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REVIEW ARTICLE

Standard methods for *Apis mellifera* royal jelly research

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Royal jelly, a honey bee secretion, plays a critical role in caste determination in honey bees because it serves as the source of nutrition for young larvae destined to become queens. It is also fed to adult queens. Royal jelly possesses numerous functional properties and thus has been used as a medication, health food, and cosmetic in many countries. In this paper, we first introduce a traditional method for producing royal jelly by artificial larvae grafting and a newly developed method that does not require grafting of larvae. We describe protocols for the storage and freeze-drying of royal jelly to preserve its biological properties. Routine methods for determination of two important quality criteria, water content and trans-10-hydroxy-2-decenoic acid content, are outlined. On a dry basis, protein, carbohydrate, and fatty acids were found to be the 3 most abundant components of royal jelly. Methods for their isolation, identification, and quantification are described. Because royal jelly is susceptible to contamination with veterinary drugs and acaricides, we also describe methods for detection and quantification of some veterinary drugs and acaricides in royal jelly.

Métodos estándar para la investigación de la jalea real de *Apis mellifera*

La jalea real, una secreción de abejas, desempeña un papel crítico en la determinación de castas en la abeja melífera, ya que sirve como fuente de nutrición para larvas jóvenes destinadas a convertirse en reinas. También alimenta a las reinas adultas. La jalea real posee numerosas propiedades funcionales y por lo tanto se ha utilizado como un medicamento, alimento saludable y cosmético en muchos países. En este artículo, introducimos un método tradicional para producir jalea real mediante el injerto artificial de larvas y un método recientemente desarrollado que no requiere injerto de larvas. Describimos protocolos para el almacenamiento y la liofilización de la jalea real para preservar sus propiedades biológicas. Se describen métodos rutinarios para la determinación de dos importantes criterios de calidad, el contenido de agua y el de ácido trans-10-hidroxi-2-decenoico. En una base seca, proteínas, carbohidratos y ácidos grasos fueron los tres componentes más abundantes de la jalea real. Se describen métodos para su aislamiento, identificación y cuantificación. Debido a que la jalea real es susceptible a la contaminación con medicamentos veterinarios y acaricidas, también describimos métodos para la detección y cuantificación de algunos medicamentos veterinarios y acaricidas en jalea real.

西方蜜蜂蜂王浆研究标准方法

蜂王浆是蜜蜂的分泌物，作为蜂王幼虫的食物在蜜蜂级型分化过程中起到了重要作用。同时，蜂王浆也是蜂王的食物。蜂王浆有丰富的功能活性，因而在很多国家被作为药物、保健品和化妆品使用。在本章节，我们首先介绍了利用人工移虫生产蜂王浆的传统方法和一种新开发的免移虫蜂王浆生产方法。我们介绍了为保留蜂王浆生物学活性所需的蜂王浆贮存方法和冷冻干燥方法，概述了蜂王浆水分含量和10-羟基-2-癸烯酸这两个重要质量指标的检测方法。蛋白质、碳水化合物和脂肪酸是蜂王浆干物质中含量最丰富的3种成分。本章还介绍了这三种成分的分离、鉴定和定量方法。蜂王浆易受到兽药和螨药的污染，因此我们还介绍了蜂王浆中一些兽药和螨药的检测和定量方法。

Keywords: royal jelly; production; storage; protein; sugar; lipid; residue

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1. Introduction

Royal jelly is a substance that is secreted by the hypopharyngeal and mandibular glands of worker honey bees. It is a yellowish, creamy, and acidic substance with a slightly pungent odor and taste, composed on a wet weight basis of 60–70% water (Karaali, Meydanoğlu, & Eke, 1988; Sabatini, Marcazzan, Caboni, Bogdanov, & De Almeida-Muradian, 2009), 9–18% proteins (Karaali et al., 1988), 3–8% lipids (Karaali et al., 1988; Sabatini et al., 2009), 6–18% hydrocarbons (Daniele & Casabianca, 2012; Sesta, 2006), 0.8–3.0% minerals (Sabatini et al., 2009), and small amounts of polyphenols and vitamins. It plays a crucial role in caste determination of honey bees because larvae that are fed copious amounts of royal jelly for a longer period develop into large, fertile, and long-lived queens rather than into smaller, sterile, and short-lived workers.

Royal jelly has been demonstrated to possess numerous functional properties such as antibacterial activity, anti-inflammatory properties, vasodilatory and hypotensive activities, disinfectant action, antioxidant effects, anti-hypercholesterolaemic activity, and antitumor properties (reviewed in Ramadan & Al-Ghamdi, 2012). As a valuable bee product, royal jelly has been incorporated into traditional human medicine and is widely promoted and commercially available as a medicament, health food, and cosmetic in many countries, especially in China and Japan. Studies on royal jelly are important for our understanding of both the physiology of honey bees and royal jelly's biological activity promoting human health.

2. Production and harvesting of royal jelly

The development of techniques for production of royal jelly has a long history. In 1921, Sherlock Holmes simply sucked royal jelly out of a naturally produced queen cell with a syringe, to harvest this substance according to principles of vacuum physics. By the 1950s, Mexican, French, and Italian beekeepers had started small-scale production and sale of royal jelly. The method for producing royal jelly they employed involved removing the queen from the colony, so that they could take royal jelly directly from the new queen cells that the worker honey bees rapidly built after detecting the absence of the queen. Nevertheless, this method has disadvantages: the decrease in colony strength (a reduction in the number of new workers) and in honey production. To improve the situation, a queen excluder was later introduced so as to maintain the production of royal jelly in a strong colony with a queen.

Because of the improvement of royal jelly production methods, the output of royal jelly increased from 200–300 to 5000–12,000 g per colony per year. This achievement is mainly attributed to successful breeding and marketing of "royal-jelly-producing bees" (Cao, Zheng, Pirk, Hu, & Xu, 2016) and to widespread use of plastic queen cells; in other words, comprehensive methods of breeding colonies with a high yield of royal jelly have been developed (Chen, 1993; Zeng, 2013).

2.1. The method for producing royal jelly by artificial larvae grafting

During 60 years of continual development, a method for production of royal jelly has been fine-tuned. It is based on artificial larvae grafting: the transfer of larvae from worker cells to a large number of (artificial) queen cells (Chen, 1993; Zeng, 2013). In this section, the tools necessary to collect royal jelly and the management of colonies used for royal jelly production are described.

2.1.1. Tools

(1) Queen-cell-base-bar: this is made of non-toxic plastic and consists of many queen cell bases (Figure 1). A common queen-cell-base-bar has 2 rows; other models can have up to 4.
(2) Grafting pen: this is a tool used to transfer worker larvae from cells in a comb to queen cell bases. A grafting pen is made of a soft tongue made of bull horn, a plastic tube, and a spring-loaded plunger (Figure 2). Compared to the grafting needles made of metal, grafting pens are more convenient to use and increase the survival of grafted larvae.
(3) Royal jelly production frame: queen-cell-base-bars can be installed in a royal jelly production frame made of fir tree wood. The size of the periphery of a royal jelly production frame is the same as that of a comb frame. A royal jelly production frame consists of a long beam (width:

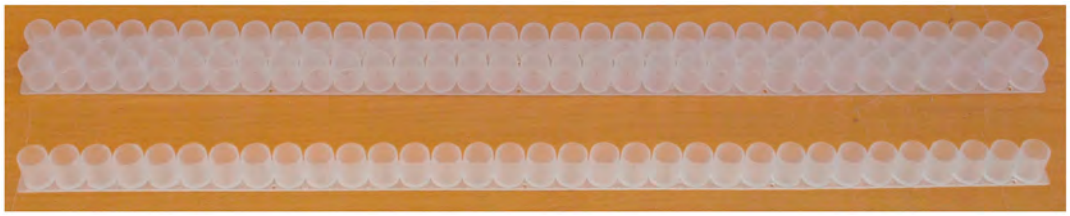

Figure 1. Plastic queen-cell-base-bar.
Photo: Linbin Zhou.

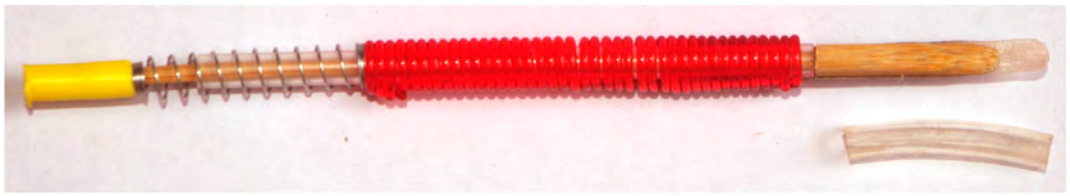

Figure 2. Grafting pen.
Photo: Qizhong Pan.

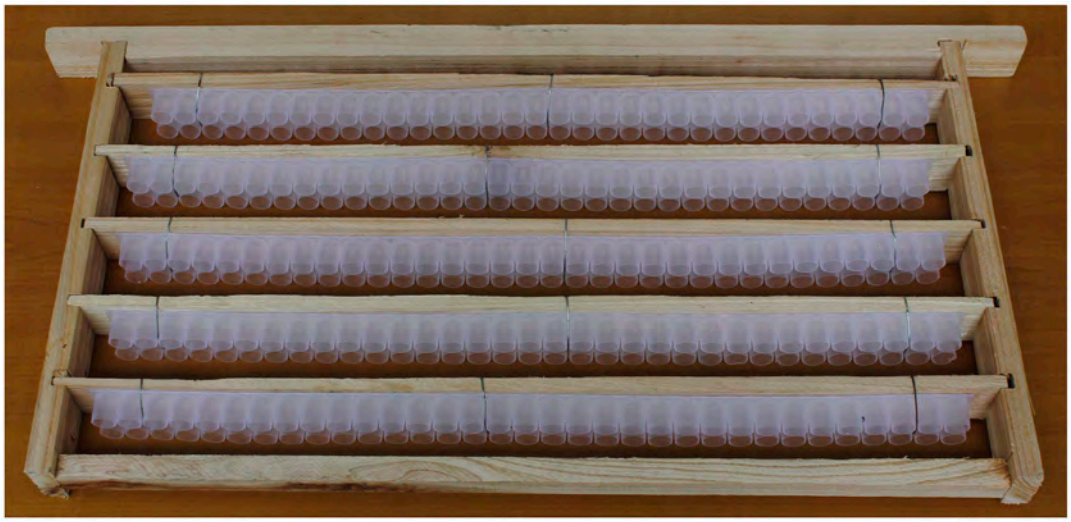

Figure 3. Royal jelly production frame.
Photo: Qizhong Pan.

Figure 4. Royal jelly scraping bar.
Photo: Qizhong Pan.

13 mm, thickness: 20 mm), 2 sidebars (width: 13 mm, thickness: 10 mm), 5 inner plates attached to queen-cell-base-bars (width: 13 mm, thickness: 5 mm), and a bottom thicker plate (width: 13 mm, thickness: 10 mm; Figure 3).

(4) Royal jelly scraping bar: composed of a royal jelly scraping tongue and a handle (Figure 4). The scraping tongue is made of a flexible plastic or rubber sheet of high tenacity, with a flat shovel-like shape. The width of the scraping tongue piece is the same as that of the longitudinal section of a queen cell. The handle is made of rigid plastic approximately 100 mm in length.

(5) Tweezers and queen-cell-wax-cleaner: stainless steel tweezers (Figure 5 top) are used to remove the larva from a queen cell. A queen-cell-wax-cleaner has a metal piece that has a similar shape to that of a royal jelly scraping tongue and a rotatable handle sleeve. This tool is useful for scraping residual wax from the inner wall of a queen cell after royal jelly collection (Figure 5, bottom).

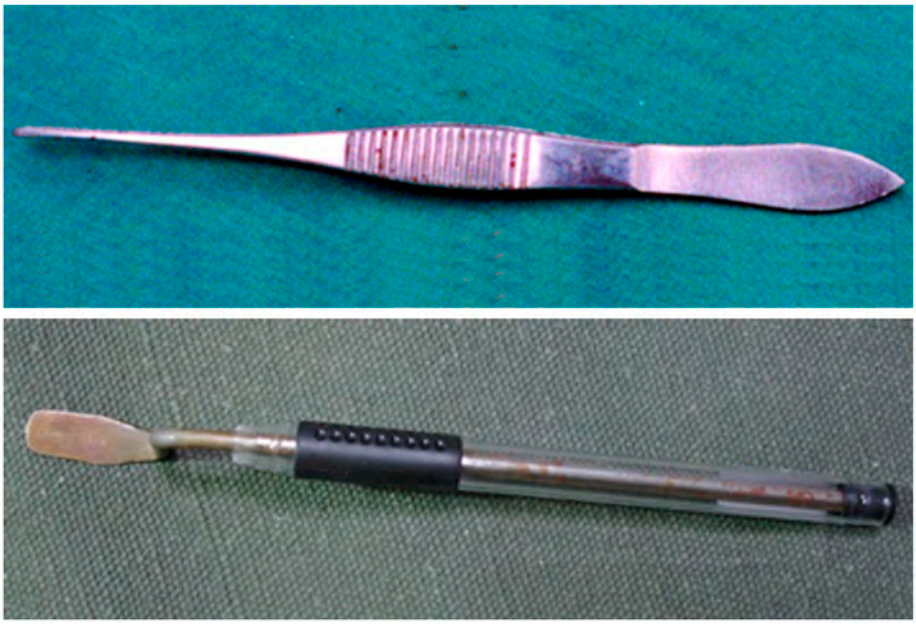

Figure 5. Tweezers (top) and queen-cell-wax-cleaner (bottom).
Photos: Zhongyin Zhang.

Figure 6. Stainless steel queen cell cutting blade.
Photo: Fei Zhang.

(6) Stainless steel queen cell cutting blade (Figure 6): before collection of royal jelly, a stainless steel queen cell cutting blade is used to cut off the protruding part of a queen cell built by workers above the plastic queen cup.

2.1.2. Management of colonies used for producing royal jelly

(1) Select strong colonies (standard colonies or colonies with an additional super) for the production of royal jelly.
(2) Divide the colony with a queen excluder into a propagation area (with a queen) and a production area (without a queen).

Note: The propagation area consists of 1 queen and 4–6 combs, including pollen combs, pupa combs, and empty combs for egg laying. The production area includes more than 5 combs with 1–2 pollen combs and several brood combs.

(3) Place brood combs in the middle of the frames, with pollen combs to their sides to favor brood tending by nurse bees.
(4) Insert the royal jelly production frame between 2 brood combs or between a brood comb and a pollen comb. Breed young larvae of the right age [see the *BEEBOOK* paper on queen rearing (Büchler et al., 2013)].

Note: To facilitate rapid larvae grafting, 2 queens are introduced into the same colony (with a shutter in the middle to separate them). Each separated area then includes 3–4 combs, with an empty comb close to the shutter for egg laying, and pollen combs close to the walls of a hive. Alternatively, a multiple-queen colony is organized to allow several queens to lay eggs on 1 comb [see the *BEEBOOK* paper on miscellaneous methods (Human et al., 2013) for the method to create multiple-queen colonies].

Preferably, the empty comb should be brown or light brown in order to cater to the queens' preference for used combs as well as to improve the visibility of larvae.

2.1.3. The procedure for producing royal jelly by artificially grafting larvae

There are five steps in the process of royal jelly production, including cleaning queen cells, grafting larvae, inserting royal jelly production frames, supplementary grafting, and harvesting.

2.1.3.1. Cleaning queen cells

(1) Clean queen cells with a detergent in warm water to remove the occasional layer of a grease-like substance on the inner wall of a new plastic queen cell.
(2) Dry the queen-cell-base-bar.
(3) Assemble the queen-cell-base-bar into a royal jelly production frame.
(4) Place the assembled frame into a colony for cleaning by the honey bees for 12–24 h.

2.1.3.2. Grafting of larvae

(1) Put the royal jelly production frame on the comb.
(2) Rotate the queen-cell-bar so that the opening of queen cells faces upward.
(3) Insert the tongue of a grafting pen along the inner wall of a worker cell until it slides underneath the body of a larva.
(4) Lift the larva together with the royal jelly.
(5) Insert the grafting pen until the tongue rests on the bottom of a queen cell.
(6) Gently press the spring-loaded plunger to force the larva and royal jelly off the tongue.
(7) Turn the openings of queen cells downward after each queen cell on the bar is loaded with a larva.
(8) Insert the frame into a royal jelly production colony.

Note: the grafting of larvae is the most important step during the process of producing royal jelly. The grafting action has to be gentle and fast and should be completed in a single lift-and-insert operation to avoid damaging the larvae. Otherwise, the larva should be discarded and a new larva, grafted. The best larvae age for the grafting is 12–18 h after they have hatched. Larvae at this age have a curvy body, a shovel-like shape, and are bathed in royal jelly.

2.1.3.3. Inserting the royal jelly production frame

(1) Insert the royal jelly production frame into a royal jelly production colony as soon as possible after all queen cells are loaded with larvae. Generally, a colony with 8–10 combs can support 1 or 2 royal jelly production frames, and a colony with more than 10 combs can have 2 or 3 royal jelly production frames.
(2) Open the hive 3–5 h after the royal jelly production frame was inserted into the royal jelly production colony, to check the acceptance of queen cells. If a larva in a queen cell has been accepted, it will be surrounded by nurse bees, and the queen cell will have fresh royal jelly secreted by workers. If there is damage during the transfer or rejection of a larva happens, the larvae will have been removed by workers. Supplementary grafting will be required in these situations, see Subsection 2.1.3.2.

2.1.3.4. Harvesting royal jelly

(1) Retrieve the royal jelly production frame from the hive 68–72 h after larvae grafting.
(2) Gently shake the frame to get rid of the bees.
(3) Brush it to remove the residual bees.
(4) Use a stainless steel queen cell cutting blade to cut off the protruding part of a queen cell.
(5) Remove the larvae from the queen cells using tweezers.
(6) Collect royal jelly from queen cells using a royal jelly scraping bar.
(7) Transfer the royal jelly into a royal jelly bottle for storage.
(8) Immediately freeze the bottle (see Subsection 3.1.2) or place it temporarily in a thermos flask with ice.

2.2. A method for producing royal jelly without grafting larvae

A new method for royal jelly production that does not require the grafting of larvae was recently described (Human et al., 2013; Pan, Wu, Guan, & Zeng, 2013; Wu et al., 2015; Zeng, 2013). The conventional method of producing royal jelly, which is based on grafting young larvae, is time-consuming, labor-intensive, and limited by the availability of larvae and the eyesight of the technician. The new method is based on a device that obviates grafting and is therefore convenient as well as efficient. The devices consist of a plastic worker foundation with regular holes (Figure 7), plastic cell bottoms mounted on a bar that can be inserted into the comb to fill the holes (Figure 8) and into the bottomless plastic queen cups on royal jelly production bars (Figure 9).

