004). This parameter has a decisive influence on viscosity, flavour and palatability, but overall on crystallization and fermentation (Turhana et al., 2008). These two alterations modify the appearance, and therefore contribute to customer rejection, consequently causing losses to the industry.

The honey packaging industry realises the importance of legal compliance but also the necessity of providing consumers with consistent quality. When the process is controlled carefully, the key to a quality end product lies in the good quality of the raw material. Knowledge about the origin of the variability of the critical parameters of physicochemical quality is essential to take measures to improve the quality of the raw honey. Consequently, the probability that the commercialized honey does not meet the required specifications will be reduced. Therefore, the objective of this paper was to evaluate the influence of the type of honey, year of collection and the beekeeper's role on the main physicochemical quality parameters analysed (HMF, moisture and colour) on reception in packaging companies.

2. Materials and Methods

2.1.-Honey samples

A total of 1593 samples of raw honey, collected over five years (from 2009 to 2013) in the routine checks that take place on reception of raw honey, were analysed. These samples came from four commercial organizations (provided by 98 beekeepers) located in the Valencian region (Spain). The samples represented the most common varieties available in Spain: 231 (14.5%) orange blossom (*Citrus* spp.); 111 (7%) lemon blossom (*Citrus limon*); 216 (13.6%) rosemary (*Rosmarinus officinalis*); 135 (8.5%) sunflower (*Helianthus annuus*); 34 (2.1%) thyme (*Thymus* spp.);27 (1.7%) lavender (*Lavandula* spp.); 14 (0.9%) lavender stoechas (*Lavandula stoechas*); 26 (1.6%) retama (*Lygos sphaerocarpa*); 76 (4.8%) eucalyptus (*Eucalyptus* spp.); 117 (7.3%) honeydew honey, and 605 (38%) polyfloral.

The botanical categorization of all the batches was carried out by means of pollinic analysis.

2.2.-Analytical Determinations

2.2.1. Melissopalynological analysis

The percentage of pollen was obtained for each sample following the recommendations of the International Commission for Bee Botany (Von Der Ohe et al., 2004). Microscopic examination was carried out at the magnification that was most suitable for identifying the various elements in the sediment (400 to 1000×). A light microscope (Zeiss Axio Imager, Göttingen, Germany) at a magnification power of x 400 with DpxView LE image analysis software attached to a DeltaPix digital camera was used.

2.2.2. Physicochemical and colour analysis

Hydroxymethylfurfural content "HMF" (*White method*), and moisture content were analyzed in accordance with the Harmonized Methods of the European Honey Commission (Bogdanov, 2002). Colour was determined using a millimetre Pfund scale C 221 Honey Color Analyzer (Hanna Instruments). All tests were performed in triplicate.

2.3. Statistical analyses

A multifactor analysis of variance (ANOVA) (using Statgraphics Centurion for Windows) was applied to study the influence of the type of honey and the year of harvesting on the HMF, moisture and colour. LSD contrast (least significant difference) with level of significance α = 5% was used to analyse the differences between means. A multiple correspondence analysis was applied using the statistical software SPAD (version 6.0), to group types of honey based on the quality parameters analysed and also to better understand the relationship between the parameters and the honeys.

3. Results and Discussion

3.1.-Botanical origin

The first step in this study was to carry out the botanical categorization of all the batches by means of pollinic analysis. As an example, Figure 1 shows several pictures corresponding to the predominant pollen present in each type of unifloral honey. Next to each botanical name, the minimum percentage of pollen required to classify a honey as belonging to a specific botanical genus considered in the present work is shown. These values represent the minimums commonly used in the industry and recommended by different authors and Quality Marks (DOGV No. 4167/2002; Von Der Ohe et al., 2004; Persano-Oddo & Piro, 2004; Saenz-Laín & Gómez-Ferreras, 2000; Persano-Oddo & Bogdanov, 2004; Gómez-Pajuelo, 2004; Soria et al., 2004). Honey was classified as honeydew, if the ratio of honeydew elements to that of pollen grains exceeded 3, and the conductivity was higher than 0.8mS/cm (Council Directive 2001/110).Finally, honey was classified as polyfloral, if it did not contain sufficient pollen from a specific botanical species.

3.2.-Influence of type of honey and year of harvest on the physicochemical parameters

Table 1 shows the descriptive statistics (mean and standard deviation) of HMF, moisture and colour parameters analysed considering the type of honey and year of harvest. In addition, this table shows the ANOVA results (F-ratio and significant differences) obtained for these two factors. The interaction between both factors was not significant. Considering that the higher the F-ratio, the greater the effect that a factor has on a variable, colour was the parameter most affected by the factors "type of honey" and "year", whereas HMF was the least affected in both cases, as was expected.

A Kolmogorov-Smirnov test (data not shown) demonstrated that data related to moisture and colour were normally distributed; however, the HMF data were not normally distributed as their descriptive statistics showed a strong positive skew (Skewness coefficient=2.7) and a high positive kurtosis (Kurtosis coefficient=10.1).

HMF ranged between a minimum value of <0.5 mg/kg (data that are present in all varieties) and a maximum of 37.4mg/kg, 37.9mg/kg and 39.8 mg/kg in sunflower honey, orange blossom honey and polyfloral honey, respectively. Although all values were below the required limit of 40mg/kg, it is obvious that some of them would be considered as unacceptable as they were raw honeys. It should be noted that these outlying values are not frequent, in fact, only 2% of samples had values higher than 20 mg HMF/kg, and less than 0.5%of samples had values higher than 30 mg HMF/kg. In contrast with the before mentioned type of honeys, retama, eucalyptus, lavender stoechas and lemon honeys had maximum values of 7.4, 9.7, 10.2 and 10.3, respectively.

With respect to the moisture, it can be observed (Table 1) that a high value of this parameter is characteristic of certain types of honey, such as thyme and rosemary with average values equal or higher than 19.5 g/100 g. Some specific batches of orange blossom, lemon tree, rosemary, sunflower, thyme, lavender, honeydew and polyfloral honeys also exceeded 20g/100g moisture. On the contrary, this percentage was quite low in some varieties such as lavender stoechas, retama and eucalyptus. In general, unifloral honeys show some typical differences in water content depending on season and climate (Persano-Oddo & Piro, 2004). Taking into account the fact that the moisture content of honey has to be lower than 20g/100g (Council Directive 2001/110), the values obtained in this work were not always satisfactory for the honey packaging industry.

With regard to colour, the results shown in this work were as expected for these varieties of honey (Piazza & Persano-Oddo, 2004). A large range of variation between the minimum and maximum was observed. Logically, the greatest difference was detected in polyfloral honey with a range of 7 to 130 mm Pfund. Some types of honey such rosemary, lemon and orange are in general characterized by a light colour which makes them highly

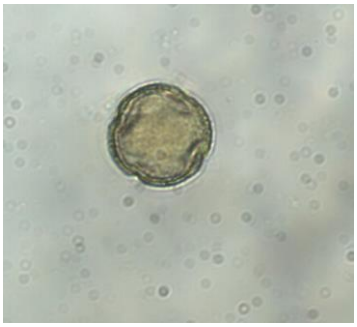
valued commercially. However, these honeys were sometimes darker than is commercially desirable, ranging from 0.1 and 2 mm, to 50, 69, 62 mm, respectively. The color of these types of honey can sometimes be strongly influenced by the nectar of other flowers that bees do to sip. On the contrary, honeydew honey and retama honey were in general the darkest (among the monovarietal honeys), reaching up to 120 mm on the Pfund scale. In these types of honey the dark colour is traditionally a highly valued characteristic. Furthermore, today it is well known that the darker a honey is, the higher the nutritional value, due to the high mineral content and the antioxidant activity (Persano-Oddo et al., 2008; Tornuka et al., 2013).

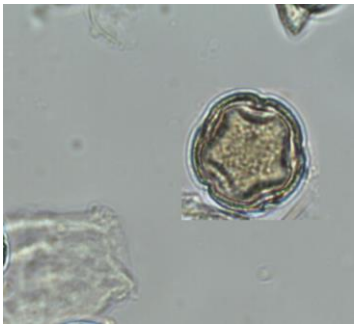
In relation to colour, although no limits are established by the general regulations (Council Directive 2001/110), specific quality marks limit values according to varieties, e.g. for citrus and rosemary less than 30mm Pfund in Valencian region regulation (DOGV No. 4167/2002) and less than 30 and 35mm Pfund, respectively in Granada PDO (BOE 24621/2002); for lavender stoechas and thyme values must be above 50 and 55 mm Pfund respectively in Granada PDO.

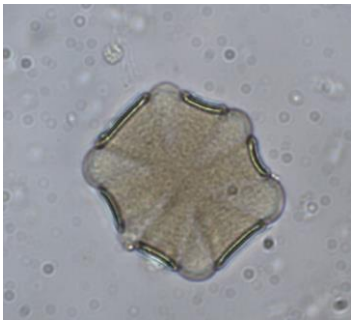
In order to detect possible grouping of the types of honeys according to the quality parameters evaluated, a multiple correspondence analysis was carried out (Figure 2) (Preys, 2007). Due to the requirements of the analysis, quality parameters were coded categorically as intervals. The parameter values were: moisture (g/100g)[<16, 16-17, 17-18, 18-19, 19-20, >20]; colour (Pfund scale)[<20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, >80]; hydroxymethylfurfural [<0.50, 0.51–1.00, 1.01–1.50, 1.51-2.00, 2.01-2.50, 2.51-3.50, 3.51-5.00, 5.01-6.50, 6.51-10.00, >10]. In addition, information about the variety of honey was projected on the biplot in order to link this factor to quality parameter intervals.

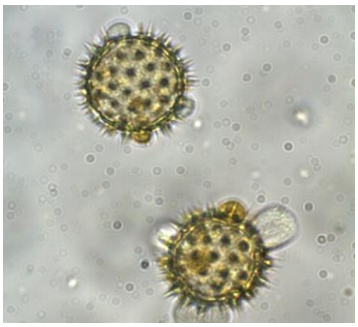
The projection of the varieties on the graph showed three clear groups: left (orange blossom, lemon and rosemary); centre (thyme, lavender and polyfloral) and right (sunflower, lavender stoechas, retama, eucalyptus and honeydew). These groups are also related to certain categories of the quality parameters: the clearest honeys with the highest moisture on the left and the darkest ones with the least moisture on the right. The remaining honeys, with intermediate values of these parameters, are located in the central area of the graph.

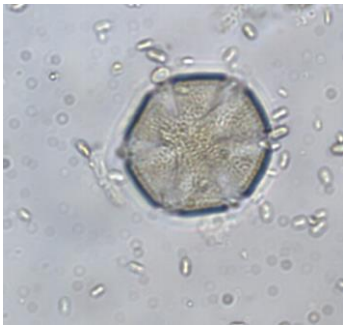
The second axis distinguishes samples depending on whether values of hydroxymethylfurfural are close to the maximum, the minimum, or remain around intermediate values. In this way, minimum values of HMF are located at the top of the Figure 2 (<0.50 HMF), whereas maximum values can be found at the bottom (>10 HMF)" (9.1%). In both cases these categories have a high relative contribution to this second axis, as shown in brackets. The remaining categories of HMF are distributed between these two reference points, but this distribution is not progressive along the axis.

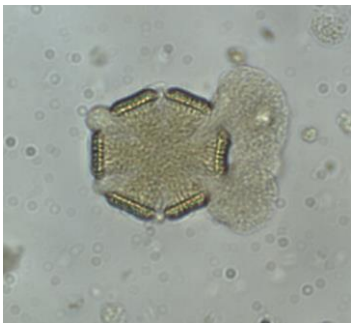

Citrus sinensis (>10 % pollen)

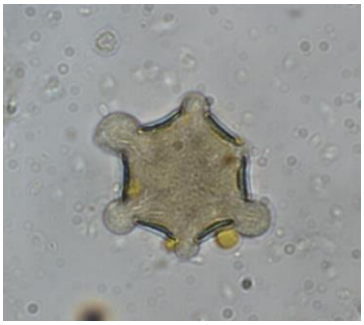

Citrus limon (>10 % pollen)

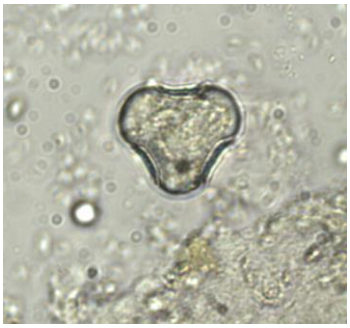

Rosmarinus officinalis (>10% pollen


Helianthus annuus (>45% pollen)


Thymus sp. (>12% pollen)


Lavandula latifolia (>12% pollen)


Lavandula stoechas (>12% pollen)


Lygos sphaerocarpa (>35% pollen)


Eucaliptus sp. (>70% pollen)

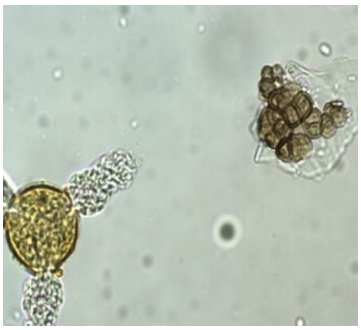

Honeydew

Figure 1.- Pictures corresponding to the predominant pollen present in each type of unifloral honey and the minimum percentage of pollen required to classify a honey as belonging to a specific botanical genus considered in the present work.

Table 1. Descriptive statistics for moisture, colour and HMF for each variety of honey and year of harvesting.ANOVA results (F-ratio and significant differences) obtained for two factors: type of honey and yearof harvest.

Type of honey	HMF (mg/kg)				Moisture (g/100g)				Colour (Pfund scale)			
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
Orange blossom	4.6^{de}	6.4	<0.5	37.9	18.7^{c}	1.3	15.5	23.3	27^{a}	17	2	62
Lemon tree	2.1^{ab}	2.3	<0.5	10.3	18.3^{b}	1.5	15.0	21.6	26^{a}	14	1	69
Rosemary	3.2^{bc}	3.2	<0.5	27.3	19.9^{d}	2.1	15.0	25.0	25^{a}	15	0	50
Sunflower	3.3^{bc}	4.3	<0.5	37.4	16.7^{a}	1.3	14.6	21.2	70^{d}	10	38	72
Thyme	4.1^{bcd}	7.2	<0.5	35.6	19.5^{d}	1.6	16.2	22.9	66^{cd}	21	18	90
Lavender	6.1^{d}	6.2	<0.5	22.9	17.9^{ab}	2.2	15.7	25.2	48^{b}	21	6	91
Lavender stoechas	3.4^{abcde}	3.5	<0.5	10.2	16.5^{a}	1.0	15.2	18.1	66^{cd}	13	36	78
Retama	1.0^{a}	1.7	<0.5	7.4	16.4^{a}	0.7	14.7	17.5	84^{e}	10	63	120
Eucalyptus	3.8^{cd}	2.7	<0.5	9.7	16.6^{a}	0.8	15.1	19.8	70^{d}	11	35	89
Honeydew	4.5^{de}	3.1	<0.5	27.2	16.5^{a}	1.4	13.9	22.0	90^{e}	13	57	127
Polyfloral	4.5^{cd}	4.8	<0.5	39.8	17.7^{ab}	1.8	14.8	24.1	64^{c}	19	7	130
ANOVA F ratio	4.42^{***}				49.0^{***}				221^{***}			
Year of harvest	**HMF(mg/kg)**				**Moisture(g/100g)**				**Colour(Pfund scale)**			
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
2009	3.1^{a}	3.4	<0.5	19.4	17.1^{a}	1.5	14.8	24.0	72^{e}	18	13	130
2010	5.0^{c}	5.9	<0.5	37.9	18.0^{bc}	1.7	15.0	23.6	62^{d}	24	10	127
2011	4.0^{b}	4.8	<0.5	37.4	18.2^{cd}	2.0	13.9	25.0	51^{c}	27	2	125
2012	2.9^{a}	3.6	<0.5	26.8	18.4^{d}	1.9	15.5	25.2	47^{b}	27	3	120
2013	4.1^{bc}	6.2	<0.5	39.8	17.9^{b}	1.7	14.2	22.1	40^{a}	26	0	110
ANOVA F ratio	7.14^{***}				17.32^{***}				55.94^{***}			

***p<0.001. For each factor, different letters in each column indicate homogeneous groups (significant differences at 95% confidence level as obtained by the LSD test)

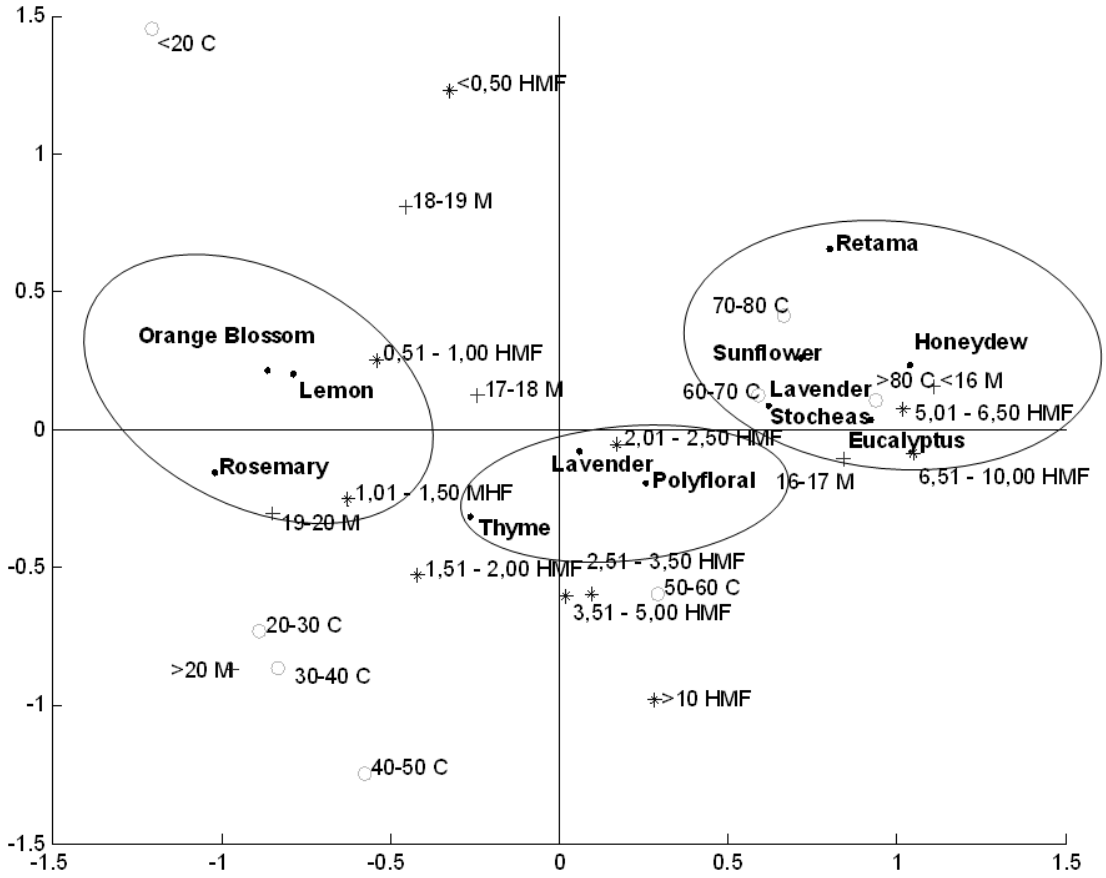


Figure 2.- Multiple correspondence analyses of the quality parameters coded as intervals with projected varieties of honey. M: moisture; C:colour; HMF: hydroxymethylfurfural.

3.3.-Influence of the beekeeper on the physicochemical parameters

In order to evaluate the influence of the beekeeper on the physicochemical parameters, the six most abundant honey types analysed in this work were considered (see section 2.1): orange blossom, lemon blossom, rosemary, sunflower, honeydew and polyfloral. An ANOVA was carried out for every type of honey and every physicochemical parameter, considering the factor beekeeper (Table 2). For this statistical analysis, beekeepers who contributed less than 10 batches to the study were not considered. It can be observed that the beekeeper has a significant influence on all the parameters evaluated and on all the types of honey studied.

In relation to the quality parameters, HMF and moisture, although only a few values exceeded the recommended limits these values came from specific types of honey and beekeepers. What is clear is that if some beekeepers can work very well, which is reflected in low values of both parameters, others should be able to also.

Colour differences are more justifiable in comparison to those observed for the before mentioned parameters. That is to say, for the same honey type the accompanying flora has a large influence on the variations in colour. However, it is important to note that beekeepers also have an influence on this parameter since they are responsible for the mix of the types of honey when cutting the honey from the honeycomb.

Due to the fact that beekeepers have a key role in honey quality parameters, it is important to monitor their handling practices, although this is not always possible if they operate in distant countries.

Table 2. Influence of the beekeeper on the physicochemical parameters (HMF, moisture and colour) and different types of honey.ANOVA results (F-ratio and significant differences) for the factor: beekeeper.

Type of Honey	HMF (mg/kg)			Moisture (g/100g)			Colour (Pfund scale)		
	Mean (SD)	Min-Max	ANOVA F ratio	Mean (SD)	Min-Max	ANOVA F ratio	Mean (SD)	Min-Max	ANOVA F ratio
Orange blossom	5.5 (7.8)	<0.5-37.8	15.3***	19 (1)	16-23	4.3***	26 (16)	2-89	5.48***
Lemon tree	2.2 (2.4)	<0.5-10.3	19.2***	18 (1)	15-22	11.2***	27 (14)	1-69	2.35*
Rosemary	19.9 (2.1)	<0.5-27.3	9.3***	20 (2)	16-25	2.1*	26 (16)	0-75	3.09***
Sunflower	3 (5)	0-10	2.06*	17 (1)	15-21	16.3***	70 (9)	43-90	1.88*
Honeydew	6 (6)	<0.5-27.3	10.18***	17 (1)	15-22	4.1***	90 (12)	57-118	15.8***
Polyfloral	4.5 (4.8)	<0.5-39.8	9.08***	18 (2)	15-24	4.5***	64.(19)	7-116	3.6***

* p<0.05; ***p<0.001

4. Conclusion

Consideration of the botanical origin of the eleven types of honey analysed permitted grouping according to the physicochemical parameters. The clearest honeys were at the same time the ones with the highest moisture while the darkest honeys were found to have the lowest moisture levels. Colour was the parameter most affected by the type of honey and year of harvesting, whereas HMF was the least affected in both cases. There were some unacceptable outliers from specific beekeepers which exceeded the permitted values for moisture and the recommended values of HMF. This indicates the important role that the beekeeper has in attaining raw honey with the correct physicochemical parameters, and even the characteristic colour which the market requires. When the process is controlled carefully, the key to the quality of the end product lies in the good quality of the raw material. Therefore, adequate training in good beekeeping practices is vital to obtain the product that the consumer expects and legislation requires.

5. References

BOE 24621. (2002) Reglamento de la Denominación de Origen Protegida "Miel de Granada" y de su Consejo Regulador. 44261-44268.

Bogdanov S. (2002). Harmonized methods of the International Honey Commission. Bern, Switzerland: Swiss Bee Research Centre, FAM, Liebefeld, CH 3003.

Codex Alimentarius. (2001). Revised Codex Standard for Honey. Codex Stan 12-1981. Codex Alimentarius Commission. Rev. 1(1987). Rev. 2.

Council Directive 2001/110 (2002) relating to honey. Official Journal of the European Communities. L10, 47-52.

DOGV No. 4167. (2002). Reglamento de la Marca de Calidad CV para miel de azahar y romero in Diario Oficial de la Generalitat Valenciana. Área de publicaciones de la Presidencia de la Generalitat Publishing, Valencia (Spain).

Escriche I., Kadar M., Domenech E. & Gil-Sánchez L. (2012).A potentiometric electronic tongue for the discrimination of honey according to the botanical origin. Comparison with traditional methodologies: Physicochemical parameters and volatile profile.J. Food Eng. 109, 449-456.

Gómez-Pajuelo A. (2004). Origen botánico de la miel, in Montagud (Eds.), Mieles de España y Portugal. Barcelona, Spain, pp. 50-56.

Panseri S., Manzo A., Chiesa L.M. & Giorgi, A. (2013). Melissopalynological and Volatile Compounds Analysis of Buckwheat Honey from Different Geographical Origins and Their Role in Botanical Determination. Journal of Chemistry, Volume 2013, Article ID 904202, 11 pages.

Persano-Oddo L. & Bogdanov S. (2004). Determination of honey botanical origin: Problems and issues. Apidologie. 35, 2-3.

Persano-Oddo L. & Piro R. (2004). Main European unifloral honeys: Descriptive sheets. Apidologie. 35, 38-81.

Persano-Oddo L., Heard T., Rodríguez-Malaver A., Pérez R.A, Fernández-Muiño M.A., Sancho M.T., Von der Ohe W., Sesta G., Lusco L. & Vit P. (2008). Composition and antioxidant activity of Trigona carbonaria honey from Australia. J. Med. Food, 11, 789-794.

Piazza M. G. & Persano-Oddo L. (2004). Bibliographical review of the main European unifloral honeys. Apidologie. 35(1), 94-111.

Preys S., Vigneau E., Mazerolles G., Cheynier V. & Bertrand D. (2007). Multivariate prototype approach for authentication of food products. Chemometr Intell Lab. 87, 200-207.

Saenz C. & Gómez C. (2000). Mieles españolas: Características e identificación mediante el análisis del polen, ed. Mundi-Prensa, España.

Soria A.C., González M., de Lorenzo C., Martínez-Castro I. & Sanz, J. (2004). Characterization of artisanal honeys from Madrid (Central Spain) on the basis of their melissopalynological, physicochemical and volatile composition data. Food Chem. 85, 121-130.

Tornuka F., Karamanb S., Ozturk, I., Toker O. S., Tastemurb B., Sagdica O., Doganb M. & Kayacierb A. (2013). Quality characterization of artisanal and retail Turkish blossom honeys: Determination of physicochemical, microbiological, bioactive properties and aroma profile. Ind Crop Prod., 46, 124-131.

Turhana I., Tetika N., Karhana M., Gurelb F. & Tavukcuoglua, H.R. (2008). Quality of honeys influenced by thermal treatment. LWT - Food Sci. Technol. 41, 1396-1399.

Visquert M., Vargas M. & Escriche I. (2014). Effect of postharvest storage conditions on the colour and freshness parameters of raw honey. Inter J. Food Sci. and Tech., 49, 181-187.

Von Der Ohe W., Persano-Oddo L., Piana M. L., Morlot M. & Martin, P. (2004). Harmonized methods of melissopalynology. Apidologie, 35

3.2. Correlation between methyl anthranilate level and percentage of pollen in Spanish citrus honey

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Abstract

The common level of methyl anthranilate (MA) in Spanish citrus honey and the correlation between this compound and the percentage of citrus pollen (sometimes underrepresented) is evaluated. The MA analysis methodology was validated before analyzing the honeys (harvested in 2011 and 2012), which were characterized by pollen, MA, hydroxymethylfurfural, electrical conductivity, moisture and colour. Pollen ranged 1-88% and MA 0.5-5.9 mg/kg and there was no quantitative correlation between both. However, significant correlations with moderate Pearson coefficients were observed: MA/electrical conductivity (-0.678); MA/colour (-0.559); pollen/electrical conductivity (-0.553) and pollen/colour (-0.556). 89.2 % of samples from 2011 and 95.4% from 2012 had the required level of citrus pollen (at least 10%), although only 53.5% and 61.4%, respectively, had the commercially required of MA (2 mg/kg,). Only about half of the samples satisfied both parameters. The MA value should be recommended only when the honey has an unexpectedly low percentage of citrus pollen, and after assessing organoleptic and physicochemical parameters.

Keywords: citrus honey, methyl anthranilate, *Citrus* spp. pollen, correlation, validation, melissopalynological analysis, electrical conductivity, colour.

1. Introduction

Traditionally, honey is authenticated by identifying and quantifying the percentage of pollen (Bogdanov, 2002). However, in the case of citrus varieties this melissopalynological analysis alone, widely used for other types of monofloral honey, is sometimes not sufficient. The problem lies in the fact that the production of pollen and nectar in citrus blossom is not always simultaneous. Which is to say that citrus trees sometimes produce nectar before the anther produces pollen. Apart from this, bees occasionally collect nectar from sterile hybrid varieties of citrus trees, which are characterized by their small amounts of pollen. For these reasons the percentage of pollen in citrus honey may be lower than expected (Molins, et al., 1995). This clearly implies a difficulty in relation to the minimum percentage of pollen from *Citrus* spp. required for this type of honey: 10% (Persano-Oddo, et al., 1995; Molins et al., 1995; DOGV, 2002); 12% (Gómez-Pajuelo,

2004); 18.6% (Persano-Oddo & Piro, 2004); ≥5 % (Rodriguez et al., 2010). This is a problem for the commercialization of this valuable type of honey since it can be unfairly rejected, even though its organoleptic characteristics are undisputed.

Methyl anthranilate (MA) is a specific compound in citrus blossom nectar; its aroma is characteristic of this type of flower (ISO 5496, 2006) which may vary depending on the citrus cultivars (Jabalpurwala, et al., 2009). The presence of this compound in a honey indicates that the bees have taken nectar from citrus trees (White, 1966; White & Bryant, 1996; Castro-Vazquez, et al., 2007). For this reason, MA has been used for decades as a marker in citrus honey and should be a good tool for the classification of citrus honey when the level of pollen is very low. However, at present, commercial transactions require that citrus honey has a minimum citrus pollen content (between 10 and 20%), together with a methyl anthranilate level of at least 2 mg/kg (Sesta, et al., 2008). According to the data reported by different authors, the quantity of MA in the nectar citrus varies, depending on the country. Citrus honey from Florida has a mean value of 3.10 mg/kg of MA (SD = 0.91) (White & Bryant, 1996). However, Sesta et al., in 2008 suggested that a minimum content of 0.5 mg/kg is sufficient for *Citrus* honey produced in Italy. There are a few old studies related to the level of this aromatic compound in Spanish citrus honey: minimum of 0.5 mg/kg (Serra-Bonvehí, 1988), and average of 2.3 mg/kg (SD=0.5) (Ferreres, et al., 1994; Serra-Bonvehí & Ventura, 1995).In addition to variation due to geographic origin, levels of MA can differ depending on storage conditions (Serra-Bonvehí & Ventura, 1995; White & Bryant, 1996; Sesta et al, 2008). Therefore, it is important to take this into consideration in order to make appropriate comparisons.

Clearly, there is a great disagreement about the required level of MA in citrus honey. However, the origin of this discrepancy could in part be in the application of different analytical techniques over the last three decades: HPLC-DAD (Ferreres, et al., 1994; Nozal, et al., 2001; Sesta et al, 2008), Photometry (White & Bryant, 1996),HS-SPME-GC (Bertelli, et al., 2008; Papotti, et al., 2009; Papotti, et al., 2012), P&T/GC-MS-thermal desorption (Escriche, et al., 2011). As there is no official methodology described for the analysis of MA, the only way to ensure the quality of the obtained results is to validate the quantification methodology.

For the above mentioned reasons, the present work aims to evaluate the level of MA in Spanish citrus honey and to determine the extent to which this level can be related to the percentage of pollen from citrus genus. In order to ensure the utility of the chromatographic procedure used to quantify this compound, it was validated before analyzing the samples.

2. Materials and Methods

2.1.-Honey samples

Ninety eight different samples of Spanish citrus honey were used in this study. The samples were harvested in the only areas (East and South) of Spain where citrus trees grow. The same beekeepers (B) directly supplied twenty eight samples from 2011 and fifty samples in 2012, ensuring that the hives had been located in citrus groves. The remaining twenty samples (10 from 2011 and 10 from 2012) were purchased, in the same period of time, in different retail outlets (R) in the city of Valencia, checking in all cases that they were sold as citrus honey harvested in Spain. A melissopalynological analysis was carried out on all samples to ascertain the percentage of pollen of *Citrus* spp.

All samples were analysed as soon as they were received in the laboratory. Samples that came from beekeepers were sent to the laboratory immediately after they were harvested. None of the samples used exhibited signs of crystallization.

2.2.-Melissopalynological analysis

Pollen analysis was performed using the recommendations of the International Commission for Bee Botany (Von der Ohe, et al., 2004), without an acetolysis solution to preserve all the components. Slides were prepared as follows: 10 g of honey were dissolved in acidulated water (H_2SO_4, 5%) on a heating plate at 40 ºC. Subsequently, it was centrifuged, the supernatant was decanted and the precipitate was suspended in 10 mL of distilled water. A second centrifugation was performed, the supernatant was decanted off and 0.2 mL of water was added to the precipitate. After stirring, 0.2 mL were deposited on a slide and dried. Finally a drop of glycerin was used to seal the coverslip. A light microscope (Zeiss Axio Imager, Göttingen, Germany) with DpxView LE image analysis software attached to a DeltaPix digital camera was used in this analysis. A count of at least 600 pollen grains was performed observing at ×400–1000 magnifications. These grains of pollen were classified according to pollen morphology as in the literature (Carretero, 1989; Saenz & Gómez, 2000).