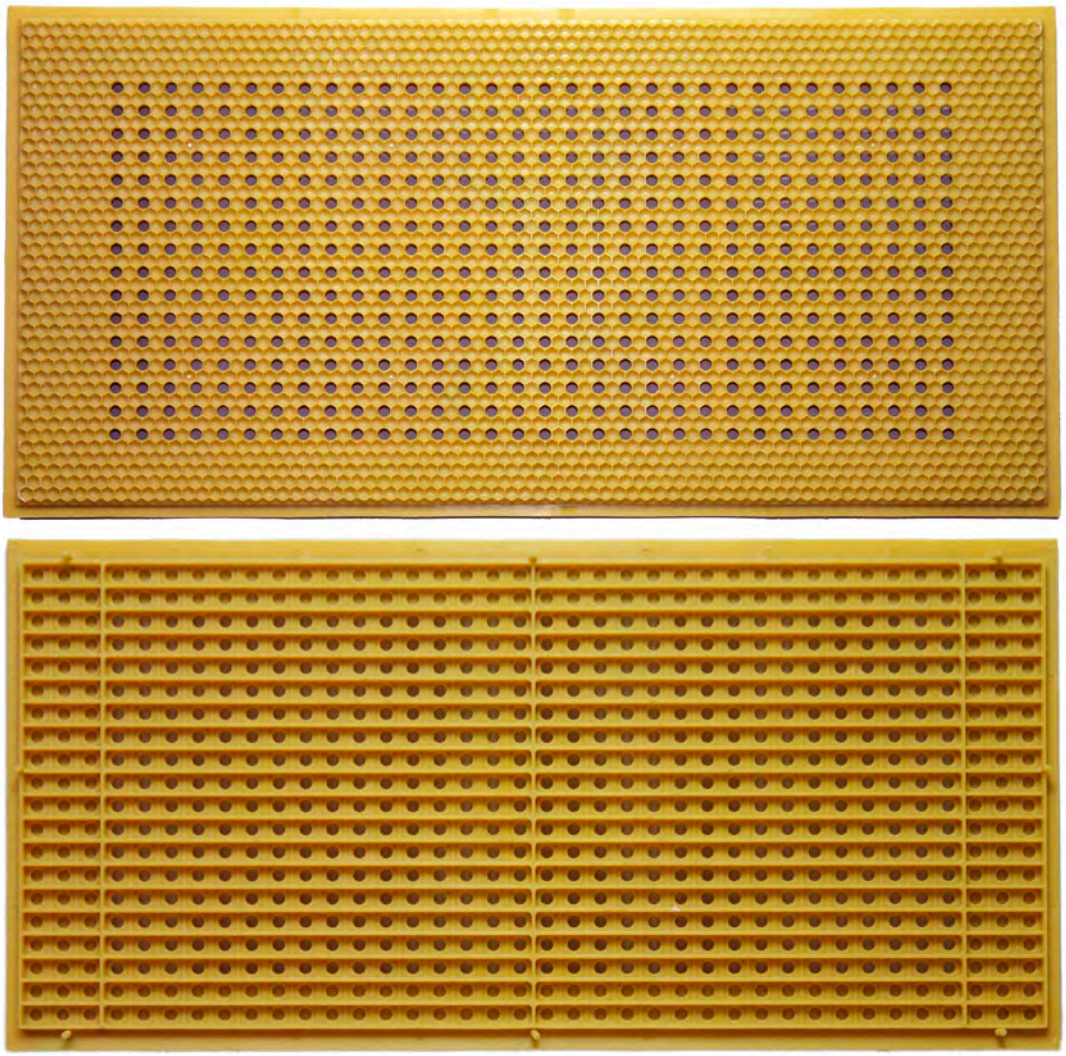

Figure 7. The plastic worker foundation with regular holes. Top: the front side. Bottom: the back side. Photo: Qizhong Pan.

Figure 8. Cell bottom bar. Top: the front side. Bottom: the back side. Photo: Qizhong Pan.

In short, the foundation and bars with cell bottoms are assembled and placed in a colony to allow for comb building by workers. A queen is then caged on this comb to lay eggs. Four days later, when the eggs hatch into larvae, the bars with cell bottoms are taken out and inserted into the openings of queen-cup-bars. The completely assembled queen-cell-bars are placed into a colony to produce royal jelly. See Figure 10 for the combination of a royal jelly production bar and supporting larva devices and Figure 11 for the assembled worker foundation in a frame by the combination of a plastic worker foundation with regular holes, cell bottom bars, and a frame. A cover plate is added to the back side of the worker foundation (Figure 11, bottom). A cover plate (Figure 12) for an assembled queen-cell-bar is present to improve the efficiency of the method of producing royal jelly without grafting larvae. When the bottomless royal jelly production bars are assembled

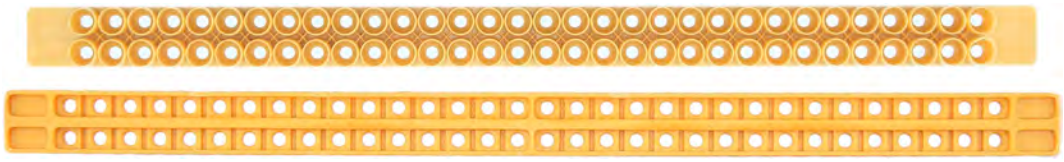

Figure 9. Royal jelly production bar. Top: the front side. Bottom: the back side.
Photo: Qizhong Pan.

Figure 10. The combination of a royal jelly production bar and cell bottom bars.
Photo: Qizhong Pan.

Figure 11. The assembled worker foundation in a frame by the combination of a plastic worker foundation with regular holes, cell bottom bars and a frame. Top: the front side. Bottom: the back side.
Photo: Qizhong Pan.

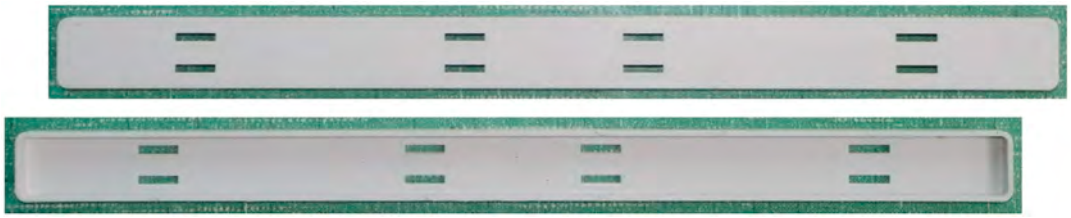

Figure 12. A cover plate for a royal jelly production bar. Top: the front side. Bottom: the back side.
Photo: Qizhong Pan.

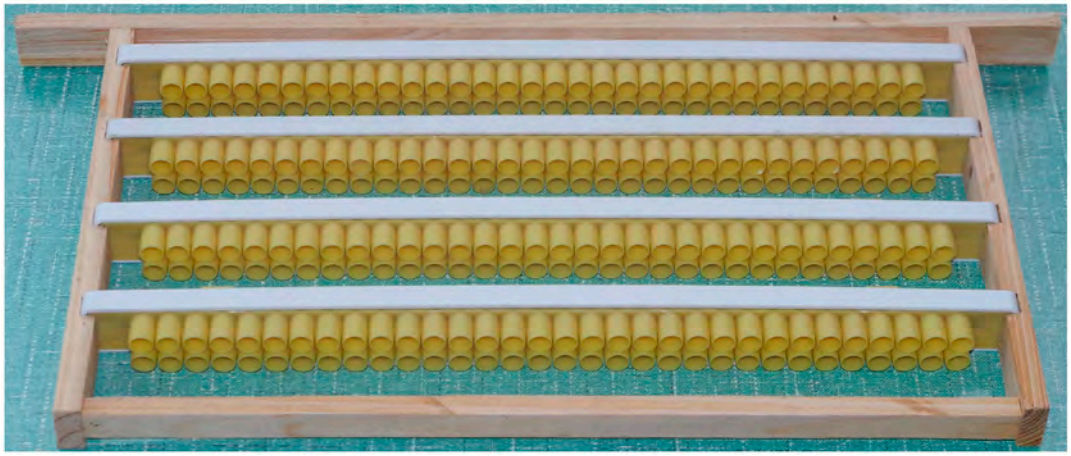

Figure 13. Royal jelly production frame.
Photo: Qizhong Pan.

Figure 14. A queen cell cleaner for producing royal jelly without grafting larvae.
Photo: Fei Zhang.

with the cell bottom bars, there may be some hidden gaps. This cover prevents workers from secreting wax in the hidden gaps. A royal jelly production frame consists of four royal jelly production bars, cell bottom bars, and cover plates (Figure 13). See Figure 14 for a queen cell cleaner for royal jelly production without grafting larvae.

There are three main procedures: building of the comb by workers, organizing an egg-laying colony, and production of royal jelly without grafting larvae.

2.2.1. Building the comb

(1) Select a strong colony with enough honey and pollen.
(2) Reduce the number of combs to 4–5 to ensure overcrowding.
(3) Boil old combs in water.
(4) Collect the water solution.
(5) Soak the plastic worker foundation with regular holes and cell bottom bars for 24 h.

(6) Let the parts dry and then assemble the foundation and cell bottom bars together.
(7) Apply a layer of beeswax to the assembled foundation.

There are 2 ways of applying wax:

- Use a broad brush to spread a thin layer of melted beeswax on the front side of the foundation.
- Alternatively, firstly, boil beeswax with a proper amount of water to achieve appropriate wax concentration (not too high) in a large iron container, then hold one side of the hollow comb foundation by hand, and dip a half of the foundation into the melted wax and pull it out immediately and shake rapidly. Do the same again for the other half of the comb foundation. Make sure that wax is in a liquid state during the whole wax application process.

These steps are meant to facilitate acceptance of the plastic foundation and comb building.

(8) Mount the assembled foundation on a frame.
(9) Place the frame in a colony, allowing bees to build the comb.
    If the procedure is not done during a strong nectar flow, feed the colony every night to promote the comb construction.
(10) Take out the comb immediately after it is built to prevent workers from using it for storage.
(11) If workers had time to store pollen and honey, place the comb in a centrifuge to remove honey.
(12) Remove pollen from cells with a tool of appropriate size.

2.2.2. *Obtaining brood*

(1) Create a multiple-queen brood provider colony [see the *BEEBOOK* paper on miscellaneous methods (Human et al., 2013)]. Such colonies ensure the availability of a large number of similarly aged larvae for producing royal jelly. Alternatively Use a colony with a new queen. This approach should ensure a high egg-laying rate.
(2) Partition the colony into an egg-laying area and a hatching area, for example, with a queen excluder covered by 2/3–3/4 with a thin wood or plastic board. A partly covered queen excluder reduces the amount of worker bees going through and results in less nectar or pollen storage in the comb in the egg-laying area.
(3) Close the entrance to the egg-laying area, while the entrance to the hatching area remains open.
(4) Place a 2-sided or a single-sided comb built from an assembled plastic foundation in the egg-laying area so as to allow a new queen or several queens to lay eggs in this comb.

2.2.3. *Production of royal jelly*

When 4 two-sided or single-sided combs built from assembled plastic foundations are prepared, colonies can be organized for royal jelly production. There are five steps involved: cleaning up queen cells, laying of eggs, removal of larvae, insertion of frames, and harvesting of royal jelly.

2.2.3.1. *Cleaning up queen cells*

(1) Boil old combs in water.
(2) Collect the water solution.
(3) Soak royal jelly production bars and cell bottom bars for royal jelly production for 24 h.
(4) Let the parts dry.
(5) Assemble the parts into a royal jelly production frame.
(6) Place the assembled frame in the royal jelly production colony to allow worker bees to clean it up for a day.

2.2.3.2. *Laying eggs*

(1) Mark 4 clean built-up combs from assembled worker foundations on their beams with numbers 1, 2, 3, and 4.
(2) Before egg-laying, allow bees in a colony to clean up comb No. 1 for 5–6 h.
(3) Insert comb No. 1 into a well-organized egg-laying colony (with several queens or a new queen laying eggs).
(4) Allow queens to lay eggs for 24 h in this comb.
(5) Take the comb out, shake off the queens, and place the comb into the hatching area.
(6) Simultaneously, put comb No. 2 into a well-organized egg-laying colony for 24 h and repeat the later step (step 5) of comb No. 1.
(7) The same steps are repeated for combs No. 3 and No. 4.
(8) When comb No. 4 is placed in the hatch area for hatching, take comb No. 1 out. Eggs on the cell bottom bars of comb No. 1 now have hatched into 1-day larvae (Figure 15).
(9) Pulled out the cell bottom bars loaded with larvae on comb No. 1 for royal jelly production.
(10) Soak comb No. 1 without cell bottom bars in clean water for 5–10 min.
(11) Spin off water, larvae, and eggs from comb No. 1 in a honey centrifuge.

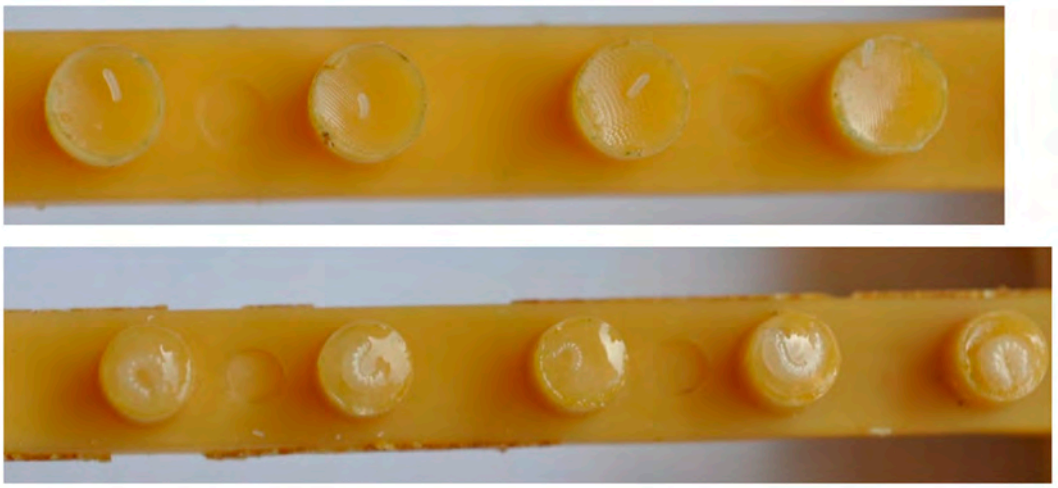

Figure 15. Eggs and larvae on cell bottom bars. Top: eggs. Bottom: larvae.
Photo: Zhijiang Zeng.

Figure 16. Royal jelly production frame covered with honey bees.
Photo: Qizhong Pan.

(12) Assemble this comb with new cell bottom bars again.
(13) Put the assembled comb in a colony for cleansing for 5–6 h.
(14) Insert it into a royal jelly production colony to allow queens to lay eggs again.

2.2.3.3. Taking out larvae

(1) Allow the larvae-loaded comb to hatch for 3 days.
(2) Pull out cell bottom bars loaded with larvae.
(3) Insert the cell bottom bars into a royal jelly production frame for royal jelly production.
(4) The action of pulling out cell bottom bars should be gentle, quick, and of regular intensity. Furthermore, there should be no gaps between cell bottom bars and royal jelly production bars to prevent a decrease in the acceptance rate of queen cells.
(5) Cover the assembled royal jelly production bars with cover plates.
(6) Assemble new unloaded cell bottom bars into the combs with holes in time for queens' continuously laying eggs.

2.2.3.4. Inserting frames

(1) Insert a royal jelly production frame between a larval comb and a pollen comb in royal jelly production colonies.

Generally, a colony with 8–11 combs can hold 1 royal jelly production frame. Nevertheless, when there are abundant nectar and pollen sources, 2 royal jelly

production frames can be inserted into a colony with more than 12 combs.

2.2.3.5. Harvesting royal jelly

(1) After 68–72 h of the frame insertion, take the royal jelly production frame out of the royal jelly production colony (Figure 16).
(2) Shake off workers remaining on the frame lightly and clean up the frame with a bee brush.
(3) Cut off the protruding part (made of wax) of queen cells (see Subsection 2.2.4.1).
(4) Pull out cell bottom bars.
(5) While harvesting royal jelly from a royal jelly production bar, clean bottomless queen cells by a queen cell cleaner (Figure 14). When bottomless queen cells in the royal jelly production bar are clean, the bar can be assembled with cell bottom bars again, and a new cycle of royal jelly production begins all over again.

2.2.4. Harvesting royal jelly by a machine

Three steps of the royal jelly production without grafting have been mechanized: cutting off the protruding part of a queen cell, removal of the larvae, and harvesting of royal jelly. The two necessary tools are a wax-cutting device and a blower for harvesting royal jelly. The process includes five steps: cutting down the protruding part of queen cells, blowing royal jelly, filtering royal jelly, collecting royal jelly, and cleaning up.

2.2.4.1. Cutting off the protruding part of queen cells

(1) Push cutter to the left side, at the starting position (Figure 17).
(2) Draw out the royal-jelly-collecting basin along the horizontal guide line to the right (Figure 17).
    A piece of warm damp cloth is used to wipe the cutter to facilitate cutting down the protruding part of a queen cell.
(3) Remove royal jelly production frames from a colony.
(4) Brush away worker bees.
(5) Pull out cell bottom bars.
    The royal jelly remains in the queen cup.
(6) Wedge the royal jelly production bars from the frames in a working platform (Figure 18).
    The cup openings of the royal jelly production bars and royal jelly are directed towards the bottom. The platform can accommodate 7 royal jelly production bars.
(7) Place the platform in the wax-cutting device. A wax-collecting basin is placed underneath.
(8) Hold the 2 handles of the cutter and pull away from the starting position as shown in Figure 18. The cutting blade is underneath the royal jelly production bars.

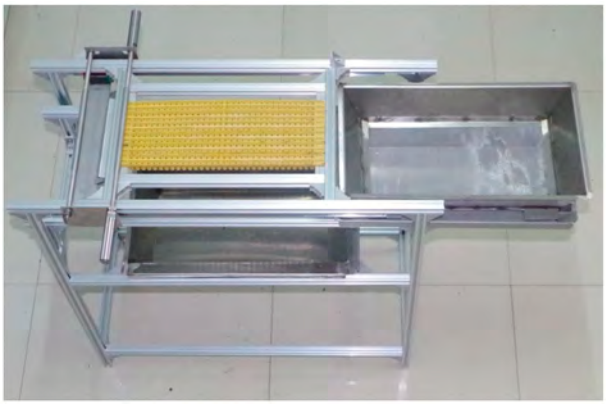

Figure 17. A wax cutting device for royal jelly production.
Photo: Qizhong Pan.