2.3.-Methyl anthranilate analysis

2.3.1. Methodology

A HPLC-DAD (Diode-Array Detection) method, based on Sesta et al. (2008), was used in the present work for MA determination. The method consists of acid hydrolysis, followed by extraction with copolymer cartridges and then chromatographic analysis. An LC Agilent 1120 Compact LC, including a binary pump, a thermostat column compartment, an auto-sampler and a UV detector were used. The chromatographic column and the software system used in the HPLC-UV method was the same as that used for the HMF analysis.

Chromatographic separation was carried out with a mobile phase consisting of water (A) and acetonitrile (B). Binary gradient conditions were used: first an isocratic step from 0 to 3.1 min with 70% A, and then a linear gradient was applied arriving at 42% A in 2 min. After that, a second linear gradient was applied arriving at 10% A, held for 2 min, and re-equilibrates to the initial conditions in 3 min. The flow-rate was 1 mL/min. The injection volume was 20 μL, and the oven column was maintained at 30 ºC. The MA was monitored at 335 nm.

A HPLC Alliance 2695, with a 2996 photodiode array detector (Waters, USA), equipped with the same column, was also used to corroborate the absorbance spectra, necessary for the identification of MA. The UV absorbance spectrum of MA presented an intense absorbance peak at 218 nm and 2 less intense peaks at 245 and 334 nm. As noted by Sesta et al. (2008), it was considered more appropriate to quantify at the absorbance of 334 nm as it presents less interference than the other ones.

Under the specified chromatographic conditions the MA peak was eluted at a retention time of about 6.8 min. Quantification was realized by means of matrix calibration curves obtained from spiked fortified blank samples. In order to ensure the quality of the results and evaluate the stability of the proposed method, an internal quality control (a spiked blank sample with a final concentration of 2 mg/kg) was injected in the equipment as a first step before each batch of sample.

In all cases a polyfloral honey with absence of MA was used as a honey blank. The absence of citrus pollen in this honey was corroborated previously.

Reagents and standards solutions

HPLC-grade acetonitrile was purchased from VWR and the standard MA (purity > 99%) from Merck (Darmstadt, Germany). Analytical grade sulphuric acid was from Scharlab (Barcelona, Spain). For Solid Phase Extraction, Oasis HLB cartridges (200 mg/6 mL) from Waters were used. De-ionized water of MilliQ quality was used throughout.

The stock standard solution of MA was prepared by weighing the appropriate amount of the pure standard and diluting it with water to obtain a final concentration of 1 mg/mL.

The working standard solution was obtained at a concentration of 0.1 mg/mL in H_2SO_4 1M in the same way as the samples. The stock standard solution was stored at -20ºC and the working standard solution was at +4ºC.

Validation of the MA analysis method

The guidelines established by Commission Decision (2002), were followed in order to validate the MA analytical methodology. To this end several parameters were studied: linearity, accuracy and precision (repeatability and reproducibility). The accuracy of the method was established through recovery studies and the precision by: repeatability or intraday precision (RSD_r) and reproducibility or interday precision (RSD_R). LODs (limit of detection) and LOQs (limit of quantification) were estimated as the amount of analyte for which signal-to-noise ratios (S/N) were higher than 3 and 10 respectively.

2.4.-Physicochemical and colour analysis

5-hydroxymethylfurfural (HMF) determined by HPLC-UV methodology and physicochemical parameters (moisture content and electrical conductivity) were analyzed as described in "Harmonized Methods of the European Honey Commission" (Bogdanov, 2002). The chromatographic column used for the analysis of HMF was a ZORBAX Eclipse Plus C18 (4.6 x 150 mm, 5 μm particle size) purchased from Agilent (Agilent Technologies, USA). The mobile phase for this analysis was water-methanol (90:10, v:v), with a flow rate of 1 mL min^{-1}. The detector was set to 285 nm. The EZChrom Elite system software was used for HPLC data processing. Colour was determined with a millimeter Pfund scale C 221 Honey Color Analyzer (Hanna Instruments, Spain). All analyses were performed in triplicate.

2.5.-Statistical analysis

The pollen percentage, physicochemical (MA, HMF, electrical conductivity) and colour data were analyzed by a multifactor analysis of variance (ANOVA) (significance level α = 0.05) (using Statgraphics Centurion for Windows) to study the influence of the year of harvesting and the type of sample (beekeeper and retail).The method used for multiple comparisons was the LSD test (least significant difference) with a significance level α = 0.05. The bivariate Pearson correlations were obtained (significance level α = 0.05) to measure the strength and direction of the linear relationships between pairs of variables using SPSS 16.0. The contingency table analysis (cross tabulations) was carried out to evaluate the interrelation between pollen and MA, considering these variables as categorical, using the same SPSS 16.0 (IBM).

3. Results and Discussion

3.1.-Validation of methyl anthranilate analytical methodology

The results from the validation procedure are shown in Table 1.In order to obtain the linearity evaluation an external standard calibration curve was constructed using spiked fortified blank honey (honey without MA) with final concentration levels of: 0.5, 1, 2, 3 and 5 mg of MA/kg honey. These concentrations covered the values of this compound which were expected to be found in the honey samples. Six replicates were carried out for each level. Injections were performed in triplicate. A calibration curve was obtained by plotting the peak area of the compound at each level versus the concentration of MA added to the sample. A good linearity response in the range of the concentration considered was observed, with a correlation coefficient (R^2=0.995) between peak areas and injected nominal concentrations.

The recovery studies were performed by adding known quantities of MA to the blank honey (0.5, 1, 2, 3 and 5 mg of MA/kg). All spiked fortified sample levels were done in triplicate and analyzed by the HPLC method. The results displayed in Table 1 show that the method used led to recovery of MA between 96 and 105% for the concentration range studied. The relative standard deviation (RSD) corresponding to recovery values ranged from 6.0 to 11.6%. As these values were less than 20%, the accuracy of the analytical method was confirmed (Commission Decision, 2002).

Repeatability (RSDr) was evaluated by performing the assay on six replicates of fortified honey samples, at the same levels (0.5, 1, 2, 3 and 5 mg of MA/kg), and performed by the same operator on the same day. To evaluate reproducibility (RSD_R) the experiment was carried out on 3 consecutive days, with 2 different operators. The results were expressed as the percentage of relative standard deviation of the measurements.

As shown in Table 1, intra-day precision (RSD_r) ranged from 1.40% to 7.20% and inter-day precision (RSD_R) from 4.50% to 13.96%. These RSD values are in complete agreement with Commission Decision (2002), requirements since they were always lower than 20% for all the concentration levels assayed. Therefore it can be concluded that the method used has good precision. The LOQ obtained was 0.1 mg of MA/kg. The results of the validation demonstrate that the applied analytical procedure guarantees satisfactory the quantitative values of MA obtained in the samples analyzed.

Table 1. Validation parameters (accuracy and precision) of methyl anthranilate (MA) methodology. The numbers in brackets are the relative standard deviation. Six replicates were carried out for each level.

MA Added (mg/kg)	Mean Recovery RSD (%)	Intra-day-precision Mean value (mg kg^{-1}) RSD_r (%)	Inter-day-precision Mean value (mg kg^{-1}) RSD_R (%)
0.5	96.0 (6.0)	0.50 (4.10)	0.49 (12.00)
1.0	105.0 (6.5)	1.08 (7.20)	1.03 (5. 80)
2.0	104.0 (10.8)	2.10 (4.11)	2.06 (13.96)
3.0	99.0 (11.6)	2.63 (4.02)	2.36 (13.70)
5.0	100.0 (6.4)	5.06 (1.40)	5.05 (4.50)

3.2.-Melissopalynological, physicochemical, colour and methyl anthranilate characterization

Table 2 shows the percentage of *Citrus* spp. pollen, the average values of MA and the physicochemical parameters quantified (HMF, electrical conductivity, and moisture), as well as the colour of each of the samples supplied by beekeepers (B) and purchased in retail outlets (R) in 2011 and in 2012. In addition, this table shows the result (*P*-value, *F*-ratio and minimum and maximum LSD values) of the multifactor ANOVA carried out considering the factors: year of collection (2011 and 2012) and "type of sample" of citrus honey (beekeepers and retail). The respective double interactions were not significant in any case (data not shown). Figure 1 shows the box and whisker plots for all the values obtained in order to facilitate the comparison of variability patterns between the four sources of citrus honey (beekeepers 2011; beekeepers 2012; retail 2011 and retail 2012).

The citrus pollen percentage varied significantly among type of samples (beekeepers and retail) but not among years of harvesting. This percentage ranged from 1 to 88 (average=34) and from 1 to 69 (average=33) in beekeeper samples from 2011 and 2012, respectively. With regard to the supermarket samples, the pollen percentage ranged from 7 to 40 (average=18) and from 8 to 42 (average=19), respectively.

Table 2.-Percentage of Citrus spp. pollen, average values (n = 3) of methyl anthranilate (MA) and physicochemical parameters (HMF, electrical conductivity, moisture) and the colour of each of the samples supplied by beekeepers (B) (28 from 2011 and 50 from 2012) and purchased in retail outlets (R) (10 from 2011 and 10 from 2012). Average and standard deviation (in brackets) for each parameter. Multifactor ANOVA results (P-value, F-ratio, minimum and maximum LSD values) obtained for the factors: year (2011 and 2012) and sample (beekeepers and retail).

	Pollen (% *Citrus* spp.)		MA (mg/kg)		HMF (mg/kg)		Electrical conductivity ($\mu S\ cm^{-1}$)		Moisture (mg/100g)		Colour (mm Pfund)	
Samples	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
B01	48	35	1.9	1.4	5.1	1.2	264	167	15.4	15.4	36	2
B02	42	48	3.2	2.5	1.1	0.5	183	150	15.5	16.7	28	3
B03	73	47	2.1	3.6	4.0	1.4	241	161	16.2	14.5	37	5
B04	48	40	1.2	3.3	3.7	1.7	243	157	17.9	17.8	27	6
B05	36	68	1.3	3.5	1.7	1.6	167	163	15.5	17.2	17	6
B06	1	47	0.5	4.5	11.8	1.6	283	164	16.8	16.4	37	7
B07	5	46	1.3	3.3	7.6	1.5	248	167	16.2	18.0	32	9
B08	30	69	0.9	3.5	9.4	2.0	271	169	15.8	17.4	43	11
B09	27	4	1.5	4.7	3.8	1.5	208	187	16.3	14.5	35	11
B10	63	48	3.8	5.9	1.7	1.1	167	161	18.6	18.2	20	11
B11	57	60	4.0	2.5	1.5	2.6	172	170	17.8	16.3	20	11
B12	33	37	2.2	1.1	1.5	0.7	194	233	17.2	15.9	22	19
B13	42	37	2.2	2.9	2.4	4.3	198	183	16.7	18.0	26	13
B14	46	23	2.5	2.2	3.2	2.6	189	210	16.6	14.3	19	18
B15	12	48	1.9	2.9	16.5	4.6	205	188	16.6	16.9	33	14
B16	36	1	2.1	3.6	7.9	2.2	202	254	16.5	14.8	25	16
B17	43	37	2.2	2.6	5.4	3.8	185	207	17.3	14.0	22	18
B18	88	30	1.3	1.6	25.0	3.0	212	290	16.3	13.2	35	20
B19	53	65	3.8	2.4	3.8	3.9	208	233	15.4	16.1	24	20
B20	8	12	1.1	1.5	5.6	7.1	215	238	16.0	17.1	33	21
B21	19	42	1.9	2.3	8.7	4.5	210	246	14.8	23.3	39	21
B22	11	35	2.9	2.3	7.6	5.2	228	222	15.8	16.7	37	22
B23	35	10	2.1	1.9	6.8	3.9	209	288	13.8	13.3	37	23
B24	17	20	1.9	2.9	4.6	4.9	266	239	16.6	14.5	40	24
B25	10	45	2.4	1.6	0.7	4.2	169	259	17.8	13.3	20	24
B26	42	26	1.9	1.7	4.9	2.8	233	273	18.6	14.7	32	25
B27	14	20	2.2	2.7	4.8	7.9	255	258	15.4	16.8	40	27
B28	12	28	2.7	0.8	0.9	5.3	220	312	15.6	14.4	37	27
B29		30		2.5		11.1		241		17.2		27
B30		19		1.4		4.1		332		13.3		29
B31		27		2.0		5.2		273		13.8		29
B32		34		1.3		4.3		278		13.6		29
B33		32		3.0		4.1		278		16.4		30
B34		12		1.9		7.2		315		16.5		32
B35		36		1.0		4.3		313		14.9		33
B36		31		1.1		6.0		288		16.9		33
B37		15		2.6		9.7		242		17.2		34
B38		16		1.1		2.9		381		13.1		36
B39		24		2.6		7.8		236		17.4		36

Table 2 *(cont.)*

	Pollen (% *Citrus* spp)		MA (mg/kg)		HMF (mg/kg)		Electrical conductivity ($\mu S\ cm^{-1}$)		Moisture (mg/100g)		Colour (mm Pfund)	
Samples	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
B40		16		2.4		9.5		224		18.1		36
B41		17		1.7		4.5		357		16.4		37
B42		15		1.4		5.8		373		15.9		40
B43		13		2.5		3.1		217		16.0		38
B44		30		1.2		10.8		284		17.1		40
B45		35		1.4		1.2		167		15.4		2
B46		48		2.5		0		150		16.7		3
B47		47		3.6		1.4		161		14.5		5
B48		40		3.3		1.7		157		17.8		6
B49		68		3.5		1.6		163		17.2		6
B50		47		4.5		1.6		164		16.4		7
Average	34(21)	33(17)	2.1(0.9)	2.4 (1.8)	5.8(5.2)	4.1(2.6)	217(7)	230 (63)	16.3(0.2)	16.0(1.7)	31(2)	20(11)
R01	15	8	0.7	2.0	34.3	25.2	175	240	16.6	16.6	50	44
R02	14	42	1.5	1.9	17.0	33.1	255	220	17.9	16.4	39	50
R03	8	9	0.8	1.8	32.0	32.2	171	210	16.3	18.7	47	26
R04	25	11	0.6	0.7	17.7	25.3	208	229	18.3	18.3	58	39
R05	21	28	1.7	1.7	21.8	30.4	320	193	16.3	16.4	58	26
R06	9	26	2.9	1.5	33.8	14.1	191	245	16.2	16.5	37	45
R07	10	11	1.2	1.4	24.3	16.0	240	244	16.9	16.3	37	40
R08	7	22	2.4	1.0	34.3	17.1	238	198	17.5	16.2	48	28
R09	26	16	1.4	2.9	42.4	22.0	228	235	15.2	17.0	51	35
R10	40	16	1.9	0.8	24.8	33.1	229	234	15.6	16.9	37	36
Average	18 (10)	19(11)	1.5(0.7)	1.6(0.7)	28.2(8.1)	25.2(7.5)	228 (12)	224 (19)	16.5(0.3)	16.9(0.8)	46(3)	37(8)
Year factor												
P-value	0.679		0.038		0.518		0.353		0.846		0.006	
F-ratio	0.17		4.41		0.42		0.87		0.04		7.72	
LSD-2011 (min/max)	22.50/31.26		1.60/2.12		13.85/16.78		202.11/239.31		16.02/16.78		33.06/40.61	
LSD-2012 (min/max)	19.08/31.51		1.97/2.71		12.40/16.57		215.33/249.95		15.96/16.96		26.01/33.04	
Sample factor												
P-value	0.0004		0.203		0.0000		0.605		0.079		0.0000	
F-ratio	13.01		1.64		242.52		0.27		3.13		25.24	
LSD-B (min/max)	29.38/36.65		2.03/2.46		3.64/6.08		217.45/242.53		15.85/16.45		24.12/29.21	
LSD-R (min/max)	12.48/25.84		1.56/2.35		22.70/27.19		201.25/245.46		16.16/17.26		35.21/44.19	

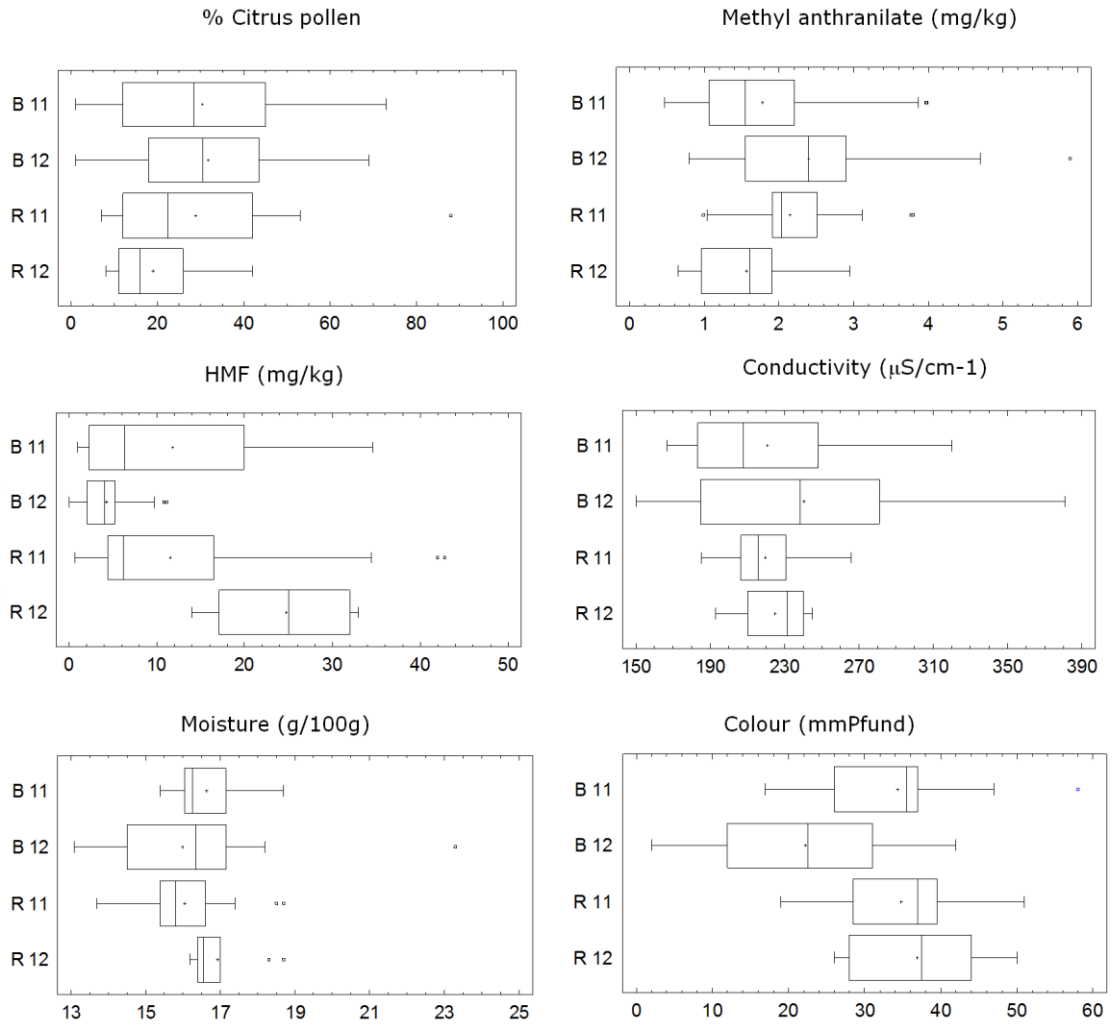

Figure 1.- Box and whisker plots for pollen, MA, HMF, electrical conductivity, moisture and colour for honeys from the bee-keepers (B) and retail outlets (R). Samples harvested in 2011 and in 2012.

Similarly, for MA the biggest dispersion between maximum and minimum corresponds to beekeeper samples (0.5-4.0 mg/kg in 2011 and 0.8-5.9 mg/kg in 2012) with means of 2.1 and 2.4 mg/kg, respectively; where as the retail samples ranged from 0.6 to 2.9 mg/kg (mean= 1.5 mg/kg) for 2011 and from 0.7 to 2.9 mg/kg (mean= 1.6 mg/kg) for 2012. In general, although not significant differences were observed for MA related to the type of sample, the lowest values and the lowest dispersion for MA (the same as for % of pollen) were detected, as expected, in retail samples. This is because the honey packaging industry mixes the raw honeys to produce relatively homogeneous batches to meet the requirements and specifications that companies have for each type of monofloral honey such as citrus honey. After reception, these industries analyse the physicochemical properties and pollen of the raw honey in order to discern the characteristics of the raw batches and be able to mix them appropriately.

The HMF of the beekeeper honeys showed relatively low average values (5.8mg/kg for 2011 and 4.1mg/kg for 2012). However, unexpectedly high values of 16.5 and 25 mg/kg were observed in 2011, probably due to sporadic bad practices. Such high values were not observed in 2012 in any case. As commercialized honeys are usually thermally treated (liquefied and pasteurized) by the industry, the highest values were usually and unsurprisingly found in the retail honeys. It should to be pointed out that one sample exceeded the overall permitted limit of 40 mg/kg for HMF (Council Directive 2001/110 relating to honey, 2002).

Electrical conductivity was quite low in the majority of the samples, as expected for the type of honeys under consideration (Persano-Oddo & Piro, 2004; Bogdanov, et al., 2004). The average values were very similar in both types of samples (217 and 230 μScm^{-1} in the beekeeper samples, and 228 and 224 μScm^{-1} in the retail ones) without significant differences neither year nor type of sample.

In the same way no significant differences were found for moisture in relation to both factors, and maximum values did not exceed 19.0 g/100g (Cano, et al., 2001), with one exception of 23.3 g/100g (in beekeeper samples of 2012). Beekeeper samples exhibited lower minimum values, reaching 13.1 g/100g, whereas the minimum value for retail samples was 15.2 g/100g. Being spring honeys, higher moisture values than those observed could be expected (Serra-Bonvehí, 1988). Again, a lesser dispersion of moisture values, reflecting greater homogeneity, can be seen for retail honey due to processing practices, as mentioned previously.

On the contrary, with regard to colour, significant differences were found both for year and supplier factors. Beekeeper samples had lower values, especially those from 2012. Retail samples reached values of up to 58 mm Pfund. These higher values could be mainly due to the influence of industrial thermal treatments (liquefaction and pasteurization) (Visquert et al., 2014). Although colour level is not a requirement for citrus honey commercialization, if this honey were sold with a specific quality mark, then particular colour requirements would have to be met. This is the case of the Valencian Quality mark (DOGV, 2002) which requires a maximum of 30 mm on the Pfund scale to benefit from this Mark. Considering this level, all of the retail samples and more than half of those from beekeepers in 2011 were above it. However, in the case of beekeeper samples from 2012, more than 75% had values lower than 30 mm on the Pfund scale.

3.3.-Relationship between the analysed parameters

In order to ascertain the possible linear dependence between the analysed variables, Pearson correlation coefficients were calculated for each pair of variables. Only the beekeeper samples were considered in this correlation since the lack of freshness of the retail samples (high HMF values) could have influenced the correlated variables (Serra-Bonvehí, 1988; Sesta et al., 2008).

Table 3 shows the correlation matrix obtained; the number in brackets is the P-value which tests the statistical significance of the estimated correlations at the 95.0% confidence level. Although some of the correlations are significant since P-values are below 0.05, the strength of the linear relationship between each pair of variables is far from the value +1 or -1. The best correlations are shown for colour and HMF (0.674 for 2011 and 0.706 for 2012) and for colour and electrical conductivity (0.596 for 2011 and 0.812 for 2012). The observed correlation between colour and HMF is coherent considering that since from harvesting, honey tends to increase HMF and colour naturally as a result of Maillard reactions (Sancho, et al., 1992). The correlation between colour and electrical conductivity is widely accepted. In general terms, the darker the honey the higher electrical conductivity. Since the samples considered for this correlation were only the unprocessed honeys, the mineral content was the main cause of this relationship (Bogdanov et al., 2004).

Previous works considered that the MA value could be related to the percentage of pollen and to other specific physicochemical parameters such as moisture and HMF (Serra-Bonvehí, 1988). These authors suggest that MA content decreases with the loss of freshness, with one year old samples showing a lower level of this compound than fresh samples.

However no good linear relationship between MA and HMF (-0.375 for 2011 and -0.336 for 2012) was observed in this present work. Similarly, there was also no correlation between MA and moisture, despite claims by Serra-Bonvehí (1988) that high moisture content can cause aromatic losses in honey to the point of reducing MA content. It is important to highlight that the range of moistures found in this work was too narrow to draw conclusions about the influence of moisture on this parameter.

In relation to the other physicochemical parameters (electrical conductivity and colour), significant correlations, with moderate Pearson coefficients were observed between them and MA, and pollen, especially for 2012 samples, with values of: MA/electrical conductivity=-0.678; MA/colour=-0.559; pollen/electrical conductivity=-0.553 and pollen/colour=-0.556. However, on the contrary to what was expected, there was no

Table 3. Correlation matrix (Pearson correlation coefficients) between percentage of pollen (*Citrus* spp.), methyl anthranilate (MA), HMF, moisture, electrical conductivity and colour. Samples harvested in 2011 and 2012.

	Pollen		MA		HMF		Moisture		Electrical conductivity	
	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
MA (mg/kg)	0.347	0.253								
	(0.062)	(0.098)								
HMF(mg/kg)	-0.292	-0.380	-0.375	-0.336						
	(0.011)	(0.011)	(0.001)	(0.026)						
Moisture (g/100g)	0.090	0.285	0.037	0.303	-0.069	0.200				
	(0.437)	(0.061)	(0.754)	(0.046)	(0.556)	(0.194)				
Electrical conductivity	-0.213	-0.553	-0.334	-0.678	0.101	0.413	-0.132	-0.352		
($\mu S/cm^{-1}$)	(0.065)	(0.000)	(0.003)	(0.000)	(0.386)	(0.005)	(0.255)	(0.019)		
Pfund colour (mm)	-0.401	-0.556	-0.519	-0.559	0.674	0.706	-0.189	-0.107	0.596	0.812
	(0.003)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.102)	(0.491)	(0.000)	(0.000)

Numbers in brackets = P-value

correlation between MA and the percentage of pollen, the coefficient being 0.347 and 0.253 for samples from 2011 and 2012, respectively.

Once the limited quantitative correlation between MA and the percentage of pollen was demonstrated, it seemed interesting to try to correlate them from a qualitative point of view. Therefore both variables were now considered to be categorical. For this purpose, the samples were classified according to whether they fulfilled the criteria for minimum level of pollen [*Citrus* spp. pollen higher than 10%: Molins et al., 1995; Persano-Oddo, et al, 1995; DOGV, 2002] and minimum level of MA [2 mg/kg: Persano-Oddo & Piro, 2004; Sesta et al., 2008 and commercial criteria according to the Spanish industry]. As a consequence, a contingency table was made (only the beekeeper samples were considered since they were always raw samples and not mixed) (Table 4), which is a double entry constructed by listing the variable "pollen" as rows and the variable "MA" as columns. Each variable has only two levels: comply or not comply. Each cell in the table represents the percentage of samples that satisfy the criterion of the row (% of *Citrus* spp pollen) or the column (MA concentration), both (% pollen and MA) or neither. Of all the observations, 89.2% (in 2011) and 95.4% (in 2012) comply with the pollen requirement (at least 10% citrus pollen) for a Mark of Quality (e.g. the Valencian Quality Mark: DOGV, 2002). In the case of methyl anthranilate the percentage of compliance (at least 2 mg/kg) was 53.5 and 61.4%, respectively. As mentioned above, in the case of citrus varieties, in addition to pollen, the methyl anthranilate content is required for commercial transactions, as was suggested several years ago by some European laboratories (Sesta et al., 2008).

Table 4.-Contingency table for pollen (comply with 10% of *Citrus* spp. pollen) and MA (comply with 2 mg/kg).Samples harvested in 2011 and 2012.

	Comply with MA≥2mg/kg		Not comply MA≥2mg/kg		Total	
	2011	2012	2011	2012	2011	2012
Comply with pollen ≥10% *Citrus* spp	53.5%	56.8%	35.7%	38.6%	89.2%	95.4%
Not comply with pollen ≥10% *Citrus* spp	0%	4.6%	10.8%	0%	10.8%	4.6%
Total	53.5%	61.4%	46.5%	38.6%	100.0%	100.0%

In this work, 53.5% for 2011 and 56.8% for 2012 of the samples fulfilled both % pollen and MA concentration. On the other hand, 35.7 % and 38.6 % of the samples met % pollen but not MA, and 4.6 % of the samples from 2012 complied with MA but not % pollen. Finally, 10.8% of samples from 2011 met neither % pollen nor MA.

It does not seem logical that MA, a parameter that has been proposed to complement the information given by pollen in citrus honeys when pollen is under-represented, is

actually an impediment to its classification. The way that MA is being applied does not seem to help this purpose. In fact, if melissopalynological analysis alone were considered in this study, as in other types of monofloral honey, 89.2 % of samples from 2011 and 95.4% from 2012 would be accepted. However, 35.7 % and 38.6% of the samples, respectively, which complied with the pollen requirement would be rejected commercially for not reaching 2 mg/kg for MA.

According to the results obtained, the criterion of 2 mg/kg for MA seems to be too demanding, and therefore not suitable for Spanish citrus honeys. Studies carried out by other authors on this type of honey from Spain and Italy (Sesta et al., 2008; Papotti et al., 2009), concluded that MA content was usually lower than the commercial requirement of 2 mg/kg. This fact was demonstrated even in honeys, which obviously had the sensory characteristics of this type of honey. Maybe, for these Mediterranean countries it would be appropriate to propose a lower value than is expected in other parts of the world (White & Bryant, 1996). According to the results, it seems more appropriate to only demand this value in the case of honeys with an unexpectedly low percentage of citrus pollen. That is, honeys with the typical physicochemical and sensory characteristics of citrus honey (light amber colour, evident notes of acidity and a very unique flavour due to the presence of specific aromatic substances) but without sufficient citrus pollen for this type of honey (Escriche, et al., 2011).

6. Conclusion

The results showed that there was a very weak linear correlation between the level of methyl anthranilate and the percentage of pollen in the samples analysed. In almost half of the cases the quantity of MA in Spanish citrus honey was lower than the commercial requirement, which was also reported by other authors for Italian citrus honey. This occurs even though the honeys have a more than sufficient commercial level of citrus pollen. This paper proposes reconsidering the level of MA required in Spanish citrus honey and applying a more realistic value. But above all, to only take this parameter into account in the case of honeys with a surprisingly low percentage of citrus pollen, and after evaluating their organoleptic and physicochemical properties.

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7. References

Bertelli D., Papotti G., Lolli M., Sabatini A.G. &Plessi M. (2008). Development of an HS_SPME_GC method to determine the methyl anthranilate in Citrus honey. *Food Chemistry*, 108, 297-303.

Bogdanov S. (2002). Harmonized methods of the International Honey Commission. Swiss Bee Research Centre, FAM, Liebefeld, CH 3003 Bern, Switzerland.

Bogdanov S., Ruoff K. & Persano-Oddo L. (2004). Physico-chemical methods for the characterization of unifloral honeys: a review. Apidologie, 35, 4-17.

Cano C. B., Felsner M. L., Matos J. R., Bruns R. E, Whatanabe H. M. & Almeida-Muradian, L. B. (2001). Comparison of Methods for Determining Moisture Content of Citrus and Eucalyptus Brazilian Honeys by Refractometry. Journal of Food Composition and Analysis, 14, 101-109.

Carretero J. L. (1989). Análisis polínico de la miel (1st ed.). Multi-Prensa, Madrid, España.

Castro-Vazque, L., Diaz-Marot, M.C. & Pérez-Coello M.S. (2007). Aroma composition and new chemical markers of Spanish citrus honeys. Food Chemistry, 103, 601-606.

Commission Decision 2002/657/EC of 12 August (2002). Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, OJEC L221, 8-36. Brusels, Belgium.

Council Directive 2001/110 relating to honey (2002). Official Journal of the European Communities, 12.1.2002.

DOGV (Valencian Region Regulation) No. 4167 (2002). Reglamento de la Marca de Calidad CV para miel de azahar y romero, in: Diario Oficial de la Generalitat Valenciana. Área de publicaciones de la Presidencia de la Generalitat Publishing.

Escriche I., Kadar M., Juan-Borrás, M. & Domenech E. (2011). Using flavonoids, phenolic compounds and headspace volatile profile for botanical authentication of lemon and orange honeys. Food Research International, 44 (5), 1504-1513.

Ferreres F., Giner J.M. & Tomás-Barberán F.A. (1994). A comparative study of hesperetin and methyl anthranilate as markers of the floral origin of citrus honey, Journal of the Science of Food and Agriculture, 65, 371-372.

Gómez-Pajuelo A. (2004). Origen botánico de la miel, In: Montagud S.A (Ed). Mieles de España y Portugal (pp. 50-56). Barcelona, Spain.

ISO 5496 (2006). Sensory Analysis. Methodology. Initiation and training of assessors in the detection and recognition of odours.

Jabalpurwala F.A., Smoot J. M. & Rouseff R. L. (2009). A comparison of citrus blossom volatiles. Phytochemistry, 70, 1428-1434.