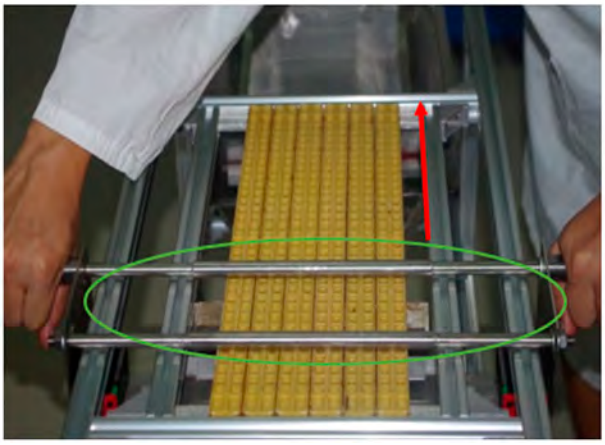

Figure 18. The operation of cutting off the protruding part of queen cells.
Photo: Qizhong Pan.

(9) Slide the cutter forward to cut the protruding part of queen cells.
(10) Slide the cutter back to the starting position.
    One back-and-forth movement of these cutters can complete wax cutting of several royal jelly production bars. The removed wax will drop into the wax-collecting basin automatically.

2.2.4.2. Blowing royal jelly

(1) Push the royal-jelly-collecting basin beneath queen cell bars.
    Note: There is a royal jelly filter screen above the royal-jelly-collecting basin (Figure 19).
(2) Turn on an oil less air compressor.
(3) Aim the spray gun to bottomless openings of royal jelly production bars at 45° (Figure 19).

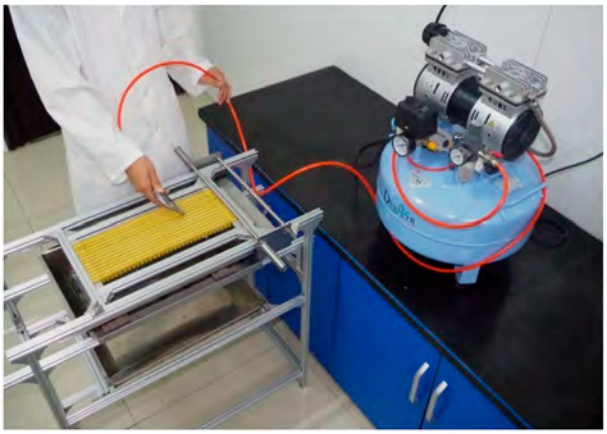

Figure 19. The operation of blowing royal jelly into the basin at the bottom.
Photo: Qizhong Pan.

Figure 20. The operation of filtering through royal jelly by a filter screen to separate royal jelly from larvae.
Photo: Linbin Zhou.

(4) Press the lever switch, and blow larvae from queen cells into the filter screen with weak airflow (3–4 kPa).
(5) Move the gun back and forth 2–3 times continuously, blowing with a stronger airflow (7–8 kPa) at the bottomless openings of royal jelly production bars, to blow royal jelly (that is attached to the queen cell wall) into the filter screen. At this stage, most of royal jelly will be filtered through the screen to the basin, and the larvae will remain above the screen.

2.2.4.3. Filtering royal jelly

(1) Blow the residual royal jelly found on the filter into the royal-jelly-collecting basin using weak airflow (Figure 20).
(2) Discard the larvae from the filter.
(3) Proceed to the next collection round.

2.2.4.4. Collecting royal jelly

(1) Collect royal jelly from the collection basin when a required amount has accumulated.
(2) Freeze or process it as required (see Subsection 3.2).

2.2.4.5. Cleaning up. Clean all components of the royal-jelly-collecting machine and air compressor according to instruction manuals.

See Table 1 for the pros and cons of the two methods for production of royal jelly.

3. Quality control of royal jelly

3.1. Storage of royal jelly

3.1.1. Freshness of royal jelly

Royal jelly is sensitive to heat, light, and air. Although there are no data on changes in its biological effectiveness for humans after inappropriate long-term storage, various changes in physical and chemical properties have been reported, such as a higher acid titre, a darker color, higher viscosity, a large insoluble-protein fraction, lower amounts of free amino acids, and less glucose oxidase (Albalasmeh, Berhe, & Ghezzehei, 2013; Kamakura, Fukuda, Fukushima, & Yonekura, 2001; Karaali et al., 1988; Takenaka, Yatsunami, & Echigo, 1986). Special precautions are thus necessary to preserve the biological properties of royal jelly during the shelf period.

3.1.2. Freezing of royal jelly

Refrigeration and freezing delay and reduce the chemical changes in royal jelly during storage. The following points should be considered for storage of fresh royal jelly.

(1) Transfer royal jelly into a dark and airtight container immediately after collection.
If royal jelly will be used rapidly,
(2) Refrigerate at 0–5 °C.

Table 1. Comparison of the two methods for producing royal jelly.

Method	Advantages	Disadvantages
Method with grafting of larvae	Beekeepers are familiar with this technology; the acceptance rate of queen cells is high; the tools for production are simple	Artificial larvae grafting is labor-intensive; beekeepers should have good eyesight
Method without grafting of larvae	Beekeepers with poor eyesight can produce royal jelly; grafting of larvae is not needed	The tools for production are costlier; the egg-laying queen needs to have high fecundity

Alternatively, if royal jelly will be stored for a longer period,

(3) Freeze at temperatures below −18 °C.
   - To protect royal jelly from light, it must be packed in dark containers.
   - To protect it from oxidation, the container must be airtight.
   - Because there are no criteria for establishing "safety" limits for product activity, storage and shelf-life should be as brief as possible.
   - After defrosting and packaging, the product should not be stored in a refrigerator for more than 12 months.
   - Repeated freeze-thaw cycles must be avoided.

3.1.3. Freeze-drying of royal jelly

Freeze-drying, also known as lyophilization or cryodesiccation, is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport. The process works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase (Franks, 1998). Compared to fresh royal jelly, a lyophilized powder of royal jelly has the following advantages:

- Royal jelly powder is less sensitive to temperature, and can be kept at room temperature for a longer period, making longer-range transportation possible.
- Royal jelly powder can be further processed into other products, such as royal jelly tablets, or mixed with other products.

The developmental and physiological activity of fresh royal jelly and freeze-dried royal jelly were compared on the fruit fly by Kayashima, Yamanashi, Sato, Kumazawa, and Yamakawa-Kobayashi (2012). The result showed that freeze-dried royal jelly retained certain properties of fresh royal jelly, suggesting that the freeze-drying process does not alter the quality and the functions of royal jelly significantly (Kayashima et al., 2012). Generally, there are several steps to freeze-dry royal jelly, as described below.

(1) Pass freshly collected royal jelly through a 100-mesh filter, to remove potential contamination with beeswax and larvae before freeze-drying. If necessary, distilled water can be added to reduce royal jelly viscosity.
(2) Transfer the filtered royal jelly to open vessels such as glass plates and pre-freeze the sample in a freezer at −18 °C or in a freeze-dryer.
(3) Set chamber temperature of a freeze-dryer to −40 °C and place the sample into the chamber of the freeze dryer.
(4) Start to vacuumize the chamber until the chamber pressure reaches 1.33 Pa.
(5) Raise the shelf temperature to −25 °C, and keep it at this temperature for 12 h.
(6) Then raise the temperature to 30 °C and maintain it for 6 h.
(7) Package the royal jelly powder in an airtight container because freeze-dried royal jelly powder is very hygroscopic.

Because the functions and capacity of different freeze-dryers vary, for detailed operations, please refer to the corresponding operation manuals.

Freeze-dried royal jelly is certainly more stable than the fresh product, but it is still best to store it by refrigeration or freezing because we still know little about the composition and changes in biological effectiveness of royal jelly powder during processing and storage.

3.1.4. Freshness parameters of royal jelly

Methods for evaluation of freshness of royal jelly are useful for the bee product industry, and identification of an indicator to measure freshness of royal jelly received a lot of attention from researchers. Trans-10-hydroxy-2-decenoic acid (10-HDA), an important quality criterion of royal jelly, has been demonstrated to be stable in royal jelly and to not be affected by the storage conditions (Antinelli et al., 2003; Kamakura et al., 2001). The enzymatic activities of superoxide dismutase (Tang & Yuan, 1999) and glucose oxidase (Wu & Zhang, 1990) were found to be affected by storage duration and conditions, but the values decreased rapidly. Chen and Chen (1995) have found that the proteins in royal jelly are affected by storage conditions (Chen & Chen, 1995; Tang & Yuan, 1999; Wu & Zhang, 1990). Kamakura et al. (2001) have stated that major royal jelly protein 1 (MRJP1, also known as royalactin) is a suitable freshness indicator because its concentration shows a good correlation with storage duration and conditions (Kamakura et al., 2001). With proteomics approaches, other MRJPs were found to be affected by storage conditions, and MRJP5 was proposed as a suitable freshness marker (Li, Feng, Zhang, Zhang, & Pan, 2008; Zhao et al., 2013). The concentration of furosine, a product of an early Maillard reaction between amino acid residues and reducing sugars, was proven to be very low in fresh royal jelly samples and increased over time with some dependence on temperature (Marconi, Caboni, Messia, & Panfili, 2002). Zheng, Wei, Wu, Hu, and Dietemann (2012) found that via an unknown mechanism, the color of the mixture of royal jelly and HCl shows a good correlation with storage duration and temperature (Zheng et al., 2012). Recently, formation of 5-hydroxymethyl-2-furaldehyde (HMF) in fresh royal jelly as a function of storage temperature and storage duration was monitored by means of an Reverse Phase-High Performance

Liquid Chromatography (RP-HPLC) method (Ciulu et al., 2015). The authors suggested the use of HMF as a possible freshness marker for royal jelly according to the finding that all the samples stored at −18 °C and 4 °C did not show any detectable levels of HMF, whereas those stored at 25 °C showed exponentially increasing amounts of HMF from the first month of storage.

Despite several criteria available as royal jelly freshness indexes, no method has been established as a standard assay of royal jelly freshness. Further research is required to clarify the variations of these criteria in different royal jelly samples, e.g., royal jelly produced in different seasons and/or areas (Wei et al., 2013) and/or by different races of bees, and to establish a threshold for each level of freshness.

3.2. Quality criteria

3.2.1. Water content

Water content is an important quality criterion of raw royal jelly. Several methods have been used for the measurement of water content of royal jelly: drying at different temperatures under reduced pressure (namely loss on drying) and refractometric analysis (Sesta & Lusco, 2008) and Karl Fischer titration (Teresa, Helena, Ewa, & Piotr, 2009). Karl Fischer titration is a classic titration method of analytical chemistry and involves colormetric or volumetric titration to determine trace amounts of water in a sample. The popularity of the Karl Fischer titration is due in large part to several practical advantages that it offers (over other methods of moisture determination), for example, accuracy, speed, and selectivity.

Presently, the titration is done with an automated Karl Fischer titrator. Elaboration of the procedures depends on the optimal sample weight and minimal homogenization time. For royal jelly, these two parameters were established at 0.02–0.05 g and 120 s, respectively, by Teresa et al. (2009).

For the cases when an automated Karl Fischer titrator is not available, the loss-on-drying method is recommended because it is a classic laboratory assay for measuring high levels of moisture in solid or semi-solid materials like royal jelly. Loss-on-drying analysis can be conducted at different temperatures (e.g., 48, 65, 90, and 105 °C) under atmospheric or reduced pressure.

A vacuum oven drying method has been described by Sesta and Lusco (2008). To enable complete water loss, the sample has to be spread in a thin layer on a wide surface. At the weighing step, however, this approach causes a visible loss of water by evaporation, producing unstable and continuously decreasing readings of weight on the scale.

To obtain an accurate measure, the method for determining the water content in royal jelly requires some care.

(1) Turn on the oven and the vacuum pump 2 h before inserting the samples.
(2) Set the temperature to 48 °C.
(3) Set the vacuum to higher than −800 mbar, resulting in a pressure lower than 200 mbar.

Due to vacuum, which accelerates water loss, a relatively low temperature (48 °C) can be employed. The 48 °C temperature was proven to be more conservative for royal jelly because it leaves the color of the sample unchanged, and proved to be sufficient for drying because it prevents formation of a superficial brown sugar crust that hampers complete evaporation (Sesta & Lusco, 2008).

(1) Weigh the Petri dish (where the sample is to be spread) on an analytical scale (precision 0.1 mg).
(2) Record the weight of the Petri dish as "Petri".
(3) Weigh the clean spatula to be used for spreading the sample.
(4) Record the weight of the spatula as "clean spatula".
(5) Transfer a homogenized royal jelly sample of approximately 3 g to the Petri dish as fast as possible.
(6) Immediately weigh it on the analytical scale.
(7) Record the weight as "Petri + R".
(8) Spread the royal jelly uniformly over the dish surface using the weighed spatula.
(9) Weigh the spatula, on which some residue of royal jelly remained.
(10) Record the weight as "dirty spatula", to take into account the weight of the residual sample left on the spreading tool.
(11) Calculate the accurate amount of royal jelly sample processed as follows: "royal jelly" = ("Petri + R" – "Petri") − ("dirty spatula" – "clean spatula").
(12) Transfer the Petri dish to a vacuum oven.
(13) Incubate for 24 h.
(14) Transfer the dried sample into a desiccator.
(15) Allow it to stand for 15 min to make sure it reaches the ambient temperature.
(16) Weigh the Petri dish on an analytical scale.
(17) Record the weight as "after drying".
(18) Calculate the water content as [("royal jelly" – "after drying")/"royal jelly"] × 100%.

In general, because the manual laboratory method is relatively slow, automated moisture analyzers based on the loss-on-drying method have been developed and can reduce the time necessary for a test from a couple hours to just a few minutes.

3.2.2. 10-HDA content

10-HDA is a major fatty acid component of royal jelly. It has many pharmacological activities such as antitumor effects (Townsend, Brown, Felauer, & Hazlett, 1961), size- and lipogenesis-inhibiting activity toward the hamster ear sebaceous gland (Maeda, Kuroda, &

Motoyoshi, 1987), collagen production-promoting effects (Koya-Miyata et al., 2004), and antibiotic properties (Blum, Novak, & Taber, 1959). The presence of 10-HDA has been regarded as a marker to differentiate royal jelly from other products. Its concentration has been used as a parameter of royal jelly quality. According to the guidelines of the Ministry of Agriculture (MOA) in China and the ISO royal jelly international standard (ISO 12824:2106, 2016), the minimum concentration of 10-HDA is 1.4% for pure royal jelly.

Many different methods have been described for determination of 10-HDA in royal jelly, including high-performance liquid chromatography (HPLC) (Bloodworth, Harn, & Hock, 1995; Genç & Aslan, 1999), ultra-performance liquid chromatography (Zhou et al., 2007), gas chromatography (Anonymous, 1989), and gas chromatography–mass spectrometry (GC/MS) (see Section 6.3.1), among which, HPLC is a routine method and is thus cited in standard regulations. In particular, as a conventional method, HPLC can be carried out using standard equipment in many laboratories and is also simple, sensitive, and suitable for monitoring 10-HDA.

Quantitative determination of 10-HDA by HPLC:
Requirements:
Reagents: 10-HDA standard, methyl-4-hydroxybenzoate as the internal standard, methanol, absolute ethanol, hydrochloric acid, and phosphoric acid.
HPLC instrument: Waters 2695 with DAD detector.

Procedure:

Prepare a 1000 μg/ml internal standard solution (methyl-4-hydroxybenzoate) in absolute ethanol.

(1) Prepare working solutions of 0.1, 0.5, 5, 10, 20, 40, 80, 160 μg/ml 10-HDA in absolute ethanol (the concentration of the internal standard solution is 100 μg/ml).
(2) Weigh 0.5 g of royal jelly cream sample or 0.15 g of lyophilized powder (see Subsection 3.1.3) into a 50-ml volumetric flask.
(3) Add 3 ml of 1 mol/l HCl.
(4) Add 5 ml of a 1 mg/ml internal standard solution.
(5) Add 40 ml of absolute ethanol in sequence.
(6) Dissolve completely by ultrasonication with occasional shaking for 15 min.
(7) Add absolute ethanol to the marked volume (50.00 ml).
(8) Pass the solution through a 0.2-μm nylon filter before injection into the HPLC apparatus.

HPLC analytical conditions:

(1) Connect the chromatograph with a Waters Nova-pak C18 column (150 × 3.9 mm, 5 μm i.d.).
(2) Set the mobile phase to the 1/1 ratio (0.3% phosphoric acid in water/methanol).
(3) Set the flow rate to 0.8 ml / min.
(4) Set the column temperature to 30 °C.
(5) Set the injection volume to 5 μl.
(6) Set the detection wavelength to 210 nm.
(7) Set the total time to 10 min.
(8) Collect the data using Waters Empower software.

3.2.3. *Protein content*

See Section 4.

3.2.4. *Sugar content*

See Section 5.

4. Royal jelly protein research

4.1. *Isolation of royal jelly proteins and peptides*

4.1.1. *Introduction*

The main protein components of royal jelly are major royal jelly proteins, accounting for 82% of total royal jelly protein, with molecular masses of 49–87 kDa assigned to one protein (and gene) family (Hanes & Šimuth, 1992; Malecová et al., 2003; Ohashi, Natori, & Kubo, 1997; Schmitzová et al., 1998). It has been suggested that this be denoted as MRJP, derived from major royal jelly protein, before their physicochemical properties were known (Hanes & Šimuth, 1992; Šimúth, 2001). The numerical symbol added to the acronym denotes the respective main protein. It was later found that all of these proteins are easily soluble in water, just as other albumin-like proteins: endosperm albumin, lactalbumin, ovalbumin, and serum albumin (Smith, 1997). The name albumin originates from the Latin term *albus;* it exists in nearly pure form in egg white, prompting the suggestion to rename major royal jelly proteins as *apalbumins* (prefix *ap* is derived from *Apis*). This means that apalbumin1 denotes MRJP1, apalbumin2 denotes MRJP2, and so on. These proteins are present not only in the larval food of a queen but also in larval food for the larvae of honey bee workers and drones. The term apalbumin implies the ubiquity and multifunctionality of royal jelly proteins; this name is a response to recent data that royal jelly proteins not only are present in royal jelly but also are synthesized in honey bee brain (Kucharski, Maleszka, Hayward, & Ball, 1998) and are authentic proteins of honey bee products (Bíliková & Šimúth, 2010; Di Girolamo, D'Amato, & Righetti, 2012; Šimúth, Bíliková, Kováčová, Kuzmová, & Schroeder, 2004). In this chapter, we will refer to the main protein of royal jelly as MRJP.

The gene family of MRJPs, encoding 9 closely related proteins is arranged in a 65-kb tandem array (The Honey bee Genome Sequencing Consortium, 2006). The most abundant royal jelly proteins consist of MRJP 1, 2, 3, 4,

and 5 representing 90% of total proteins of royal jelly. MRJPs 6–9 are present in royal jelly in trace amounts. The molecular properties of MRJP (6–9) as authentic proteins isolated in natural form have been unknown until now. No conclusions about molecular properties of MRJPs 6–9 can be drawn from modern bioinformatics data. Other proteins such as alpha-glucosidase, glucose oxidase, and alpha-amylase have also been detected in royal jelly (Ohashi, Sawata, Takeuchi, Natori, & Kubo, 1996; Scheparts, 1965), but these enzymes are also present in honey. Proteins and peptides present in honey bee larval food play a significant role in honey bee life. They are a source of nutrition because of high concentrations of essential amino acids (Schmitzová et al., 1998). MRJPs are synthesized in cephalic glands of the honey bee (Hanes & Šimuth, 1992). Most recently, shotgun proteomics were used to identify the royal jelly proteome as well as proteomes of the hypopharyngeal gland, post-cerebral gland, and thoracic gland, from which royal jelly proteins are assumed to be derived (Fujita et al., 2013). MRJPs can be considered ubiquitous proteins, with multifunctional properties.

MRJP1 mRNA was found to be differentially expressed in heads of early emerged honey bees (Kucharski et al., 1998). The nurse brain showed increased expression of major royal jelly proteins (MRJP1, MRJP2, and MRJP7), which are related to the determination of castes during honey bee larvae differentiation (Garcia et al., 2009; Hojo, Kagami, Sasaki, Nakamura, & Sasaki, 2010). Unlike MRJPs 1–7, MRJPs 8 and 9 are expressed in the honey bee venom gland (Blank, Bantleon, McIntyre, Ollert, & Spillner, 2012).

The various health-promoting properties of royal jelly (Ramadan & Al-Ghamdi, 2012) and new proteomic data on royal jelly proteins initiated extensive research into the physiological functions of royal jelly proteins (Buttstedt, Moritz, & Erler, 2014). These proteins on a global scale are expected to have a significant impact on human immunotherapy and as potential proteinaceous antibiotics (Fontana, Mendes, Souza, & Konno, 2004; Šimúth et al., 2004; Tamura et al., 2009; Watanabe et al., 1998). The royalactin isoform of apalbumin1 is a queen determinant (Kamakura et al., 2001).

Recently, physiological properties of royal jelly and MRJPs were uncovered in another laboratory. Consequences of this fact are often problems with verification of experimental data for objective evaluation of the biological potential of royal jelly and MRJP1 from a methodological point of view. Therefore, the overview of the methods referred to in this chapter should contribute to standardization of methods used in research on royal jelly proteins.

4.1.2. Isolation of major royal jelly proteins

The systematic research on royal jelly proteins started with isolation of the individual major proteins of royal jelly using size exclusion and ion exchange column chromatography, one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1DE), immunoblotting, isoelectrofocusing, cDNA cloning, and N-terminal amino acid sequencing (Bíliková et al., 2002; Schmitzová et al., 1998). These methods enabled characterization of the most abundant proteins of royal jelly, namely MRJP 1–5.

Differences in molecular and chemical properties among MRJPs are used for their isolation. The high molecular weight of the oligomeric form of MRJP1 is used for its isolation by size exclusion chromatography (see Subsection 4.1.2.2, Figure 21). MRJP1 is the most abundant protein in royal jelly and its acidic properties contribute to the acidic nature of royal jelly. MRJP2 is a slightly basic protein, and MRJP3, 4, and 5 are almost neutral proteins. These differences in polarity of royal jelly proteins are utilized in column ion exchange chromatography, where the acidic MRJP1 is eluted at 0.25 M NaCl, while the other MRJPs are present in the fraction eluted at low ionic strength (0.05 M NaCl).

These analyses formed the basis for further characterization of royal jelly proteins by proteomic methods. Before proteomic methods became available, conventional methods were applied to describe only the most abundant royal jelly proteins. Now, due to the proteomic approach, direct data on minor components at the molecular level may be derived. Latest discoveries about molecular properties and physiological function of royal jelly proteins during the last two decades help to provide new knowledge about royal jelly for proteomics and nutrigenomics and potential use for human health.

4.1.2.1. Preparation of the water-soluble protein fraction from royal jelly. The fraction of water-soluble proteins is prepared from fresh royal jelly according to Hanes and Šimuth (1992).

(1) Homogenize 30 g of royal jelly in a beaker (on a magnetic stirrer) containing 90 ml of phosphate buffer (50 mM NaH_2PO_4, 50 mM Na_2HPO_4, pH 7.0, 100 mM NaCl, 20 mM EDTA).

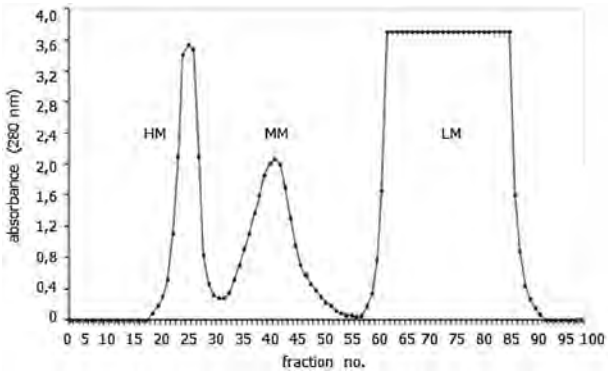

Figure 21. Fractionation of water-soluble fraction of royal jelly by size-exclusion chromatography. Sephadex G-200, elution by 50 mM NaCl in Tris/EDTA buffer, obtained high (HM), middle (MM), and low (LM) molecular weight protein fraction, respectively.

(2) Vortex the mixture for 30 min at room temperature.
(3) Centrifuge it 15,250*g* for 30 min, collect the supernatant.
(4) Dialyze the supernatant against 1 l of Tris-EDTA buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA) in tubing with molecular weight cut-off (MWCO) 10 kDa, at 4 °C.
(5) Change diffusates every 12 h, 4 times in total.
(6) Centrifuge at 15,250*g* for 30 min at 4 °C.
(7) Use the supernatant containing the water-soluble proteins for subsequent purification or concentrate the fractions by lyophilization (see Subsection 3.1.3) or by Macrosep 10 K (MWCO 10 kDa) centrifugal concentrators (Pall Gelman Sciences, Germany).
(8) Store solid protein samples at 4 °C, or protein solutions at −20 °C for further analysis.

4.1.2.2. Fractionation of proteins of royal jelly by size exclusion chromatography. Royal jelly proteins are purified by size exclusion chromatography from a water-soluble fraction of royal jelly (see Subsection 4.1.2.1), according to the following steps.

(1) Apply 30 ml of a sample (supernatant obtained by centrifugation of royal jelly) containing 2 g of proteins in Tris-EDTA buffer (20 mM Tris–HCl and 1 mM EDTA, pH 7) on a Sephadex G-200 (Pharmacia Biotech, Sweden) column (46 × 750 mm) at the flow rate of 1 ml/min.
(2) Wash the column with Tris-EDTA buffer.
(3) Perform elution by means of 50 mM NaCl in Tris-EDTA buffer at the flow rate of 1 ml/min.
(4) Collect 10-ml fractions.
(5) Monitor the absorbance of the fractions at 280 nm.
(6) Pool fractions corresponding to the peak with absorbance at 280 nm, in order to obtain 3 fractions: high (HM)-, middle (MM)-, and low (LM)-molecular-weight protein fraction (Figure 21).
(7) Dialyze the fractions in tubing with MWCO 3 kDa against Milli-Q water at 4 °C.
(8) Concentrate fractions by lyophilization (see Subsection 3.1.3) or using Macrosep 10 K (MWCO 10 kDa) centrifugal concentrators (Pall Gelman Sciences, Germany).
(9) Store solid protein samples at 4 °C or protein solutions at −20 °C for further analysis.

4.1.2.3. Fractionation of royal jelly proteins by ion exchange chromatography. Water-soluble royal jelly proteins (see Subsection 4.1.2.1) are used for purification of major royal jelly proteins by ion exchange chromatography.

(1) Load 150 ml of a dialyzed sample of water-soluble royal jelly proteins (1 mg protein per ml) at the flow rate of 1 ml/min on a DEAE (Diethylaminoethyl) cellulose column (35 × 400 mm) equilibrated with Tris-EDTA buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA).
(2) Wash the column with Tris-EDTA buffer.
(3) Use a linear gradient from 0 to 0.4 M NaCl in Tris-EDTA buffer for elution of the proteins.
(4) Collect 7-ml fractions, monitor the elution at 280 nm.
(5) Pool the fractions of the first peak eluted at approximately 0.05 M NaCl in Tris-EDTA buffer. The first peak corresponds to the weakly bound neutral and basic proteins, mainly MRJP2–MRJP5.
(6) Pool the fractions of the second peak eluted approximately at 0.2 M NaCl in Tris-EDTA buffer; it corresponds to the acidic protein MRJP1.
(7) Dialyze the protein solutions in tubing with MWCO 10 kDa against Milli-Q water.
(8) Concentrate fractions by lyophilization (see Section 3.1.3) or by means of Macrosep 10 K (MWCO 10 kDa) centrifugal concentrators with (Pall Gelman Sciences, Germany).
(9) Store the solid protein samples at 4 °C or protein solutions at −20 °C for further analysis.

4.1.2.4. Isolation of oligomeric MRJP1 by HPLC. Preparation of a royal jelly protein sample for HPLC analysis:

(1) Resuspend fresh royal jelly in phosphate-buffered saline (1:20, w/v) immediately before use.
(2) Dialyze 3 ml of the royal jelly suspension in cassettes with MWCO 3.5 kDa (PIERCE, Rockford, Illinois, USA) against distilled water for 1 week at 4 °C.
(3) Add 1.5 ml of distilled water to the dialyzed royal jelly.
(4) Incubate the royal jelly solution for 30 min at 37 °C.
(5) Centrifuge it at 12,500× *g* for 30 min at room temperature.
(6) Centrifuge the supernatant at 12,500× *g* for 30 min at 4 °C.
(7) Store the supernatant at 4 °C until analysis.
(8) Measure the total protein concentration in the sample using the Micro BCA protein Assay Kit (PIERCE), with human serum albumin (WAKO, Tokyo, Japan) as a standard protein.

Size exclusion HPLC analysis:

(1) Calibrate the column (Superose 12 column, 10 × 300 mm, GE Healthcare, Buckinghamshire, England) using Gel Filtration Calibration Kits with low-molecular-weight and high-molecular-weight proteins (GE Healthcare).
(2) Inject 100 μl of a sample into the column.

(3) Perform elution with phosphate-buffered saline (20 mM Na_2HPO_4, 2 mM $NaH_2PO_4{\cdot}2H_2O$, 150 Mm NaCl, pH 7.5) at the flow rate 0.5 ml/min.
(4) Collect 0.8-ml fractions and monitor HPLC elution profiles at 280 nm using the Akta Explorer System (GE Healthcare).
For anion exchange HPLC analysis, a miniQcolumn (4.6 × 50 mm, GE Healthcare) is used.
(1) Inject 1 ml of the sample in binding buffer (20 mM Tris–HCl pH 8.0) into the column.
(2) Perform elution with a buffer consisting of 20 mM Tris–HCl pH 8.0, 1 M NaCl, with NaCl gradient from 0 to 0.5 M at the flow rate 1 ml/min.
(3) Collect 1-mL fractions and monitor HPLC elution profiles at 280 nm using the Akta Explorer System (GE Healthcare).
(4) Desalt the samples by dialysis against distilled water.
(5) Concentrate the samples with Minicon (Millipore; Billerica, MA, USA).

4.1.3. Isolation of royal jelly peptides

Some honey bee antimicrobial peptides (apidaecin, abaecin, and hymenoptaecin) are induced specifically by pathogens and are released into haemolymph only after a bacterial infection, whereas others such as royalisin and apisimin are secreted into royal jelly. The molecular weight of royalisin is 5523 Da, and it consists of 51 amino acid residues, where six cysteine residues form three intramolecular disulphide linkages resulting in a compact globular structure. Royalisin belongs to the family of insect defensins: cysteine-rich cationic antimicrobial peptides.

Apisimin was found in the high-molecular-weight fraction, in the presence of MRJP1 (apalbumin1). Apalbumin1 tends to create a self-assembled structure and to form a gel. It appears that apisimin interacts with apalbumin1 and forms an oligomeric [apalbumin:apisimin] complex of yet unknown stoichiometry and structure. This complex is stable under the conditions used during size exclusion chromatography and ion exchange column chromatography. Native apisimin was obtained by separation of the high-molecular-weight fraction using ion exchange FPLC (Fast Protein Liquid Chromatography) in the presence of 100 mM glycine. In the absence of glycine, apisimin is eluted together with apalbumin at 0.2 M NaCl.

4.1.3.1. Purification of cationic royal jelly peptides by a 2-step dialysis. Dialysis is a simple method that can be used for separation of royal jelly proteins and peptides with appropriate buffers. Purification of royal jelly peptides according to Bíliková, Wu, and Šimúth (2001) is based on two step dialysis (Bíliková et al., 2001). The first dialysis of a royal jelly suspension in tubing with MWCO 2 kDa enables removal of sugars and the low-molecular-weight compounds. The second dialysis in tubing with MWCO 10 kDa allows for the diffusion of peptides from the retentate (royal jelly suspension) to the diffusate that are collected and concentrated. A simple scheme of 2-step dialysis of a royal jelly suspension is presented in Figure 22.

Wear gloves throughout the procedure to prevent contamination of the samples.

(1) Homogenize 30 g of royal jelly in a beaker (on a magnetic stirrer) containing 90 ml of phosphate buffer (50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 100 mM NaCl, 20 mM EDTA, pH 7.0).
(2) Dialyze the royal jelly suspension in tubing with MWCO 2 kDa (Serva Electrophoresis, Heidelberg, Germany) at 15 °C against 1 l of 20 mM EDTA, pH 7.0.
(3) Change diffusates every 2 h, while monitoring the decrease in the amount of low-molecular-weight substances by measuring UV spectra in the range 210–350 nm, until the UV absorbance of diffusate at 210–350 nm cannot be observed, which means all low-molecular-weight compounds from retenate have been removed.
(4) Re-place the retenate into the dialysis tubing with a MWCO 10 kDa (Serva Electrophoresis, Heidelberg, Germany).
(5) Continue dialysis at 15 °C against 500 ml of Milli-Q water adjusted to pH 2 by addition of HCl.
(6) Change the diffusate every 12 h, 4 times.
(7) Concentrate diffusates by means of Macrosep 1 K (MWCO 1 kDa) centrifugal concentrators (Pall Gelman Sciences, Germany). Alternatively,
(8) Dry diffusates by lyophilization (see Subsection 3.1.3).
(9) Store dry peptide fractions at 4 °C or dissolve in an appropriate buffer according to the next experiments.

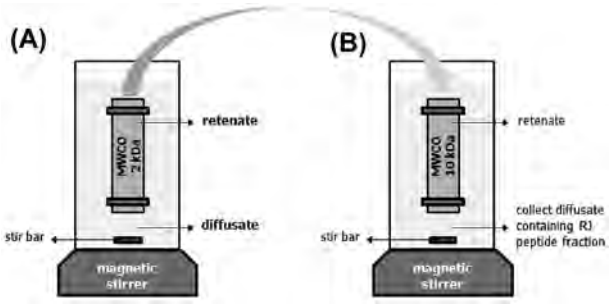

Figure 22. Scheme of two step dialysis of royal jelly suspension (Bíliková et al., 2001). (A) First dialysis performed at MWCO 2 kDa removes sugars and other low molecular weight compounds. (B) Diffusates obtained after dialysis of royal jelly suspension at MWCO 10 kDa containing peptide fraction, are collected and concentrated.

4.1.3.2. Purification of royal jelly peptide apisimin by ion exchange FPLC. Royal jelly peptide apisimin was purified from the HM fraction obtained by fractionation of a water-soluble fraction of royal jelly by size exclusion chromatography (see Subsection 4.1.2.2) according to Bíliková et al. (2002). All procedures for isolation of apisimin were carried out at 4 °C.

(1) Load 120 ml of the HM fraction at the concentration of 0.8 mg/ml, in Tris-glycine buffer (20 mM Tris-HCl, 100 mM glycine, pH 7.0) onto a DEAE Sepharose fast flow column (28 × 200 mm; Pharmacia Biotech, Sweden) at a flow rate of 2 ml/min.
(2) Wash the column with Tris-glycine buffer.
(3) Perform stepwise elution with 0.1 M NaCl, 0.15 M NaCl, and 0.2 M NaCl.
(4) Collect 7 ml fractions.
(5) Monitor the presence of apisimin by dot-blot analysis (see Subsection 4.2.4.1).
(6) Pool fractions containing apisimin.
(7) Concentrate and desalt the peptide fraction by means of Macrosep 1 K (MWCO 1 kDa) centrifugal concentrators (Pall Gelman Sciences, Germany).

4.1.3.3. Electroelution of royal jelly proteins/peptides from the gel after preparative PAGE. The royal jelly proteins or peptides can be purified in µg and/or mg quantities from the gel after preparative SDS-PAGE or native PAGE. The prepared protein or peptide can be used for immunization of experimental animals in order to raise antibodies, for characterization of the proteins, or for testing their physiological activity.

Wear gloves throughout the procedure to prevent contamination of the samples.

Preparation of a protein sample for electroelution:

(1) Dissolve 5 mg of the HM fraction (see Subsection 4.1.2.2) in 200 µl of Milli-Q water.
(2) Add 200 µl of 2× SDS-PAGE sample buffer and incubate for 10 min at 100 °C.
(3) Centrifuge at 18,000× *g* for 1 min.
(4) Load the supernatant on the gel of preparative native PAGE (160 × 140 × 1 mm).
(5) Run the electroseparation at a constant current, 12 mA.
(6) Stain the gel with 1 M KCl for 5–10 min, until white bands appear.
(7) Excise the peptide band of interest and wash the gel strip in Milli-Q water to remove the staining solution.

Protein/peptide elution:

(1) Cut the gel strip into smaller pieces.
(2) Place the gel pieces into the electro-eluter glass tubes with membrane caps of MWCO appropriate for the MW of the eluted protein or peptide (Figure 23).
(3) Perform electroelution using the Electro-Eluter instrument (Model 422, Bio-Rad, USA), in 1× elution buffer (Table 2) according to the instructions of the manufacturer. In general, constant current at 10 mA per glass tube for 3–5 h with vigorous stirring is sufficient. Increase elution time when using high-percentage gels or eluting high-molecular-weight proteins.
(4) Concentrate the eluate (containing the royal jelly protein or peptide) on a Microsep centrifugal column.
(5) Desalt by washing the column with Milli-Q water.

4.2. Characterization of major royal jelly proteins and peptides

The first necessary information for protein/peptide characterization is its concentration, which can be determined by several methods described previously in Section 1.2 of the *BEEBOOK* paper by Hartfelder et al. (2013). The principal molecular characteristics of proteins are determined by electrophoretic and immunochemical methods. Gel electrophoresis can provide information about the molecular weight, polarity, and subunit structure of proteins, and about the purity of a particular protein preparation. Isoelectric focusing (IEF) provides important information about ion properties of a protein.

Analysis by immunochemical methods is an indispensable part of characterization of bee proteins. Determination of the identity of proteins/peptides is possible by immunochemical methods with specific antibodies.