Molins J. L., Perea F., Montilla J., Martinez E. & Guerra E. (1995). Characterization du miel d'orange (Citrus sp.) produit en Espagne, au moyen de son spectre pollinique. Lagascalia, 18, 71-82.

Nozal M., Bernal J., Toribio L., Jiménez J. J. & Martín M.T. (2001). High-performance liquid chromatographic determination of methyl anthranilate, hydroxymethylfurfural and related compounds in honey. Journal of Chromatography A, 917, 95-103.

Papotti G., Bertelli D., Lolli M., Sabatini A.G. & Plessi M. (2009). Methyl anthranilate content in Italian citrus honeys determined by HS-SPME-GC. International Journal of Food Science and Technology, 44, 1933-1938.

Papotti G., Bertelli D. & Plessi M. (2012). Use of HS-SPME-GC-MS for the classification of Italian lemon, orange and citrus spp. Honeys. International Journal of Food Science and Technology, 47, 2352 -2358.

Persano-Oddo L., Piazza M.G., Sabatini A.G. &Accorti M. (1995). Characterization of unifloral honeys. Apidologie, 26, 453-465.

Persano-Oddo L. & Piro R. (2004). Main European unifloral honeys: descriptive sheets. Apidologie, 35, S38–S81.

Rodriguez I., Serrano S., Galán H., Ubera J. L. & Jodral M. (2010). Characterisation of Sierra Morena citrus blossom honey (Citrus sp). International Journal of Food Science and Technology, 45, 2008-2015.

Saenz C. & Gómez C. (2000). Mieles españolas. Características e identificación mediante el análisis del polen. (1st ed.). Multi-Prensa, Madrid, España.

Sancho M.T., Muniategui S., Huidobro J. F. & Simal J. (1992). Aging of honey. Journal of Agricultural and Food Chemistry, 40, 134–138.

Serra-Bonvehí J. (1988). Determinación de antranilato de metilo en la miel de citricos (Citrus sp.) del Levante Español, y su influencia en la actividad diastásica de la miel. Alimentaria, 197, 37–40.

Serra-Bonvehí J. & Ventura F. (1995). Characterization of Citrus honey produced in Spain. Journal of Agricultural and Food Chemistry, 43, 2053–2057.

Sesta G., Piana M.L., Persano-Oddo L., Lusco L. & Belligoli P. (2008). Methyl anthranilate in Citrus honey. Analytical method and suitability as a chemical marker. Apidologie, 39, 334-342.

Visquert M., Vargas M. & Escriche I. (2014). Effect of postharvest storage conditions on the colour and freshness parameters of raw honey. International Journal of Food Science and Technology, 49, 181–187.

Von der Ohe W., Persano-Oddo L., Piana M.L., Morlot M. & Martin P. (2004). Harmonized methods of melissopalynology. Apidologie, 35, 18-25.

White J. W. (1966). Methyl anthranilate content of citrus honeys. Journal of Food Science, 31, 102-104.

White J.W. &Bryant V.M. (1996). Assessing Citrus honey quality: pollen and methyl anthranilate content. Journal of Agricultural and Food Chemistry, 44, 3423–3425.

3.3. Potenciometric tongues with noble and non-noble metals for the differentiation of Spanish honeys considering the antioxidant capacity

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Food Control (En revisión)

Abstract

Knowledge about the antioxidant level of honey is increasingly important, due to its implications for health, and as a marketing tool for the industry. The aim of this work was to evaluate the capacity of a potentiometric electronic tongue, with a combination of four noble (Au, Pt, Ir and Rh) and four non-noble metals (Ag, Ni, Co and Cu), to differentiate between types of Spanish honey (orange blossom, rosemary,thyme,sunflower,winter savory and honeydew honey) according to their antioxidant level. The botanical categorization of each of the batches was carried out by means of pollen analysis. The electronic tongue system made with Ag, Ni, Co, Cu and Au was able to not only differentiate between types of honey but also to predict their total antioxidant capacity. The discrimination ability of the system was proved by means of a Neural Network Analysis fuzzy artmap type, with 100% classification success.A prediction MLR model showed that the best correlation coefficient was for antioxidant activity (0.9666), then for electrical conductivity (0.8959) and to a lesser extent for the other parameters considered (a_w, moisture and colour). The proposed measurement system could be a quick, easy option for the honey packaging sector to provide continuous in line information about a characteristic as important as the antioxidant level.

Keywords: Potentiometric tongue; honey; antioxidant capacity

1. Introduction

Honey has been a highly valued food since ancient times for its organoleptic and therapeutic characteristics and also nowadays for its antioxidant properties. The demand for natural antioxidants is increasing constantly as they play an important role in human health avoiding damage caused by oxidizing agents. It is well known that honey is an important source of natural antioxidants, one of the reasons why honey consumption is recommended. Recent research has shown that the antioxidant properties of honey depend on the nectar of blossoms or exudates of trees and plants visited by bees. In turn, this is conditioned by the geographical and climatic conditions (Escricheet al., 2014). At present, their information available to the consumer through labeling is very incomplete. This is limited at the most to the botanical origin, allowing honey to be classified as monofloral. There is growing interest in assessing the specific healthy properties of honey (e.g. antioxidant level), in order to inform consumers and to increase producers profit margins.

A large number of procedures are used to evaluate the antioxidant potential of honey. Although these methodologies show good precision, accuracy and reliability, the problem is that they are inappropriate for in situ monitoring. In addition, these techniques are destructive, time-consuming and very tedious, requiring highly expert analysts and specialized equipment (Oroian & Escriche, 2015).

Among the fastest, easy-to-handle measurement techniques that have been tested in recent years, electronic tongues (based mainly on voltammetry, potentiometry or impedance spectroscopy), together with multivariate chemometric tools, are the most promising. The effectiveness of a potentiometric tongue (measuring the electrochemical balance) made of various metals and metallic compounds (metal oxides and insoluble metal salts) was successfully applied for the classification of honey according to its botanical origin although it was less efficient at discriminating between applied thermal treatments (raw, liquefied and pasteurized) (Escriche et al., 2012). Tiwari et al. (2013) used an electronic tongue based on the electrochemical information analysis of the voltammetric scan of a single platinum electrode as a sensor element for discrimination between several monofloral honey samples. Ulloa et al. in 2013 described the differentiation of various Portuguese honeys using a sensor obtained by the fusion of an impedance electronic tongue and optical spectroscopy (UV–Vis–NIR).

The application of a commercially available potentiometric electronic tongue (Astree, Alpha M.O.S.), either as a unique process (Major et al., 2011) or in combination with a voltammetric electronic tongue (Wei & Wang, 2014) has been described to classify types of honey according to botanical and geographical origin.

Using potentiometric electrodes (with metal/metal oxides and insoluble metal salts) provides information about the presence of compounds through interaction with them (Soto et al., 2006). For example, metal/metal oxide electrodes are sensors that afford information about the pH of the medium, the presence of anions which form low soluble salts with the oxidized metal and the presence of dissolved substances which can form compounds with the corresponding metals (Escriche et al., 2012). Noble metals, such as gold or platinum, have been used to determine the redox potential in food systems. The measurement of the redox potential can be directly related to the content of total reducing or oxidizing agents present in the sample. Antioxidants, such as polyphenols or flavonoids, are compounds with reducing character that can cause changes in the potential of metal polarization as a consequence of both their potential redox and their relative concentrations (Soto et al., 2013). This suggests that metal sensors could be useful for the determination of the antioxidant activity of honey.

For this reason the goal of the present study was to evaluate whether a potentiometric electronic tongue made with a combination of metal electrodes (noble and non-noble) is able to differentiate between types of Spanish honey according to their antioxidant level. The specific contribution of each electrode sensor is evaluated, together with the

possibility of a correlation between the potentiometric measures of honey and the physicochemical parameters and antioxidant capacity.

2. Materials and Methods

2.1.-Honey samples

Six types raw honey harvested in 2014 in different areas of Spain were used in this study. They are among the most common varieties available in Spain: orange blossom (*Citrus* spp.), rosemary (*Rosmarinus officinalis*), thyme (*Thymus* spp.), sunflower (*Helianthus annuus*), winter savory honey (*Satureja montana*), and honeydew honey. Three batches of each type of honey were directly supplied by beekeepers. All samples were sent to the laboratory immediately after they were harvested.

2.2.-Analytical determinations

2.2.1. Melissopalynological analysis

The botanical categorization of each of the batches were performed by means of pollen analysis. Quantification of pollen was carried out following the recommendations of the International Commission for Bee Botany (Von Der Ohe et al., 2004). A light microscope (Zeiss Axio Imager, Gottingen, Germany) at a magnification power of x400 with DpxView LE image analysis software attached to a DeltaPix digital camera was used in this analysis.

2.2.2. Colour and physicochemical analysis

Color was determined using a millimeter Pfund scale C 221 Honey Color Analyzer (Hanna Instruments). Moisture content (by refractrometry: Abbe-type model T1 Atago, USA and the Chataway table) and electrical conductivity (by conductimetry: Crison Instrument, Barcelona, Spain, model C830) were analyzed in accordance with the Harmonized Methods of the European Honey Commission (Bogdanov, 2002). Water activity (aw) was determined at 25 °C (± 0.2 °C) using an electronic dewpoint water activity meter, Aqualab

Series 4 model TE (Decagon Devices, Pullman,Washington, USA), equipped with a temperature-control system (Chirife et al., 2006).

2.2.3. Sugar Analysis

Fructose, glucose and sucrose were analyzed as described by Bogdanov et al. (1997) using a HPAEC high-resolution ionic chromatograph with a pulsed amperometric detector (PAD) (Bioscan, Methrom, Switzerland) and a Metrosep Carb chromatographic column (styrene divinylbenzene copolymer, 4.6 × 250 mm). Carbohydrates were eluted with NaOH 0.1N at a flow rate of 1 mL min-1. Quantification of sugars was realized using external standards constructing the corresponding calibration curves.

2.2.4. Antioxidant activity

The antioxidant activity (AA) was measured based on the scavenging activities of the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich, Germany) free radical as described by Shahidi et al. (2006). Accordingly, 1g of the honey sample (diluted in 25 mL methanol: water, 80:20) was mixed with 3.9 mL of a methanolic solution of DPPH (0.025mg/mL, prepared in methanol: water (80:20)). After 30 min, the absorbance of the samples was measured at 515 nm with methanol as a blank, using a spectrophotometer (JASCO V-630). The quantification was carried out using a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The results were expressed as mg of Trolox equivalent per gram of honey.All analyses were performed in triplicate.

2.2.5. Potenciometric tongue

Measurement system

Measurements were performed with tailor-made equipment formed basically by the following parts: a set of metal electrodes, an electronic system (Martínez-Mañez et al., 2005), a computer system for digitalization and acquisition of the signal and data analysis software.

Set of metallic electrodes

The electrochemical measurements were obtained using an electronic tongue consisting of an array of eight metallic electrodes (1 mm diameter from Aldrich): Noble metals (gold, platinum, iridium and rhodium); and non-noble metals (copper, silver, nickel and cobalt). All electrodes were placed in two homemade stainless steel tube (one for the 4 noble metals and another for the rest) that were used as the body of the electronic tongue. The different wire electrodes were fixed inside the tube using RS 199-1468 epoxy polymer. Before use, the surfaces of the electrodes were polished with emery paper, and rinsed with distilled water. Then they were polished on a felt pad with 0.05 μm alumina polish

from BAS, washed with distilled water and polished again on a nylon pad with 15.3 and μm diamond polishes. During the measurements, only a simple diamond polishing was carried out.

Electronic Measurement Equipment

The measuring equipment permitted multiplexed potentiometric measures for 16 independent channels. The potential was measured automatically, sequentially and cyclically for each of the eight electrodes with respect to the Ag/AgCl reference electrode. The potentiometric measurements were simply the potential difference between each electrode and the reference electrode.

Implementation of measures

To carry out a measurement, the set of eight electrodes and the reference electrode were dipped in a vessel containing 5 g of honey in 25 mL water. Then, the multiplexed reading of the potential was carried out for 15 min. The time required to reach equilibrium was usually less than 10 minutes. The data used for multivariate analysis were the average values of the last 5 min of acquisition (between 10 and 15 min). This process was repeated three times for each of the 18 samples (6 types of honey x 3 batches).

2.3.-Statistical analysis

An analysis of variance (ANOVA) (using Statgraphics Centurion for Windows) was applied to study the influence of the type of honey on colour, physicochemical parameters (a_w, moisture and electrical conductivity), sugars and total antioxidant activity. LSD (least significant difference) at significance level α = 5%was used to analyze the differences between means.

The data matrix obtained with the potentiometric tongue was analyzed using multivariate statistical techniques: principal component analysis (PCA) and Fuzzy ARTMAP-type artificial neural network (ANN) were applied to evaluate the possible classification (non-supervised and supervised, respectively) of the samples. Fuzzy ARTMAP type ANN (Carpente et al., 1992) is a self-organizing supervised classifier that has been previously used with nose and tongue electronic systems, with good results for a limited number of samples, as is the case of the present work (Llobet et al., 1999). The network was implemented in-house using function macros from basic functions of MATLAB with a graphical environment (GUI) (Garcia-Breijo et al., 2013) using a simplified version of the original algorithm (Kasuba, 1993). Multiple linear regression (MLR) was used to model the relationship between the potentiometric measurement and the physicochemical parameters. PCA and MLR were performed using the annex SOLO of the MATLAB program (Eigenvectors Research, Inc.).

3. Results and Discussion

3.1.-Melissopalynological, colour, physicochemical parameters, sugars and antioxidant activity

Pollen analysis of the honey samples was made as a first step in order to ascertain their botanical origin. Table 1 illustrates the predominant pollen, colour, physicochemical parameters, sugars and total antioxidant activity of each honey sample used in this study. In addition, this table shows the ANOVA results (F-ratio and significant differences) obtained for the factor "type of honey". The information about the homogeneous groups obtained from the ANOVA for every one of the parameters appears (in superscript letters) in this table next to the information of the first batch of each type of honey. Taking into account that the higher the F-ratio, the greater the effect that a factor has on a variable, electrical conductivity, colour and total antioxidant activity were the parameters most affected by "type of honey". Moisture, a_w, and glucose where less affected, whereas fructose and sucrose did not show significant differences between types of honey.
Among all the honey samples considered in this study, honeydew honey, as expected, had the highest electrical conductivity (up to 814μS/cm). In fact, this honey has to have more than 800μS/cm to be classed as this type of honey.

Table 1. Predominant pollen of each sample, colour, physicochemical parameters, sugars, total antioxidant activity and ANOVA results (F-ratio and significant differences) obtained for the factor type of honey. The number before of each type of honey refers to the batch.

Type of honey	Predominant pollen	Colour (Pfund scale)	a_w	Moisture g/100 g	Electrical conductivity µS/cm	Glucose g/100g	Fructose g/100g	Sucrose g/100g	Total antioxidant activity mg trolox/100g
1. Orange	60% *Citrus* spp., *Diplotaxis* spp. , *Taraxacum type*	$10^{(a)}$	$0.585^{(a.b)}$	$17.7^{(c)}$	$128^{(a)}$	$32^{(bc)}$	43	1	$15.81^{(a)}$
2. Orange	31% *Citrus* spp., *Taraxacum type*	6	0.591	18.0	128	32	44	1	15.81
3.Orange	60% *Citrus* spp., *Taraxacum type,Diplotaxis* spp., *Quercus* spp., *Olea europaea*	8	0.567	17.2	150	30	42	2	15.06
1. Rosemary	36% *Rosmarinus officinalis,Taraxacum type ,Thymus* spp., *Anthyllis citysoides, Quercus* spp., *Citrus* spp.	$16^{(a)}$	$0.593^{(b)}$	$17.2^{(b,c)}$	$178^{(a)}$	$32^{(bc)}$	45	2	$16.04^{(a)}$
2. Rosemary	21% *Rosmarinus officinalis, Vicia* spp., *Echium* spp.	13	0.594	17.3	118	32	44	2	16.04
3. Rosemary	17% *Rosmarinus officinalis, Vicia* spp., *Echium* spp.	0	0.568	16.9	97	30	41	1	11.72
1. Thyme	28% *Thymus* spp., *Prunus dulcis, Centaurea* spp., Compositae, *Satureja montana, Onobrychis viciifolia*	$78^{(c)}$	$0.561^{(a)}$	$15.9^{(a)}$	$517^{(c)}$	$30^{(b)}$	42	1	$356.15^{(c)}$
2. Thyme	23% *Thymus* spp., 2% *Satureja montana, Onobrychis viciifolia*	69	0.560	15.4	392	31	44	2	257.75
3. Thyme	29 % *Thymus* spp., *Onobrychis viciifolia*	89	0.556	15.0	451	30	42	1	318.05
1. Sunflower	51 % *Helianthus annuus,* 23% *Xanthium* spp.	$55^{(b)}$	$0.584^{(b)}$	$16.6^{(b,c)}$	$316^{(b)}$	$32^{(c)}$	48	2	$39.91^{(a,b)}$
2. Sunflower	74% *Helianthus annuus*	67	0.594	16.8	369	36	46	1	51.96
3. Sunflower	70% *Helianthus annuus*	60	0.618	18.2	359	33	42	2	48.56

Table 1 (cont.)

Type of honey	Predominant pollen	Colour (Pfund scale)	a_w	Moisture g/100 g	Electrical conductivity µS/cm	Glucose g/100g	Fructose g/100g	Sucrose g/100g	Total antioxidant activity mg trolox/100g
1. Winter savory	35% *Satureja montana, Diplotaxis* spp., *Onobrychis viciifolia, Centaurea* spp., *Lavandula latifolia, Thymus* spp.	$86^{(c)}$	$0.573^{(b)}$	$16.2^{(b,c)}$	$278^{(b)}$	$30^{(b,c)}$	42	1	$85.56^{(b)}$
2. Winter savory	52% *Satureja montana, Onobrychis viciifolia, Diplotaxis* spp., Apiaceae, *Echinops* spp., Compositae	90	0.606	17.8	292	32	49	2	67.64
3. Winter savory	49% *Satureja montana, Centaurea* spp., *Diplotaxis* spp., Apiaceae, *Citrus* spp.	90	0.602	17.6	300	31	45	3	63.66
1. Honeydew	8% *Retama sphaerocarpa, Rubus ulmifolius*, *Genista type, Erica* spp., *Diplotaxis* spp.	$88^{(c)}$	$0.578^{(a,b)}$	$16.4^{(a,b)}$	$809^{(d)}$	$25^{(a)}$	42	1	$55.65^{(b)}$
2. Honeydew	30% *Rubus ulmifolius*, 17% *Echium* spp., 12% *Castanea sativa,* Compositae	86	0.580	16.5	809	28	46	3	57.09
3. Honeydew	49% *Rubus ulmifolius*, 29% *Echium* spp.,9% *Castanea sativa,* Apiaceae, *Erica* spp., Labiatae, *Genista type*	87	0.584	16.4	814	25	39	1	53.76
ANOVA F-ratio		115.10***	3.35*	5.87**	170***	7.8**	n.s.	n.s.	85***

***p< 0.001. For each factor, different letters in each column indicate homogeneous groups (significant differences at 95% confidence level as obtained by the LSD test). The information about the homogeneous groups appears between brackets and superscript letters in the first row of each group

Thyme honey stands out from the other monofloral honeys due to its high electrical conductivity values, which reached 517μS/cm. This is a very high value considering its floral origin, much higher than that observed by Karabagias et al. (2014) in thyme honey from Greece (average value: 399 μS/cm). Orange blossom and rosemary honey had the lowest conductivity (no more than 150 and 178μS/cm, respectively), without significant differences between them.

With respect to colour, the results show that orange blossom and rosemary honey had the lightest colour with a range of 0 to 16 mm Pfund, and winter savory honey the darkest showing values up to 90 mm Pfund. The dark colour in honey is traditionally a highly valued characteristic because it is well known that the darker a honey is, the higher the mineral content (González-Miret et al., 2005). Therefore, it follows that darker honey has higher conductivity, to the point that this relationship has been extrapolated to antioxidant activity, however the results of the present work do not support this idea. In fact, although the lowest values of total antioxidant activity correspond to the lightest honeys (orange blossom and rosemary honeys), the darkest honey (winter savory honey) did not exhibit the greatest antioxidant activity. Of all the analyzed samples, thyme honey differs significantly in relation to antioxidant activity, as the values of this parameter were between 4-5 times more than that of winter savory honey and 5-7 times more than honeydew honey, traditionally considered to have a high antioxidant level.

The PCA bi-plot of scores and loading obtained considering the 18 different samples (6 types of honey x 3 batches), the physicochemical parameters and color variables is shown in Figure 1, with the aim of evaluating the global effect of the type of honey on these variables from a descriptive position. The predominant pollen in each sample is placed next to the code of the botanical name.

Two principal components explained 66% of the variations in the data set: PC1 (44%) and PC2 (22%). It can be observed that honeydew honey and thyme honey are located in the right quadrant (although not totally differentiated), next to the highest values of colour, electrical conductivity and antioxidant activity. The other samples on the left quadrant without a clear distinction between them. Although the botanical origin of honey had a clear impact on some of the parameters studied, in general terms a good differentiation between the types of honey studied was not achieved.

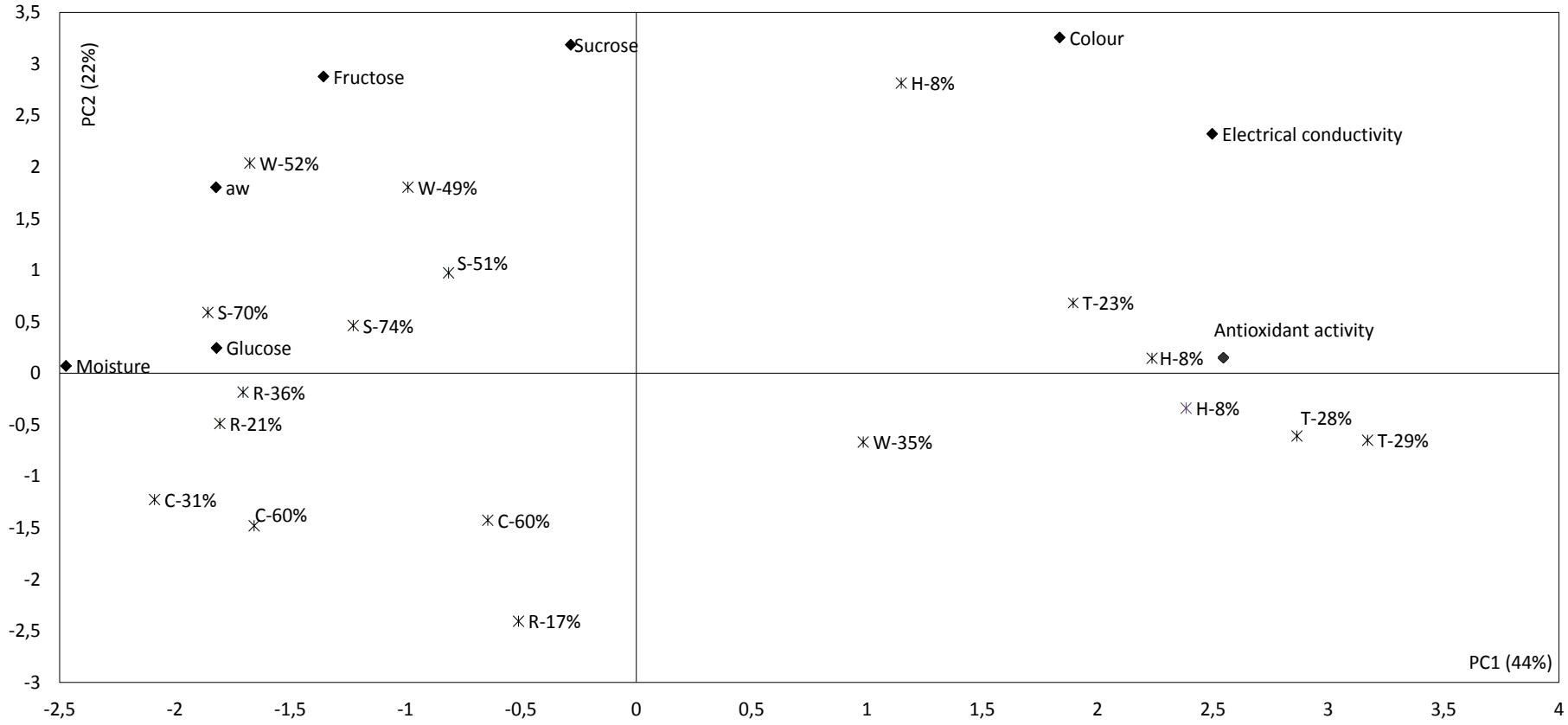

Figure 1. PCA biplot (scores and loadings) of the variables (physicochemical parameters, sugars, color and total antioxidant activity) and honey samples: C (orange blossom); R (rosemary); T (thyme); S (sunflower); W (winter savory honey); H (honeydew honey). Next to each sample the percentage of the relevant predominant pollen is shown.

3.2.-Potentiometric electronic tongue information: PCA and artificial neural networks classifications

A PCA analysis (unsupervised procedure) was applied to check if there was a spontaneous classification from the data generated by the eight electrodes, without previously defining the categories of the samples. Figure 2 shows the PCA biplot (scores and loadings) from the measurements obtained with the eight electrodes together, noble and non-noble metals. Two components explained 92% of the total variance (PC1=72% and PC2=20%). It can be observed that a good discrimination was found between types of honey. Samples of thyme honey and honeydew honey showed the clearest separation between them and with the rest of the samples; PC1 promotes the best discrimination of thyme honey and PC2 of the honeydew honey. This figure also shows that sunflower and winter savory are well differentiated, whereas citrus and rosemary have the most similar behavior in terms of potentiometric tongue response.

With respect to the contribution of each electrode in the calculation of PC1andPC2values, it can be seen that two electrodes (Co and Cu) have very different behavior to the rest. This is particularly important with regards to their influence on the PC2, since the variation of the contribution of these two electrodes is not relevant to PC1. This figure shows that the location of the four noble metal electrodes (Au, Pt, Ir and Rh) are very close to each other, which implies that their behavior is quite similar. Furthermore, the position of these four metals is relatively close to the other two non-noble metals: especially Ag, and to a lesser extent Ni. Consequently, it was considered appropriate to simplify the electrode system, reducing the four noble metals to one, Au, which was selected because is easier to obtain and to work with in comparison to the other three noble metals (Pt, Ir, Rh). Therefore, a new PCA was carried out (Figure 3) only using the information from five electrodes: the four non-noble metals (Ag, Ni, Co, Cu) plus Au. It can be observed that the position of the samples in Figure 3 is quite similar to Figure 2. In the case of Figure 3 the three noble electrodes: Au, Ag and Ni, exhibited similar but slightly different behaviour, while the other two metals(Co and Cu) still presented different behaviour to each other and the rest. In this way it was possible to simplify the measure system without losing information.

With the aim of complementing the information given by the PCA, an artificial neural network analysis, as a supervised procedure, was applied. The type of neural network used in this work was Fuzzy Artmap (Carpenter, Gossberg, Markuzon, Reynolds & Rosen, 1992) that has been previously used with nose and tongue electronic systems, with good results for a limited number of samples (Llobet et al., 1999), as is the case of the present work. In this analysis the available data were divided into two groups; one group was used

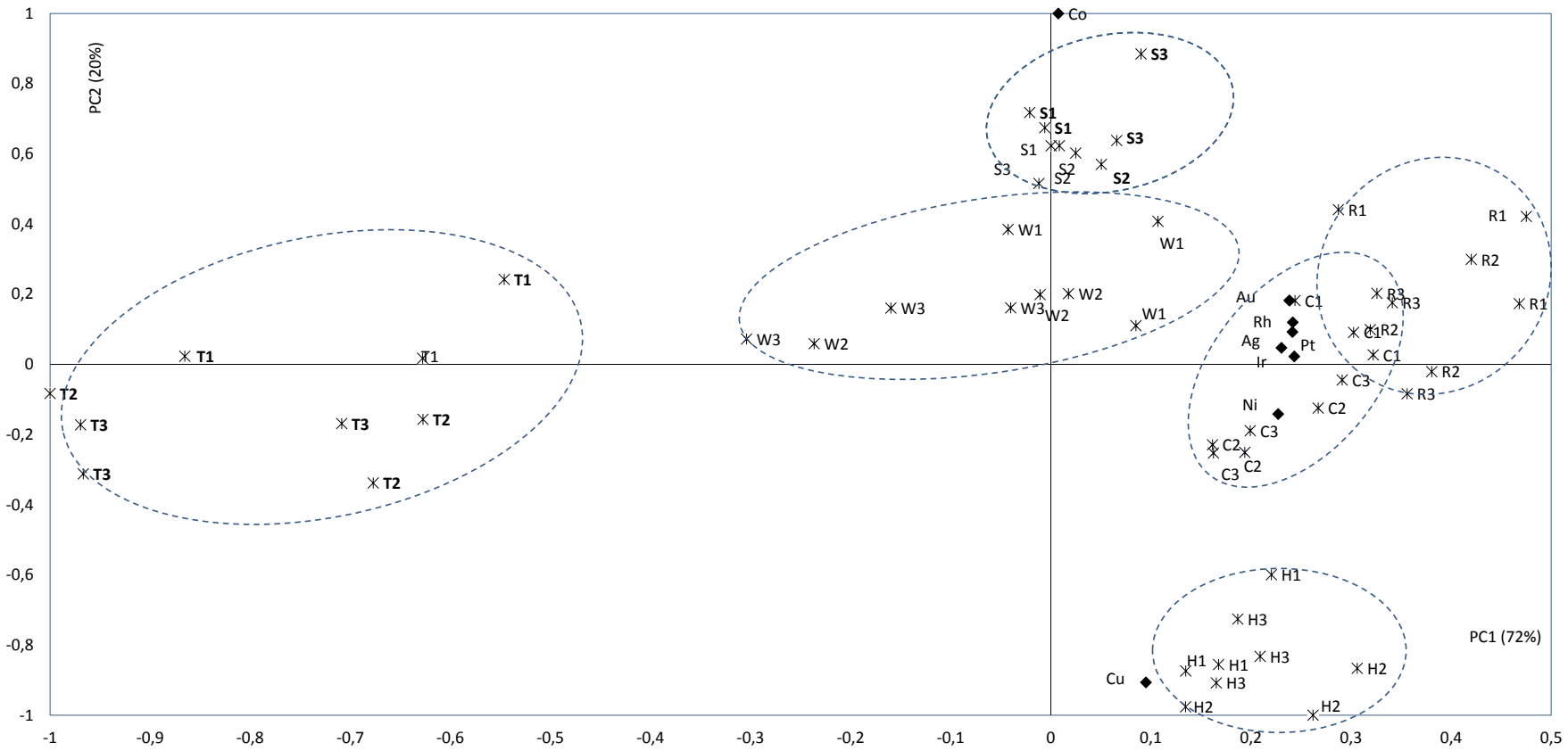

Figure 2. PCA biplot (scores and loadings) from the measurements obtained with the 8 electrodes together (4 noble and 4 non-noble metals: "Au, Pt, Ir, Rh, Ag, Ni, Co and Cu"). C (orange blossom); R (rosemary); T (thyme); S (sunflower); W (winter savory honey); H (honeydew honey).