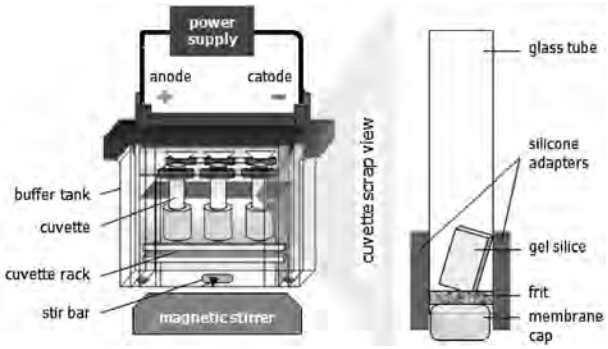

Figure 23. Electroelution performed on Elektro-Eluter (Model 422, Bio-Rad). The cuvette rack is placed into the main buffer tank. 1X elution buffer is added to the main tank until the assembly joints are covered in buffer. Likewise, 1X buffer is added to the upper buffer tank. Using a stir bar, the buffer in the main tank is gently agitated. The electrode plug is inserted into the power source. In general, each cuvette requires 10 mA of power (e.g., if three cuvettes are used, the power should be set at 30 mA).

Table 2. Composition of elution buffer for electroelution of a protein or peptide from the gel after SDS-PAGE. Store a stock solution (10×) at room temperature. Prepare a working solution (1×) by diluting the stock solution before use. In case of PAGE and electroelution under native conditions, do not add SDS to elution buffer.

	10×		1×
Elution buffer	Composition	Concentration	Concentration
Tris	30 g	0.250 M	25 mM
Glycine	144 g	1.920 M	192 mM
SDS	10 g	1%	0.1%
Milli-Q water	To 1000 ml		

Note: pH of the working solution is adjusted to 8.3.

Physical spectrophotometric methods, e.g., ultraviolet (UV), circular dichroism (CD), and mass spectrometry [MS (MALDI-TOF MS and nanoLC MALDI-TOF/TOF MS)], provide important information on the secondary structure of the protein or peptide.

4.2.1. Determination of protein concentration

Protein quantitation is often necessary before processing of protein samples for isolation, separation, or analysis by chromatographic electrophoretic, or immunochemical techniques. Depending on the accuracy required and the amount and purity of the protein, different methods for determining protein concentration are available.

For determination of the concentration of royal jelly protein/peptides, the Bradford assay (Bradford, 1976) and bicinchoninic acid (BCA) method (Smith et al., 1985) are mostly used. These methods are described in detail in Subsection 1.2 of the article by Hartfelder et al. (2013).

4.2.2. One-dimensional gel electrophoresis for identification of major royal jelly proteins and their (protein) isoforms

SDS-PAGE is the most commonly practiced gel electrophoretic method for protein characterization. The typical electrophoretic profile of major proteins of royal jelly (*Apis mellifera*) is presented in Figure 24. For separation of proteins with a molecular mass lower than 30 kDa and peptides, high resolution can be achieved using Tricine SDS-PAGE (Schägger & von Jagow, 1987).

SDS-PAGE requires that proteins be denatured to their constituent polypeptide chains; therefore, this method provides limited information. In those situations where it is desirable to maintain biological activity or antigenicity, non-denaturing (native) electrophoresis systems must be employed. Nonetheless, the gel patterns from non-denaturing gels are more difficult to interpret than those from SDS-PAGE. Native or non-denaturing electrophoresis in the absence of denaturants allows for determination of the native size or subunit structure and optimal separation of proteins. Mobility depends on the size, shape, and intrinsic charge of the protein. As an example, the electrophoretic pattern of royal jelly proteins and of peptide apisimin is presented. Separation by Tricine SDS-PAGE under denaturing conditions (Figure 25(a)) shows apisimin as 1 distinct peptide band, while under non-denaturing conditions of native PAGE (Figure 25(b)), several bands of the peptide can be explained by the different degrees of oligomerization (Bíliková et al., 2002). Non-denaturing systems also provide information about the charge isomers of proteins, but this information is best obtained by IEF (*see* Subsection 4.2.3). An IEF run will often show heterogeneity (due to structural modifications) that is not apparent in other types of electrophoresis. Proteins thought to be a single species according to SDS-PAGE analysis are sometimes found by IEF to consist of multiple species. IEF is a method for separating molecules by differences in their isoelectric point (pI). Proteins or peptides are introduced into an immobilized pH gradient gel composed of polyacrylamide, starch, or agarose where a pH gradient has been established. A protein that is in a pH region below its pI will be positively charged and so will migrate towards the cathode (negatively charged electrode). As it migrates through a gradient of increasing pH, however, the protein's overall charge will decrease until the protein reaches the pH region that corresponds to its pI. As a result, the proteins become focused into sharp stationary bands, with each protein positioned at a point in the pH gradient corresponding to its pI. The technique is capable of high resolution,

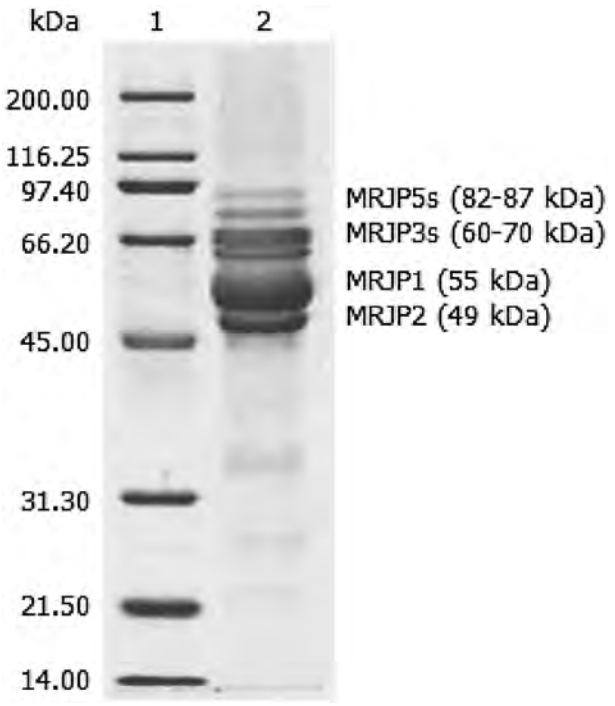

Figure 24. Electrophoretic pattern of royal jelly proteins (*Apis mellifera*) separated by SDS-PAGE (10%), lane 1: molecular mass marker; lane 2: major proteins of royal jelly. Gel stained by Coomassie Brilliant Blue.

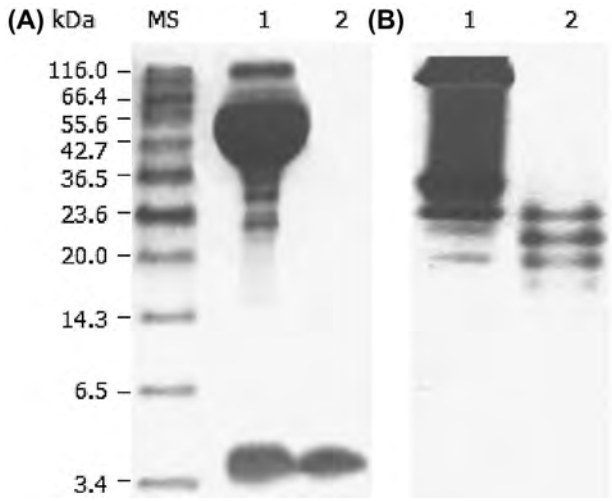

Figure 25. Protein patterns of royal jelly proteins and peptide apisimin by Tricine SDS-PAGE (A) and native Tricine PAGE (B); lane MS: protein mass standards; lane 1: royal jelly; lane 2: apisimin (From Bíliková et al., 2002. Reproduced with permission).

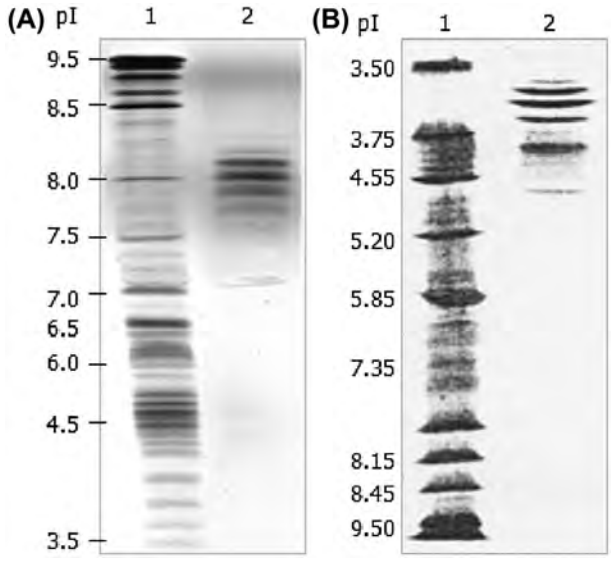

Figure 26. Isoelectrophocusing of major basic royal jelly protein MRJP2 and acidic royal jelly peptide apisimin. A: Servalyt Precotes 3–10 IEF gel, lane 1: pI standard; lane 2: MRJP2, Coomassie Blue G-250 staining. B: PhastGel Dry IEF gel, line1: pI standard; line2: apisimin, silver staining (B is from Bíliková et al., 2002. Reproduced with permission).

where proteins differing by a single charge can be fractionated into separate bands.

IEF of royal jelly proteins and peptides usually shows several isoelectrofocusing variants. In case of MRJP2, there are IEF bands in the range of pI from 7.5 to 8.5 corresponding to different posttranslational modifications of the protein (Figure 26(a)) (Bíliková, Klaudiny, & Šimúth, 1999), while in the case of royal jelly peptide apisimin, several IEF bands in the acidic range of pI (3.55–4.55) correspond to the oligomeric structures of the peptide (Figure 26(b)) (Bíliková et al., 2002).

4.2.2.1. SDS-PAGE. Royal jelly proteins are usually separated by SDS-PAGE in a 10 or 12% gel (Laemmli, 1970), already described in *Section* 1.3 of the *BEEBOOK* paper by Hartfelder et al. (2013).

4.2.2.2. Tricine SDS-PAGE. Royal jelly peptides can be separated by Tricine SDS-PAGE, which is commonly used for separation of molecules smaller than 30 kDa. Electrophoretic separation of royal jelly peptides can be performed by Tricine SDS-PAGE according to Schägger and von Jagow (1987)) (Schägger & von Jagow, 1987).

Sample preparation

(1) Add 10 µl of 2× Tricine sample buffer (4% SDS, 12% glycerol w/v, 50 mM Tris pH 6.8, 1.5% dithiothreitol, 0.01% Serva Blue G250) to 10 µl of a peptide sample.
(2) Incubate for 40 min at 40 °C.
(3) Centrifuge at 18,000× *g* for 1 min.
(4) Load 20 µl on the Tricine SDS polyacrylamide gel.

Preparing and running vertical slab gels

(1) Prepare stock solutions for making gels and electrolytes for Tricine SDS-PAGE according to Table 3.
(2) Prepare the stacking (4% T/3% C) and separating (16.5% T/3% C) gels for Tricine SDS-PAGE according to Table 4.
(3) Load samples onto the gel.
(4) Run electrophoresis at a constant current, 20 mA, in the presence of 2 electrolytes, the cathode and anode buffer (Table 3, Figure 27).

Staining and destaining gels

(1) Stain the gels with Serva Blue G250 in 10% acetic acid.
(2) Destain in 10% acetic acid.

In case of expecting a low concentration of peptides, the silver staining can be used, following Subsection 1.3.4 in the *BEEBOOK* paper by Hartfelder et al. (2013).

4.2.2.3. Native-PAGE (n-PAGE). Separation of the proteins under non-denaturing conditions requires the same type of equipment used for denaturing gels as described before (see Subsection 4.2.2), but without SDS in stock solutions, including the sample buffer.

Table 3. Recipes for stock solutions and electrolytes of Tricine SDS-PAGE.

	Tris (mol l^{-1})	Tricin (mol l^{-1})	pH	SDS (%)
Anode buffer	0.1	–	8.9[a]	–
Cathode buffer	0.1	0.1	8.25[b]	0.1
3× gel buffer	3	–	8.45[a]	0.3
AB-mix 49.5%T/3%C	(1) Weigh 48 g of acrylamide and 1.5 g of bisacrylamide (2) Dissolve in 80 ml of Milli-Q water (3) Adjust volume to 100 ml (4) Pass through a 0.22-μm filter (5) Keep in the dark at 4 °C			

[a]pH adjusted by HCl.
[b]without adjustment of pH. % T, total concentration of both monomers (acrylamide and bisacrylamide). % C, the percentage of cross-linked polymer relative to the total concentration.

Table 4. Recipes for Tricine peptide-separating and stacking gels.

Stock solution	Stacking gel 4% T/3% C	Separating gel 16.5% T/3% C
AB-mix*	5.0 ml	0.1 ml
3× gel buffer	5.0 ml	1.5 ml
Glycerol	1.6 ml	–
H_2O	3.4 ml	4.0 ml
10% (APS)	100 μl	50 μl
TEMED	10 μl	5 μl

*AB-mix, a stock solution of the acrylamide-bisacrylamide mixture, for composition see Table 3.

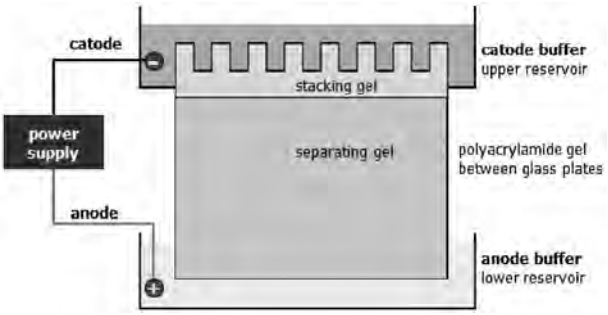

Figure 27. Setup of vertical Tricine-SDS-PAGE. The upper buffer compartment is filled by 1X cathode buffer, the lower reservoir contains 1X anode buffer.

4.2.3. *IEF*

IEF of royal jelly protein samples is performed on 5% polyacrylamide gels (0.15 mm, 125 × 125 mm, Servalyt Precotes 3–10, Serva) using SERVALYT™ ampholytes according to the instructions of producer (Serva Electrophoresis, GmbH, Germany).

(1) Run the IEF gel for 30 min at 30 V cm^{-1} without samples.
(2) Load the samples and run the gel for 2 h at 120 V cm^{-1}.
(3) Stain the IEF gel with Coomassie brilliant blue (CBBR-250) or by silver staining. The detailed methods for staining the gels are given in Section 4.3 of the *BEEBOOK* paper by Hartfelder et al. (2013).

IEF separation of the proteins using PhastGel Dry IEF (PhastSystem-200 V, Pharmacia LKB, Uppsala, Sweden) is carried out on 5% polyacrylamide gels (0.45 mm, 40 × 50 mm, PhastGel IEF 3–9, Pharmacia Biotech, Sweden) for 30 min at 2.2 mA.

4.2.4. *Immunochemical methods*

Immunoassays are analytical methods based on highly specific binding between an antigen and an antibody. An epitope (immunodeterminant region) on the antigen surface is recognized by the antibody's binding site. The type of antibody and its affinity and avidity for the antigen determine assay sensitivity and specificity. Immunochemical methods offer simple, rapid, and sensitive methods for routine protein or peptide analyses. The most popular either quantitative or qualitative formats are enzyme linked immunosorbent assays (ELISAs), immunochromatography, dot blot, or western blot assays used to interpret data from protein analysis by gel electrophoresis.

4.2.4.1. Dot blot analysis. A dot blot is a simple and quick assay that can be used to determine if antibodies and a detection system are effective and can identify the appropriate starting concentration of a primary antibody for western blot analysis, as well as for detection of the

protein of interest in the fractions after chromatographic separation.

(1) Assemble the dot blot equipment according to Figure 28.
(2) Spot up to 500 µl of a fraction solution (after chromatographic separation) on the wall of the dot blot equipment.
(3) Leave the sample to be soaked up by the polyvinylidene difluoride (PVDF) or nitrocellulose (NC) membrane.

    Note: An NC membrane is commonly used and cheaper but the material is brittle. PVDF is more durable and has higher chemical resistance making it ideal for reprobing and sequencing applications. A PVDF membrane has higher protein binding capacity and sensitivity; an NC membrane, on the other hand, produces less background noise.
(4) Block the membrane with 5% dry milk in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) for 1 h at room temperature.
(5) Decant the block buffer, but keep the membrane wet at all times for the remainder of the procedure.
(6) Incubate the membrane with a primary antibody in 5% dry milk in TBS for 1 h at room temperature.
(7) Wash the membrane 3 times (10 min each) in TBS buffer on a rocker.
(8) Incubate the membrane with a secondary antibody in 5% dry milk in TBS for 1 h at room temperature.
(9) Wash the membrane 3 times (10 min each) in TBS buffer on a rocker.
(10) Place the membrane in the chromogenic DAB/$NiCl_2$ solution (0.8 mg/ml DAB; 0.4 mg/ml $NiCl_2$; 0.1% H_2O_2 in 100 mM Tris–HCl pH 7.4).

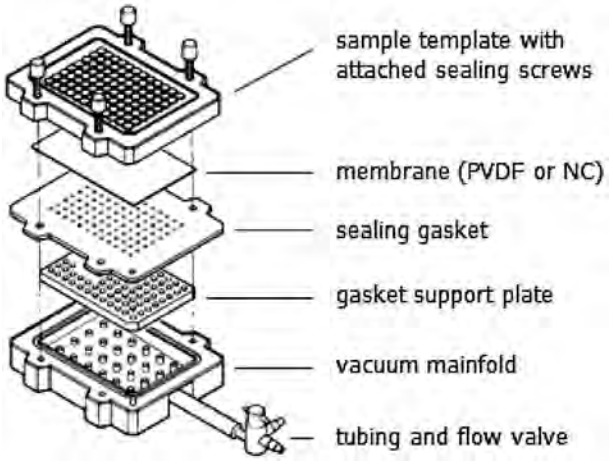

Figure 28. Set up of Dot Blot system.

(11) Incubate for 5 min for detection of the immunoactive protein/peptide spots.
(12) Wash the membrane with Milli-Q water and dry it off.

    Colored spots show us in which chromatographic fraction the protein of interest is present.

4.2.4.2. Western blot. As already described in Section 1.4 of the *BEEBOOK* paper by Hartfelder et al. (2013), the samples are transferred after SDS-PAGE to a PVDF or NC membrane using the tank method (MiniTrans-Blot Electrophoretic Transfer Cell, Bio-Rad Laboratories, USA). The membranes are first incubated overnight in TBS buffer containing 5% powdered non-fat milk with polyclonal rabbit antiserum against recombinant royal jelly protein or peptide at a dilution of 1:2000, then 2 h in the same buffer with a peroxidase-conjugated secondary antibody (in case of a rabbit primary *anti*-MRJP antibody, the swine or goat secondary *anti*-rabbit IgG can be used) at a dilution of 1:5000. Visualization of the immunoactive protein/peptide bands is performed by incubating the blots in the chromogenic DAB/$NiCl_2$ solution (*see* Subsection 4.2.4.1).