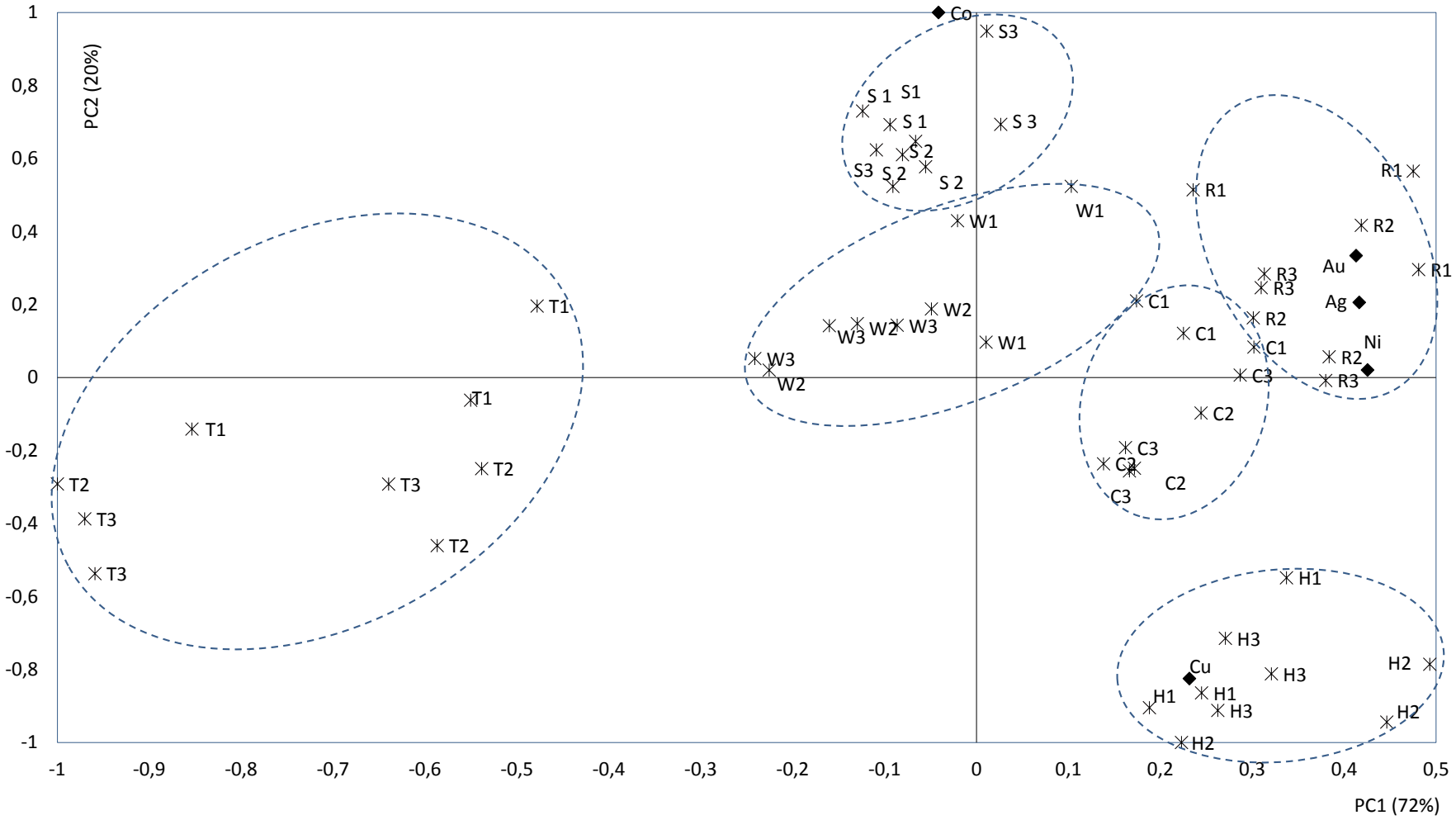

Figure 3. PCA biplot (scores and loadings) from the measurements obtained with the 5 electrodes together (4 non-noble metals "Ag, Ni, Co and Cu", plus Au). C (orange blossom); R (rosemary); T (thyme); S (sunflower); W (winter savory honey); H (honeydew honey).

for network training and obtaining the prediction model of the categories, and the other group to verify the neural network and to estimate the degree of success in the classification of new data into the established categories. The data distribution was as follows: of the54 total input data 6types of honey x 3 batches x 3repetitions), two-thirds(36) of the data were used for training and a third (18) for validation; ensuring that each category(types of honey) was represented proportionally in both the training and the validation groups. That is to say, for training the matrix of measurements of each electrode was: 6 types of honey x 2 batches x 3repetitions, and for validation: 6 types of honey x 1 batches x 3repetitions.-

The optimum model was achieved by obtaining a scan of the operating parameters of the neural network. After that, the data of verification were applied to check the rate of success/failures for the identification of each sample in the appropriate category. The classification success (data not shown) was 100%. The same result was obtained considering the starting 8 electrodes (4 noble and 4 non-noble metals). This demonstrates that the electrode measurement system made with the 5 metals was useful for the classification of samples of honey depending on the floral origin.

3.3.-Correlation of potentiometric data with physicochemical parameters: MLR analysis

With the aim of verifying whether the data given by the electronic tongue system is useful to predict relevant information provided by the physicochemical parameters, a MLR analysis (Multiple Linear Regression) was applied. To perform the MLR analysis, the data of 36 samples were taken to create the prediction model and the remaining 18 samples were used to verify the performed model. That is to say, the same separation of samples previously used in the neural network analysis.

A prediction MLR model was carried out for each the physicochemical parameters (a_w, conductivity, moisture), colour and antioxidant activity, and the potentiometric experimental data from the metallic electrodes. The analysis was made twice, on one hand considering the eight electrodes (4 noble metals plus 4 non-noble metals) and on the other hand considering just the 5 selected electrodes (4 non-noble metals plus Au). Table 2 shows the MLR results including the values of the slope, intercept, regression coefficient and number of latent variables. A good correlation exists for total antioxidant activity and electrical conductivity.

Table 2. MLR prediction results for the physicochemical parameters (aw, conductivity, moisture), colour and antioxidant activity

	4 non-noble metals +4 noble metals			4 non-noble metals +Au		
Parameters	Correlation coefficient	Slope	Intercept	Correlation coefficient	Slope	Intercept
aw	0.4860	0.516	0.279	0.4790	0.445	0.319
Electrical conductivity	0.9050	0.850	25.359	0.8959	0.813	51.126
Moisture	0.7380	0.646	5.905	0.7040	0.558	7.311
Colour	0.5300	0.634	14.529	0.7400	0.755	8.895
Antioxidant Activity	0.9665	0.970	11.162	0.9666	0.911	22.256

The best correlation coefficient was for the antioxidant activity using the 4 non-noble metals plus Au (0.9666), although it was very similar to that obtained with all the electrodes (0.9665). In the case of electrical conductivity, the correlation coefficients were 0.8959 and 0.9049, respectively. A weaker correlation exists for the rest of the parameters, especially for aw. Figure 4 shows the MLR graph corresponding to measured values vs. predicted values of the antioxidant activity in both cases.

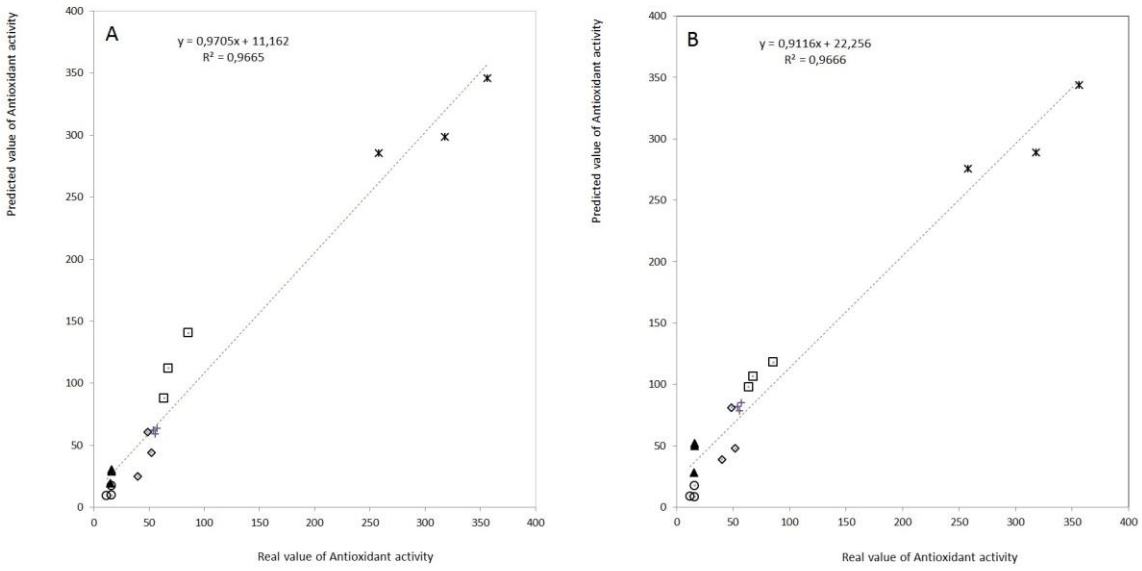

Figure 4. Predicted versus actual values of total antioxidant capacity given by the MLR model. A) 8 electrodes: 4 noble metals "Au, Pt, Ir and Rh" plus 4 non-noble metals "Ag, Ni, Co and Cu" and B) 4 non-noble "Ag, Ni, Co and Cu" metals plus Au.

A previous study found a remarkable correlation between physicochemical parameters such as colour Pfund (0.9580) and diastase activity (0.9260) and data from an electronic tongue, made of three metals (Au, Ag and Cu) and four metal compounds (Ag_2O, AgCl, Ag_2CO_3 and Cu_2O) (Escriche et al., 2012). However, the correlation with total antioxidant activity was much weaker (0.7660). The new electronic tongue proposed here is a great improvement over the previous one from the point of view of its ability to differentiate honey from the point of view of antioxidant capacity.

4. Conclusion

An electronic tongue system made of 5 metals: 4 non-noble metals (copper, silver, nickel and cobalt) and just one noble metal (Au) is able to not only differentiate between types of honey but also to predict their total antioxidant capacity.
The differentiation was most marked for thyme and honeydew honey and much weaker for citrus and rosemary honey. The discrimination ability of the measurement system was evaluated by means of an ANN fuzzy artmap type analysis, showing that the classification success was 100%.

In summary, the proposed measurement system could be a good tool for the honey packaging industry to provide continuous information about a factor as important as antioxidant capacity. The importance of knowledge about this component in honey is increasing, not only due to its implications for health, but also in terms of marketing.

The promising results obtained highlight the need to continue researching the validation of this approach to corroborate the efficacy of the proposed electronic tongue, using a wide variety of honey types with different botanical and geographical origins.

Acknowledgment
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5. References

Bogdanov S. (2002). Harmonized methods of the International Honey Commission Swiss Bee Research Centre, FAM, Liebefeld, CH 3003 Bern, Switzerland.

Bogdanov S., Martin P. & Lüllman C. (1997). Harmonised methods of the European Honey Commission. Apidologie, extra issue, 1–59.

Carpenter G. A., Gossberg S., Markuzon N., Reynolds I, & Rosen D. (1992). Fuzzy Artmap: a neural network architecture for incremental supervised learning of analog multidimensional maps. IEEE Trans Neural Network, 3, 698-713.

Chirife J., Zamora M. C. & Motto A. (2006). The correlation between water activity and % moisture in honey. Fundamental aspects and application to Argentine honeys Journal of Food Engineering, 72, 287-292.

Escriche I., Kadar M., Domenech E. & Gil-Sánchez L. (2012). A potentiometric electronic tongue for the discrimination of honey according to the botanical origin. Comparison with traditional methodologies: Physicochemical parameters and volatile profile. Journal of Food Engineering, 109, 449-456.

Escriche I., Kadar M., Juan-Borrás M. & Domenech E. (2014). Suitability of antioxidant capacity, flavonoids and phenolic acids for floral authentication of Spanish honey. Impact of industrial thermal treatment. Food Chemistry, 142, 135–143.

Garcia-Breijo E., Garrigues J. & Gil-Sánchez L. (2013). Optimization of an Embedded Simplified Fuzzy ARTMAP Implemented on a Microcontroller using MATLAB GUI Environment".Pattern Recognition: Practices, Perspectives and Challenges. Nova Science Publishers (Hauppauge NY, EEUU).

González-Miret M .L., Terrab A., Hernanz D., Fernández-Recamales M. A. & Heredia F.J. (2005). Multivariate correlation between color and mineral composition of honeys and by their botanical origin.Journal of Agriculture and Food Chemistry, 6, 53(7), 2574-80

Karabagias I. K., Vavoura M. V., Nikolaou C., Badeka A. V., Kontakos S. & Kontominas M.G. (2014). Floral authentication of Greek unifloral honeys based on the combination of phenolic compounds, physicochemical parameters and chemometrics. Food Research International, 62, 753–760.

Kasuba T. (1993). Simplified Fuzzy ARTMAP. Al Expert, 18-25.

Llobet E., Hines E.L., Gardner J.W., Bartlett P.N. & Mottram T.T. (1999). Fuzzy ARTMAP based electronic nose data analysis. Sensors and actuators b-chemical, 61, 183-190.

Major N., Markovic K., Krpan M., Saric G., Hruskar M. & Vahcic N. (2011). Rapid honey characterization and botanical classification by an electronic tongue. Talanta, 85, 569–574.

Martınez-Máñez R., Soto J., Garcia-Breijo E., Gil L., Ibáñez J. & Llobet E. (2005). An "electronic tongue" design for the qualitative analysis of natural waters. Sensors and Actuators, B 104, 302–307.

Oroian M. & Escriche I. (2015). Antioxidants: characterization, natural sources, extraction and analysis. Food Research International, 74, 10-36.

Shahidi F., Liyana-Pathirana C. M. & Wall D. S. (2006). Antioxidant activity of white and black sesame seeds and their hull fractions. Food Chemistry, 99, 478–483.

Soto J., Labrador R. H., Marcos M. D., Martınez-Máñez R., Coll C., García-Breijo E. & Gil L. (2006). Introduction of a model for describing the redox potential in faradic electrodes. Journal of Electroanalytical Chemistry, 594, 96–104.

Soto J., Campos I. & Martínez-Máñez R. (2013). Monitoring Wastewater Treatment Using Voltammetric Electronic Tongues. In S.C. Mukhopadhyay & A. Mason (Eds.).Smart Sensors for Real-Time Water Quality Monitoring (pp. 65–103). Springer- Verlag Berlin Heidelberg.

Tiwari K., Tudu B., Bandyopadhyay R., & Chatterjee A. (2013). Identificattion of monofloral honey using voltammetric electronic tongue. Journal of Food Engineering, 117, 205-210.

Ulloa P. A., Guerra R., Cavaco A.M., Rosa da Costa A. M. & Figueira A.C. (2013). Determination of the botanical origin of honey by sensor fusion of impedance e-tongue and optical spectroscopy. Computers and Electronics in Agriculture, 94, 1-11.

Von Der Ohe W., Persano-Oddo L., Piana M. L. Morlot M. & Martin P. (2004). Harmonized methods of melissopalynology, Apidologie, 35, 18-25.

Wei Z. & Wang J. (2014). Tracing floral and geographical origins of honeys by potentiometric and voltammetric electronic tongue. Computers and Electronics in Agriculture, 108, 112-122.

3.4. Effect of country origin on physicochemical, sugar and volatile composition of acacia, sunflower and tilia honeys

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Food Research International 60, 86-94. (2014)

Abstract

The aim of this study was to evaluate the influence of country (Spain, Romania, and Czech Republic) and botanical origin, on the physicochemical (HMF, diastase activity, moisture content, electrical conductivity), colour (Pfund scale and CIEL*a*b*), principal sugars (glucose, fructose and sucrose) and volatile composition of acacia, sunflower and tilia honeys. PCA analyses considering these variables showed that honey type had a far greater influence on the differentiation of samples (above all due to the presence of certain volatile compounds such as carvacrol and α-terpinene for tilia honey;α-pinene and 3-methyl-2-butanol for sunflower honey, and cis-linalool oxide for acacia honey) than geographical origin. Discriminant models obtained for each kind of botanical honey (classified 100% for acacia and tilia honeys and 93.8% for sunflower of the cross-validated cases) confirmed that differentiation of honeys according to their country was mainly based on volatile compounds (For instance: 2-methyl-2-butenal and 2-methyl-2-propanol, for acacia honeys; 1-hexanol and α-pinene, for sunflower honeys and 3-methyl-1-butanol and hotrienol, for tilia honey) and to a lesser extent on certain physicochemical parameters such as diastase, sucrose and conductivity, respectively. Correct classification of all samples was achieved with the exception of 10% of the sunflower honeys from the Czech Republic. The results suggest that the presented models are potentially useful tools for the classification of acacia, sunflower and tilia honeys according to the country of origin.

Keywords

Acacia honey; sunflower honey; tilia honey; country origin; physicochemical parameters; volatile compounds

1. Introduction

Consumers appreciate the possibility to choose between different unifloral honeys as they have specific organoleptic characteristics and different attributable therapeutic properties. Since these unifloral honeys are part of the import-export market, they offer beekeepers and the industry the opportunity to obtain higher prices in comparison to those without a determined botanical origin. Physicochemical properties and colour are taken into account when the market price of honey is fixed, and they can be measured

to classify and typify the raw batches before entering the packaging process. Specifically, colour is one of the most valuable attributes since it is considered to represent the preferred honey flavour, and therefore directly contributes to consumer acceptability (Visquert et al., 2014).

The physicochemical properties of honeys with the same floral source can vary to some extent as a consequence of different climatic conditions or different geographical origins (Anklam, 1998).The use of botanical appellation of honey together with geographical origin is becoming a good option to protect and promote this traditional food in different countries.

Melissopalynological characterization is commonly used for the classification of honey according to its uniflorality, and sometimes its geographical origin. However, in some cases the percentage of pollen is not always decisive because the production of pollen and nectar by flowers is not always simultaneous, varying between countries and even within the same country according to the geographical area (Feás et al., 2010a; Feás et al., 2010b). For this reason, in addition to the quantification of pollen, the combination of multi-component analysis and chemometric techniques is now the most efficient approach to guarantee the authentication of honey (Anklam, 1998; Terrab et al., 2003; Ruoff et al., 2007; Kropf et al., 2010). Among these procedures, physicochemical (electrical conductivity,diastase activity, moisture, etc.), colour and chemical analyses (such as sugars, among others) have been widely used in the characterization of unifloral honeys (Persano-Oddo & Bogdanow, 2004; Ruoff et al., 2007; Escriche et al., 2011;Oroian et al., 2013).

However, the discriminative power of the physicochemical properties and colour varies according to the botanical origin, and the geographical and climatic conditions as a consequence of their influence on the flowering or secretions of plants. For this reason, as suggested by Persano-Oddo & Bogdanow, 2004, the broader the analytical scope considered, the more accurate the classification of a specific honey.

Hence, considering that the flavour and aroma of honey are directly related to its volatile compounds, it is reasonable to consider that volatile fraction analysis could be of great importance to reach a better understanding of the intrinsic characteristics of honey (Cuevas-Glory et al., 2007; Aliferis et al., 2010). The importance of this analytical determination on its own or as a complement to the information provided by other methodologies is reflected in different studies published in the last decade (Radovic et al., 2001; Serra-Bonvehí & Ventura-Coll, 2003).

There are many works focused on the characterization of honey from different botanical or geographical origins. However, to our knowledge there are no publications about the combined use of physicochemical, sugar and volatile composition for this purpose, nor the comparison of specific unifloral honeys (with the same botanical origin), from

different countries. For this reason, the aim of this study was to determine the influence of the country (Spain, Romania, and Czech Republic) on the physicochemical, sugar and volatile composition of acacia, sunflower and tilia honeys.

2. Materials and Methods

2.1.-Honey samples

A total of 80 raw unifloral honey samples (collected from beekeepers in 2011) with different botanical origins: Acacia (*Robinia pseudoacacia*), sunflower (*Helianthus annuus)* and tilia or lime (*Tilia sp*), and from different countries (Spain, Romania, and the Czech Republic) were analysed. The acacia and sunflower honeys came from the three countries mentioned above, whereas tilia honey was only from Romania and the Czech Republic since it is practically inexistent in Spain. In summary, of the 80 raw samples, 30 came from Romania (10 acacia, 10 sunflower and 10 tilia, all of them from the Transylvanian region); another 30 came from the Czech Republic (10 acacia, 10 sunflower and 10 tilia, all of them from the Central Bohemian region) and 20 from Spain (10 acacia from northern Spain and 10 sunflower from central Spain).

In order to guarantee the botanical origin of the samples, the percentage of pollen was measured for each one, following the recommendations of the International Commission for Bee Botany (Von Der Ohe et al., 2004). A light microscope (Zeiss Axio Imager, Göttingen, Germany) at a magnification power of x 400 with DpxView LE image analysis software attached to a DeltaPix digital camera was used in this analysis. According to this analysis, a honey was considered to be from acacia trees if the pollen from *Robinia pseudoacacia* L. was not lower than 45%; from sunflower, if the pollen from *Helianthus annuus* L. was not lower than 60% and from tilia trees if the pollen from *Tilia* spp. was not lower than 45% (Saenz & Gómez, 1999; Gómez-Pajuelo, 2004; Von Der Ohe, et al., 2004;Persano-Oddo & Piro, 2004). Samples were classified on arrival at the laboratory and were preserved at 12ºC until they were analysed. None of the samples exhibited signs of fermentation or granulation before initiating the analyses.

2.2.-Analytical determinations

2.2.1. Physicochemical and colour analyses

Diastase activity (*Phadebas method*), 5-Hydroxymethylfurfural content "HMF*" (White method),* electrical conductivity (by conductimetry), and moisture content (by refractrometry) were analysed in accordance with the Harmonized Methods of the European Honey Commission (Bogdanov, 2002). Colour was determined using a millimetre Pfund scale C 221 Honey Color Analyzer (Hanna Instruments) and a spectrocolorimeter Minolta CM-3600d (Osaka, Japan). Translucency was determined by applying the Kubelka-Munk theory for multiple scattering of the reflection spectra (Hutchings, 1999). Colour coordinates were obtained from R∞, between 400 and 700 nm for D65 illuminant and 2º observer. All tests were performed in triplicate.

Chromatic parameters Chroma (eq. 1) and hue (eq. 2), were calculated from L*, a* and b* coordinates.

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \qquad \text{(eq.1)}$$

$$h^*_{ab} = arctg \frac{b^*}{a^*} \qquad \text{(eq.2)}$$

2.2.2.Sugar determination

Sugar (fructose, glucose and sucrose) analysis was carried out as described by Bogdanov et al. (1997). Separation of carbohydrates took place in a HPAEC-PAD high-resolution ionic chromatograph with a pulsed amperometric detector (PAD) (Bioscan, Methrom, Switzerland) and a Metrosep Carb chromatographic column (styrene divinylbenzene copolymer, 4.6 x 250 mm). Carbohydrates were eluted with NaOH 0.1N at a flow rate of 1 mL min^{-1}. Quantification of sugars was carried out using external standards. The corresponding calibration curves were constructed covering the values of the three sugars which were expected to be found in the honey samples. For fructose, glucose and sucrose, respectively, the correlation coefficients (R^2) were: 0.995, 0.996 and 0.996; the LODs (limit of detection) were: 0.01g/100g,0.01g/100g and 0.05g/100g and the LOQs (limit of quantification) were: 0.05g/100g, 0.05g/100g and 0.1g/100g.

All analyses were carried out in triplicate.

2.2.3. Volatil compounds analysis

Extraction

Volatile compounds were extracted by purge and trap at 45ºC for 20 minutes and trapped in a glass tube packed with Tenax TA (20-35 mesh), bubbling purified nitrogen (100 mL min^{-1}) through the sample (Escriche et al., 2011). Next, the compounds were thermally desorbed at 220ºC for 10 minutes (at 10 mL min^{-1} helium flow) (TurboMatrix TD, Perkin ElmerTM, CT-USA), then cryofocused in a cold trap at -30ºC and transferred onto the capillary column by heating the cold trap to 250ºC (at a rate of 99ºC/s).

GC-MS analysis

A GC-MS (Finnigan TRACETM MS, TermoQuest, Austin, USA) with a DB-WAX capillary column (SGE, Australia) (60 m length, 0.32 mm i.d., 1.0 µm film thickness) was used to separate the volatile compounds. The carrier gas was Helium at a flow rate of 1 mL min-1. The temperature programme was: from 40ºC (2-minute hold time) to 190ºC at 4ºC min^{-1} (11-minute hold time) and finally to 220ºC at 8 ºC min^{-1} (8-minute hold time). Electron impact mass spectra were recorded in impact ionization mode at 70 eV, with a mass range of m/z 33-433. A total of 3 extracts were obtained for each sample.

2-pentanol was used as an internal standard. The identification of isolated volatile compounds was performed by comparing their mass spectra, retention times and linear retention indices against those obtained from authentic standards: acetic acid (ethanoic acid);nonanal; decanal; benzaldehyde; 6-methyl-5-hepten-2-one (6-methyl-hept-5-en-2-one); 2-methyl-3-buten-2-ol (Sigma-Aldrich, San Louis, Missouri and Acros Organics, Geel, Belgium); 2-methyl-1-propanol (2-methylpropan-1-ol); 3-methyl-3-buten-1-ol; octane; 3-hydroxy-2-butanone (3-hydroxybutan-2-one); 2-furanmethanol (furan-2-ylmethanol); furfural (furan-2-carbaldehyde); dimethyl sulphide; β-linalool (3,7-dimethylocta-1,6-dien-3-ol) (Fluka Buchs, Schwiez, Switzerland). The compounds, for which it was not possible to find authentic standards, were tentatively identified by comparing their mass spectra (m/z values of the most important ions) with spectral data from the National Institute of Standards and Technology 2002 library, as well as retention indices and spectral data published in the literature(Kondjoyan & Berdagué, 1996; Radovic et al., 2001; Soria et al., 2004; De la Fuente et al., 2005; Bianchi et al., 2005; Alissandrakis et al., 2005). A mixture of a homogenous series of alkanes (C8-C20 by Fluka Buchs, Schwiez, Switzerland) was injection into the Tenax in the same temperature-programmed run, as described above in order to determine the Kovàts retention indices of all the compounds. Due to fact that was not possible to obtain authentic commercial standards for all the identified compounds, the variables used in the statistical analysis for differentiation between honeys corresponded to semiquantified compounds. These data were calculated (µg/100 g of honey) using the amount of internal standard, the relative area

between the peak areas of each compound and the peak area of the internal standard, assuming a response factor equal to one (Castro-Vazquez et al., 2009).

2.3.-Statistical analysis

A multifactor analysis of variance (ANOVA) (using Statgraphics Centurion for Windows) was carried out to study the influence of the type of honey and the country of harvesting on the physicochemical parameters, colour, sugars and volatile compounds. The method used for multiple comparisons was the LSD test (least significant difference) with a significance level $\alpha = 0.05$. In addition, data were analyzed using a Principal Component Analysis (PCA) applying the software Unscrambler X.10 and a Stepwise Linear Discriminant Analyses (SLDA) using "forward" procedure (SPSS 16.0). This analysis selects the variables that allow differentiation between honeys. The classification functions corresponding to each group of honeys were calculated. The statistical F function was used as a criterion for variable selection.

3. Results and Discussion

3.1.-Physicochemical, colour and sugar analyses

Table 1 shows the results of the analysis of the three types of honey harvested in the different countries: the average values and standard deviation of the physicochemical parameters (HMF, diastase activity, moisture content, electrical conductivity); colour (Pfund and CIEL*a*b*); the percentage content of the principal sugars (glucose, fructose and sucrose) and the fructose/glucose ratio. In addition, this table shows the ANOVA results (F-ratio and significant differences) obtained for the factors "type of honey" and "country". For the country factor, each type of honey was considered separately.

Although raw honey was used, hydroximethylfurfural (HMF) was evaluated to corroborate the freshness. All the analyzed honeys complied with the international maximum limit of 40 mg/kg (Council Directive 2001/110 relating to honey, 2002). Acacia honey had the lowest average values (from 3.3 to 7.2mg/kg), and sunflower honey,

especially from Romania, and the Czech Republic, had surprisingly high average values (23.4 and 21.9mg/kg, respectively), taking into account that they were fresh, non-thermally treated samples. These values are in accordance with Kádár et al. (2010) and Oroian (2012).

Diastase is one of the most important enzymes in honey. Its concentration varies not only according to its botanical origin, but also due to aging and extreme temperatures (Fallico et al., 2006). In this study samples ranged from 8.7 ºGoethe in acacia honey from the Czech Republic to 19.1 ºGoethe for Spanish sunflower honey. All the samples complied with the Council Directive 2001/110 relating to honey (2002), which stipulates that these types of honeys should have a value higher than 8ºGoethe. The only exception is acacia honey for which a minimum of 3.1ºGoethe is admitted, as it is considered to have low enzyme content. However, in this paper such low values were not found in any of the analyzed acacia honeys.

Honey moisture content, which can vary from year to year, depends not only on environmental conditions, but also on beekeeping practices (Acquarone et al., 2007). Taking into account the fact that the moisture content of honey has to be lower than 20 g/100g (Council Directive 2001/110 relating to honey, 2002), the values obtained in this work were satisfactory as they ranged from 15.3g/100g in sunflower honey from Spain to 17.5g/100g in tilia honey from Romania. Spanish acacia and sunflower honeys showed the best moisture values, lower than 16g/100g.

As expected, tilia honey had the highest levels of conductivity, with average values of 0.80 and 0.50 mS/cm from the Czech Republic and Romania, respectively. Values higher than 0.80mS/cm are not acceptable for floral honeys in general; however there are some specific honeys that can exceed this value. This is the case of tilia honey and others such as Calluna, Erica or Arbustus, because of the mineral content of these honeys. On the contrary, the low level of minerals in acacia honey is reflected by its low electrical conductivity (0.17-0.19 mS/cm) (Feás et al., 2010a). No significant differences were observed between countries.

Table.1 Physicochemical parameters, colour and principal sugars (average values and standard deviation) in acacia, sunflower and tilia honeys harvested in different countries: Spain (Sp), Romania (Ro), and Czech Republic (Cz). ANOVA results (F-ratio and significant differences) obtained for two factors: country and type of honey. For the country factor, each type of honey was considered separately.

Physico-chemical Parameters	COUNTRY FACTOR											TYPE OF HONEY FACTOR			
	Acacia				Sunflower				Tilia						
	Sp	Ro	Cz	F-ratio	Sp	Ro	Cz	F-ratio	Ro	Cz	F-ratio	Acacia	Sunflower	Tilia	F-ratio
HMF (mg kg^{-1})	3.3(1.8)	7.2(11.9)	3.3(2.2)	0.79ns	16.4(3.5)	23.4(0.4)	21.9(7.9)	0.57ns	7.1(6.5)	18.8(14.9)	2.22ns	4.8(7.6)	21.3(7.0)	15.5(13.9)	15.17***
Diastase activity (ºGoethe)	17.3(4.8)	10.4(4.6)	8.7(1.7)	9.93***	19.1(1.2)	10.1(0.8)	11.9(3.7)	4.33*	8.8(0.9)	14.6(5.2)	4.66ns	11.3(4.9)	12.7(4.2)	12.9(5.1)	0.66ns
Moisture (g/100g)	15.9 (0.2)	16.9(1.5)	17.0(1.0)	1.91ns	15.3(0)	17.3(0.14)	16.6(1.04)	2.33ns	17.5(0.12)	16.4(1.05)	3.92ns	16.7(1.16)	16.5(1.0)	16.7(1.1)	0.24ns
Conductivity (μS cm^{-1})	0.19(0.04)	0.17(0.03)	0.17(0.08)	0.32ns	0.44(0.0)	0.35(0.0)	0.43(0.12)	0.52ns	0.50(0.08)	0.80(0.12)	18.29**	0.17(0.05)	0.42(0.10)	0.71(0.17)	106.37***
Colour															
Pfund	9.1(2.5)	10.6 (2.2)	4.3 (1.3)	1.7ns	66.7 (0.5)	51.0 (3)	53.2 (14.6)	0.86ns	37.3(3.9)	42.2(17)	0.15ns	6.9(4.3)	56.3(12.6)	40.8(13.7)	24.8***
L*	50.5(4.3)	56.6(5.8)	54.6(1.8)	2.22ns	43.6(3.4)	48.1(0.1)	44.9(3.5)	0.53ns	49.6(1.2)	48.3(7.6)	0.05ns	54.1(4.7)	44.9(3.3)	48.6(6.2)	9.86***
Chroma (C^*_{ab})	19.6(3.1)	17.8(5.9)	17.8(2.6)	1.60ns	22.9(4.6)	27.4(2.5)	23.8(5.2)	0.27ns	27.2(1.3)	24.5(4.3)	0.68ns	17.3(4.4)	24.1(4.5)	25.4(3.7)	10.36***
Hue (h^*_{ab})	84.4(9.1)	93.2(7.2)	94.9(3.6)	2.96ns	70.4(2.8)	78.5(3.4)	74.6(8.3)	1.56ns	82.7(2.,2)	81.2(7.9)	0.06ns	91.3(7.7)	74.0(6.9)	81.6(6.2)	14.98***
Sugars (g/100g)															
Glucose	26.8(2.7)	26.9(2.8)	31.0(4.5)	7.86**	37.1(0.8)	33.9(1.62)	38.3(7.2)	1.33ns	29.7(0.8)	33.1(1.4)	18.8***	28.5(4.4)	36.3(6.6)	32.2(2.02)	24.49***
Fructose	40.2(3.6)	45.7(2.8)	49.2(6.6)	8.16**	39.3(0.6)	39.5(0.98)	43.0(6.9)	0.68ns	41.3(1.6)	41.9(1.4)	0.38ns	45.2(6.)	40.3(6.1)	41.7(1.5)	3.16ns
Sucrose	2.2(0.8)	1.7(0.6)	1.6 (0.3)	1.63ns	1.04(0.54)	0.60(0.37)	0.7(0.9)	4.92*	0.9(0.4)	1.5(0.1)	0.15ns	1.7(0.5)	0.8(0.5)	0.3(0.2)	7.80**
Fructose/Glucose ratio	1.5(0.1)	1.7(0.2)	1.5 (0.1)	4.49*	1.0(0.1)	1.16(0.02)	1.1(0.1)	3.48ns	1.3(0.1)	1.2(0.03)	38.68***	1.6(0.17)	1.06(0.07)	1.3(0.06)	68.28***

ns: Non significant; * p<0.05; ** p<0.01; ***p<0.001

With regard to colour, semi-qualitative Pfund scale and colour coordinates CIEL* a* b* were measured (Table 1). CIEL* a* b*colour coordinates, and chromatic parameters (hue and chroma), are not commonly regulated. However, they are often used in research studies to supplement the information provided by the Pfund scale. In this work Pfund values ranged from 4.3 mm for the acacia honey from the Czech Republic to 66.7 mm for sunflower honey from Spain. Acacia honey is characterized by a very light colour together with low conductivity. This is logical as honey colour is mainly related to mineral content. Light coloured honeys usually have low mineral levels, while dark coloured honeys normally have high mineral content (Al et al., 2009).

In relation to CIEL* a* b* values, acacia honey had the highest lightness (especially from Romania: L*= 56.6), a yellowish hue (average value of 91.3) and the lowest chroma (average value of 17.3) which is associated with the lowest colour purity. On the contrary, sunflower honey was the darkest (lowest L*, with an average value of 44.9), with the same chroma as tilia honey and the lowest hue of the three types of analyzed honeys: 74.0, 81.6 and 91.3 for sunflower, tilia and acacia, respectively. In general, the tilia honey had intermediate L* values (average= 48.6), lower than those found by Kropf, et. al., 2010 (between 60.3 and 62.3), who analyzed this type of honey from three different geographical regions of Slovenia.

In general, the colour values obtained with the Pfund scale as well as CIEL*a*b, were as expected for these varieties of honey (Persano-Oddo et al., 1995; Piazza& Persano-Oddo, 2004).

The sugar composition of honey depends of the type of flowers used by the bees, and therefore varies according to the type of honey and geographical and climatic conditions (Mateo & Bosch-Reig, 1998; Al et al., 2009; Kaskonienè et al., 2010). For this reason, the level of some sugars and even the ratios between them are used to ascertain honey authenticity (Nozal et al., 2005). As expected, fructose was the most dominant sugar followed by glucose in all cases (Persano-Oddo & Piro, 2004). Acacia had high fructose (49.2g/100g for acacia from the Czech Republic) and low glucose content (26.8g/100g for the acacia honey from Spain). Acacia honeys showed the highest sucrose content, as reported by Persano-Oddo et al. in 1995. In this study the Spanish acacia had the highest sucrose level: 2.2 g/100g. Sunflower had a very high glucose level (average=36.3 g/100g) compared to both the other honeys and therefore a very low F/G ratio (average=1.06).

In respect to the fructose-glucose ratio F/G ratio, acacia and tilia honeys are characterized by high F/G values in contrast to sunflower honeys, as reported in previous works (Persano-Oddo et al., 1995) and as established by European legislation (Council Directive 2001/110 relating to honey, 2002). The values obtained in the present work (averages=1.6, 1.3 and 1.06 for acacia, tilia and sunflower) are in accordance with these.

Besides that, it is important to point out that the fructose-glucose ratio (F/G) indicates whether a honey will granulate; the lower the ratio, the quicker the crystallization.

Accordingly, the order of crystallization of the three types of honey analyzed in this study is: sunflower honey (F/G= 1.06), tilia honey (F/G= 1.3), and acacia honey (F/G= 1.6).

Almost all the physicochemical, colour and sugar parameters differed significantly between the three botanical types of honey studied. However, considering each type of honey separately, significant differences between countries were only found for diastase activity (for acacia and sunflower honeys), conductivity (for tilia honey) as well as for some sugars (glucose for acacia and tilia, fructose for acacia, and sucrose for sunflower). In the same way, the F/G ratio differed significantly between countries for acacia and tilia.

In order to evaluate the global effect of the type of honey on the physicochemical parameters, colour, and F/G ratio from a descriptive point of view, a principal component analysis (PCA) was performed. Figure 1 shows the PCA bi-plot of scores and loading obtained considering the eight analysed honeys and the different parameters. The values of HMF and moisture were not taken into account, as both are mainly related to the quality of honey and not to the botanical origin, and therefore are not useful for differentiation between honeys. This analysis was carried out considering the average values of each parameter obtained from each type of honey and country (the code for each point in the figure corresponds to: kind of honey-country). In the score plot, proximity between samples reflects similarity in relation to the analysed parameters.

Two principal components explained 74% of the variations in the data set: PC1 (55%) and PC2 (19%). The first principal component differentiates the three kinds of honeys to a certain extent. Acacia located on the left was differentiated clearly from the others, while sunflower and tilia on the right, are not so obviously separated from each other. This indicates that although the botanical origin of honey has an influence on the parameters studied, these are not sufficient for differentiation between the three varieties studied here. On the other hand, the country seems to imply a minor effect on the analysed parameters as the samples were principally grouped according to type of honey.

3.2.-Volatile compounds

The average values and standard deviation of the volatile compounds analyzed in the three types of honey harvested in the different countries are showed in Table 2. Of the 51 identified compounds, only 17 compounds in acacia honey, 9 in sunflower and 8 in tilia honeys, showed significant differences between countries. However, considering the type of honey as a factor, significant differences were found for 45 volatile compounds.

Another PCA (Figure 2) was conducted to evaluate the global effect of the type of honey and country, but in this case for the volatile compounds. The distribution of the samples was similar but clearer than the previous bi-plot obtained from the FQ parameters. In this case, the first principal component clearly differentiates acacia honey (bottom left

quadrant) from tilia (bottom right quadrant) and the second principal component differentiates quite well between sunflower (upper quadrants) and acacia and tilia honeys (lower quadrants).

The loading plot shows that certain compounds are to some extent responsible for this differentiation. This is the case of compounds such as carvacrol (Lusic et al., 2007; Plutowska et al., 2011) and α-terpinene (Radovic et al, 2001) which were attributed as markers for tilia honey, and were only found in this kind of honey in this work. Plutowska et al. (2011), also only identified α-terpinene in tilia honeys, when analyzing 7 varieties of honeys. The same occurs for other compounds, such asα-pinene and 3-methyl-2-butanol in the case of sunflower, and cis-linalool oxide in the case of acacia which were essential in this work to differentiate these honeys. This is in line with (Radovic et al., 2001) for these two varieties of honey.

The aforementioned authors reported phenylacetaldehyde as a typical volatile compound for acacia honeys and phenylethyl alcohol for tilia honey, though this is not consistent with this study, nor with others (Plutowska et al., 2011).

As observed before for physicochemical parameters, volatile compounds seem to contribute more to the differentiation of honey according to botanical origin, than country of origin.

However, it is logical that honeys with the same botanical origin (*Robinia pseudoacacia* in the case of acacia honeys, *Helianthus annuus* in the case of sunflower honeys and *Tilia* sp in the case of tilia honeys), but from different countries are relatively similar. Nevertheless, there are obvious differences between the geographic sources which could be attributed to climatic conditions, but above all to the surrounding flora. The nectar of other plants may contribute to the variability of the analysed parameters: physicochemical, sugar and volatile compounds. This should not be considered a negative aspect; instead it confers a certain singularity to the same type of honey with different geographical origins.

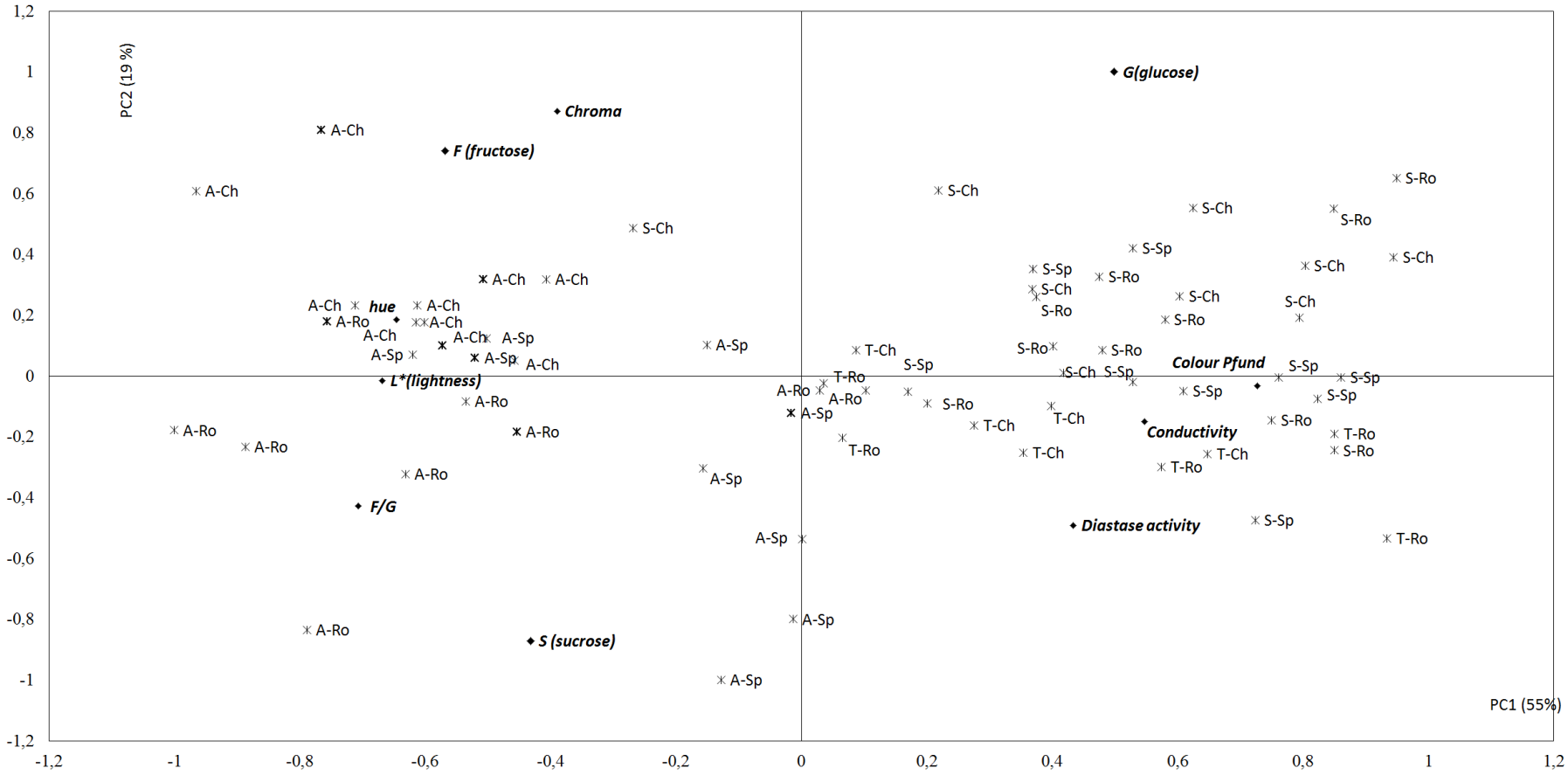

Figure 1. Biplot for the two principal components of the PCA model for the physicochemical parameters, sugars (fructose "F", glucose "G" sucrose "S" and F/G ratio) and colour (Pfund and CIEL*a*b) in acacia, sunflower and tilia honeys harvested in the different countries: Spain (Sp), Romania (Ro), and Czech Republic (Cz).

Table.2. Volatile compounds (average values and standard deviation) in acacia, sunflower and tilia honeys harvested in different countries: Spain (Sp), Romania (Ro), and Czech Republic (Cz). ANOVA results (F-ratio and significant differences) obtained for two factors: country and type of honey. For the country factor, each type of honey was considered separately.

COMPOUNDS	RI	COUNTRY FACTOR											TYPE OF HONEY FACTOR			
		ACACIA				SUNFLOWER				TILIA			AC	SUN	TIL	ANOVA F ratio
		Sp	Ro	Cz	ANOVA F ratio	Sp	Ro	Cz	ANOVA F ratio	Ro	Cz	ANOVA F ratio				
ACIDS																
Ethanoic acid	1584	0.01(0.02)	<0.001	0.03(0.04)	3.80*	0.12(0.06)	0.19(0.13)	0.22(0.18)	0.5ns	0.02(0.02)	0.13(0.12)	3.17ns	0.01c	0.20a	0.10b	15.11***
Propanoic acid 2-methyl-	1697	0.06(0.02)	0.04(0.04)	0.01(0.01)	5.93***	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.03a	0.00b	0.00b	15.53***
ALDEHYDES																
3-Methyl-butenal	935	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.21(0.12)	0.19(0.08)	0.20ns	0.00b	0.00b	0.19a	84.27***
2-Pentanal	937	0.03(0.01)	0.04(0.02)	0.02(0.00)	2.60ns	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.03a	0.00b	0.00b	61.41***
2-Methyl-2-butenal	1129	0.06(0.03)	0.01(0.00)	0.02(0.01)	10.70***	0.13(0.01)	0.09(0.0)	0.14(0.18)	0.09ns	0.02(0.04)	0.13(0.06)	9.80**	0.02b	0.13a	0.02b	9.70***
3-Methyl-2-butenal	1236	0.07(0.02)	0.08(0.12)	0.06(0.01)	0.18ns	0.13(0.01)	0.05(0.00)	0.07(0.06)	1.23ns	0.02(0.00)	0.02(0.01)	0.24ns	0.07a	0.08a	0.09a	0.67ns
Octanal	1417	0.01(0.00)	0.01(0.00)	<0.001	2.69ns	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.01a	0.00b	0.00b	52.91***
Nonanal	1523	0.07(0.01)	0.06(0.04)	0.04(0.01)	2.27ns	0.02(0.01)	0.03(0.0)	0.03(0.02)	0.19ns	0.09(0.02)	0.18(0.13)	1.50ns	0.05b	0.03b	0.15a	14.73***
Decanal	1630	0.01(0.00)	0.03(0.01)	0.02(0.00)	8.11**	0.04(0.0)	0.04(0.0)	0.03(0.02)	0.03ns	0.04(0.00)	0.04(0.02)	0.48ns	0.02b	0.03a	0.04a	7.34**
Benzaldehyde	1675	0.13(0.06)	0.25(0.15)	0.20(0.05)	2.15ns	0.12(0.02)	0.14(0.01)	0.13(0.09)	0.02ns	0.14(0.05)	0.37(0.43)	1.04ns	0.2ab	0.13b	0.31a	2.42ns
ALCOHOLS																
2-Methyl-2-propanol	920	0.03(0.02)	0.04(0.04)	0.10(0.02)	8.07**	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.06a	0.00b	0.00b	25.74***
2-Propanol	947	0.03(0.01)	0.10(0.12)	0.02(0.01)	2.91ns	0.25(0.14)	0.19(0.01)	0.21(0.12)	0.11ns	<0.001	<0.001	-	0.05b	0.21a	0.00c	26.23***
Ethanol	956	0.40(0.20)	0.56(0.75)	0.38(0.33)	0.32ns	0.51(0.35)	0.38(0.06)	0.79(0.98)	0.23ns	1.25(0.24)	0.73(0.56)	3.01ns	0.45b	0.69ab	0.88a	2.25ns
2-Butanol	1047	0.20(0.14)	0.03(0.02)	0.05(0.13)	5.19*	0.73(0.90)	0.01(0.0)	0.12(0.21)	3.11ns	0.25(0.08)	0.06(0.02)	50.84***	0.08a	0.19a	0.11a	1.2ns
2-Methyl-3-buten-2-ol	1063	0.11(0.05)	0.11(0.12)	0.16(0.06)	0.89ns	<0.001	<0.001	<0.001	-	0.62(0.48)	0.14(0.06)	10.29**	0.13b	0.00c	0.28a	8.84***
2-Methyl-3-buten-1-ol	1062	<0.001	<0.001	<0.001	-	0.26(0.00)	0.20(0.06)	0.21(0.21)	0.08ns	0.31(0.07)	0.30(0.12)	0.01ns	0.00b	0.21a	0.00b	30.8***
2-Methyl-1-propanol	1119	0.05(0.02)	0.07(0.07)	0.05(0.02)	0.47ns	0.05(0.0)	0.01(0.0)	0.01(0.01)	5.60*	0.27(0.08)	0.16(0.10)	3.52ns	0.06b	0.01a	0.19a	28.99***
3-Methyl-2-butanol	1137	<0.001	<0.001	<0.001	-	0.42(0.04)	0.25(0.01)	0.36(0.04)	6.2*	0.01(0.00)	0.00(0.00)	1.16ns	0.00b	0.36a	0.00b	61.12***
1-Butanol	1175	0.10(0.03)	0.10(0.10)	0.08(0.02)	0.34ns	0.33(0.27)	0.15(0.01)	0.39(0.37)	0.41ns	0.11(0.02)	0.06(0.05)	2.55ns	0.09b	0.35a	0.08b	11.56***
3-Methyl-1-butanol	1233	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.61(0.04)	0.30(0.10)	32.03***	0.00b	0.00b	0.39a	114.95***
2-Penten-1-ol	1268	0.00(0.00)	0.14(0.10)	0.05(0.04)	8.18**	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.07a	0.00b	0.00b	10.72***
3-Methyl-3-buten-1-ol	1277	0.19(0.04)	0.11(0.08)	0.16(0.04)	1.59ns	0.44(0.02)	0.21(0.01)	0.50(0.21)	1.93ns	0.31(0.02)	0.30(0.11)	0.01ns	0.15c	0.45a	0.31b	27.38***
2-Heptanol	1449	<0.001	<0.001	<0.001	-	0.03(0.03)	0.01(0.0)	0.04(0.05)	0.37ns	<0.001	<0.001	-	0.00b	0.04a	0.00b	16.78***
2-Methyl-2-buten-1-ol	1449	0.14(0.03)	0.06(0.05)	0.13(0.05)	7.15**	0.09(0.00)	0.16(0.0)	0.15(0.09)	0.42ns	0.16(0.01)	0.19(0.13)	0.09ns	0.11b	0.14ab	0.19a	3.96*
1-Hexanol	1476	<0.001	<0.001	<0.001	-	0.10(0.0)	0.01(0.0)	0.01(0.01)	36.67***	0.02(0.00)	0.04(0.04)	0.52ns	0.00b	0.03a	0.03a	12.69***
3-Hexen-1-ol	1511	0.01(0.00)	0.03(0.04)	0.00(0.00)	2.97ns	<0.001	0.02(0.0)	0.01(0.0)	13.07**	<0.001	<0.001	-	0.018a	0.012ab	0.00b	3.54*

Table 2 (cont.)

COMPOUNDS	RI	COUNTRY FACTOR											TYPE OF HONEY FACTOR			
		ACACIA				SUNFLOWER				TILIA			AC	SUN	TIL	ANOVA F ratio
		Sp	Ro	Cz	ANOVA F ratio	Sp	Ro	Cz	ANOVA F ratio	Ro	Cz	ANOVA F ratio				
KETONES																
Acetone	836	0.45(0.27)	0.28(0.16)	0.09(0.01)	9.35**	0.33(0.05)	0.45(0.12)	0.23(0.08)	5.32*	0.85(0.27)	1.05(0.51)	0.54ns	0.24b	0.27b	0.99a	35.41***
2-Butanone	921	<0.001	<0.001	<0.001	-	0.66(0.56)	0.17(0.03)	1.19(1.72)	0.40ns	0.10(0.03)	0.42(0.18)	10.73**	0.00b	0.97a	0.33b	7.38**
2-Pentanone	1003	<0.001	<0.001	<0.001	-	0.13(0.11)	0.00(0)	0.26(0.32)	0.74ns	0.37(0.05)	0.57(0.31)	1.65ns	0.00c	0.21b	0.51a	30.11***
3-Hepten-2-one	1020	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.03(0.00)	0.08(0.04)	4.65ns	0.00b	0.00b	0.07a	46.32***
2-Heptanone	1212	<0.001	<0.001	<0.001	-	0.02(0.00)	0.01(0.00)	0.00(0.0)	1.48ns	<0.001	<0.001	-	0.00b	0.01a	0.00b	20.64***
3-Hydroxy-2-butanone	1425	0.06(0.03)	0.10(0.18)	0.01(0.00)	1.69ns	0.25(0.02)	0.10(0.02)	0.05(0.05)	13.43**	0.19(0.05)	0.31(0.16)	2.26ns	0.05b	0.08b	0.28a	16.42***
6-Methyl-5-hepten-2-one	1469	0.00(0.00)	0.01(0.01)	0.00(0.01)	0.80ns	0.02(0.0)	0.0(0.0)	0.19(0.0)	24.88***	0.01(0.00)	0.01(0.00)	0.67ns	0.01b	0.018a	0.01b	3.96*
Ethanone-1-4-methyl phenyl	1869	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.06(0.02)	0.32(0.25)	4.07ns	0.001b	0.001b	0.25a	21.65***
HYDROCARBONS																
Octane	802	0.07(0.04)	0.03(0.01)	0.03(0.0)	7.14**	0.12(0.01)	0.06(0.01)	0.04(0.06)	1.51ns	<0.001	<0.001	-	0.04a	0.05a	0.00b	10.2***
Nonane	902	0.01(0.00)	0.01(0.00)	0.01(0.00)	0.38ns	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.01a	0.00b	0.00b	125.97***
n-Decane	1004	0.17(0.05)	0.16(0.05)	0.10(0.03)	5.16*	1.88(0.85)	1.00(0.0)	1.69(1.62)	0.22ns	<0.001	<0.001	-	0.14b	1.62a	0.00b	24.58***
Toluene	1069	0.07(0.02)	0.06(0.05)	0.08(0.02)	0.63ns	<0.001	<0.001	<0.001	-	0.06(0.02)	0.12(0.13)	0.75ns	0.07a	0.00b	0.11a	10.86***
p-Xylene	1164	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	0.00	0.22(0.10)	0.25(0.34)	0.50ns	0.01a	0.00a	0.18a	2.11ns
ESTERS																
Ethyl acetate	909	0.01(0.00)	1.42(1.36)	0.01(0.00)	8.32*	<0.001	<0.001	<0.001	-	1.17(0.91)	1.63(0.92)	0.71ns	0.55b	0.00b	1.50a	10.51***
Acetic acid butyl-ester	1098	0.05(0.02)	0.02(0.03)	0.11(0.05)	11.91***	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.06a	0.001b	0.001b	21.53***
SULFUR COMPOUNDS																
Dimethyl sulphide	<800	0.09(0.06)	0.08(0.08)	0.16(0.06)	3.20ns	0.62(0.16)	0.54(0.17)	0.40(0.20)	1.25ns	0.35(0.08)	0.27(0.23)	0.43ns	0.11c	0.30b	0.45a	21.92***
Dimethyl disulfide	1104	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.08(0.03)	0.32(0.37)	1.54sn	0.00b	0.00b	0.25a	11.63***
FURANES																
Furanmethanol	1576	0.06(0.05)	0.43(0.52)	0.08(0.04)	3.57*	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.21a	0.00b	0.00b	4.84*
Furfural	1606	0.32(0.06)	0.56(0.47)	0.18(0.06)	4.04*	1.13(0.17)	1.38(0.18)	0.71(0.29)	5.93*	1.15(0.11)	1.33(0.94)	0.14ns	0.36c	0.86b	1.28a	16.07***
TERPENES																
Carvacrol	1803	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.45(0.16)	1.32(0.18)	5.2*	0.00b	0.00b	1.07a	20.68***
α-Terpinene	1267	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.45(0.34)	0.10(0.05)	10.94**	0.00b	0.00b	0.20a	14.8***
α-Pinene	1024	<0.001	<0.001	<0.001	-	0.05(0.02)	0.08(0.02)	0.33(0.12)	5.80*	<0.001	<0.001	-	0.00b	0.25a	0.00b	4.58*
Borneol	1822	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.11(0.07)	0.22(0.18)	1.41ns	0.00b	0.00b	0.19a	29.72***
β-Linalool	1670	0.06(0.03)	0.09(0.04)	0.04(0.01)	5.09*	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.07a	0.00b	0.00b	51.32***
Hotrienol	1737	0.71(0.27)	0.39(0.49)	0.07(0.02)	2.66**	0.15(0.04)	0.24(0.03)	0.39(0.40)	0.41ns	0.24(0.02)	0.91(0.48)	7.33*	0.34b	0.33ab	0.72a	2.84ns

ns: Non significant; * p<0.05; ** p<0.01; ***p<0.001

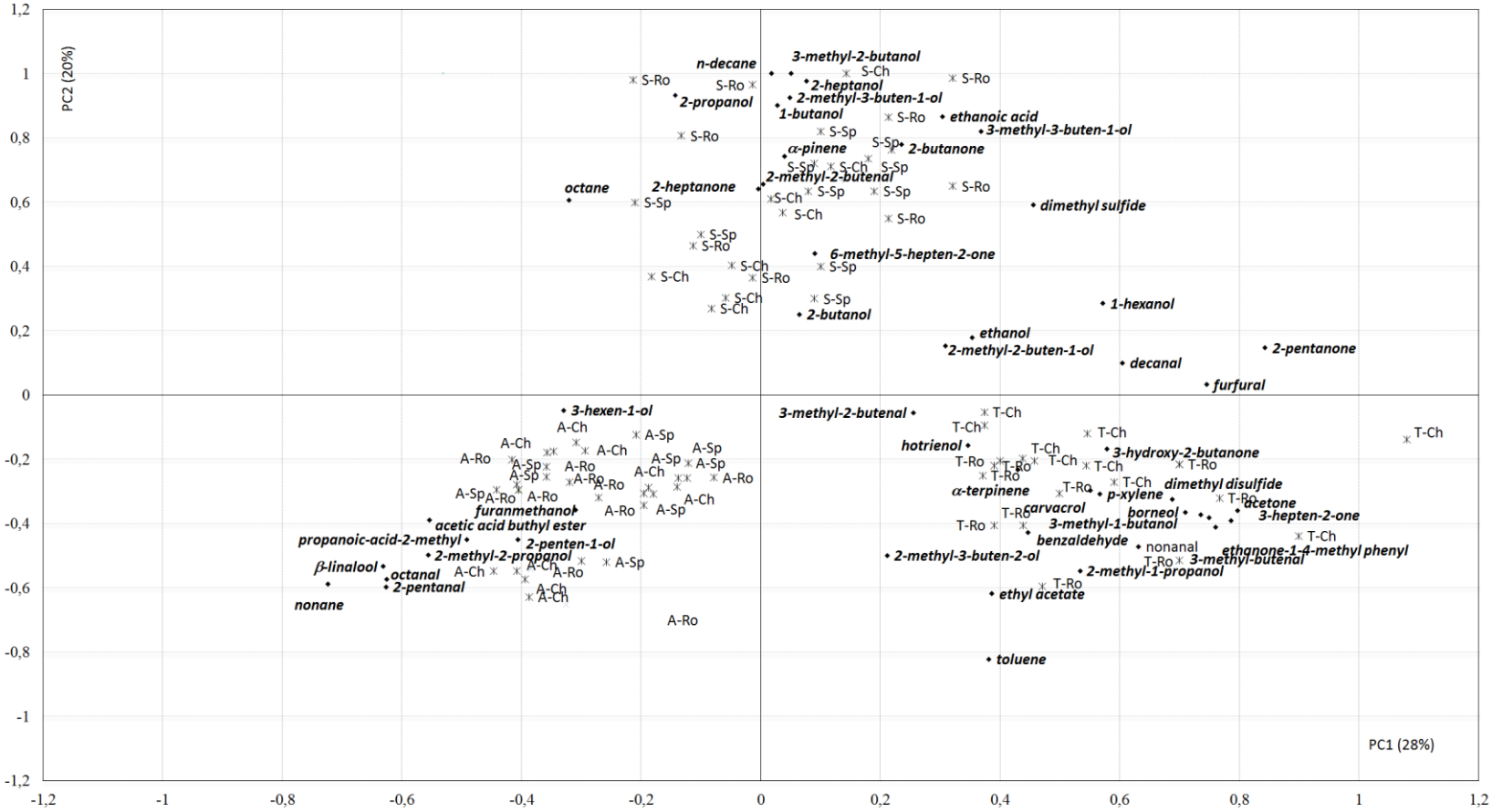

Figure 2. Biplot for the two principal components of the PCA model for the volatiles compounds identified in acacia, sunflower and tilia honeys harvested in the different countries: Spain (Sp), Romania (Ro), and Czech Republic (Cz).

3.3.-Identification of the variables with the highest discriminant power

The information provided by both ANOVA and PCA analyses carried out for physicochemical parameters and volatile compounds, shows that certain variables are to some extent more important in the differentiation of honeys. To discern which variables contribute the most to the differentiation of honeys from different countries but from the same botanical origin a discriminant analysis was applied separately for every botanical type of honey (acacia, sunflower and tilia).

Only the variables with significant differences between countries (in ANOVA results) were included in the models. These models, obtained using the physicochemical and volatile compound variables jointly and applying a stepwise method, permitted the classification of 100% of acacia and tilia honeys and 93.8% of sunflower for the cross-validated cases. Kadar et al. in 2011 reported that a discriminant model obtained with volatile compounds and physicochemical parameters used jointly and applying a cross-validated procedure was effective for the differentiation of two types of honeys (between lemon blossom honey and orange blossom honey).

Table 3 shows the standardized canonical discriminant function coefficients obtained in the selected models for every type of honey. In the construction of the two discriminant functions, different variables were used in each case. Specifically, 7, 6 and 3 volatile compounds and 1 physicochemical parameter (diastase activity, sucrose and conductivity) for acacia, sunflower and tilia honeys, respectively.

The higher the absolute value of a standardized canonical coefficient, the more significant a variable is. The first canonical function was the one that discriminated best between honey groups, given that it represented the highest variability. Accordingly, the variables that most contributed to the discrimination of honeys according to their country of origin were: for acacia honeys (which function 1 explained 88.2% of the total variance), 2-methyl-2-butenal, 2-methyl-2-propanol and acetic acid butyl ester; for sunflower honeys (function 1 explained 94.3%), 1-hexanol, sucrose and α–pinene; and for tilia honey (function 1 explained 100%), 3-methyl-1-butanol, hotrienol y 2-butanone. It should be highlighted that despite the appearance of a physicochemical variable in each model, this was not the one which contributed the most in any case.

Table 3. Standardized canonical discriminant function coefficients

Acacia honey Variables	Function 1 88.8%	Function 2 11.2%
Diastase activity	1.588	1.304
Octane	2.592	0.575
2-Methyl-2-propanol	-2.815	0.533
2-Butanol	0.693	0.443
Acetic acid butyl ester	2.380	-0.905
2-Methyl-2-butenal	4.148	2.179
2-Penten-1-ol	1.699	0.876
2-Methyl-2-buten-1-ol	-1.402	-2.546
Sunflower honey Variables	**Function 1 94.3%**	**Function 2 5.7%**
Sucrose	17.416	9.760
α-Pinene	-16.045	-4.218
2-Methyl-1-propanol	-5.927	7.059
3-Hydroxy-2-butanone	4,744	-4.320
6-Methyl-5-hepten-2-one	11.603	4.685
1-Hexanol	22.218	0.474
3-Hexen-1-ol	5.139	14.851
Tilia honey Variables	**Function 1 100%**	**Function 2**
Conductivity	0.758	
2-Butanone	1.036	
3-Methyl-1-butanol	-2.120	
Hotrienol	1.827	

The classification results (expressed as percentages) of the discriminant analysis carried out by cross validated procedure demonstrated a very good classification of the acacia and tilia honeys according to their country (Table 4). This was also true of sunflower honey from Spain and Romania. However, 10% of sunflower honey from the Czech Republic was incorrectly classified as sunflower honey from Romania.

Table 4. Classification results of the discriminant analysis carried out by cross validated procedure. Percentage of samples well classified by the model. Spain (Sp), Romania (Ro), and Czech Republic (Cz).

	Predicted Group Membership							
Floral and Country origin								
	Acacia Sp	**Acacia Ro**	**Acacia Cz**	**Sunflower Sp**	**Sunflower Ro**	**Sunflower Cz**	**Tilia Ro**	**Tilia Cz**
Acacia Sp	100	0	0	-	-	-	-	-
Acacia Ro	0	100	0	-	-	-	-	-
Acacia Cz	0	0	100	-	-	-	-	-
Sunflower Sp	-	-	-	100	0	0	-	-
Sunflower Ro	-	-	-	0	100	0	-	-
Sunflower Cz	-	-	-	0	10	90	-	-
Tilia Ro	-	-	-	-	-	-	100	0
Tilia Cz	-	-	-	-	-	-	0	100

4. Conclusion

The information obtained about physicochemical parameters and volatile compounds is a useful complement to that provided by the percentage of pollen to distinguish acacia, sunflower and tilia monofloral honeys, with subsequent benefits for beekeepers and the industry. Although it was found that the country (Spain, Romania, and the Czech Republic) may lead to significant variations in the levels of certain parameters and compounds, it is the type of honey that has by far the greatest influence on the differentiation of honeys, above all due to the presence of certain volatile compounds such as carvacrol and α-terpinene in the case of tilia honey, α-pinene and 3-methyl-2-butanol in sunflower honey, and cis-linalool oxide in acacia honey. Discriminant models obtained for each kind of botanical honey confirmed that the differentiation of honeys according to their country of origin was principally based on volatile compounds (2-methyl-2-butenal, 2-methyl-2-propanol, acetic acid butyl ester, etc., for acacia honeys; 1-hexanol, α-pinene, etc., for sunflower honeys and 3-methyl-1-butanol, hotrienol, 2-butanone, etc., for tilia honey) and to a lesser extent on certain physicochemical parameters such as, diastase, sucrose and conductivity, respectively.