4.2.4.3. Preparation of a polyclonal antibody. Antibodies are serum immunoglobulins produced by a body's immune system and bind specifically to particular antigens. The methods for eliciting antibodies involve immunization of experimental animals with a purified antigen. Polyclonal antibodies are valuable for immunochemical methods such as immunoblotting (dot blot or western blot analysis), immunoprecipitation, and ELISA. Production of good antibodies (antisera) depends in large part upon the quality, purity, and amount of available antigens as well as on the specificity of the assay. For protein antigens, the material should be biochemically homogeneous and, depending on the intended use, should be in either a native or denatured conformation.

The choice of the animal for production of antibodies depends on the amount of antiserum desired. Rabbits are the usual animal to be immunized and provide as much as 25 mL of serum from each bleed. For smaller-scale experiments, the mouse may be used, where the volume of antigen suspension used for immunization is significantly less and the amount of serum obtained from a single bleed does not exceed 0.5 ml.

For preparation of antibodies against MRJPs, one can use purified royal jelly protein or peptide as an antigen. Another way is to use the water-soluble protein fraction of royal jelly for immunization of experimental animals (see Subsection 4.1.2.1) and subsequently, the individual *anti*-MRJP antibody is purified from the obtained pool of antibodies by immunoprecipitation.

A pure protein can be prepared by the chromatographic methods already described in Subsection 4.1.2. For immunization, the protein is excised from the gel strip after preparative SDS-PAGE or Tricine-PAGE

directly or is purified by electroelution of the protein from the gel (see Subsection 4.1.3.3).

4.2.4.3.1. Preparation of the MRJP antigen in an SDS-PAGE gel

(1) Run preparative SDS-PAGE (160 × 140 × 1 mm gel; 10% separating gel; 5% stacking gel) of MRJP, at 20 mA.
(2) Stain the gel with 1 M KCl.
(3) Excise the protein band of interest.
(4) Homogenize the gel slice in 0.15 M NaCl by vortexing.
(5) Use the homogenized soft gel suspension for immunization of a rabbit or mouse directly or purify the protein from the gel by electroelution (see Subsection 4.1.3.3).

4.2.4.3.2. Immunization with a purified protein. Use 400 μl of the antigen (the protein of interest) and complete Freund's Adjuvant (CFA) (1:1) for the first immunization and next in incomplete Freund's Adjuvant (IFA) for 1 injection per animal.

(1) Inoculate the rabbit with 500 μg/ml antigen (the protein) in CFA.
(2) Inoculate, every 2 weeks, 3 times with 250 μg/ml protein in IFA.
(3) Collect the animal's blood a week after the last immunization.
(4) Centrifuge the blood at 4000*g* for 5 min.
(5) Collect the supernatant, where the *anti*-MRJP antibody is.
(6) Store in aliquots at −80 °C.
(7) Test antibody titre by a dot blot or western blot analysis (see Subsections 4.2.4.1 and 4.2.4.2).

4.2.4.3.3. Immunization with WSP antigen

(1) Administer 400 μl of the water-soluble royal jelly proteins (WSPs, see Subsection 4.1.2.1) plus Freund's complete adjuvant (1:1) as antigen samples at the concentration of 500 mg/ml to a rabbit or at 100 mg/ml to a mouse.
(2) After 15 days, administer a booster injection of 250 mg/ml (in the rabbit) or 25 mg/ml protein (in the mouse).
(3) Remove the animal's blood by cardiac puncture.
(4) Centrifuge the blood at 4,000*g* for 3 min.
(5) Store the serum at −80 °C.

4.2.4.3.4. Antibody purification. MRJP1 is used as an example.

(1) Separate (2 μg/μl) WSP fraction by SDS-PAGE in a 5–22% gradient gel (300 V, 2 h).
(2) Stain the gel with Coomassie brilliant blue-R250, for 1 h.
(3) Excise the bands of interest.
(4) Electrotransfer onto a nitrocellulose membrane (300 V, overnight, see Subsection 4.2.4.2).
(5) Incubate the membranes in TBS-T (50 mm Tris–HCl pH 7.4, 150 mM NaCl, 0.5% Tween 20) containing 5% of dried milk overnight at room temperature.
(6) Incubate with diluted serum (*anti*-MRJP1) in TBS (1:1) for 2 h.
(7) Wash the membranes 3 times for 5 min with TBS-T.
(8) Elute the bound antibodies (*anti*-MRJP1) with 1.4% triethylamine (1 min).
(9) Transfer the eluted antibodies to vials containing 1 M Tris–HCl (pH 8.5) at room temperature.
(10) Dialyze the antibody against TBS containing 0.1% sodium azide for 24 h.

4.2.4.3.5. Immunoprecipitation of MRJPs

(1) Incubate the protein extracts overnight at 4 °C with 20 mg/ml *anti*-MRJP1 and 150 mM NaCl 0.2% Triton X-100.
(2) Add the protein A Sepharose beads (2 mg/ml).
(3) Gently mix the suspension on a rocking platform for 30 min at 4 °C.
(4) Centrifuge this suspension at 18,000× *g* for 10 min to obtain the immunoprecipitation fractions.
(5) Transfer the supernatant.
(6) Wash the pellet with TBS plus 0.5 M NaCl.
(7) Repeat washing 6 times with TBS without salt addition.
(8) Dilute the pellets and the supernatant collected at step 5 with SDS sample buffer.
(9) Boil at 100 °C for 5 min.
(10) Analyze them separately for MRJPs content by immunoblotting using a rabbit *anti*-MRJPs antibody (see Section 4.2.4.2).

4.2.5. Physical methods

Physical methods including UV, circular dichroism (CD), and mass spectrometry (MS) provide important information on the molecular properties and secondary structure of the protein or peptide. A combination of high-resolution two-dimensional (2D) polyacrylamide gel electrophoresis (2DE), highly sensitive biological MS, and the rapidly growing protein and DNA databases have paved the way for high-throughput proteomics of MRJPs. Protein electroblotting and sequencing of N-terminal amino acids are described as a tool for *de novo* sequencing and protein identification. In the second part of this section, we highlight matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) as one of the main contemporary analytical methods for linking gel-separated proteins to entries in sequence databases. CD is a method for rapid evaluation of the secondary structure of

proteins. In the far UV region (240–180 nm), which corresponds to peptide bond absorption, the CD spectrum can be analyzed to determine regular secondary structural features (Kelly, Jess, & Price, 2005). As an example, the CD spectrum of apisimin is presented (Figure 29); this protein was isolated from royal jelly (Bíliková et al., 2002). MS is an analytical tool useful for measuring the mass-to-charge ratio (*m*/*z*) of one or more molecules present in a sample. It is used to determine the exact molecular mass of the analyzed molecule as well as for characterization of a wide variety of post-translational modifications such as phosphorylation and glycosylation; when the protein/peptide sample is pure, it is possible to determine the N-terminal amino acid sequences (Figure 30) (Bíliková et al., 2002). The *nano* LC MALDI TOF/TOF MS analysis of the peptides obtained after tryptic digest of the protein showed that apalbumin2a has 2 fully occupied N-glycosylation sites, 1 with high-mannose structure, HexNAc2Hex9, and another carrying a complex type of antennary structures, HexNAc4Hex3 and HexNAc5Hex4; this structure is different from that of MRJP2 (Figure 31). This posttranslational modification of apalbumin2a leads to changes in physiological activity of the protein. Apalbumin2a inhibits growth of *Paenibacillus larvae* sub. *larvae*, the primary honey bee pathogen of American foulbrood disease, while MRJP2 has no antimicrobial activity (Bíliková et al., 2009).

4.2.5.1. CD analysis. For structural studies, the CD spectra of a protein or peptide at the concentration of 0.5 μg/ml in water are recorded on a Jasco J-600 Spectrophotopolarimeter (Japan Spectroscopic Co., Ltd., Japan).

(1) Dilute a sample solution to 0.5 μg/ml in water.
(2) Transfer the sample solution to 1-cm quartz cuvettes thermostated at 25 °C.
(3) Record the spectra from 190 to 250 nm, with 3 scans at 50 nm/min, time constant 1 s, and bandwidth 1 nm.

Ellipticity and photomultiplier voltage baselines for the protein are measured using deionized water in a 1-cm cuvette. The CD spectrum for apisimin in water was obtained at peak ellipticity 50 mdeg and HT < 700 V. The obtained CD spectra are evaluated with the software provided by the instrument manufacturer, Jasco.

4.2.5.2. N-terminal amino acid sequencing. This sequence can be determined directly from the lyophilized sample (see Subsection 3.1.3) as well as from the individual protein/peptide bands after SDS-PAGE or Tricine SDS-PAGE.

(1) Separate the proteins or peptides by SDS-PAGE (see Section 1.3 of the *BEEBOOK* paper by Hartfelder et al. (2013)).

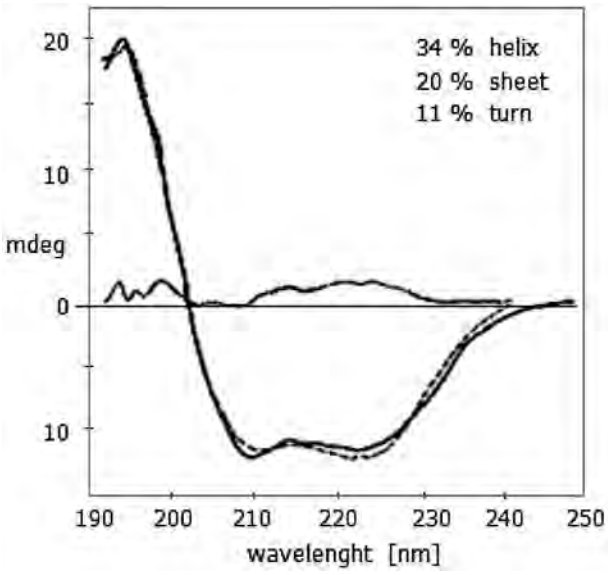

Figure 29. CD spectrum of apisimin purified from royal jelly fraction using ion-exchange FPLC. measured CD spectrum; —— calculated CD spectrum according to the software protein standards with known secondary structure; ..... differences between measured and calculated CD data. The analysis of the spectrum performed by the Jasco software showed that apisimin contains 34% K-helical, 20% *alpha*-sheet, 11% *beta* -turn, 30% random structure, that is in good agreement with the theoretically calculated parameters, typical for proteins with a predominantly helical structure. The occurrence of 65% of well-defined secondary structure in such a small peptide with only one aromatic amino acid and without disulfide bridges is unique and can be used as a new model for research into mechanisms of action of antibiotic peptides (Bíliková et al., 2002).

(2) Electroblot the proteins or peptides from the polyacrylamide gel onto a PVDF membrane (ProBlott, Applied Biosystems, USA) using the tank method of protein transfer (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad Laboratories, USA), in electroblotting buffer [10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) in 10% methanol] according to the procedure recommended by the manufacturer [see Section 1.4 of the *BEEBOOK* paper by Hartfelder et al. (2013)].
(3) Perform visualization by Coomassie brilliant blue R250 staining (0.1% CBBR-250 in 40% methanol, 1% acetic acid).
(4) Destain the membrane with 50% methanol.
(5) Wash the membrane with Milli-Q water.
(6) Let it dry in ambient air.
(7) Excise the protein/peptide bands of interest and subject them to sequencing by automated Edman degradation on an LF3600D Protein Sequenator (Beckman, USA).
(8) Identify amino acid sequences by ExPASy BLAST program (http://br.expasy.org/tools/blast) and the Swiss-Prot database for protein searches.

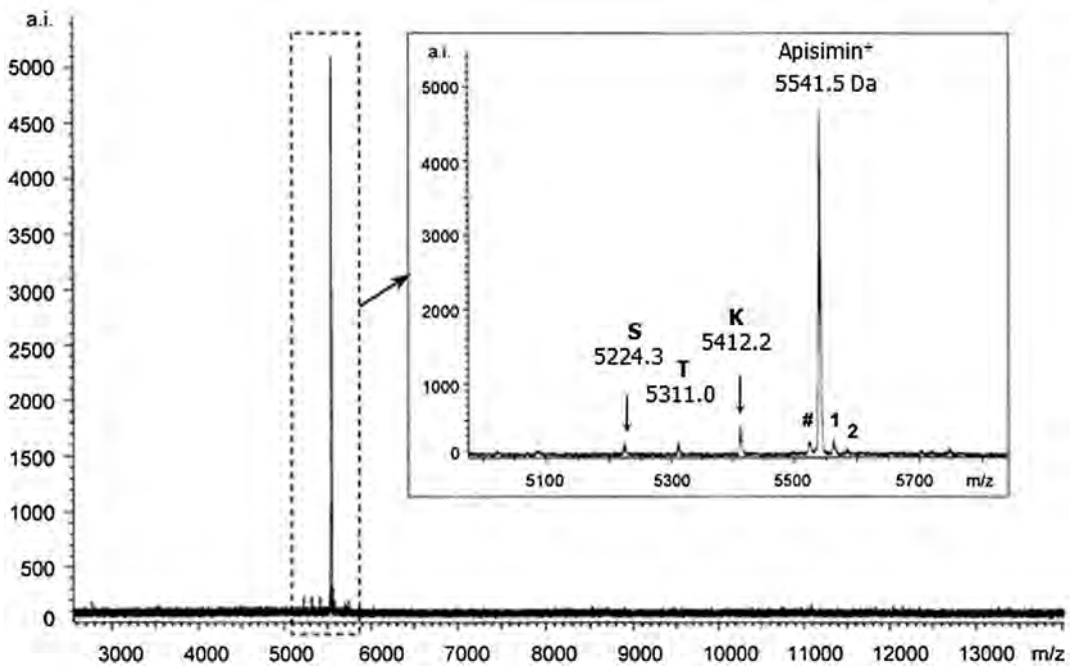

Figure 30. MALDI-TOF mass spectrum of apisimin purified from royal jelly fraction by ion-exchange FPLC. The molecular mass of apisimin determined by MALDI-TOF MS analysis (5540.5 Da) is in agreement with calculated one from amino acid sequence of the peptide (5540.4 Da), which indicates that apisimin is not post-translationally modified. The three satellite signals at 5413.2, 5312.0 and 5225.3 Da correspond to a consecutive loss of the N-terminal amino acids: Lysine, Threonine and Serine residues (From Bíliková et al., 2002. Reproduced with permission).

4.2.5.3. Mass spectrum analysis. See Subsection 4.3.

4.3. Methods for analysis of the proteome of royal jelly and its post-translational modifications

4.3.1. Introduction

Proteins are the executors of biological functions, however, the activities of most eukaryotic proteins are modulated by posttranslational modifications (PTMs). Among hundreds of PTMs, phosphorylation and glycosylation are the most widespread and play critical roles in all parts of cellular life. Since the PTMs became a hotspot of proteomics, PTM research on royal jelly proteins has also been advancing rapidly.

Although phosphorylation is considered the reason for the high heterogeneity of MRJPs, the 2DE-based phosphoprotein fluorescent staining method only detects MRJP2, and MRJP7 carries potential phosphorylation sites without well-defined phosphorylation locations (Furusawa et al., 2008). In the research involving 2DE, shotgun analysis in combination with high-sensitivity MS and bioinformatics, phosphorylation of MRJP1, MRJP2, and apolipophorin-III-like protein were identified for the first time and a new site was localized in venom protein 2 (Zhang et al., 2012). Recently, 2 complementary phosphopeptide enrichment materials (Ti^{4+}-IMAC and TiO_2) and high-sensitivity MS have been applied to map the phosphoproteomes of royal jelly produced by *A. m. ligustica* and *A. cerana cerana*. In total, 16 phosphoproteins carrying 67 phosphorylation sites and 9 proteins phosphorylated on 71 sites were identified in royal jelly derived from Western and Eastern honey bees, respectively (Han et al., 2014). As another important protein post-translational modification, protein glycosylation mediates many important biological processes involved in cell adhesion, cell differentiation, cell growth, and immunity (Rudd, Elliott, Cresswell, Wilson, & Dwek, 2001). N-glycosylation of proteins has been reported to promote protein folding, stability, solubility, oligomerization, quality control, sorting, and transport (Lis & Sharon, 1993). Using liquid chromatography tandem mass spectrometry (LC-MS/MS) together with hydrazide chemistry and lectin enrichment technology, 13 novel proteins and 42 N-linked glycosylation sites were mapped on royal jelly proteins for the first time (Zhang et al., 2014). A 350-kDa royal jelly protein that can stimulate the cell growth (Kimura et al., 2003) is reported to bear the high-mannose type N-glycans with 4 different structures, as analyzed by NMR spectroscopy (Kimura, Washino, & Yonekura, 1995), while a 55-kDa royal jelly protein that can maintain high viability of rat liver primary cultured cells, only possesses 1 kind of N-linked sugar chain (Kimura, Kajiyama, Kanaeda, Izukawa, & Yonekura, 1996). Apalbumin2a also known as isoform MRJP2, carries 2 fully occupied N-glycosylation sites and has an antimicrobial activity (Bíliková et al., 2009).

Although the above efforts have been made to explore the unknown proteins and PTMs in royal jelly,

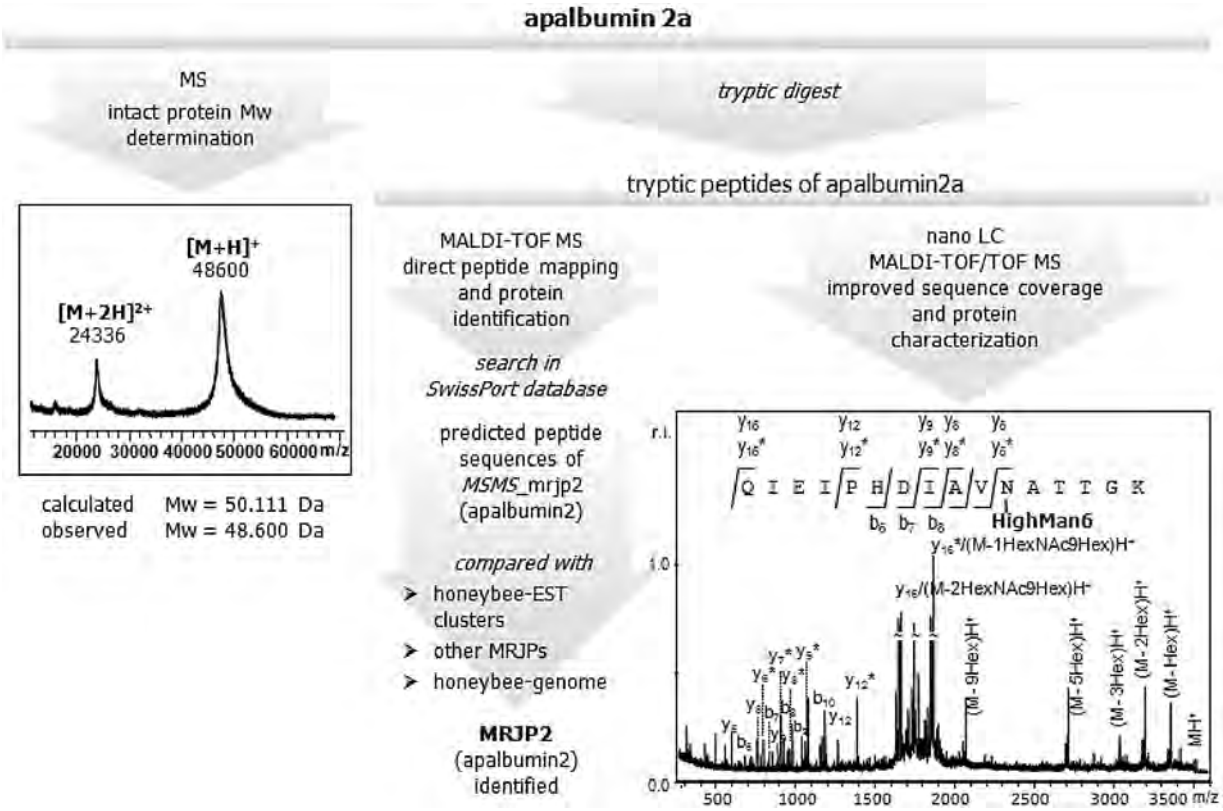

Figure 31. Simple scheme of molecular characterization of apalbumin2a, the minority homologue of major basic royal jelly protein apalbumin2 (MRJP2) using MS, MALDI-TOF MS and nanoLC MALDI-TOF MS analysis. MS analysis provide the exact molecular mass of the protein. Triptic peptides obtained after trypsin digestion of analyzed protein can be compared in protein database search to select the optimal candidate of the protein. The nanoLC MALDI TOF MS analysis of the triptic peptides showed that apalbumin2a carrying two fully occupied N-glycosylation sites, one with high-mannose structure, HexNAc2Hex9, and another carrying complex type antennary structures, HexNAc4Hex3 and HexNAc5Hex4 (Bíliková et al., 2009).

this research is still at the infancy stage. Therefore, there is a high demand for cutting-edge knowledge and state-of-the-art technology to identify the unknown proteins and the PTM status in royal jelly. This is important for gaining new insights into the new roles of such proteins both for honey bee biology and for promotion of human health.

4.3.2. Two-dimensional gel electrophoresis (2DE) for identification of major royal jelly proteins and their (protein) isoforms

4.3.2.1. Sample preparation

(1) Mix the royal jelly sample and lysis buffer (LB: 8 M urea, 2 M thiourea, 4% 3-[(3–151 cholamidopropyl) dimethylammonio]-1-propanesulfonate [CHAPS], 20 mM Tris base, 30 mM dithiothreitol [DTT], store at −20 °C before use) at the ratio of 1:10 (w/v).
(2) Homogenize for 30 min on ice.
(3) Sonicate 3–5 times for 10 s.
(4) Centrifuge at 12,000*g*, 4 °C for 10 min.
(5) Further centrifuge at 15,000*g*, 4 °C for 10 min.
(6) Collect the supernatant (be careful not to pipette the lipid layer).
(7) Add a 3-fold volume of acetone (pre-cooled) to the supernatant.
(8) Mix.
(9) Keep on ice for 30 min for protein precipitation.
(10) Centrifuge at 15,000*g*, 4 °C for 2 × 10 min.
(11) Discard the supernatant.
(12) Dry the protein pellet at room temperature.
(13) Dissolve the pellet in LB.

4.3.2.2. Measurement of protein concentration. Protein concentration can be determined according to previously described methods, see Section 1.2 of the *BEEBOOK* paper by Hartfelder et al. (2013).

4.3.2.3. IEF and SDS-PAGE. IEF is described in Section 4.2.3. SDS-PAGE is described in Section 1.3 of the *BEEBOOK* paper by Hartfelder et al. (2013). The following requirements need to be noted. IPG strips of different length require different volume of the rehydration solution. The IPG strips for the first dimensions have to be rehydrated for ~14 h in an aqueous solution consisting of 8 M urea, 2% CHAPS, 0.001% bromophenol blue,

45 mM DTT, and 0.2% Bio-lyte. After IEF, the strips should be washed with electrophoresis buffer consisting of 0.3% Tris base, 1.44% glycine, and 0.1% SDS, and then equilibrated with equilibration buffer 1 [an aqueous solution consisting of 0.375 M Tris–HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, 2% DTT, store at −20 °C before use] and equilibration buffer 2 [prepare an aqueous solution consisting of 0.375 M Tris–HCl (pH 8.8), 6 M urea 20% glycerol, 2% SDS, 2.5% iodoacetamide [IAA], store at −20 °C before use]. As for the second-dimension SDS-PAGE, 12% T linear gradient polyacrylamide gels are commonly used. Then, the well-equilibrated gel strips are transferred to the top of the gels and subjected to SDS-PAGE at constant voltage or current.

4.3.2.4. Gel fixing and gel staining

(1) Fix the gels with an aqueous solution consisting of 40% ethanol and 10% acetic acid for more than 4 h.
(2) Stain the gels using one of the following methods.

4.3.2.4.1. Coomassie brilliant blue (CBB) G-250 staining. This method is described in Subsection 1.3.3 of the *BEEBOOK* paper by Hartfelder et al. (2013).

4.3.2.4.2. Fluorescent staining. Different methods are needed for gels with different types of proteins of interest.

Pro-Q Diamond staining for phosphorylated proteins:

(1) Wash with double-distilled H_2O (ddH_2O), 2 × 15 min.
(2) Stain with a Pro-Q solution for 3 h (Pro-Q: ddH_2O = 1:2, V/V).
    Note: The staining solution should be kept in darkness!
(3) Destain the gel with the destaining solution consisting of 20% acetonitrile (ACN) and 5% 1 M sodium acetate (pH 4.0) for 4 × 15 min.
(4) Wash with ddH_2O, 2 × 5 min.

Pro-Q Emerald staining for glycoproteins:

(1) Add 6 ml of N,N-dimethylformamide (DMF) to the vial containing the pro-Q Emerald reagent.
(2) Mix gently and thoroughly.
(3) Store this Pro-Q Emerald stock solution at −20 °C or lower.
(4) Prepare the oxidizing solution by adding 250 ml of 3% acetic acid to the bottle containing the periodic acid.
(5) Mix until completely dissolved.
(6) Incubate the gel in an appropriate volume (according to the gel size) of the oxidizing solution with gentle agitation for 30 min.
    For a large gel, the incubation time should be 1 h.
(7) Wash the gel with 3% glacial acetic acid with gentle agitation for 10–20 min.
(8) Repeat this step 2 more times.
(9) Dilute the pro-Q Emerald stock solution 50-fold to prepare the staining buffer.
(10) Incubate the gel in an appropriate volume of staining buffer in the dark with gentle agitation for 90–150 min.
    The signal can be seen after ~20 min. Do not stain overnight.
(11) Wash the stained gel with a wash solution at room temperature for 15–20 min.
(12) Repeat this step once.
    Do not leave the gel in the wash solution for more than 2 h because the staining will start to decrease.

SYPRO RUBY staining for total protein

(1) Wash the gel with ddH_2O, 2 × 5 min.
(2) Stain with SYPRO RUBY overnight, keep in the dark.
(3) Destain the gel with a destaining solution consisting of 10% (v/v) methanol and 7% (v/v) acetic acid.
(4) Agitate for 15 min.
(5) Repeat steps 3 and 4 four times.
(6) Wash with ddH_2O, 2 × 5 min.

4.3.2.4.3. Silver staining. Silver staining is less compatible with mass spectrometric analysis. Therefore, you have to choose a MS-compatible silver stain protocol for any samples that will subsequently be subjected to MS analysis. The detailed methods for silver staining are given in Section 1.3.4 in the article by Hartfelder et al. (2013).

4.3.2.5. Gel scanning and gel analysis. For gels stained with CBB G-250 and a silver solution, a common gel scanner can be used.

For gels stained with Pro-Q Emerald, use an excitation maximum at ~280 nm and emission maximum at ~530 nm. Bands can be visualized using a 300-nm UV transilluminator.

For gels stained with Pro-Q Diamond, bands can be visualized using excitation at 532 nm with a 640-nm bandpass emission filter (Pharos FX Plus system, Bio-Rad), or 532 nm with a 560-nm longpass emission filter (Typhoon system, Amersham Biosciences). For gels stained with SYPRO RUBY, bands can be visualized using excitation at 582 nm with a 610 bandpass emission filter (Pharos FX Plus system, Bio-Rad). The well-scanned gels can be used for qualitative and quantitative analysis in commercially available software such as PDQuest from Bio-Rad and Imagemaster from GE. It is

helpful to follow the instructions of these software programs for gel analysis.

4.3.2.6. Protein identification

4.3.2.6.1. Gel cutting. Excise the gel spots with a gel-cutting machine, or cut manually using pipette tips.

4.3.2.6.2. Destaining. For gels stained with CBB-G250

(1) Prepare the destaining solution: an aqueous solution consisting of 50% (V/V) ACN and 40 mM NH_4HCO_3.
(2) Add 100 μl of this solution.
(3) Agitate.
(4) Repeat steps 2 and 3 until the gel becomes transparent.
(5) Add 100 μl of 100% ACN.
(6) Agitate for 10 min.
(7) Remove ACN.
(8) Dry the gel spot with a Speed-Vac system.

For silver staining

(1) Prepare 30 mM $K_3Fe(CN)_6$ (solution A) and 100 mM $Na_2S_2O_3$ (solution B).
(2) Wash the gel spot with ddH_2O for 1 min 3 times.
(3) Mix solution A and B with equal volume to get the fresh destining solution
(4) Add 100 μl destaining solution to each gel spot.
(5) Agitate until the brown color disappears.
(6) Discard the supernatant.
(7) Rinse the gel spot with ddH_2O 3 times.
(8) Discard the supernatant.
(9) Rinse the gel spot with 100 μl of 40 mM NH_4HCO_3 3 times.
(10) Discard the supernatant.
(11) Add 100 μl of 100% ACN.
(12) Agitate for 10 min.
(13) Remove ACN.
(14) Dry the gel spot in a Speed-Vac system.

4.3.2.7. Trypsin digestion

(1) Prepare the trypsin solution with 40 mM NH_4HCO_3 at the final concentration 10 ng/μl.
(2) Add 10 μl of the trypsin solution to each dried gel spot with pipetting.
(3) Incubate for 60 min at 4 °C.
(4) Discard the supernatant to minimize the autodigestion of trypsin.
(5) Incubate at 37 °C for 12–14 h keeping the Eppendorf tube upside down.
(6) Add 50% (v/v) ACN [containing 2.5% (v/v) TFA] using an appropriate volume to cover the gel spot.
(7) Incubate for 60 min at 30 °C.
(8) Pool the supernatants.
(9) Dry them by means of a Speed-Vac system.

4.3.2.8. Mass spectrometric analysis

The royal jelly sample is analyzed by means of an LC-MS system (QTOF G6520, Agilent Technologies). The LC-Chip (Agilent Technologies) consists of a Zorbax 300SB-C18 enrichment column (40 nl, 5 μm) and a Zorbax 300SB-C18 analytical column (75 μm × 43 mm, 5 μm). The parameter settings of liquid chromatography and the above-mentioned MS are as follows.

(1) Set the loading flow rate to 4 μl/min.
(2) Use water with 0.1% formic acid as a loading mobile phase.
(3) Perform sample elution with a binary solvent mixture composed of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B).
(4) Use the following gradient program:
  • from 3 to 8% B in 1 min,
  • from 8 to 40% B in 5 min,
  • from 40 to 85% B in 1 min,
  • 85% B for 1 min.
(5) Set chip flow rate to 300 nl/min.
(6) MS conditions are
  • positive ion mode;
  • Vcap: 1900 V;
  • drying gas flow rate: 5 l/min;
  • drying gas temperature: 350 °C;
  • fragmentor voltage: 175 V;
  • skimmer voltage: 65 V;
  • precursors: 3.

Tandem mass spectra can be retrieved using the MassHunter software (Version B. 02. 01, Agilent Technologies).

4.3.2.9. Database searches. Mascot Distiller software (Matrix Science) is used to generate a peak list and to store it in a combined .mgf file.

4.3.2.9.1. Online Mascot search. (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS).

(1) Fill the Email address.
(2) Database: NCBInr.
(3) Enzyme: trypsin.
(4) Allow up to 1 missed cleavage.
(5) Taxonomy: all entries.
(6) Modifications: carbamidomethyl (C).
(7) Variable modifications: oxidation (M).
(8) Peptide tol. ± 50 ppm (change according to the accuracy of the instrument).

(9) MS/MS tol. ± 0.05 Da (change according to the accuracy of the instrument).
(10) Peptide charge: 2+, 3+, and 4+.
(11) Loading the .mgf file, start the search.

4.3.2.9.2. In-house Mascot search. The parameter settings are the same as for the on-line mode, except that the protein database of *Apis* can be downloaded from NCBI in FASTA format and then loaded into the Mascot software.

4.3.2.9.3. PEAKS Studio. The raw data of MS/MS can be searched using the PEAKS Studio software (Bioinformatics Solutions Inc.). The parameter settings are the same as those for Mascot.

According to the 2DE method described above, the proteomes of royal jelly derived from the Western honey bee (*A. mellifera ligustica*) and the Eastern honey bee (*A. cerana cerana*) were compared (Yu, Mao, & Li, 2010).

4.3.3. Gel-free proteomic technology for royal jelly protein identification and quantification

Although 2DE in combination with MS is effective at identification of royal jelly proteins, especially their isoforms, it excludes low-abundance proteins because of the restrictions of its sensitivity, as well as the extreme pI and molecular mass (Mr) of the proteins in question (Ong & Pandey, 2001). Nevertheless, gel-free proteomic technology involving high-resolution and high-mass-accuracy MS is one of the most powerful proteomic strategies for comprehensive protein identification.

4.3.3.1. Sample preparation

(1) Dissolve the protein pellet (see Subsection 4.2.1) in 100 μl of 5 M Urea.
(2) Add 400 μl of 40 mM NH_4HCO_3.
(3) Reduce the protein sample with 10 mM DTT for 1 h.
(4) Alkylate the protein sample with 50 mM IAA for 1 h in the dark.
(5) Digest the protein with trypsin at a 1:50 enzyme/protein ratio (w/w) at 37 °C for 14 h.
(6) Add 1 μl of formic acid to the solution to stop the reaction.
(7) Dry the solution using a Speed-Vac system.
(8) Dissolve the dried peptides with 0.1% formic acid.
(9) Centrifuge at 12,000*g* for 10 min.
(10) Transfer the supernatant into a new tube.
(11) Freeze for storage at −80 °C for further LC-MS/MS analysis.

4.3.3.2. HPLC-MS/MS analysis. Currently, a variety of instruments can be used to perform HPLC-MS/MS analysis. Here, we review the following system as an example: the EASY-nLC 1000 (Thermo Fisher Scientific) nano-liquid chromatography system coupled with a Q-Exactive via the nanoelectrospray source.

(1) Prepare buffer A (0.5% acetic acid) and buffer B (80% ACN in 0.5% acetic acid).
(2) Set the flow rate to 350 nl/min.
(3) The peptides are separated by the following gradient program:
- from 3 to 8% buffer B for 5 min,
- from 8 to 20% buffer B for 55 min,
- from 20 to 30% buffer B for 10 min,
- from 30 to 90% buffer B for 5 min,
- 90% buffer B for 15 min.

The Q-Exactive is operated in data-dependent mode with survey scans acquired at the resolution of 70,000 at *m/z* 400. The top 10 most abundant ions with the charge ≥2 from the survey scan are selected and fragmented by higher-energy collisional dissociation with normalized collision energies of 25. Xcalibur (version 2.2, Thermo Fisher Scientific) is used to retrieve MS-MS spectra.

4.3.3.3. Database search. MS/MS data extracted in RAW format can be searched using such software as PEAKS (Bioinformatics Solutions Inc.), against a composite database containing protein sequences of *Apis* and a common repository of adventitious proteins (cRAP, from The Global Proteome Machine Organization). The following modifications can be applied:

- carbamidomethylation (C)/+57.02 Da is selected as a fixed modification,
- oxidation (M)/+15.99 Da is selected as a variable modification.

The other parameters are as follows:

- Parent ion mass tolerance, 15.0 ppm;
- Fragment ion mass tolerance, 0.05 Da;
- Enzyme, trypsin;
- Allow non-specific cleavage at one end of the peptide;
- Maximum missed cleavages per peptide, 2;
- Maximum allowed variable PTM per peptide, 3;
- The false discovery rate (FDR) is filtered to ≤1.0% with a target-decoy database searching strategy to distinguish positive and negative identification.

4.3.3.4. Protein quantification

4.3.3.4.1. Quantification by labelling. In the gel-free proteomic experiment, a number of labelling approaches can be used for quantitative comparison of protein

abundance, including stable isotope labelling by amino acids in cell culture, stable isotope labelled peptides, radiolabeled amino acid incorporation, isotope-coded affinity tags (ICAT), and more recently, isobaric tags for relative and absolute quantification (iTRAQ) (Patel et al., 2009). The iTRAQ system is now commercially available and has been widely accepted as a reliable method for proteomic studies.

(1) Prepare a protein pellet as described in Subsection 4.3.2.1.
(2) Add 20 μl of dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5) to each sample containing 100 μg of the protein pellet.
(3) Add 1 μl of the denaturant (2% SDS).
(4) Vortex.
(5) Reduce the protein sample with 10 mM DTT for 1 h.
(6) Alkylate the protein sample with 50 mM IAA for 1 h in the dark.
(7) Add 1 μl of Cysteine Blocking Reagent to each tube.
(8) Vortex to mix.
(9) Centrifuge.
(10) Incubate the tubes at RT for 10 min.
(11) Digest the protein using trypsin at a 1:50 enzyme/protein (w/w) concentration at 37 °C for 14 h.
(12) Allow each vial of iTRAQ™ Reagent to be used to reach RT.
(13) Spin each vial to bring the solution to the bottom of the tube.
(14) Add 70 μl of ethanol to each RT iTRAQ™ Reagent vial.
(15) Vortex each vial to mix the contents.
(16) Centrifuge.
(17) Transfer the contents of 1 iTRAQ™ Reagent vial to 1 sample tube.

For example, for a duplex-type experiment, transfer the contents of the iTRAQ™ Reagent 114 vial to the sample 1 protein digest tube and transfer the contents of the iTRAQ™ Reagent 117 vial to the sample 2 protein digest tube.