A correct classification of all the samples was achieved with the exception of 10% of the sunflower honeys from the Czech Republic. The main advantage of the model presented

is to support the classification of the acacia, sunflower and tilia honeys according to the country of origin. In order to be totally conclusive, it would be advisable to check the predictive capacity of the proposed classification model with additional batches with the same botanical and country origin but from different years.

5. References

Acquarone C., Buer, P. & Elizalde B. (2007). Pattern of pH and electrical conductivity upon honey dilution as a complementary tool for discriminating geographical origin of honeys. Food Chemistry, 101, 695-703.

Al M.L., Daniel D., Moise A., Bobis O., Laslo L., & Bogdanov S. (2009). Physico-chemical and bioactive properties of different floral origin honeys from Romania. Food Chemistry. 112 (4), 863–867.

Aliferis K.A., Ttarantilis P.A., Harizanis P.C., & Alissandrakis E. (2010). Botanical discrimination and classification of honey samples applying gas chromatography/mass spectrometry fingerprinting of headspace volatile compounds. Food Chemistry, 121, 856-862.

Alissandrakis E., Tarantilis P.A, Harizanis P.C. & Polissiou M. (2005). Evaluation of four isolation techniques for honey aroma compounds. Journal of the Science of Food and Agriculture, 85, 91-97.

Anklam E., (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. Food Chemistry, 63, 549-562.

Bianchi F., Careri M. & Musci M. (2005). Volatile norisoprenoids as markers of botanical origin of Sardinian strawberry-tree (Arbutus unedo L.) honey: characterisation of aroma compounds by dynamic headspace extraction and gas chromatography–mass spectrometry. Food Chemistry, 89, 527–535.

Bogdanov S., Martin P. & Lüllman C. (1997). Harmonised methods of the European Honey Commission. Apidologie, Extra Issue, 1–59.

Bogdanov S. (2002). Harmonized methods of the International Honey Commission. Swiss Bee Research Centre, FAM, Liebefeld, CH 3003 Bern, Switzerland.

Castro-Vázquez L., Diaz-Maroto M. C., González-Viñas M. A. & Pérez-Coello M. S. (2009). Differentiation on monofloral citrus, rosemary, eucalyptus, lavender, thyme and heather honeys based on volatile composition and sensory descriptive analysis. Food Chemistry, 112(4), 1022-1030.

Council Directive 2001/110 relating to honey (2002). Official Journal of the European Communities, 12.1.2002.

Cuevas-Glory L.F., Pino J.A., Santiago L. S., & Sauri-Duch E. (2007). A review of volatile analytical methods for determining the botanical origin of honey.Food Chemistry, 103, 1032–1043.

De la Fuente E., Martinez-Castro I. & Sanz J. (2005). Characterization of Spanish unifloral honeys by solid phase microextraction and gas chromatography-mass spectrometry. Journal of Separation Science 28, 1093-1100.

Escriche I., Kadar M., Juan-Borrás M. & Domenech E. (2011). Using flavonoids, phenolic compounds and headspace volatile profile for botanical authentication of lemon and orange honeys. Food Research International, 44 (5), 1504-1513.

Fallico B., Arena E., Verzera A. & Zappalà M. (2006). The European Food Legislation and its impact on honey sector. Accreditation and Quality Assurance11, 49-54.

Feás X., Pires J., Iglesias A. & Estevinho M.L. (2010a). Characterization of artisanal honey produce don the Nirthwest of Portugal by melissopalynological and physic-chemical data. Food and Chemical Toxicology, 48, 3462-3470.

Feás X., Pires J., Estevinho M.L., Iglesias A., & Pinto de Araujo J.P. (2010b). Palynological and physicochemical data characterisation of honeys produced in the Entre-Douro e Minho region of Portugal. International Journal of Food Science & Technology, 45, 1255-1262.

Gómez-Pajuelo A. (2004). Origen botánico de la miel. In Montagud S.A (Eds), Mieles de España y Portugal (pp. 50-56). Barcelona, Spain.

Hutchings J.B. (1999). Food colour and appearance. Gaithersburg, Maryland (USA).

Kádár M., Juan-Borrás M., Hellebrandova E., Doménech& I. Escriche. (2010). Differentiation of acacia, sunflower and tilia honeys from different countries based on sugar composition, physicochemical and color parameters. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 67(2), 252-258.

Kaskonienè V., Venskutonis P.R. & Ceksterytè V. (2010). Carbohydrate composition and electrical conductivity of different origin honeys from Lithuania. LWT-Food Science and Technology, 43, 801-807.

Kondjoyan N. & Berdagué J.L. (1996). A compilation of relative retention indices for the analysis of aromatic compounds. Laboratoire Flaveur (INRA), Theix, France.

Kropf U., Korosec M., Bertoncelj J., Ogrinc N., Necemer M. & Kump P. (2010). Determination of the geographical origin of Slovenian black locust, lime and chestnut honey. Food Chemistry, 121, 839–846.

Lusic D., Koprivnjak O., Curic D., Sabatini A. G. & Conte L. S. (2007). Volatile profile of Croatian lime tree (Tilia sp.), fir honeydew (Abies alba) and sage (Salvia officinalis) honey. Food Technology and Biotechnology, 45(2), 156–165.

Mateo R. & Bosch-Reig F. (1998). Classification of Spanish unifloral honeys by discriminant analysis of electrical conductivity, color, water content, sugars and pH. Journal of Agricultural and Food Chemistry, 46, 393-400.

Oroian M. (2012). Physicochemical and Rheological Properties of Romanian Honeys. Food Biophysics. 7, 296-307.

Oroian M., Amariei S., Escriche I. & Gutt G. (2013). Rheological Aspects of Spanish Honeys. Food Bioprocess Technology, 6, 228–241.

Nozal M. J., Bernal J. L., Toribio L., Alamo M., Diego J. C. & Tapia J. (2005). The use of carbohydrate profiles and chemometrics in the characterization of natural honeys of identical geographical origin. Journal of Agricultural and Food Chemistry, 53, 3095-3100.

Piazza M.G. & Persano-Oddo L. (2004). Bibliographical review of the main European unifloral honeys. Apidologie,35 (1), 94-111.

Persano-Oddo L., Piazza M. G., Sabatini A. G. & Accorti M. (1995). Characterization of unifloral honeys. Apidologie, 26, 453–465.

Persano-Oddo L. & Bogdanov S. (2004). Determination of honey botanical origin: problems and issues. Apidologie, 35, 2–3.

Persano-Oddo L. & Piro R. (2004). Main European unifloral honeys: descriptive sheets. Apidologie35, 38–81.

Plutowska B., Chmiel T., Dymerski T. & Wardencki W. (2011). A headspace solid-phase microextraction method development and its application in the determination of volatiles in honeys by gas chromatography. Food Chemistry, 126, 1288-1298.

Radovic B. S., Careri M., Manglia A., Musci M., Gerboles M. & Anklam E. (2001). Contribution of dynamic headspace GC–MS analysis of aroma compounds to authenticity testing of honey. Food Chemistry, 72(4), 511–520.

Ruoff K., Luginbuehl W., Bogdanov S., Bosset J.O., Esterman B. & Ziorko T. (2007). Quantitative determination of physical and chemical measurands in honey by near-infrared spectrometry. European Food Research and Technology, 225, 415–423.

Saenz-Laín C. & Gómez-Ferreras C. (2000). Mieles españolas: Características e identificación mediante el análisis del polen. España: Mundi-Prensa.

Serra Bonvehí J. & Ventura-Coll F. (2003). Flavour index aroma profiles of fresh and processed honeys. Journal of the Science of Food and Agriculture, 83, 275–282.

Soria A. C., Gonzalez M., de Lorenzo C., Martinez-Castro I. & Sanz J. (2004). Characterisation of artisanal honeys from Madrid (Central Spain) on the basis of their melissopalynological, physicochemical and volatile composition data. Food Chemistry, 85, 121-130.

Terrab A., Gustavo-González A., Díez M. J. & Heredia F.J. (2003). Characterization of Moroccan unifloral honeys using multivariate analysis. European Food Research and Technology, 218, 88-95.

Visquert M., Vargas M. & Escriche I. (2013). Effect of postharvest storage conditions on the colour and freshness parameters of raw honey. International Journal of Food Science and Technology, 49(1), 181-187.

Von Der Ohe W., Persano-Oddo L., Piana M. L., Morlot M. & Martin P. (2004). Harmonized methods of melissopalynology. Apidologie, 35, 18-25.

3.5. Routine quality control in honey packaging companies as a key to guarantee consumer safety. The case of the presence of sulfonamides analyzed with LC-MS-MS.

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Abstract

One of the main challenges in the Horizon 2020 framework is to ensure sufficient food and feed, while monitoring safety throughout the food chain. In this context, the objective of this paper was to evaluate the efficacy of the routine quality control that honey companies carry out on raw batches (before entering the industrial packaging process) considering the presence of sulfonamides. A total of 279 honey samples were analyzed in this study: 178 raw honey samples were taken on reception in different companies, and 101 samples (from the same industries) were purchased locally. The validation of the methodology applied (LC–MS/MS) before analyzing the samples, confirm the reliability of the results obtained. All the purchased samples were found to be negative for sulfonamides, however, in 9 raw samples sulfathiazole (6 samples) and sulfadiazine (3 samples) were found, which represents 3.4% and 1.7 % of the 178 raw samples analysed, respectively. Therefore, if monitoring is carried out routinely at reception, risk can be decreased to a negligible level. The results confirm that using a suitable analytical methodology and implementing an appropriate routine quality control on reception is totally effective to avoid the presence of sulfonamides in the commercialized product, thereby ensuring consumer safety.

Keywords: sulfonamides; honey; LC-MS-MS; consumer safety

1. Introduction

Honey is a very healthy, nutritious food, however, in recent years it has been the focus of food alerts due to the presence of chemical hazards such as antibiotics or pesticides. The origin of these residues in honey is mainly veterinary treatments (acaricides, sulfonamides, antibiotics, etc.) required to treat bee parasites and bacterial diseases such as European foulbrood (*Streptococcus pluton*) or American foulbrood (*Bacillus larvae*) which can destroy an apiary, and propagate to other bee-hives very easily; although these compounds are often used in bee-keeping as preventive or therapeutic treatments to protect an apiary (Staub-Spörri et al., 2014).

Chemical hazards has become a major concern for the administration and the honey sector due to both the important consequences for public health (allergic reactions, bacterial resistance, changes in intestinal flora, etc.), and the impact on bees. In fact, the European Commission states that if a food-producing animal has to be treated with medicines to prevent or cure disease, the veterinary residues in these food products should not harm the consumer (European Commission, 2007). In the new societal challenges proposed by The EU Framework Programme for Research and Innovation, Horizon 2020 (Commission Decision, 2014), meeting consumer needs and preferences, but minimising the related impact on health and the environment is included as one of the main goals. The point "Food security, sustainable agriculture and forestry, marine, maritime and inland water research, and the bioeconomy" highlights that research should address food and feed safety, covering the whole food chain and related services from primary production to consumption. In truth, the control of all stages of the food chain «from farm to fork» is a shared responsibility, including primary production (agricultural and livestock), and industrial processing. It is essential to ensure consumer protection, the last link of the chain.

In order to minimize consumer exposure to residues, the Commission requires EU countries to implement residue monitoring plans through official control to monitor the illegal use of substances and misuse of authorized veterinary medicines (Commission Decision C 4995, 2014). Thus, Council Directive 96/23/EC (1996) and Commission Decision 97/747/EC (1997)establish the frequency of sampling and the levels of the groups of substances to be monitored, considering veterinary medicines, pesticides and contaminants in food of animal origin. This situation calls for the development of a quantitative framework based on risk assessment (the tool for science-based decision-making) to estimate the impact on health, and to increase the efficiency and effectiveness of safety evaluations.

Bearing all of this in mind, the objective of the current study was to evaluate the effectiveness of the routine quality control sampling which companies carry out on raw batches of honey (before entering the industrial packaging process) considering the presence of sulfonamides. To this end, both raw samples (unprocessed honey collected randomly from the initial stage of the different industries) and commercialized samples (from the same industries but bought locally) were evaluated. Before analyzing the samples, the methodology applied (LC–MS/MS) was developed and validated to guarantee the reliability of the results. As a first step in the validation process, the matrix effect of the proposed method was studied.

2. Materials and Methods

2.1.-Chemicals and Reagents

Sulfanilamide, sulfathiazole, sulfamerazine, sulfadiazine, sulfapyridine, sulfamethazine, sulfamethizole, sulfachloropyridazine, sulfamethoxazole, sulfadimethoxine, and sulfaquinoxaline; where purchased from Sigma (Steinheim, Germany), with a purity ≥95% in all cases. Hydrochloric acid (37%), formic acid (FA, 99%), acetonitrile (ACN) and methanol (MeOH) were obtained from Prolabo (VWR, Fontenay-sous-Bois, France); ammonia solution was purchased from Sharlau (Barcelona, Spain) and citric acid monohydrate was acquired from Merck (Darmstadt, Germany). The solid phase extraction (SPE) columns Strata X-CW (33µm, 100 mg, 3mL) were obtained from Phenomenex (Torrance, CA). Ultrapure water was generated in-house from a Milli-Q system (Millipore Corp., Billerica, MA).All reagents were MS, HPLC or analytical grade.

Individual stock solutions of all standards were prepared in methanol at a concentration of 1mg/mL and stored in a freezer at -20ºC, the concentrations were corrected for purity and salt form. The stock solutions were stable for at least 6 months (Kaufmann et al., 2002). A working standard mix solution of the 11 sulfonamides, in a concentration of 1µg/mL, was prepared in water. This solution was used to construct the calibration curves and to prepare the spiking experiments. Before each use it was left to reach room temperature. The stability of the 11 sulfonamides in the mixed working standard solution was checked to ensure that the standard could be stored at +4ºC for at least 3 months, with no decrease in response or degradation.

2.2.-Honey samples

A total of 279 multifloral honey samples from the Valencian Region (Spain) were used in this study. 178 of them were taken from the routine quality control sampling which companies carry out on every batch of raw honey before entering the industrial packaging process. The other 101 samples (from the same industries), were purchased locally. All the samples were stored in a dark, dry place at room temperature until analysis.

A mixture of 10 multifloral honeys without the compounds analyzed in this study was selected as a "blank honey" in order to perform the validation procedure of the methodology. Multifloral honeys with very different physicochemical characteristics (colour and texture) were specifically selected in order to cover the widest range of variability, using both light and dark honeys. This is a common procedure used by different authors to obtain a blank honey (Hammel et al., 2008; Martinez Vidal, et al., 2009; Dubreil-Chéneau et al., 2014). It is important to point out that our experience on honey analysis, as well as the results observed by other authors, showed that, in general, the types of honey don't affect the accuracy of the method (Dubreil-Chéneau et al., 2014). Although in specific cases some modifications could occur to certain analyte signals (ion suppression or enhancement) for particular types of honey, these differences are less important than those due to the intrinsic inter-day variation of the method (Dubreil-Chéneau et al., 2014). Notwithstanding this, in the case of very dark honeys, like chestnut honeys, a matrix effect for some analytes could be observed (Galarini et al., 2014). This may lead to the conclusion that for the specific case of very dark honeys it would be advisable to use this same type of honey as a "blank honey".

2.3.-Analytical determinations

2.3.1. Sulfonamide extraction method in honey

Samples of honey (1.0g) were placed in beaker flasks. The fortified samples were prepared by adding the mixed working standard solution (1μg/mL) to the blank honey to obtain the appropriate levels for validation of the method. Then, they were shaken well and allowed to stand for at least 1 hour to permit sufficient absorption of the different standards. After addition of 1mL 0.1M HCl, the samples were dissolved using a magnetic stirrer and left to stand at room temperature for at least 20 minutes to allow hydrolization of the sulfonamides (80-90% of sulfonamides are bound to sugars). Then, 5 mL of 3M citric acid were added and stirred for 30 s. Next, 5mL of the honey solution was passed through the SPE column, previously conditioned with 3mL of MeOH and 3mL of ultrapure water. The cartridges were then washed by adding 3 mL MeOH/ACN (1/1) twice. The cartridges were vacuum drained, by passing air through them, for 2 min at a pressure of 10 mmHg. The elution was accomplished with 3 mL of 2% ammonium hydroxide in MeOH, and the analytes were collected in 6mL glass tubes. The SPE procedure was performed in a Lichrolut vacuum manifold coupled to a vacuum pump (Merck, Darmstadt, Germany). Finally, the eluates were evaporated to dryness under a stream of nitrogen while being maintained at 40 ºC in a thermostatic bath (Grant GR, Cambridge, England). After evaporation, 100 µL of mobile phase was added to each tube, and thoroughly mixed to ensure the complete dissolution of the extract. Finally, the re-dissolved extracts were injected into the LC-MS/MS system.

2.3.2. LC/MS/MS Analysis

The chromatography system consisted of a HPLC Agilent 1200 Infinity Series coupled to an Agilent 6420 Triple Quadrupole detector, equipped with a source set in positive electrospray ionization mode. The column used was a Zorbax Eclipse XDB-98 (4.6 x 50 mm, 1.8 microns) supplied from Agilent. Chromatographic separation was carried out with a mobile phase consisting of 0.5% formic acid in water (mobile phase A) and ACN (mobile phase B) with a flow rate of 0.4 mL/min. The gradient used started with mobile phase A at 20%, then at minute 4 was 30%, reaching 40% at minute 7. These conditions were maintained until minute 9. After that, the system was left for 4 min to re-equilibrate before the next injection. The oven column was set at 30 ºC, and the injection volume was 5 μL.

The system was equilibrated at the beginning of each day for 1h, and three injections of the standard solution were made to check its stability and the response of the equipment, a solvent blank was then injected to assess the cross-talk. The operating parameters for the mass spectrometer were as follows: capillary voltage 4 kV; source temperature 350 ºC; nebulization gas (nitrogen) at a flow rate of 12 L/min and collision gas (nitrogen) at a flow rate of 3 L/min and 40 psi. The optimization of the MS/MS operating parameters were performed by the automatic optimization function of the MS software (Optimizer, Agilent), using direct infusion, without column, of the mixed working standard solution of the 11 sulfonamides, at a concentration of 40 μg/L. The most important LC-ESI-MS parameters for the acquisition and identification of the 11 target compounds are summarized in Table 1.

Table 1.- MS/MS operating parameters used in Sulfonamide analysis.

Analyte	Quantification Transition	Confirmation Transition	Fragmentor	CE[a](V)	RT[b] (min)
Sulfanilamide	173.0>93.1	173.0>156.1	100	5/15	1.78
Sulfathiazole	256.0>156.1	256.0>92.1	91	8/24	2.36
Sulfadiazine	251.5>156.0	251.5>92.1	91	12/24	2.45
Sulfapyridine	250.1>156.1	250.1>92.1	121	12/28	2.55
Sulfamerazine	265.1>172.1	265.1>92.1	121	12/28	2.95
Sulfamethazine	279.1>186.1	279.1>124.1	121	12/24	3.40
Sulfamethizole	271.0>156.1	271.0>92.1	120	8/24	3.59
Sulfachloropyridazine	285.0>156.0	285.0>92.1	91	8/24	5.60
Sulfamethoxazole	254.0>156.1	254.0>108.1	91	12/24	6.50
Sulfadimethoxine	311.1>156.1	311.1>124.1	121	16/32	8.60
Sulfaquinoxaline	301.1>156.0	301.1>108.1	121	12/24	8.70

[a] =collision energy; [b] = retention time

2.3.3. Matrix effect evaluation and quantification

Food matrices can vary in terms of complexity and content, and it is well established that co-eluting matrix constituents may interfere with the ionization process of the analytes (Sterner et al., 2000; Lopez et al., 2008). In order to evaluate the matrix effect, the calibration curve in solvent should be compared with the calibration curve in matrix, and a quantitative measure of the ion suppression or enhancement can be obtained comparing the peak areas of the analyte standards in solvent and the peak areas of the analyte standards spiked in honey before extraction.

The calibration method of standard addition was used to quantify the sulfonamides. Therefore, a 7-point standard curve (including zero) was constructed for each sulfonamide by plotting the peak area of the SRM transitions showing the most intense signal of each analyte versus its nominal concentration. As honey has no MRLs (maximum residue levels) for these compounds, the European Commission (Regulation (EC) No 470/2009) states that if sulfonamides are present, they must be below the limit of quantitation according to the analytical method used. Due to the fact that this limit differs between laboratories and that there is no legislation or official recommendation, in this study the target limit considered was 10 μg/kg, as this is the most demanding action limit or tolerated level found in the literature in Europe (Muñoz de la Peña et al., 2007; Sajid et al., 2013). In addition to this, a further lower level of 5μg/kg was considered in order to evaluate values lower than the target limit.

Therefore, to construct the curves, the blank honey was fortified with 0, 5, 10, 20, 40, 60 and 100 μg/kg of each compound, injected in duplicate into the LC-MS/MS system. This process was carried out in triplicate.

To identify a sample as positive, three criteria were considered: first, the signal-to-noise ratio (S/N) of the product ions selected must be greater than or equal to three; second, the allowable deviation of the retention time of the target matter and that of its corresponding standard should be within ±2.5%; third, the allowable deviation of the relative abundance of the characteristic ions of the target matter and those of the characteristic ions corresponding standard should be within ±20%.

2.3.4. Validation of the sulfonamide analytical method in honey

The analytical methodology applied in this work was validated as a first step in order to ensure the reliability of the results for every compound in the quantification range considered. The present validation study was performed in accordance with Commission Decision 2002/657/EC (2002). To this end several parameters were studied: selectivity, linearity, recovery, precision (repeatability or intraday precision "RSDr" and

reproducibility or interday precision "RSD_R"), accuracy, decision limit (CCα) and detection capability (CCβ).

The selectivity, or ability of the method to differentiate and quantify each analyte in the presence of potentially interfering substances in honey samples, was evaluated by analyzing the blank honey 20 times. To this end, the absence of any interference in the segment of the retention window of each product ion was verified for each analyte. Linearity (R^2) was tested in the 0-100 μg/kg range drawing seven-point calibration curves for fortified honey blanks. The accuracy of this method was evaluated through recovery experiments, carried out by spiking a honey blank with aliquots of the mixed working standard solution before the extraction procedure to obtain the seven concentration levels (0, 5, 10, 20, 40, 60 and 100 μg/kg). Six replicates were performed at each level. Recoveries for each analyte were determined by comparing the concentrations obtained from the calibration curves for the fortified blanks with their nominal concentrations. Recovery was measured as a percentage and RSD. The precision of the developed method was evaluated in two stages: intra-day precision (RSDr) and inter-day precision (RSD_R). To determine intraday precision six samples per level were spiked just before analysis and extracted on the same day, at the same levels as for recovery. The experiment was repeated on two further days to determine inter-day precision. Intra-day precision was expressed as the RSD of samples extracted the same day, at the same concentration, inter-day was expressed as the RSD of samples extracted on different days, at the same concentration.

CCα (decision limit) was carried out by analyzing the blank honey 20 times and calculating the signal to noise ratio at the time window in which each analyte was expected. Three times the signal to noise ratio was used as the decision limit. CCβ (detection capability) was determined by analyzing the blank honey fortified with the analytes at the decision limit at least 20 times. Detection capability is equal to the value of the decision limit plus 1.64 times the corresponding standard deviation of the within-laboratory reproducibility.

With the strategy described above, linearity, recovery, and precision were determined through 42 measurements.

3. Results and Discussion

3.1.-Matrix effect

LC-MS/MS detection is considered to be the best tool for good selectivity and speed of analysis (Cirić et al., 2012). However, it should be taken into account that the results may be adversely affected by lack of selectivity (Rogatsky & Stein, 2005) due to ion suppressions or ion enhancement caused by the sample matrix, interferences from metabolites, and "cross-talk" effects (Matuszewski et al., 2003). Because the matrix effect may compromise the quantitative results as well as the reproducibility of the method, as a first step in the validation process, the matrix effect (ME) of the proposed method was carefully studied and calculated as described in Eq.1. If ME(%)=100, no matrix effect is present; if ME(%)>100 there is a signal enhancement and if ME(%)<100 there is a signal suppression.

$$ME\ \% = \frac{peak\ area\ of\ standard\ in\ solvent}{peak\ area\ of\ standard\ spiked\ before\ extraction} \times 100 \qquad (1)$$

Three nominal concentration levels were considered to calculate the matrix effect: low-level=5 μg/kg, medium level= 20 μg/kg and high level= 40 μg/kg (Table 2) (Sajid et al., 2013). All the analytes showed a signal enhancement for the 3 concentration levels to a greater or lesser extent. Sulfanilamide was the least affected by the matrix effect because ME was 100% at the highest concentration level, and very close to it at the lowest and medium concentration levels (113 and 114, respectively). On the contrary, sulfamethoxazole with values of 463, 354 and 316, showed the most marked signal enhancement effect.

To estimate the matrix effect it is also possible to compare the slopes of calibration plots built both for the standards in methanol solution and for the standards additions in blank honey samples, which is more visual (Taylor, 2005; Gosetti et al., 2010). As an example, Figure 1 shows these calibration curves obtained for sulfanilamide and sulfamethoxazole.

Table 2. Matrix effect of the 11 sulfonamides studied.

	Nominal Concentration		
	Low-level: 5 µg/kg	Medium-level: 20 µg/kg	High-level: 40 µg/kg
Sulfanilamide	113	114	100
Sulfathiazole	179	206	185
Sulfadiazine	183	173	181
Sulfapyridine	212	168	152
Sulfamerazine	294	262	242
Sulfamethazine	340	278	270
Sulfamethizole	329	313	340
Sulfachloropyridazine	335	322	323
Sulfamethoxazole	463	354	316
Sulfadimethoxine	398	349	241
Sulfaquinoxaline	209	149	135

Due to the fact that an enhancement phenomenon was observed in this study for the eleven sulfonamides studied, it was decided to carry out the quantification step using the standard addition method (that is to say, quantification based on matrix-spiked calibration solution). In this way, the matrix effect was efficiently minimized (Economoui et al., 2012).

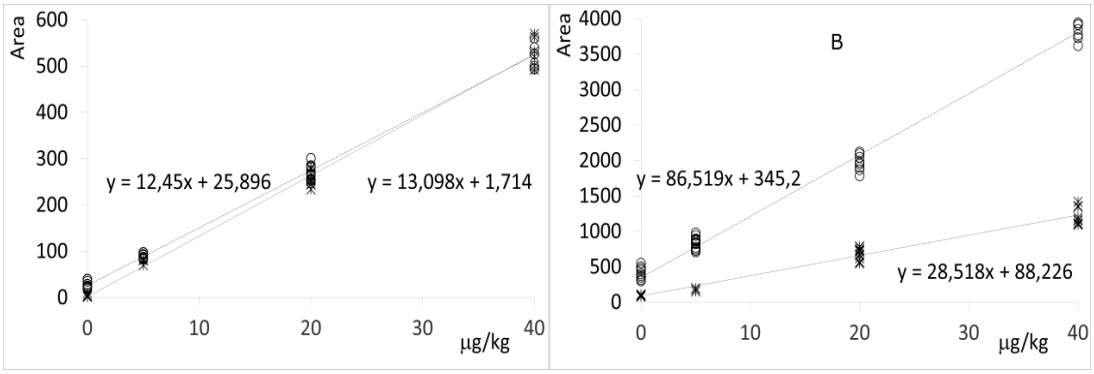

Figure 1. Calibration curves obtained for sulfanilamide (A) and sulfamethoxazole (B) in methanol solution (white circles) and in standard additions in blank honey sample (asterisks).

3.2.-In-house validation method

The analytical methodology used to perform the sulfonamide analyses of the honey samples was subjected to an in-house validation method. The selectivity, as mentioned before, was evaluated by comparing 20 chromatograms obtained from the analysis of the corresponding blank honey sample and those obtained from blank honey fortified with 11 sulfonamides. Figure 2 shows as an example the selected reaction monitoring (SRM) chromatogram of a blank honey and the same honey fortified at 20 μg/kg with all the sulfonamides studied. The absence of interference, which could compromise the

identification and quantitation of the analytes, was verified near the retention time of each sulfonamide. Regarding the linearity, the results demonstrated that in the range studied 5-100 μg/kg, the method showed a good linearity for all the sulfonamides, with a linear coefficient between 0.993 and 0.999. This is considered adequate according to the recommendations of regulatory agencies such as the Commission Decision 2002/657/EC.

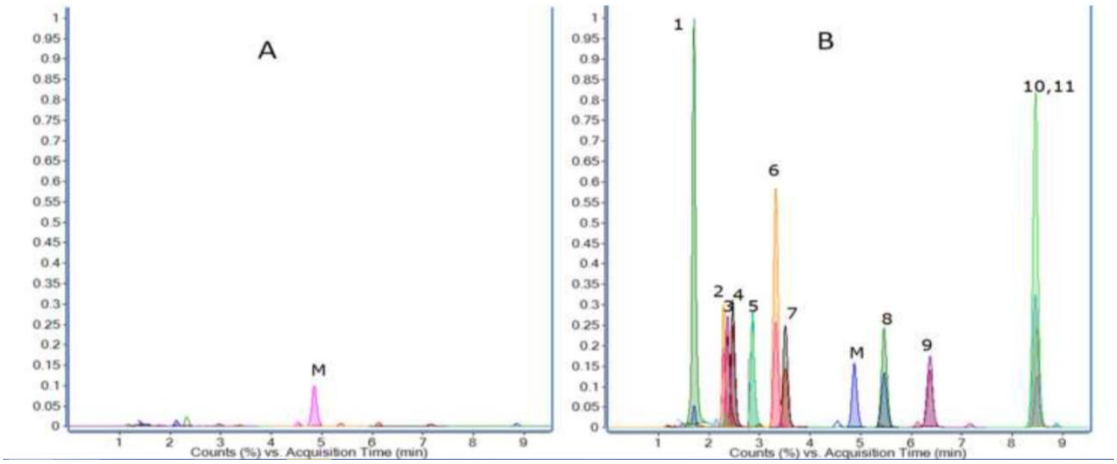

Figure 2. Selected reaction monitoring (SRM) chromatogram of a blank honey extract (A) and the same honey fortified at 20 μg/kg (B) with all the sulfonamides studied.(1)Sulfanilamide; (2) Sulfathiazole; (3) Sulfadiazine; (4) Sulfapyridine; (5) Sulfamerazine; (6) Sulfamethazine; (7) Sulfamethizole; (8) Sulfachloropyridazine; (9) Sulfamethoxazole; (10) Sulfadimethoxine; (11) Sulfaquinoxaline; (M) Matrix.

The data about the other validation parameters are shown in Table 3. These parameters provide information regarding the recovery, precision (repeatability or intra-day precision RSDr and reproducibility or inter-day precision RSD_R), decision limit (CCα) and detection capability (CCβ). The recoveries of all sulfonamides were in a range of 89-114%, complying with the requirements of the Commission Decision 2002/657/EC, which concludes that the proposed method shows good accuracy for all the studied analytes.

The repeatability (RSD_r) for all the sulfonamides studied ranged from 3.0 to 19.5%, in agreement with this Commission Decision. In the case of reproducibility (RSD_R), for 6 of the 11 compounds this parameter was below the required value: lower than 20%. In a few cases for the other four compounds, this parameter was exceeded slightly but its value was very close to 20% (always lower than 24%): Sulfanilamide at the 10 μg/kg level showed 22.8 %; sulfamerazine at the 20 μg/kg level showed 22.3%; sulfachloropiridazine 20.8% at the 10 μg/kg level, sulfamethazine at the 10 and the 20 μg/kg levels showed 23.7 and 22.1% and sulfaquinoxaline 22.7 and 22.9% at the 20 and the 40μg/kg level respectively. Therefore it can be concluded that the method used has good precision (repeatability and reproducibility) (Bohm et al., 2013).

The limit of decision (CCα) values ranged from 0.7 μg/kg (sulfamethoxazole) to 4.5 μg/kg (sulfamethazine) and the detection capability (CCβ) limit from 2.3 μg/kg (sulfamethoxazole) to 4.3 μg/kg (sulfadiazine). It is noticeable that in all the cases the 2 limits are below 5μg/kg, which is the target minimum level in this paper, as mentioned before. The results of the validation demonstrate that the applied analytical procedure guarantees the quantitative values of sulfonamides in the samples analyzed.

Table 3. Validation parameters for the analytical method.

Analytes	Nivel (μg/kg)	Recovery %	RSD_r[a] %	RSD_R[b] %	CCα (μg/kg)	CCβ (μg/kg)
Sulfanilamide	5	98	15.3	12.4	1.5	3.1
	10	105	16.9	22.8		
	20	95	10.5	17.8		
	40	89	3.5	16.5		
	60	104	10.9	14.8		
	100	104	7.3	9.0		
Sulfathiazole	5	114	4.6	14.2	2.4	4.0
	10	108	4.5	11.6		
	20	96	7.2	14.0		
	40	95	8.6	16.6		
	60	93	7.3	14.6		
	100	98	8.5	10.3		
Sulfadiazine	5	104	9.9	8.9	2.8	4.3
	10	106	13.8	14.8		
	20	97	10.8	18.4		
	40	94	3.5	11.9		
	60	98	5.1	7.2		
	100	104	6.0	8.1		
Sulfapyridine	5	101	6.2	9.6	2.2	3.0
	10	104	6.1	18.9		
	20	98	7.0	18.1		
	40	99	3.9	15.4		
	60	95	5.6	7.3		
	100	102	4.6	13.3		

Table 3 (Cont)

Analytes	Nivel (µg/kg)	Recovery %	RSD_r[a] %	RSD_R[b] %	CCα (µg/kg)	CCβ (µg/kg)
Sulfamerazine	5	109	7.4	6.8	1.4	3.0
	10	101	4.5	19.5		
	20	96	9.7	22.3		
	40	89	8.8	15.0		
	60	97	6.3	10.1		
	100	101	6.6	14.1		
Sulfamethazine	5	93	7.5	16.3	4.5	4.1
	10	110	6.3	23.7		
	20	96	10.5	22.1		
	40	99	9.2	17.8		
	60	95	6.6	8.1		
	100	101	6.3	17.7		
Sulfamethizole	5	99	5.7	12.5	2.0	3.0
	10	101	6.8	8.1		
	20	96	6.5	13.5		
	40	97	9.6	15.2		
	60	98	16.9	17.1		
	100	102	4.5	7.4		
Sulfachloropyridazine	5	109	10.3	11.5	2.0	3.5
	10	101	6.3	20.8		
	20	95	7.3	18.7		
	40	97	8.8	17.0		
	60	98	8.8	11.9		
	100	101	4.9	16.3		
Sulfamethoxazole	5	101	11.3	11.5	0.7	2.3
	10	101	8.6	16.3		
	20	95	10.2	19.3		
	40	93	6.5	10.5		
	60	99	6.6	10.3		
	100	101	5.4	10.0		
Sulfadimethoxine	5	94	8.6	11.6	2.0	3.5
	10	98	9.4	17.2		
	20	97	11.0	19.9		
	40	99	3.0	13.9		
	60	100	6.9	9.9		
	100	101	3.2	15.7		
Sulfaquinoxaline	5	89	6.4	12.2	2.4	3.9
	10	93	8.5	19.4		
	20	96	8.8	22.7		
	40	100	12.6	22.9		
	60	98	19.5	16.4		
	100	99	7.7	17.9		

[a]RSD_r= repeteatability; [b]RSD_R = reproducibility

3.3.-Samples analyses

Of the 279 honey samples analysed for the presence of 11 sulfonamides, 64% of them were from the routine sampling of every batch of raw honey which companies realize before the industrial packaging process and the other 36% samples (from the same industries), were purchased in local shops. The values of the percentage of positive samples and quantitative results of the sulfonamides found are shown inTable 4. The sulfonamide levels reported are the mean of three replicates obtained by subjecting the sample to the extraction and the analysis process. This was done to confirm that the results were not derived from incidental sample contamination. All the purchased samples had a "negative result" for all the sulfonamides, which means that the values obtained were under the CCα. On the contrary, in 9 raw samples sulfathiazole (6 samples) and sulfadiazine (3 samples) were found, which represents 3.4 % and 1.7 % of the 178 raw samples analysed, respectively. For sulfathiazole the levels ranged between 5 and 9 μg/kg, whereas for sulfadiazine a minimum of 13 μg/kg was found and a maximum that exceeded the maximum limit of quantification (100 μg/kg).

Table 4. Samples analyzed, percentage of positives for sulfonamides and concentration range.

	Honey samples purchased locally	Raw honey samples from the routine quality control in companies	Concentration range (μg/kg) of the positive compounds
Nº of analysed samples	101	178	
Nº of positive samples	0	9	
% of *samples positive for sulfathiazole*	0	3.4	5-9
% of samples positive for *sulfadiazine*	0	1.7	13-(100≤)

The "positive" samples found in the present work on raw honey are clearly in violation of current European directives. Although in the Commission regulation (Commission Decision 2010/37/EC) for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin MRLs are not included for honey, some EU countries have established internal MRLs for some substances. In the case of sulfonamides (based on the sum of sulfonamide family), the permitted level ranges from 20 μg/kg in Belgium to 50 μg/kg in the UK (Maudens et al., 2004). There is an obvious discrepancy between countries which affects commercial transactions. At present the limit for sulfonamides and other antibiotics in honey is established taking into account the limit of quantification of the methodology used. For instance, 10 μg/kg for sulfonamide in honey, a value which is only attained by techniques such as LC-MS/MS (Sheridan et al., 2008; Martinez Vidal et al., 2009). In fact, the afore mentioned Council regulation (Commission Decision 2010/37/EC) recognizes that it is becoming easier to detect the presence of residues of veterinary medicines in foodstuffs (meat, fish, milk,

eggs and honey) at ever lower levels as a consequence of scientific and technical progress.

In other studies carried out with a similar detection technique to that used in the present work, sulfathiazole is also one of the most present sulfonamides in honey. For instance, in 116 honey samples analyzed from Eastern Europe sulfathiazole was present in 47% of the cases (Sheridan et. al, 2008). In a set of honey samples from the USA, Asia and Europe, the presence of sulfadiazine, among other sulfonamides, and the presence of sulfathiazole in 2 samples (Hammel et al., 2008). In the report "Monitoring of veterinary medicinal product residues and other substances in live animals and animal products" published by European Food Safety Authority about the results obtained in 2011 it is noteworthy that the highest frequency of non-compliant samples for antibacterials (including sulfonamides) was observed in honey (European Food Safety Authority (2013). In relation to the specific case of sulfonamides the study mentions positive cases in 4 out of 129 samples from Poland. Sulfadimethoxine was found in 2 out of 67 samples from Hungary and sulfathiazole in 1 out of 5 from Lithuania. However, it is important to mention that in some countries there are specific control programs, applied to different live animals and animal products, which use microbiological tests (inhibitor tests), and sometimes the positive results are not confirmed by the most appropriate technique and thus there is no conclusive quantification of the substance concerned (European Food Safety Authority, 2013).

More recent results were reported by Galarini et al. (2014) based on 74 honey samples acquired in the Italian market. The samples had both different botanical and geographical origins (such as Italy, Hungary, Argentina, Bulgaria, Romania, Spain, and other EU and non EU countries). 12% of the samples analyzed by LC-MS/MS had traces of sulfonamides. More specifically, in 5 samples concentrations between 0.3 to 1.7 μg/kg of sulfathiazole, and in 4 samples concentrations between 0.2 to 1.7 μg/kg of sulfadimethoxine were confirmed. These authors pointed out that their results were in agreement with those reported by the Italian National Reference Laboratory for Beekeeping. This laboratory analyzed over 1500 honeys during a time period of six years, observing that 11% of the samples contained sulfonamide residues.

In Spain the most recent data from the official monitoring of antibiotics in honey are published by the Spanish Agency for Food Safety in the 2012 and 2013 reports (AESAN, 2012; AESAN 2013). In both years no antimicrobials were detected in more than 700 honey samples analyzed every year.

The above mentioned information shows that although the use of sulfonamides in beekeeping is banned in the European Union, the occurrence of residues of these compounds in honey samples is significant when sensitive analytical methods are used. However, when these compounds are present in honey they occur at very low levels,

even lower than in the tissues of farm animals. Therefore, they are not important from a toxicological point of view (Baran et al., 2011) given that honey is consumed in very small quantities. In this context, it is possible to estimate the risk to the consumer associated with the presence to this chemical hazard in honey.

This risk to the consumer is defined as a combination of the probability of occurrence of a hazard and the severity of this hazard in terms of human health: Risk=Probability*Severity (FAO/WHO 1995; Doménech et al., 2007). Asselt et al. (2013) considered that this probability must be established as the probability of consumption and the probability of exposure. They estimated the severity of a hazard associated with an antibiotic residue as both the intrinsic toxicity of the antibiotic and the consequences for human health related to the development of antimicrobial resistance. Taking this into account, these authors attributed scores to the above mentioned factors for antibiotics in different foods, including sulfonamides in honey, assigning a value in the range 0-3 (where 0 is low and 3 is high) for every factor. In the case of sulfonamides in honey, they established a value of 1 for the severity. Considering this value and the results obtained in the present paper, the risk to the consumer associated with eating commercial honey samples is 0 because no positive samples were found. However, this value would be 1 if the companies did not monitor the raw honey samples before the industrial packaging process as 3.4% and 1.7% of the analyzed raw honey samples were positive for sulfathiazole and sulfadiazine, respectively. This value concurs with the European Food Safety Authority report (2013), which remarked that a real risk of exposure to different sulfonamides in honey exists. Therefore, if adequate monitoring is carried out routinely at reception, the risk can decrease from 1 to 0 in a range of 0 to 3. This highlights the importance of the routine quality control that each company is expected to carry out.

4. Conclusion

A quantitative LC-MS/MS method for the determination of 11 sulfonamides in honey was validated with good results in agreement with the Commission Decision 2002/657/EC, which confirm the results of sulfonamides obtained in the honey samples analyzed. Monitoring the raw honey samples, before the industrial packaging process, showed that a real risk to the consumer exists due to the presence of sulfonamides. However, the results from honey sampled at retail confirmed that correct monitoring by the company is able to reduce the risk to an acceptable level. To sum up, the results indicate that using a suitable analytical methodology and implementing the routine quality control that each company is expected to carry out, it is possible to avoid the presence of sulfonamides, and therefore to ensure consumer safety. This can be extrapolated to other chemical hazards that could be present in any kind of honey.

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5. References

AESAN (Agencia Española de Seguridad Alimentaria) (2012). Memoria 2012. Available:

http://aesan.msssi.gob.es/AESAN/docs/docs/publicaciones_estudios/memoria/memoria_2012.pdf

AESAN (Agencia Española de Seguridad Alimentaria) (2013). Memoria 2013. Available at: http://aesan.msssi.gob.es/AESAN/docs/docs/publicaciones_estudios/memoria/memoria_2013.pdf

Asselt E.D. van, Spiegel M. van der, Noordam M.Y., Pikkemaat M.G. & Fels-Klerx H.J. van der. (2013). Risk ranking of chemical hazards in food. A case study on antibiotics in the Netherlands. Food Research International, 54, 1636-1642.

Cirić A., Prosen H., Jelikić-Stankov M. & Durdevic P. (2012). Evaluation of matrix effect in determination of some bioflavonoids in food samples by LC–MS/MS method. Talanta, 99, 780-790.

Baran W., Adamek E., Ziemian J. & Sobczak A. (2011). Effects of the presence of sulfonamides in the environment and their influence on human health. Journal of Hazardous Materials, 196, 1-15.

Bohm D.A, Stachel C.S. & Gowick P. (2013). Development and validation of a confirmatory method for the determination of tranquilisers and a β-blocker in porcine and bovine kidney by LC-MS/MS. Food Additives & Contaminants,30 (6), 919-920.

Commission Decision 97/747/EC. (1997). Fixing the levels and frequencies of sampling provided for by Council Directive 96/23/EC for the monitoring of certain substances and residues thereof in certain animal products. Official Journal of European Commission,L303/12 Available at:http://ec.europa.eu/food/food/chemicalsafety/residues/comm_dec1997_747ec.pdf

Commission Decision 2002/657/EC. (2002). Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of European Commission, L221, 8–36. Available at: http://crl.fougeres.anses.fr/publicdoc/decision2002-657-EC crit%C3%A8resvalidation.pdf

Commission Decision 2010/37/EC. (2010). On pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.

Official Journal of European Commission, L15/1. Available at:http://ec.europa.eu/health/files/eudralex/vol/reg_2010_37/reg_2010_37_en.pdf

Commission Decision C 4995. (2014). Horizon 2020 Work Programme 2014-2015. 9. Food security, sustainable agriculture and forestry, marine and maritime and inland water research and the bioeconomy. Available at: http://ec.europa.eu/research/participants/data/ref/h2020/wp/2014_2015/main/h2020-wp1415-intro_en.pdf

Council Directive 96/23/EC (1996). On measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. L 125/10-31. Available at: http://ec.europa.eu/food/food/chemicalsafety/residues/council_directive_96_23ec.pdf.

Domenech E., Escriche I. & Martorell S. (2007). Quantification of risks to consumers' health and to company's incomes due to failures in food safety. *Food Control, 18,* 1419-1427.

Dubreil-Chéneau E., Pirotais Y., Verdon E. & Hurtaud-Pessel D. (2014). Confirmation of 13 sulfonamides in honey by liquid chromatography-tandem mass spectrometry for monitoring plans: Validation according to European Union Decision 2002/657/EC. Journal of Chromatography A, 1339, 128–136.

Economou A., Petraki O., Tsipi D. & Botitsi E. (2012). Determination of a liquid chromatography–tandem mass spectrometry method for the determination of sulfonamides, trimethoprim and dapsone in honey and validation according to Commission Decision 2002/657/EC for banned compounds. Talanta, 97, 32-41.

European Commission (2007). A new Animal Health Strategy for the European Union (2007-2013) where "Prevention is better than cure". Available at: http://ec.europa.eu/food/animal/animalproducts/index_en.htm.Assessed April 2014.

European Food Safety Authority (2013). Report for 2011 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products1. EFSA supporting publication 2013, EN-363. Available at: www.efsa.europa.eu/publications. Assessed April 2014.

FAO/WHO (1995). Application of risk analysis to food standards issues. Report Joint FAO/WHO Expert Consultation. WHO Document WHO/FNU/FOS 195.3. Geneva. Available at: http://www.who.int/foodsafety/publications/micro/en/march1995.pdf?ua=1. Assessed April 2014.

Galarini R., Saluti G., Giusepponi D., Rossi R. & Moretti S. (2014). Multiclass determination of 27 antibiotics in honey. Food Control. Available online April 2014.

Gosetti F., Mazzucco E., Zampieri D., & Gennaro M. (2010). Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. Journal of Chromatography A, 1217, 3929-3937

Hammel Y.A., Mohamed R., Gremaud E., LeBreton M.H. & Guy P. (2008). Multi-screening approach to monitor and quantify 42 antibiotic residues inhoneyby liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1177(1), 58-76.

Kaufmann A., Roth S., Ryser B. & Widmer M. (2002). Quantitative LC/MS-MS determination of sulfonamides and some other antibiotics in honey. Journal of AOAC International, 85 (4), 853-860.

Lopez M.I., Pettis J.S., Smith I.B. & Chu P. (2008). Multiclass Determination and confirmation of antibiotics residues in honey using LC-MS/MS. Journal of Agriculture and Food Chemistry, 56, 1553-1559.

Martinez Vidal J.L., Aguilera-Luiz M.M., Romero-Gónzalez R., & Garrido A. (2009).Multiclass analysis of antibiotics residues in honey by ultraperformance liquid chromatography-tandem mass spectrometry. Journal of Agriculture and Food Chemistry, 57, 1760-1767.

Matuszewski B. K., Constanzer M. L. & Chavez-Eng C. M. (2003). Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS. Journal of Analytical Chemistry, 75, 3019-3030.

Maudens K., Zhang G-F. & Lambert W. (2004). Quantitative analysis of twelve sulfonamides in honey after acidic hydrolysis by high-performance liquid chromatography with post-column derivatization and fluorescence detection. Journal of Chromatography A, 1047, 85-92.

Muñoz de la Peña A., Mora Diez N., Mahedero García M.C., Bohoyo Gil D. & Cañada-Cañada F. (2007). A chemometric sensor for determining sulphaguanidine residues in honey samples. Talanta 73, 304-313.

Sajid M., Na N., Safdarb M., Lu X., Ma L., Hec L. & Ouyanga J. (2013). Rapid trace level determination of sulfonamide residues in honey with online extraction using short C-18 column by high-performance liquid chromatography with fluorescence detection. Journal of Chromatography A, 1314, 173-179.

Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin. Official Journal L 152, 16 June 2009, pp. 11-22. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:152:0011:0022:en:PDF

Rogatsky E., & Stein D. (2005). Evaluation of matrix effect and chromatography efficiency: new parameters for validation of method development. Journal of the American Society for Mass Spectrometry, 6, 1757-1759.

Sheridan R., Policastro B., Thomas S. & Rice D. (2008). Analysis and occurrence of 14 sulfonamide antibacterials and chloramphenicol in honey by solid-phase extraction followed by LC/MS/MS analysis. Journal of Agriculture and Food Chemistry, 56, 3509-3516.

Staub-Spörri A., Jan P., Cognard E., Ortelli D. & Edder P. (Published online 07 Apr 2014). Comprehensive screening of veterinary drugs in honey by ultra-high-performance liquid chromatography coupled to mass spectrometry. Food Additives & Contaminants.

Sterner J.L., Johnston M.V., Nicol G.R. & Ridge D.P. (2000). Signal suppression in electrospray ionization Fourier transform mass spectrometry of multi-component samples. Journal of Mass Spectrometry, 35, 385–391.

Taylor P. (2005). Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. Clinical Biochemistry 38, 328-334.

3.6. Mixture-risk-assessment of pesticide residues in retail polyfloral honey

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Abstract

The presence of even tiny quantities of pesticide residues in honey, a traditional healthy product, is a matter of concern for producers, packers and consumers. The aim of this paper was to quantify the different pesticides in retail brands of polyfloral honey, and to calculate the mixture risk assessment of honey for consumers (due to exposure to pesticides), according to the results obtained from the analyzed samples. A LC-MS/MS multi-residue method based on QuEChERS extraction was developed and validated for 13 compounds: 11 pesticides (chlorfenvinphos, coumaphos, tau-fluvalinate, amitraz, very common in veterinary treatments and imidacloprid, acetamiprid, simazine, cyproconazole, tebuconazole, chlorpiryphos-methyl, chlorpiryphos, widely used in agricultural practices), and 2 metabolites of amitraz (2,4-DMA and 2,4-DMF). Results showed that the samples contained pesticide residues at different concentration levels; however, the MRL in honey for each of the 11 pesticides was not exceeded in any of the cases. All the pesticides studied were found in at least one of the samples analysed; the most common were amitraz (from 1 to 50 μg/kg) present in 100% of the samples and coumaphos (up to 14 μg/kg) in 63%. The hazard index (HI) for adults was less than 0.002 in all cases, a long way from 1, the value established as the limit of acceptability. Therefore, commercial honey does not represent any significant risk to health. However, taking into account that the residue levels should be present "as low as reasonably achievable" it is deemed necessary to make an effort to reduce the presence of these residues by appropriate agricultural and, above all, beekeeping practices, since acaridae treatments are the main source of pesticides in honey.

Keywords

Honey; pesticides; Mixture-risk-assessment; MRL; hazard index

1. Introduction

Honey is a highly valued natural product due to its nutritional properties and appreciated therapeutic applications. However, recently, food alerts caused by the detection of antibiotics, pesticides or heavy metals in honey have jeopardized its healthy image (Juan-Borrás et al., 2015). Pesticide residues in honey come from environmental pollution and veterinary practices.

Honeybees come into contact with pesticides because veterinary practices expose them to pesticides such as acaricides required to control bee parasites like V*arroa destructor and Varroa jacobsonie* (Li et al., 2015)) and fungicides to control *Ascosphera apis*, as well as other chemical agents such as antibiotics, and sulphonamides used to control bacterial diseases like European foulbrood (*Streptococcus pluton*) or American foulbrood (*Bacillus larvae*).

On the other hand, another source of contamination is a wide range of pathways such as contaminated water, pollen and nectar from treated plants and crops, or even by direct contact during flight (García-Chao et al., 2010; Rodriguez-Lopez et al., 2014). Currently in Europe, the majority of insecticides used in agricultural practices are organophosphates, carbamates and neonicotinoids, although in some countries the use of a number of them is forbidden. These compounds affect the central nervous system of beneficial insects such as honeybees, by inhibiting the activity of the enzyme acetylcholinesterase (Blasco et al., 2011); Tanner & Czerwenka, 2011).

Combinations of sub-lethal doses of modern pesticides, of any origin, often produce additive or even synergistic effects on the mortality and behaviour of animals, which contributes to Colony Collapse Disorder (CCD) (Laetz et al., 2009; Van Engelsdorp et al., 2009).These pesticides not only affect the insects, but also contaminate bee products like honey. The determination of contaminants and residues in honey and other bee products has become a growing concern in recent years, especially as these compounds may diminish the beneficial properties of honey and, if present in significant amounts, may pose a serious threat to human health (Kujawski & Namiesnik, 2011).

In order to protect human health, chemical hazards must be controlled to stop pesticides reaching the food chain (Blasco et al., 2011; Barganska et al., 2013). With this aim in mind, Regulation (EC) Nº 396/2005 and Regulation (EC) Nº 37/2010 established MRLs for residues of certain specific pharmacologically active substances in foodstuffs of animal origin (for instance, coumaphos and amitraz in honey). This lowering of the limits of detection in a matrix as complex as honey is only possible thanks to modern analytical techniques (GC-MS-MS and LC-MS/MS) and adequate extraction and cleaning of analytes using QuEChERs (Blasco et al., 2011; Barganska et al., 2013; Hou et al., 2013).

Currently, chemicals are routinely assessed on a chemical-by-chemical basis, however, consumers could be exposed to multiple chemicals via different food types, and consequently, this approach may not be sufficiently protective. Hence, a new metric known as Mixture Risk Assessment (MRA) has been introduced to assess the cumulative risk to human health (Zheng et al., 2007; Boobis et al., 2008; Kortenkamp et al., 2009); Evans et al., 2015; Yu et al., 2016). The existence of a mixture is not always an indication of a risk to human or environmental health, but indicates the necessity of examining whether more accurate estimations of risk will be produced by considering all of the chemicals that are present.

Therefore, the objective of this paper was to quantify the presence of different pesticides in polyfloral labeled brands, and to estimate the mixture risk assessment of the honey for consumers (due to exposure to pesticides) based on the results obtained from the analyzed samples. As a first step, the analytical procedure was validated in order to guarantee the quality of the results obtained.

2. Materials and Methods

2.1.-Honey samples

This study was carried out with supermarket own-brands and well-known brands, which represent almost all of the retail sales in the Spanish market. A total of 22 honey samples, labelled as polyfloral were purchased across Spain from different retail outlets.
A mixture of 5 polyfloral honeys without the compounds analyzed in this study was selected as a "blank honey" in order to perform the validation procedure of the methodology.These samples were provided directly by Spanish beekeepers.
In all the samples the percentage of pollen of the different botanical species was evaluated in order to estimate their geographical origin. There was only one sample for which it was not possible to perform this characterization due to the insufficient presence of pollen.

2.2.-Analytical determinations

2.2.1. Pesticide analysis

Standards and reagents

Table 1 shows the 11 pesticides analysed in the present work. All of them [including the two metabolites of amitraz: 2,4-DMA (2,4-dimethylaniline) and 2,4-DMF (N-2,4-dimethylphenyl formamide)], were purchased from Sigma-Aldrich, (Steinheim, Germany), with a purity of ≥99%.Individual stock standard solutions (200 µg/mL) were prepared in methanol (MeOH) or acetonitrile (MeCN), depending on the solubility of each pesticide, and stored at -20º C. These solutions are stable for at least 1 year. From them,

a stock standard mixture of 13 pesticides was made in MeCN (each analyte at a concentration of 40µg/mL) and stored in amber glass vials at -20º C.

The spiking solutions (10µg/mL and 1µg/mL) were prepared in ultrapure water from the stock standard mixture solution and stored in amber glass vials at -4º C.

The MeOH used for the mobile phase was MS-Grade (Scharlab, Barcelona). The MeOH and MeCN used for sample extraction (Quechers methodology) and for the standards preparation was HPLC grade and was obtained from Prolabo (VWR, France).

Table 1. Pesticides studied in the present work.

Common name	Biocide action	Pesticide family	MRL[a]	ADI [b]	WHO[c]
Veterinary practices					
Amitraz	Acaricide	Formamidine	200[(1)]	10	III
Chlorfenvinphos	Acaricide	Organophosphorus	10	0.5	IB
Coumaphos	Acaricide	Organophosphorus	100	0.25	IB
Tau-fluvalinate	Acaricide	Pyrethroid	50	5	U
Agricultural practices					
Acetamiprid	Insecticide	Neonicotinoid	50	70	II
Chlorpyrifos	Insecticide	Organophosphorus	10	10	II
Chlorpyriphos-methyl	Insecticide	Organophosphorus	10	10	III
Cyproconazole	Fungicide	Azole	50	10	III
Imidacloprid	Insecticide	Neonicotinoid	50	60	II
Simazine	Herbicide	Triazine	10	5	U
Tebuconazole	Fungicide	Azole	50	30	III

[a] *MRL (Maximun Residues Level); mg kg^{-1}* (EU Pesticides database, 2015; Regulation EU 37/2010)
[b] *ADI (Acceptable Daily Intake); mg kg^{-1} d^{-1} (WHO, 2012)*
[c] *(WHO, 2010); IB = Highly hazardous; II = Moderately hazardous; III = slightly hazardous; U = Unlikely to present acute hazard in normal use*
[(1)] *The metabolites of amitraz (2,4-DMA and 2,4-DMF) are included*

The formic acid (purity 99%) for LC-MS analysis, analytical grade sodium chloride (NaCl), anhydrous magnesium sulphate (MgSO4), disodium hydrogen citrate sesquihydrate (di-Na), trisodium citrate dihydrate (tri-Na) and Bondesil Primary-Secondary Amine (PSA) were provided by Sigma Aldrich (Saint Quentin Fallavier, France). Ultrapure water was obtained from a Milli-Q® water purification system connected to a LC-PAK cartridge to remove the remaining organic contaminants at trace levels (Millipore, Molsheim, France).

Extraction procedure

Extracts were prepared using QuEChERS as it is one of the most commonly applied procedures for pesticide residue extraction. 5g of sample were weighed into a polypropylene tube. After addition of 10 mL of MeCN, the sample was extracted by shaking by hand for at least 1 min, and by vortex 1 more minute. Then a salt mixture (NaCl, MgSO4, di-Na, tri-Na) was added and the tube was shaken vigorously for a few seconds to prevent agglomeration. After shaking for 1 min the tubes were centrifuged at 1500g to obtain a clarified MeCN extract. The supernatant was transferred into another tube (PSA) shaken for 1 min and centrifuged at 1500g for 4 min. A 1 mL aliquot was taken and filtered through a nylon 0.45 μm membrane filter. This final extract was injected in the LC-MS/MS system.

LC-MS/MS analysis

The analyses were performed using an Agilent 1200 Series Rapid Resolution Liquid Chromatograph (Agilent, Palo Alto, CA) equipped with a Cortecs column (100 mm × 2.1 mm I.D., 2.7 μm particle size, Waters; Milford, MA, USA) maintained at 30 ºC. The mobile phase consisted of MeOH and water acidified with 0.1% of formic acid. The gradient was applied at a flow rate of 0.4 mL/min as follows: initial conditions of 5% MeCN increased linearly to 20% for 30s, then increased linearly to 100% at min 6, held at 100% for 2 min and returned to initial conditions for 5 min. The inject volume was 5 μL.
The MS–MS detection was performed using an Agilent 6410 Series Triple Quadruple (Agilent, Palo Alto, CA). The mass spectrometer was operated using electrospray ionization in the positive ion mode (ESI+). The capillary voltage was set to 4.0 kV. The source temperature was 350 ºC and the desolvation temperature was 350 °C. Nitrogen was used as the desolvation gas (flow 12 L/min) and collision gas at a pressure of 40 psi. Detection was performed in the multiple reaction monitoring (MRM) mode, this function automatically optimized the dwell times according to the number of simultaneously detected MRM transitions. The transition with the highest intensity was used as a quantifier and the second transition as a qualifier. Fragment voltage and collision energy were optimized for each pesticide, masses of precursor ions and product ions are summarized in Table 2.

2.2.2. Validation of the analytical method

The pesticide analytical methodology applied in this work was validated for every compound to ensure the reliability of the results in the quantification range considered. To this end, following the SANCO 12571/2013 guidance, the parameters: linearity, recovery, precision (repeatability or intraday precision “RSDr” and reproducibility or

interday precision "RSDR"), limit of detection (LOD) and limit of quantification (LOQ) were calculated.

Table 2. Settings for the ion transitions of the thirteen selected pesticides studied in MRM mode

Compound	Precursor Ion	Product Ion 1 (m/z)	Product Ion 2 (m/z)	Collision energy (V)	Retention Time (min)
Amitraz	294.2	163.0	122.0	10;35	10.20
2,4-DMA	122.1	77.0	107.0	30	6.20
2,4-DMF	150.0	107.0	123.2	18;13	8.40
Chlorfenvinphos	359.0	154.7	126.8	10;15	9.49
Coumaphos	363.0	306.6	226.4	10;40	9.52
Tau-fluvalinate	503.1	180.9	207.7	37;5	10.30
Acetamiprid	223.0	126.0	56.0	15	7.72
Chlorpiryphos	351.6	200.0	97.0	15	9.99
Chlorpiryphos-methyl	324.0	125.0	292.0	15	9.66
Cyproconazole	292.1	69.9	124.7	17;37	9.28
Imidacloprid	256.1	209.3	175.4	15;20	7.30
Simazine	202.1	132.0	123.9	20	8.63
Tebuconazole	308.2	69.9	124.7	21;5	9.48

2.2.3. Risk Evaluation

Estimated daily intake (EDI)

This parameter, expressed as μg kg^{-1} d^{-1}, is obtained as follows Eq 1:

$$EDI = \frac{C * Con}{Bw} \quad (1)$$

Where C (μg kg^{-1}) is the average concentration of a given pesticide in the collected honeys; C_{on} (kg $person^{-1}$ d^{-1}) is the daily average consumption of honey in Spain; Bw (kg $person^{-1}$) represents body weight. Based on the report by the *Instituto Nacional de Estadística* (Spanish National Institute of Statistics), the average honey consumption for adults is 0.7 kg per person per year, and the average body weight is 70 kg for adults (*INE*, 2012).

Hazard quotient (HQ)

This parameter is calculated for each pesticide by dividing the estimated daily intake (EDI) by the acceptable daily intake (ADI) (μg kg^{-1} d^{-1}) for each pesticide (Table 1), (USEPA, 2007; Evans et al., 2015), Eq 2.

$$HQ = \frac{EDI}{ADI} \quad (2)$$

Evaluation of hazard index (HI)

The HI is a measurement of the potential risk of adverse health effects from a mixture of chemical constituents (Zheng et al., 2007; Evans et al., 2015).The Hazard Index (HI) is used in most MRA (Mixture risk Assessment) approaches. The HI due to daily average consumption of honey for a human being is obtained as the sum of the hazard quotient (HQ) calculated for each chemical, Eq 3:

$$HI = \sum_{n=1}^{i} HQn \qquad 3$$

Conventionally, a HI less than 1 indicates that the total exposure does not exceed the level considered to be "acceptable", and people are unlikely to be exposed at a toxic level with possible consequences for health. On the contrary, if it exceeds one, there is a possibility of suffering adverse effects, (Evans et al., 2015; Yu et al., 2016).

3. Results and Discussion

3.1.-Matrix effect and in-house validation method

Since co-extracted matrix constituents may cause ion suppressions or ion enhancement, therefore interfering with the quantitative result, the first step in the validation process was the evaluation of the matrix effect. To this end, for every studied compound, matrix effects were tested by comparing the slopes of the calibration curves obtained for the standard solutions prepared in solvent (MeCN) with those prepared in blank matrix extracts (mix of 5 honeys). In general, medium or low matrix effects were observed, and therefore the calibration curves were constructed using the blank matrix extracts to avoid these effects. The levels of concentrations considered were: 3, 10, 20, 50, 100 and, 200 μg/kg. In addition, the level of 400 μg/kg was included in the calibration curve for amitraz; this is because this value corresponds to twice the MRL of this compound, as specified by SANCO guidelines.

Table 3 shows the data for the validation parameters. Linearity (expressed as R^2), was in all cases higher than 0.9977 throughout the concentration range considered.

The recoveries of most of the studied compounds were in a range between 70 and 120%, complying with the requirements of SANCO 12571/2013. On very few occasions the recoveries were less than 70% (60% and 66% for coumaphos at levels 3 μg/kg and 10 μg/kg, respectively, and 66% for 2,4-DMA at the level 50 μg/kg). Amitraz was the most problematic compound because the recoveries ranged between 60 and 76% for 4 of the concentration levels used.

With respect to repeatability (RSDr) and reproducibility (RSD_R), all the pesticides studied were in agreement with SANCO 12571/2013, since the values were less than 20%. The only exception was coumaphos with 22.2% at the level of 10 μg/kg for repeatability, and coumaphos and amitraz, at the level of 20 μg/kg, with 21.0% and 24.5% in the case of reproducibility.

In the present study 3 μg/kgwas considered to be the LOQ (limit of quantification) because it was the lowest validated point of the calibration curve for all the compounds, with acceptable trueness and precision results (SANCO 12571/2013). As ten times LOD is equal to three times LOQ, the LOD of the method was set at 1μg/kg, in all cases.

Being a multi-residue analysis, in general, the values obtained for all the validation parameters can be considered acceptable. It is usual to reach a compromise in order to analyze all the compounds considered together (Kujawski & Namiesnik, 2011).

Table 3. Validation parameters for the chemical compounds studied.

Analyte		3 µg/kg		10 µg/kg		20 µg/kg			50 µg/kg		100 µg/kg		200 µg/kg		400 µg/kg	
	R^2	Recovery	RSDr	Recovery	RSDr	Recovery	RSDr	RSD_R	Recovery	RSDr	Recovery	RSDr	Recovery	RSDr	Recovery	RSDr
Amitraz	0.9988	60 (10)	12.8	66 (6)	16.8	67 (13)	12.6	24.5	76 (5)	15.4	70 (8)	10.7	63 (5)	10.0	69 (8)	8.4
2,4-DMA	0.9990	76 (3)	5.0	70 (4)	5.1	74 (3)	4.1	7.2	66 (4)	5.4	70 (2)	2.2	79 (1)	0.8		
2,4-DMF	0.9996	98 (3)	3.5	101 (7)	4.8	92 (4)	4.8	0.9	86 (2)	2.4	86 (2)	2.5	91 (4)	4.9		
Chlorfenvinphos	0.9989	93 (4)	9.0	96 (13)	13.9	97 (6)	5.8	7.6	89 (6)	7.3	98 (4)	4.2	102 (2)	1.7		
Coumaphos	0.9993	60 (14)	20.0	66 (34)	22.2	90 (10)	11.2	21.0	94 (10)	10.8	95 (8)	8.0	101 (2)	1.8		
Tau-fluvalinate	0.9977	80 (5)	8.2	87 (6)	7.4	84 (3)	4.0	8.9	82 (2)	2.8	81 (2)	2.0	87 (3)	3.4		
Acetamiprid	0.9997	98 (6)	4.6	101 (7)	7.2	97 (2)	2.6	3.8	91 (3)	3.1	95 (2)	2.1	99 (1)	0.8		
Chlorpiryphos	0.9990	85 (2)	10.2	89 (17)	18.5	86 (7)	6.5	17.1	78 (6)	8.1	86 (3)	3.4	92 (3)	2.9		
Chlorpiryphos-methyl	0.9988	82 (12)	5.0	80 (14)	5.2	87 (2)	2.5	16.8	79 (4)	5.3	87 (2)	2.7	91 (2)	2.0		
Cyproconazole	0.9998	90 (4)	6.6	91 (7)	7.4	93 (5)	4.9	5.1	85 (4)	4.5	90 (2)	2.1	95 (1)	0.9		
Imidacloprid	0.9998	102 (4)	5.2	113 (9)	8.1	102 (3)	2.8	5.0	98 (3)	3.6	96 (2)	1.9	98 (1)	0.7		
Simazine	0.9997	95 (2)	6.0	101 (7)	7.0	92 (3)	3.0	5.6	89 (3)	3.1	94 (1)	1.1	96 (1)	0.9		
Tebuconazole,	0.9996	101 (8)	9.0	104 (2)	11.7	100 (4)	4.4	4.3	96 (5)	4.7	97 (3)	2.7	102 (2)	1.7		

3.2.-Analysis of the honey samples

Few studies regarding the monitoring of pesticide residue levels in honey produced in Spain have been published previously (Blasco et al., 2003; Blasco et al., 2011); what is more, own-brands which contain mixtures from EU and non-EU countries honey have not been studied at all. Blasco et al. 2003 analyzed 42 pesticide residues (organochlorine, carbamate, and organophosphorus) in 50 samples of honey collected from local markets in Spain and Portugal during 2002; they found that of the 26 honey samples from Spain 16 (61%) samples were contaminated with at least one pesticide, and of the 24 Portuguese samples, pesticide residues were detected in 23 (95%) samples.

Table 4 shows the concentration levels of pesticide residues obtained in the present work. All the pesticides studied were found in at least one of the samples investigated. In all cases the MRL established by the EU was satisfied. The pesticide which was present in all of the samples was amitraz (100%), the next most frequently occurring was coumaphos (63.6%), followed by pesticides such as acetamiprid (45.4%) and simazine (40.9%) used in agricultural practices.

Amitraz and coumaphos are widely used in veterinary practices, in several European countries and the United States, due to their effectiveness against the varroa acarus. This results in the habitual presence of these compounds in honey (Lambert et al., 2013). In the present work amitraz was found at an average value of 12.4 µg/kg (n=22), followed by coumaphos 4 µg/kg (n=14). Similar results were reported by Gómez-Pérez et al.in 2012, who detected 5.1 µg/kg of coumaphos in one Spanish sample. Barganska et al. (2013) quantified coumaphos in 6 out of 45 (13%) Polish samples, where the concentration ranged from less than the limit of quantification (4.95 µg/kg) to 16.7 µg/kg. A higher percentage of coumaphos in honey (n=186) was found by the USDA 32.3% (USDA, 2015).

Lambert et al., 2013 studied the presence of 80 pesticides in honey, pollen and honey bees, obtained directly from apiaries in France. These authors found that the frequency of detection of these compounds was higher in the honey samples (28/141) than in pollen (23/128) or honey bee (20/141) samples. These authors observed that the acaricides, coumaphos (78.0%) and amitraz (68.8%) were the most frequently detected residues in honey.

In relation to chlorfenvinphos and tau-fluvalinate, in the present work the results showed that the first one was detected in 36.4% (n=8) of the samples with a mean concentration of 1.8 µg/kg and tau-fluvalinate 13.6% (n= 3) with an average value of 1.3 µg/kg. Similar values were found (12.3%) for tau-fluvalinate by the USDA pesticide data program (USDA, 2015).

Acetamiprid and simazine, which are related to agricultural practices, were found in the present study at mean values of 2.9 and 2.6 µg/kg, respectively. Similar values were found

by other authors who detected compounds that come from crop treatment which were also below the limits (Rissato et al., 2007; Barganska et al., 2013). In the present work chlorpyriphos was present in 10 samples (18.2%) and imidacloprid in 9 (13.6%) samples; with mean concentrations of 1.3 µg/kg and 2 µg/kg, respectively. Comparing these results with published data, Rodriguez-Lopez et al. in 2014, detected chlorpyriphos in 50.1% (31 out of 61 samples), however only two samples were higher than the limit of quantification (LOQ=5 µg/kg), with mean values of 6 and 21 µg/kg, respectively. Panseri et al., 2014 detected chlorpyriphos in 33% of the samples with a mean concentration of 5.6 µg/kg. The results obtained by Rissato et al. in 2007, indicated a low level of contamination by pesticide residues, nevertheless, honey sampled in 2003 and 2004 had a concentration of 10 and 15 µg/kg of chlorpyrifos, respectively, while tau-fluvalinate was not detected.

In summary, 100% of honey samples contained at least one of the pesticides studied. Particularly, around 40% of the honey samples presented less than three pesticides and 18% only one pesticide. However, it is highlighted that in any case, the MRL was exceeded.

3.3.-Mix risk assessment

Nowadays, there is concern that the chemical by chemical approach may not be sufficiently protective, especially when it is well known that humans are exposed to more than one chemical at a time (Evans et al., 2015). The measure of the potential risk of adverse health to consumers due to the presence of a mixture of pesticides in honey is evaluated in the present work through the estimation of the hazard index (HI). This is shown in Figure 1 for the mixture of pesticides in the 22 honey samples (own-brands and well-known brands labelled as polyfloral), calculated as the sum of the hazard quotient (HQ) for each pesticide. The different colours in the bars refer to the contribution of each pesticide (HQ) to the HI value of each brand. On the x axis, next to the code for each sample (from B1 to B22) appears the information about the countries of origin. It is well known that honey companies mix raw material from different sources to make up a production batch.

The HI values ranged between 5.5 10^{-6} in B9, and 1.8 10^{-3}in B8. This means that in the worst case, the HI value was 500 times lower than 1, the limit of acceptability. Few studies have been reported about the HI values for pesticides in other types of food. Among them, the study carried out by Yu *et al*. in 2016 should be highlighted. These authors conclude that the HI for adults for fresh vegetables (n= 214) due to 11 pesticides in Changchun (China) was 0.44. This value is less than half of the limit of acceptability; however it is much higher than that estimated for honey here. This is mainly due to the large quantitative difference in the consumption of both types of food. Therefore citizens

are not exposed to a toxic level of pesticides via honey in sufficiently large quantities to cause possible health consequences (Evans et al., 2015; Yu et al., 2016).

Table 4. Average concentration (µg/kg) of pesticides present in the 22 own-brand and well-known brand samples of honey (n=3).

Honey branch	Amitraz*	Chlorfenvinphos	Coumaphos	Tau-fluvalinate	Acetamiprid	Chlorpyriphos	Chlorpyriphos-methyl	Cyproconazole	Imidacloprid	Simazine	Tebuconazole
B1	8	2	8	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B2	21	n.d.	2	n.d.	3	n.d.	n.d.	n.d.	n.d	2	n.d.
B3	5	1	5	1	3	n.d.	n.d.	5	3	3	4
B4	3	n.d.	1	n.d.	6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B5	7	1	7	n.d.	2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B6	43	2	2	n.d.	n.d.	1	n.d.	n.d.	n.d.	1	n.d.
B7	12	n.d.	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1	n.d.
B8	50	4	13	2	2	2	2	2	2	3	2
B9	2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B10	2	n.d.	n.d.	n.d.	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B11	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B12	13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2	n.d.
B13	3	1	n.d.	n.d.	2	n.d.	n.d.	n.d.	n.d.	4	n.d.
B14	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B15	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B16	8	n.d.	n.d.	n.d.	3	1	n.d.	n.d.	n.d.	2	n.d.
B17	3	n.d.	6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B18	6	n.d.	2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B19	7	1	2	n.d.	1	n.d.	n.d.	n.d.	1	n.d.	n.d.
B20	4	n.d.	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B21	30	n.d.	3	n.d.	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B22	31	2	4	n.d.	n.d.	1	n.d.	n.d.	n.d.	5	n.d.
N	22	8	14	3	10	4	1	2	3	9	2
	(100%)	(36.4%)	(63.6%)	(13.6%)	(45.4%)	(18.1%)	(4.5%)	(9.1%)	(13.6%)	(40.9%)	(9.1%)

n.d.= non detected

N= Number of samples with presence of pesticides

*= amitraz and its metabolites

In general, coumaphos followed by chlorfenvinphos had the highest contribution to the HI values, 71% and 15%, respectively. It should be noted that both pesticides are classified as "highly hazardous" by the WHO and "highly toxic" by the WHO (2010). Third was amitraz HQ (8%), which despite being classified as "moderately hazardous" by OMS, in the present paper had the highest exposure (100% of the honey brands sampled).

Simazine represented 2%, followed by chorpyrifos-methyl, chlorpyrifos, cyproconazole and tau-fluvalinate, all of them with 1%. Therefore, 94% are due to veterinary practices.

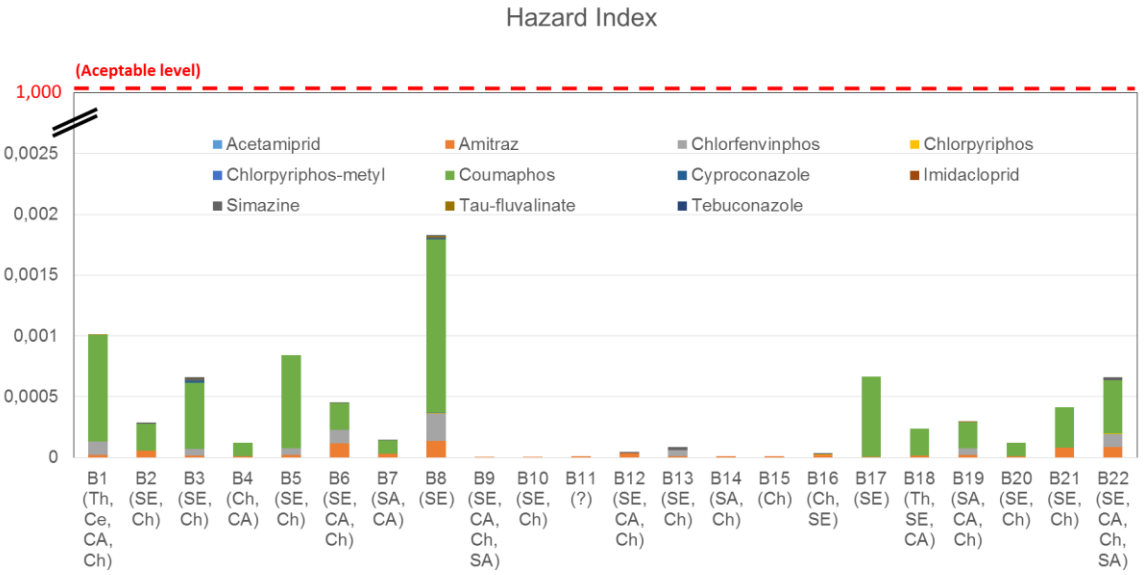

Figure 1. Hazard Index of the mixture of 11 pesticides in the 22 samples (own-brands and well-known brands labelled as polyfloral). Different colours in the bars refer to the contribution of each pesticide (HQ) in the HI of each brand. Country origin: SE (Southern Europe); Ch (China); CA (Central America); SA (South America); Th (Thailand); Ce (Chile); ? (unknown origin).

Considering the country of origin of honey, it is observed that the presence of pesticide residues does not conform to a geographic pattern. In this regard, it is noted that a country can be associated with both high HI values and very low HI values. Therefore, the presence of pesticides may be associated with specific beekeeping practices carried out against the varroa acarus. This can vary from year to year because the propagation of this parasite is greatly influenced by weather conditions (Garrido-Bailón et al., 2012).

4. Conclusion

The analytical procedure developed to determine eleven pesticide residues in honey permits a level of quantification of 3 μg/kg and detection of 1μg/kg. All the pesticides studied were found in at least one of the retail polyfloral brands analysed. The highest percentages correspond to those that come from veterinary treatments, especially amitraz and coumaphos, followed by pesticides such as acetamiprid and simazine used in agricultural practices. The samples contained pesticide residues at different concentration levels, however, the MRL in honey for each of the 11 pesticides was not exceeded, in any of the cases.

In relation to the individual hazard quotient, coumaphos followed by chlorfenvinphos and amitraz had the highest contribution to the HI values, which means that, veterinary treatments are the main source of pesticides in honey. However, the hazard index (HI) for adults was always less than 0.002, infinitesimal compared to the value of 1 recognized as the level considered to be "acceptable". Therefore, it is concluded that the daily intake of pesticides through brands labelled as polyfloral honey is not an important pathway for the dietary exposure of citizens and consequently does not entail a significant health risk. However, we should not be satisfied with this, but strive to attain the ALARA principle (As Low As Reasonably Achievable), by which residues have to be eliminated or minimized as much as possible. This requires an effort by the primary sector: farmers and beekeepers, as their practices have the greatest influence on this problem.

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5. References

Barganska Z., Slebioda M. & Namiesnik J. (2013).Pesticide residues levels in honey from apiaries located of Northern Poland. Food Control, *31*, 196-201.

Blasco C., Fernandez M., Pena A., Lino C., Silveira I., Font G. & Pico Y. (2003). Assessment of Pesticide Residues in Honey Samples from Portugal and Spain. Journal of Agricultural and Food Chemistry, 51, 8132-8138.

Blasco C., Vázquez-Roig P., Onghena M., Masia A. & Picó Y. (2011). Analysis of insecticides in honey by liquid chromatography-ion trap-mass spectrometry: Comparison of different extraction procedures. Journal of Chromatography A, 1218, 4892-4901.

Boobis A. R., Ossendorp B. C., Banasiak U., Hamey P. Y., Sebestyen I., & Moretto A. (2008). Cumulative risk assessment of pesticide residues in food. Toxicology letters 180 (2), 137-150.

EU Pesticides database European Commission. Available at: http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database.

Evans R. M., Scholze M. & Kortenkamp A. (2015). Examining the feasibility of mixture risk assessment: A case study using a tiered approach with data of 67 pesticides from the Joint FAO/WHO Meeting on Pesticide Residues (JMPR). Food and Chemical Toxicology, 84, 260-269.

García-Chao M., Agruña M. J., Flores Calvete G., Sakkas V., Llompart M. & Dagnac T. (2010). Validation of an off line solid phase extraction liquid chromatography-tandem mass spectrometry method for the determination of systemic insecticide residues in honey and pollen samples collected in apiaries from NW Spain. Analytica Chimica Acta. 672, 107-113.

Garrido-Bailón E., Botías C., Martin-Hernandez R., Meartinez-Salvador A., Meana A. & Higes M. (2012). Prevalencia de los principales Agentes Patógenos de Apis mellifera iberiensis en la cabaña apícola Española. Anales Real Academia de Ciencias Veterinarias de Andalucía Oriental. 25 (1), 109-130.

Gómez-Pérez M. L., Plaza-Bolaños P., Romero-González R. Martínez-Vidal, J.L. & Garrido-Frenich A. (2012). Comprehensive qualitative and quantitative determination of pesticides and veterinary drugs in honey using liquid chromatography-Orbitrap high resolution mass spectrometry. Journal of ChromatographyA, 1248, 130-138

Hou X., Han M., Dai X., Yang X. & Yi S. (2013). A multi-residue method for the determination of 124 pesticides in rice by modified QuEChERS extraction and gas chromatography–tandem mass spectrometry. Food Chemistry, 138, 1198–1205.

Juan-Borrás M., Periche A., Domenech E. & Escriche I. (2015). Routine quality control in honey packaging companies as a key to guarantee consumer safety. The case of the presence of sulfonamides analyzed with LC-MS-MS. Food Control, 50, 243-249.

Kortenkamp A., Backhaus T. & Faust M. (2009). State of the Art on Mixture Toxicity.Report. Available at: http://ec.europa.eu/environment/chemicals/pdf/report_Mixture%20toxicity.pdf.

Kujawski M. W. & Namiesnik J. (2011). Levels of 13 multi-class pesticide residues in Polish honeys determined by LC-ESI-MS/MS. Food Control. 22, 914-919.

Lambert O, Piroux M, Puyo S, Thorin C, L'Hostis M, Wiest L., Bulete A., Delbac F. & Pouliquen H. (2013) Widespread Occurrence of Chemical Residues in Beehive Matrices from Apiaries Located in Different Landscapes of Western France. Available at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0067007

Li Y., Kelley R. A., Anderson T. D. & Lydy M. J. (2015). Development and comparison of two multi-residue methods for the analysis of select pesticides in honey bees, pollen, and wax by gas chromatography-quadrupole mass spectrometry. Talanta. 140; 81-87.

Panseri S., Catalano A., Giorgi A., Arioli F., Procopio A., Britti D. & Chiesa L.M. (2014). Occurrence of pesticide residues in Italian honey from different areas in relation to its potential contamination sources. Food Control, 38, 150-156.

Regulation (EU) Nº 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Available at:http://ec.europa.eu/health/files/eudralex/vol-5/reg_2010_37/reg_2010_37_en.pdf.

Regulation (EC) Nº 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC, European Community, Brussels, 16-3-2005.Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:070:0001:0016:en:PDF.

Rissato S. R., Galhiane M. S., de Almeida M. V., Gerenutti M. & Apon B. M. (2007). Multiresidue determination of pesticides in honey samples by gas chromatography–mass spectrometry and application in environmental contamination. Food Chemistry, 101(4), 1719-1726.

Rodriguez-López D., Ahumada D. A., Díaz A. C. & Guerrero J. A. (2014). Evaluation of pesticide residues in honey from different geographic regions of Colombia. Food Control, 37, 33-40.

SANCO 12571/2013. (2013). Method Validation and Quality Control Procedures for Pesticide Residue Analysis in Food and Feed. Available at: http://ec.europa.eu/food/plant/pesticides/guidance_documents/docs/qualcontrol_en.pdf.

Tanner G. & Czerwenka C. (2011). LC-MS/MS analysis of neonicotinoid insecticides in honey: methodology and residue findings in Austrian honeys. Journal of Agricultural and Food Chemistry, 59 (23), 12271-12277.

USEPA U.S. (Enviromental Protection Agency). (2007). Integrated risk information system-database (Philadelphia PA; Washington, DC).

USDA 2015. Pesticides data program. Available at: http://www.ams.usda.gov/datasets/pdp.

Van Engelsdorp D., Evans J., Saegerman C., Mullin C., Haubruge E, Nguyen B.K., Frazier M., Frazier J., Cox-Foster D., Chen Y., Underwood R., Tarpy D.R.& Pettis J.S. (2009). Colony Collapse Disorder: A Descriptive Study. Available at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0006481.

WHO (World Health Organization) (2010). The WHO recommended classification of pesticides by hazard. Available at: http://www.who.int/ipcs/publications/pesticides_hazard/en/

WHO (World Health Organization) (2012). Inventory of evaluations performed by the Joint Meeting on Pesticide Residues (JMPR). Available at: http://apps.who.int/pesticide-residues-jmpr-database/Home/Search

Yu R., Liu Q., Liu J., Wang Q. & Wang Y. (2016). Concentrations of organophosphorus pesticides in fresh vegetables and related human health risk assessment in Changchun, Northeast China. Food Control, 60, 353-360.

Zheng N., Wang Q., Zhang X., Zheng D., Zhang Z. & Zhang S. (2007). Population health risk due to dietary intake of heavy metals in the industrial area of Huludao city, China. Science of the Total Environment, 387(1), 96-104.

4. CONCLUSIONES

4. Conclusiones

4.1.-Conclusiones del objetivo general 1

Aportaciones:

Journal of Chemistry (2015), http://dx.doi.org/10.1155/2015/929658.
International Journal of Food Science and Technology (2015), 50(7), 1690-1696.
Food Research International (2014), 60, 86-94.
Food Control (En revisión).

4.1.1. El control de determinados parámetros físico-químicos en la etapa de recepción de la industria de envasado de miel, es fundamental para garantizar la calidad del producto final y el cumplimiento de los requisitos legales. Llegar a conocer el origen de la variabilidad de estos parámetros es esencial para tomar medidas en la mejora de la calidad de la miel. En este sentido, en la etapa de recepción se evaluó la influencia de la variedad de miel, año de cosecha, así como el papel del apicultor sobre la variabilidad del HMF, la humedad y el color. Se analizaron 1593 muestras de 11 variedades de miel (botánicamente clasificadas), de 98 apicultores, cosechadas entre 2009 y 2013. Las mieles más claras presentaron mayor humedad, mientras que las mieles más oscuras mostraron valores de humedad más bajos. El color fue el parámetro más afectado tanto por la variedad de la miel como por el año de la cosecha, mientras que el HMF fue el menos influenciado por ambos atributos. Para el HMF y la humedad, se observaron muestras que presentaban algunos valores extremos, legalmente inaceptables, provenientes en todos los casos de ciertos apicultores. Esto demuestra el importante papel que el apicultor tiene sobre ciertos parámetros que afectan a la calidad de la miel, e incluso sobre el color varietal característico que el mercado requiere. Por lo tanto, unas buenas prácticas apícolas son vitales para obtener el producto que el consumidor espera y la legislación exige.

4.1.2. Se ha evaluado el nivel de antranilato de metilo (MA) que comúnmente tienen las mieles de cítricos españolas y la posible correlación con el porcentaje de polen de *Citrus* spp. El análisis melisopalinológico por sí solo no es suficiente para la clasificación de algunas variedades de miel, como es el caso de la miel de cítrico, ya que el porcentaje de polen en este tipo de miel puede ser menor de lo esperado (variedades híbridas estériles

o producción de polen y néctar no simultánea). Por ello, se analizaron muestras de mieles vendidas como cítrico y procedentes de apicultor, ambas de las campañas de 2011 y 2012. El polen osciló entre 1 y 88% y el MA entre 0.5 y 5.9 mg/kg, no habiendo correlación cuantitativa entre ambos. Sin embargo, se observaron correlaciones significativas con coeficientes de Pearson moderados entre algunos de los parámetros fisicoquímicos evaluados en las muestras [MA/conductividad eléctrica (-0.678); MA/de color (-0.559); polen /conductividad eléctrica (-0.553) y polen color (-0.556)]. Un análisis de tabla de contingencia (tabulaciones cruzadas) llevado a cabo para evaluar la interrelación entre el polen y MA (teniendo en cuenta estas variables como categóricas: al menos un 10% de polen de *Citrus* spp.; mínimo contenido de MA: 2 mg/kg) mostró que el 53.5% de las muestras en 2011 y el 56.8% en 2012 cumplieron tanto con el porcentaje de polen como con la concentración de MA requeridos. Por otro lado, el 35.7% en 2011 y el 38.6% de las muestras en 2012, aun cumpliendo con el porcentaje de polen no cumplían con el MA. Además el 4.6% de las muestras que cumplían con MA, no lo hacían con el polen. Este hecho también ha sido observado por otros autores en mieles de cítrico italianas. En este trabajo se propone reconsiderar el nivel de MA requerido para la miel de cítricos españoles, aplicando un valor más realista. Pero, sobre todo, sólo tener en cuenta este parámetro en el caso de las mieles con un sorprendente bajo porcentaje de polen de cítricos, y después de la evaluación de sus propiedades organolépticas y fisicoquímicas.

4.1.3. Un sistema de lengua electrónica construido con 5 metales: 4 metales no nobles (cobre, plata, níquel y cobalto) y únicamente oro como metal noble, es capaz de diferenciar no sólo entre tipos de miel (azahar, romero, tomillo, girasol, ajedrea y mielada), sino también predecir su capacidad antioxidante total.

Aunque el origen botánico de las mieles tiene un claro impacto en algunos de los parámetros químicos y fisicoquímicos estudiados (especialmente el color, la conductividad eléctrica y la actividad antioxidante), en términos generales con estos parámetros no se ha logrado una buena diferenciación entre todos los tipos de mieles estudiadas.

La combinación de la información generada por la lengua electrónica junto con la aplicación de adecuadas técnicas estadísticas multivariantes, como el PCA y las redes neuronales (Neural Network Analysis fuzzy artmap type), ha demostrado que este sistema permite la diferenciación de mieles según su origen botánico con un porcentaje de éxito del 100%.

Un modelo de predicción MLR demostró una buena correlación entre la lengua electrónica y la capacidad antioxidante de las mieles (0.9666). Esta correlación fue peor para otros parámetros evaluados como la conductividad eléctrica (0.8959) e incluso menor para a_w, humedad y color.

En definitiva, el sistema de medición de lengua electrónica propuesto podría ser una opción rápida y fácil para el sector de envasado de la miel, ya que puede proporcionar información rápida sobre el origen botánico de una miel e incluso sobre una característica tan importante como es su capacidad antioxidante.

4.1.4. Se ha estudiado la influencia que tienen determinados parámetros físico-químicos (HMF, actividad diastásica, humedad, conductividad eléctrica), color (escala Pfund y CIEL a* b*), azúcares mayoritarios (glucosa, fructosa y sacarosa) y fracción volátil, sobre el origen geográfico (España, Rumania y República Checa) así como el origen botánico de mieles de acacia, girasol y tilo. Un análisis de componentes principales (PCA) mostró que la variedad tiene mayor influencia en la diferenciación de las mieles que el origen geográfico, debido especialmente a ciertos compuestos volátiles tales como carvacrol y α-terpineno para la miel de tilo; α-pineno y 3-methyl-2 butanol para la miel de girasol, y óxido de cis-linalool para la miel de acacia.

Un análisis discriminante obtenido para cada variedad permitió diferenciar las mieles de acuerdo a su país de origen, en base principalmente a los compuestos volátiles (2-metil-2-butenal, 2-metil-2-propanol, éster butílico del ácido acético, etc., para mieles de acacia; 1-hexanol, α-pineno, etc., para mieles de girasol y 3-metil-1-butanol, hotrienol, 2-butanona, etc., para la miel tilo) y, en menor medida a ciertos parámetros fisicoquímicos tales como, diastasa, sacarosa y conductividad, respectivamente. Los resultados sugieren que los modelos multivariantes presentados son herramientas potencialmente útiles para la clasificación de mieles ya que pueden complementar la información obtenida con el análisis polínico.

4.2.-Conclusiones del objetivo general 2

Aportaciones:

-*Food Control* (2015), 50, 243-249.
-*Food Control* (En revisión).

4.2.1. Un análisis multi-residuos de sulfamidas (por LC-MS/MS) fue desarrollado, como ejemplo, para evaluar la eficacia del control que de forma rutinaria, llevan a cabo las empresas envasadoras sobre la materia prima, en relación a la presencia de residuos químicos. Siguiendo los requisitos del correspondiente Reglamento Europeo, se validaron 11 sufamidas (sulfanilamide, sulfathiazole, sulfamerazine, sulfadiazine, sulfapyridine, sulfamethazine, sulfamethizole, sulfachloropyridazine, sulfamethoxazole, sulfadimethoxine y sulfaquinoxaline). Se analizaron muestras comercializadas (101)

procedentes de diferentes empresas, así como lotes de materia prima (178 muestras crudas) de las mismas empresas después de la recepción y antes de su aceptación para la entrada en el proceso industrial. Todas las muestras comerciales dieron negativo a la presencia de sulfamidas, sin embargo, de los 178 lotes crudos analizados, en 9 de ellos se encontró sulfathiazole (3.4% de los casos) y en 3 de ellos sulfadiazine (1.7 % de los casos). Los resultados confirman que la aplicación en la recepción de un control de calidad apropiado aplicando una metodología analítica adecuada y validada, resulta eficaz para reducir en la miel comercializada el riesgo de exposición por la presencia de sulfonamidas. Este estudio concluye que está garantizada la seguridad del consumidor de miel en lo que respecta no solo a la presencia de sulfamidas, sino también de otras sustancias químicas como antibióticos y pesticidas, ya que el control que las empresas llevan a cabo de forma rutinaria los engloba a todos.

4.2.2. Un análisis de multi-residuos de pesticidas (por LC-MS/MS) fue desarrollado para evaluar el riesgo de exposición a pesticidas a través del consumo de miel. Siguiendo los requisitos de la guía SANCO, se validaron (en un rango comprendido entre 1 y 400 μg/kg) 11 pesticidas (chlorfenvinphos, coumaphos, tau-fluvalinate, amitraz, muy comunes en tratamientos veterinarios y imidacloprid, acetamiprid, simazine, cyproconazole, tebuconazole, chlorpiryphos-methyl, chlorpiryphos, ampliamente utilizados en las prácticas agrícolas), y 2 metabolitos del amitraz (2,4-DMA and 2,4-DMF). Se evaluaron 22 marcas blancas y primeras marcas del mercado, por representar la casi totalidad de la miel que se comercializa en España. De este trabajo se concluye que:

-No se superó el LMR para ninguno de los pesticidas analizados, sin embargo, todas las muestras contenían residuos de alguno de ellos en diferentes concentraciones. Los pesticidas más abundantes fueron los correspondientes a los tratamientos veterinarios contra la varroa: amitraz y coumaphos; el primero presente en el 100% de las muestras y el segundo en el 63% de las mismas. Los pesticidas procedentes de los tratamientos agrícolas, como acetamiprid y simazine, solo se encontraron en pocos casos y en concentraciones muy bajas.

-La medida del riesgo potencial para la salud de los consumidores, debido a la presencia de cantidades de residuos de pesticidas inferiores al LMR, se estimó en las diferentes muestras comerciales mediante el cálculo del “hazar index” (HI), como sumatorio de "hazard quotient (HQ)”individual para cada pesticida presente en ellas. Los valores de HI obtenidos para todas las mieles analizadas fueron en el peor de los casos 500 veces inferior al valor de 1, considerado como límite de aceptabilidad.

-La presencia de residuos de pesticidas no sigue un comportamiento geográfico, ya que un mismo país puede asociarse con muestras con altos valores de HI o con muestras con bajos valores de HI. Esto hace pensar que la presencia de pesticidas puede estar asociada, más bien, a las prácticas apícolas, especialmente aquellas que se llevan a cabo contra el ácaro varroa.

-Aunque los consumidores no están expuestos a niveles tóxicos de pesticidas a través del consumo de miel, sin embargo, siguiendo el principio de que una exposición tiene que ser "tan baja como sea razonablemente posible", el sector primario, apicultores y agricultores, deben hacer un mayor esfuerzo ya que sus prácticas pueden influir directamente en el problema de la presencia de residuos en la miel.

Conclusión General de la Tesis

La presente tesis doctoral pone en evidencia que, las técnicas alternativas ensayadas en el presente estudio para la clasificación industrial de mieles (compuestos volátiles, lenguas electrónicas) son esperanzadoras, ya que han demostrado ser útiles para complementar los resultados obtenidos mediante métodos convencionales: melisopalinológico, fisicoquímicos y sensorial.

Este trabajo demuestra el papel fundamental que juegan los diferentes eslabones de la cadena de producción de miel (apicultor e industria de envasado), en la obtención del producto que el consumidor espera y la legislación exige, atendiendo a criterios de calidad e inocuidad. Para poder garantizar la inocuidad del producto, la industria debe llevar a cabo de forma rutinaria controles eficaces de la materia prima, especialmente en lo que respecta a los residuos químicos que pudieran afectar la salud del consumidor. Por su parte el sector primario, tanto apicultores como agricultores, deben hacer un mayor esfuerzo, ya que sus prácticas influyen directamente en el problema de la presencia de residuos en la miel.