(18) Vortex each tube to mix.
(19) Centrifuge.
(20) Incubate the tubes at RT for 1 h.
(21) Combine the contents of each iTRAQ™ Reagent-labelled sample tube into 1 tube.
(22) Vortex to mix.
(23) Centrifuge.

After labelling, the sample is used for HPLC-MS/MS analysis. Concentration ratios of iTRAQ-labelled proteins are calculated on the basis of signal intensities of reporter ions observed in peptide fragmentation spectra, with the relative areas of the peaks corresponding to proportions of the labelled peptides.

4.3.3.4.2. Label-free quantification. Label-free quantitation can be subdivided into two distinct groups: (i) area under the curve (AUC) or signal intensity measurement based on precursor ion spectra, which measures the ion abundance levels at specific retention time points for the given ionized peptides without a stable isotope standard; (ii) spectral counting, which is based on counting the peptides assigned to a protein in an MS/MS experiment (Neilson et al., 2011). A large number of commercial and open-source software packages are available for label-free quantitation, such as Progenesis-LC, Scaffold, emPAI Calc, and PEAKS. The reader is directed to the respective manuals for data analyses.

4.3.4. Identification and quantification of phosphorylated royal jelly proteins

Generally, the reversible phosphorylation of proteins at serine, threonine, and tyrosine residues is one of the most important and pleiotropic modifications. Protein phosphorylation plays crucial roles in enzyme activity regulation, function modulation of structural proteins, subcellular localization, protein interactions with other molecules, and in capacity for further covalent modification. Although as many as one-third of eukaryotic proteins are phosphorylated, the stoichiometry of phosphopeptides on phosphoproteins is considerably lower than that of their non-phosphorylated counterparts. Therefore, the enrichment techniques are necessary for successful phosphoproteomic experiments. A wide variety of approaches are available for phosphopeptide enrichment, among which, immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO_2) are the most widely used.

4.3.4.1. Sample preparation. Prepare a digested protein sample as described in Subsection 4.3.3.1, steps 1–7.

4.3.4.2. Phosphopeptide enrichment

4.3.4.2.1. TiO_2

(1) Prepare binding buffer consisting of 6.0% TFA, 80% ACN, and 0.2 M dihydroxy-benzoic acid (DHB).
(2) Prepare a TiO_2 slurry by adding 10 mg TiO_2 to 1 mL of binding buffer.
(3) Add 500 μl of binding buffer to the digested sample.
(4) Add 50 μl of the prepared TiO_2 slurry to the above mixture.
(5) Incubate at RT for 60 min with vigorous shaking.
(6) Centrifuge at 12,000*g* for 5 min.
(7) Remove the supernatant.
(8) Rinse the pellet in 1 ml of binding buffer for 30 min at RT with shaking.

(9) Centrifuge at 12,000*g* for 5 min.
(10) Remove the supernatant.
(11) Rinse the pellet in 1 ml of washing buffer I (0.5% TFA, 50% ACN solution) for 30 min at RT with shaking.
(12) Centrifuge at 12,000*g* for 5 min.
(13) Remove the supernatant.
(14) Rinse the pellet in 1 ml of washing buffer II (0.1% TFA, 30% ACN solution) for 30 min at RT with shaking.
(15) Elute phosphopeptides from the pellet twice with 100 μl of a 0.5 mM K_2HPO_4 solution.

4.3.4.2.2. Ti^{4+}-IMAC

(1) Prepare Ti^{4+}-IMAC
  (1.1.) Incubate 10 mg of poly-microspheres in a 100 mM $Ti(SO_4)_2$ solution at RT overnight with gentle stirring.
  (1.2.) Centrifuge at 13,000*g* for 3 min.
  (1.3.) Remove the supernatant.
  (1.4.) Wash the Ti^{4+}-IMAC beads 6 times with distilled water to remove the residual titanium ions.
  (1.5.) Wash the Ti^{4+}-IMAC beads 2 times with 200 mM NaCl.
  (1.6.) Wash the Ti^{4+}-IMAC beads 2 times with distilled water.
  (1.7.) Dry the beads using a Speed-Vac system.
(2) Prepare binding buffer consisting of 6.0% TFA, 80% ACN.
(3) Add 5 mg of Ti^{4+}-IMAC beads to 500 μl of binding buffer.
(4) Add 500 μl of the digested sample to the above mixture.
(5) Incubate at RT for 60 min with vigorous shaking.
(6) Centrifuge at 12,000*g* for 5 min.
(7) Remove the supernatant.
(8) Rinse the pellet in 1 ml of binding buffer for 30 min at RT with shaking.
(9) Centrifuge at 12,000*g* for 5 min.
(10) Remove the supernatant.
(11) Rinse the pellet in 1 ml of washing buffer I (0.5% TFA, 50% ACN solution) for 30 min at RT with shaking.
(12) Centrifuge at 12,000*g* for 5 min.
(13) Remove the supernatant.
(14) Elute phosphopeptides from the pellet twice with 100 μl of a 10% ammonia solution.

4.3.4.3. Mass spectrometric analysis. Refer to Subsection 4.3.3.2 "*HPLC-MS/MS analysis*".

4.3.4.4. Data analysis

4.3.4.4.1. Database search and site localization. MS/MS data extracted in RAW format are searched using in-house PEAKS software (version 6.0, Bioinformatics Solutions Inc.) against a composite database containing protein sequences of *Apis* and a common repository of adventitious proteins (cRAP, from The Global Proteome Machine Organization). The following modifications are applied:

- carbamidomethylation (C)/+57.02 Da is selected as a fixed modification,
- Oxidation (M)/+15.99 Da and Phospho (S, T, Y)/ + 79.96 Da are selected as variable modifications.

The other parameters are as follows:

- Parent ion mass tolerance, 15.0 ppm;
- Fragment ion mass tolerance, 0.05 Da;
- Enzyme, trypsin;
- Allowing non-specific cleavage at neither end of the peptide;
- Maximum missed cleavages per peptide, 2;
- Maximum allowed variable PTMs per peptide, 3.
- The false discovery rate (FDR) is filtered to ≤1.0% with a target-decoy database searching strategy to distinguish positive and negative identification.

The phosphorylation sites are assigned using Scaffold PTM (version 1.1.3; Proteome Software, Portland, OR, USA) on the basis of the Ascore algorithm. Only the site confidence more than 95% implies a mapped phosphosite.

4.3.4.4.2. Quantitative analysis. Refer to Subsection 4.3.3.4 "Protein quantification".

4.3.5. Identification and quantification of glycosylated royal jelly proteins

4.3.5.1. Sample preparation

(1) Prepare the protein pellet as described in Subsection 4.3.2.1.

Note: Keep the protein pellet dry without dissolving it in LB.

4.3.5.2. N-linked glycopeptide enrichment

4.3.5.2.1. Hydrazide enrichment

(1) Resuspend the protein pellet (~1 mg) in 250 μl of coupling buffer (100 mM NaAc and 150 mM NaCl, pH 5.5)
(2) Add 100 μl of 50 mM sodium periodate for glycoprotein oxidation at RT.

The solution should be protected from light.

(3) Agitate for 1 h.

(4) Hydrazide equilibration
  (4.1.) Add 250 μl of hydrazide and 750 μL of ddH_2O,
  (4.2.) Centrifuge at 15,000*g* for 5 min,
  (4.3.) Discard the supernatant,
  (4.4.) Repeat steps 4.2 and 4.3 thrice,
  (4.5.) Add 750 μl of coupling buffer,
  (4.6.) Centrifuge at 15,000*g* for 5 min,
  (4.7.) Discard the supernatant,
  (4.8.) Repeat steps 4.6 and 4.7 three times,
  (4.9.) Add 250 μl of coupling buffer,
  (4.10.) Agitate thoroughly.
(5) Add 500 μl of an equilibrated hydrazide solution to the dissolved protein solution.
(6) Agitate at RT for 24 h.
(7) Centrifuge at 15,000*g* for 5 min.
(8) Discard the supernatant.
(9) Desalting
  (9.1.) Add 1 ml of ddH_2O,
  (9.2.) Mix,
  (9.3.) Centrifuge at 15,000*g* and 4 °C for 5 min.
  (9.4.) Discard the supernatant,
  (9.5.) Repeat steps 9.1–9.5 twice,
  (9.6.) Add 1 ml of 40 mM NH_4HCO_3,
  (9.7.) Mix,
  (9.8.) Centrifuge at 15,000*g* and 4 °C for 5 min,
  (9.9.) Discard the supernatant,
  (9.10.) Repeat steps 9.6–9.9 twice.
(10) Resuspend the precipitate in 250 μl of 40 mM NH_4HCO_3.
(11) Add 50 μl of 100 mM DTT for protein reduction.
(12) Leave at RT for 30 min.
(13) Add 125 μl of 100 mM IAA for protein alkylation.
(14) Leave at RT for 30 min.
  Note: The solution should be kept in darkness.
(15) Add trypsin in the trypsin:protein ratio 1:50 (w/w).
(16) Keep at 37 °C overnight for digestion.
(17) Add 1 μl of formic acid to stop the digestion.
(18) Centrifuge at 15,000*g*, at 4 °C for 5 min.
(19) Retrieve the pellet.
(20) Wash the pellet with 1 ml of ddH_2O.
(21) Centrifuge at 15,000*g* and 4 °C for 5 min.
(22) Retrieve the pellet.
(23) Repeat steps 20–22 three times.
(24) Wash with 1 ml of 30% ACN.
(25) Centrifuge at 15,000*g* and 4 °C for 5 min.
(26) Retrieve the pellet.
(27) Repeat steps 24–26 three times.
(28) Wash with 1 ml of dd H_2O.
(29) Centrifuge at 15,000*g*, at 4 °C for 5 min.
(30) Retrieve the pellet.
(31) Repeat steps 28–30 three times.
(32) Wash with 1 ml of 40 mM NH_4HCO_3.
(33) Centrifuge at 15,000*g* and 4 °C for 5 min.
(34) Retrieve the pellet.
(35) Repeat steps 32–34 three times.
(36) Resuspend the pellet in 200 μl of 40 mM $NH_4HC^{18}O_3$ (prepared by dissolving NH_4HCO_3 in $H_2{}^{18}O$).
(37) Add 5 μl of PNGase F to release the N-linked glycopeptides.
(38) Incubate at 37 °C overnight.
(39) Add 1 μl of formic acid to stop the digestion.
(40) Centrifuge at 15,000*g* at 4 °C for 15 min.
(41) Keep the supernatant.
(42) Wash the pellet with 80% ACN (diluted with $H_2{}^{18}O$).
(43) Centrifuge at 15,000*g*, at 4 °C for 15 min.
(44) Keep the supernatant.
(45) Repeat steps 42–44 twice.
(46) Pool the supernatant and concentrate the solution by means of a Speed-Vac system for MS analysis.

4.3.5.2.2. Lectin enrichment

(1) Resuspend the protein pellet (~1 mg) in 200 μl of UA buffer (8 M urea, 100 mM Tris–HCl, pH 8.5).
(2) Centrifuge at 14,000*g*, at 4 °C for 15 min.
(3) Pipette the supernatant onto the 0.5-ml filter unit.
(4) Add 200 μl of UA buffer.
(5) Centrifuge at 14,000*g* for 15 min.
(6) Discard the solution in the collection tube.
(7) Add 100 μl of a 100 mM IAA solution and mix for 1 min.
(8) Incubate without mixing for 30 min in the dark.
(9) Centrifuge the filter unit at 14,000*g* for 15 min.
(10) Add 200 μl of UA buffer to the filter unit.
(11) Centrifuge at 14,000*g* for 15 min.
(12) Repeat step 11 twice.
(13) Add 200 μl of 40 mM NH_4CO_3 to the filter unit.
(14) Centrifuge at 14,000*g* for 15 min.
(15) Repeat step 14 twice.
(16) Add trypsin (enzyme to protein ratio 1:50, w/w) to the filter unit.
(17) Place the filter unit into a new collection tube.
(18) Mix for 1 min.
(19) Incubate at 37 °C overnight.
(20) Add 1 μl of formic acid to stop the reaction.
(21) Centrifuge at 14,000*g* for 15 min.
(22) Add 50 μl of 1× binding buffer (see Table 5) to the filter unit.
(23) Centrifuge at 14,000*g* for 15 min.
(24) Repeat step 23.
(25) Transfer the solution from the collection tube to a new filter unit.
(26) Add 36 μl of lectin CWR (Table 6).
(27) Agitate for 1 h at RT.

(28) Centrifuge at 14,000*g* for 15 min.
(29) Add 200 μl of 1× binding buffer to the filter unit.
(30) Centrifuge at 14,000*g* for 15 min.
(31) Repeat step 30 four times.
(32) Add 200 μl of 40 mM NH_4CO_3 to the filter unit.
(33) Centrifuge at 14,000*g* for 15 min.
(34) Repeat step 33 three times.
(35) Add 50 μl of 40 mM NH_4CO_3 (prepared by dissolving NH_4CO_3 in $H_2{}^{18}O$) to the filter unit.
(36) Centrifuge at 14,000*g*, for 5 min.
(37) Add 200 μl of 40 mM NH_4CO_3 (prepared by dissolving NH_4CO_3 in $H_2{}^{18}O$) to the filter unit.
(38) Add 5 μl of PNGase F to the filter unit to release the N-linked glycopeptides.
(39) Incubate at 37 °C overnight.
(40) Add 1 μl of formic acid to stop the digestion.
(41) Transfer the filter unit to a new collection tube.
(42) Centrifuge at 14,000*g* for 15 min.
(43) Add 50 μl of 40 mM NH_4CO_3 (prepared by dissolving NH_4CO_3 in $H_2{}^{18}O$).
(44) Centrifuge at 14,000*g*, for 10 min.
(45) Collect the solution in the collection tube.
(46) Concentrate the solution by means of a Speed-Vac system for MS analysis.

4.3.5.3. Mass spectrometric analysis. The enriched glycopeptides are analyzed according to the method in Subsection 4.3.3.2. "HPLC-MS/MS analysis". The parameters are similar except the elution gradient program is as follows:

- from 3 to 8% buffer B for 10 min,
- from 8 to 23% buffer B for 130 min,
- from 23 to 30% buffer B for 20 min,
- from 30 to 90% buffer B for 8 min,
- 90% buffer B for 12 min.

Table 5. Composition of Binding Buffer, pH 7.6.

Component	Concentration	
	2×	1×
$MnCL_2$	2 mM	1 mM
$CaCl_2$	2 mM	1 mM
NaCl	1 M	500 mM
Tris	40 mM	20 mM

4.3.5.4. Data analysis

4.3.5.4.1. Database search and glycosite assignment. The raw data on glycoprotein MS/MS analysis are used in searches within the PEAKS Studio (Bioinformatics Solutions Inc.). The parameter settings are similar to the phosphoprotein procedure except for the selection of variable modifications:

- Oxidation (M)/+15.99 Da,
- Deamidation_O^{18} (2.9883),
- Deamidation (NQ).

The glycosites are assigned using Scaffold PTM (version 1.1.3; Proteome Software, Portland, OR, USA) on the basis of the Ascore algorithm. Only those having the consensus sequence N-X-S/T ($X \neq P$) and a site confidence over 95% are considered mapped glycosites.

4.3.5.4.2. Quantification. Refer to Section 4.3.3.4. "Protein quantification".

4.3.6. Conclusion

A major challenge for honey bee proteomics is the research on low-abundance proteins in royal jelly and different organs and glands of the honey bee. The amounts of the most abundant proteins can be million-fold greater than those of the low-abundance royal jelly proteins. Many important families of honey bee and royal jelly proteins (that may be promising drug targets) such as transcription factors, protein kinases, and regulatory proteins are low-copy proteins. These proteins will not be detected in the analysis of crude soluble fractions of honey bee glands or royal jelly without some purification. Therefore, new methods must be devised for sub-proteome isolation. Despite these limitations, proteomics, when combined with other complementary technologies such as conventional separation methods, has an enormous potential in terms of new insights into physiological functions of honey bee proteins.

5. Royal jelly sugar research

5.1. Introduction

As for any food or dietary products, the knowledge about the levels and composition of carbohydrates in royal jelly offers important information on the product,

Table 6. Composition of Lectin CWR (Wisniewski et al., 2009; Zielinska et al., 2010; Zhang et al., 2014).

Component	Concentration	Volume
Concanavalin A (prepare with 2× Binding buffer)	6 mg/ml	15 μl
Wheat germ agglutinin (prepare with 2× Binding buffer)	6 mg/ml	15 μl
RCA 120 agglutinin solution		6 μl
Pool together, agitate thoroughly, store at −20 °C		

such as its caloric content calories. Sugar content and profile can also indicate the use of exogenous sugars because of abnormal composition or too high a concentration of carbohydrates.

In contrast to honey from nectar or honeydew, few articles dealing with the sugar composition of royal jelly are available. Determination of sugar content of royal jelly is based on methods used for honey (See the *BEEBOOK* paper on honey research methods (in prep)). Nevertheless, carbohydrate physico-analytical methods applicable to honey samples are not so easily transferable to this complex matrix. Methods involved in honey analysis can show lower robustness when applied to royal jelly. The presence of higher water content (See Subsection 3.2.1), high-molecular-weight molecules (proteins, see Section 4), and lipids (triglycerides, free fatty acids, see Section 6) can cause incompatibility with specific analytical instruments or sample preparation procedures. As a result, there are fewer methods commonly employed for royal jelly carbohydrate analysis.

The relevant studies on royal jelly are mainly based on 2 approaches: either a total sugar analysis (in general by colourimetric methods) or a carbohydrate profile using chromatographic separation: HPLC, ionic chromatography (IC), capillary zone electrophoresis (CZE), or gas chromatography. These methods are described below.

5.2. General methods for sugar analysis applied to royal jelly

5.2.1. Methods for quantification of total carbohydrates

One of the cheapest, easiest, and universally used approaches to analysis of the total carbohydrate content of a product is the colourimetric method (See the *BEEBOOK* paper on honey research methods (in prep)). It is based on the reaction between a hydrolyzed carbohydrate solution and a coloring reagent leading to a color detectable with a spectrometer in the visible range. Reagents commonly used for coloration include phenol (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956), alkaline ferricyanide (Englis & Becker, 1943), alkaline cupric tartrate (Munson & Walker, 1906), and anthrone (Dreywood, 1946).

The phenol-sulfuric acid method of DuBois et al. (1956) is so far the most reliable and has been extensively used in many fields, including honey bee products.

(1) Place a clear aqueous solution of the sample in a test-tube.
(2) Add 1 ml of phenol and sulfuric acid solution (5% w/v).

    The solution turns yellow-orange as a result of the interaction between the carbohydrates and phenol.
(3) Measure the absorbance at 420 nm.

    The absorbance is proportional to the carbohydrate concentration in the sample.
(4) Prepare a calibration curve with standards of known carbohydrate concentrations for quantification.

Because sulfuric acid converts all non-reducing sugars into reducing sugars, this method determines the total sugar content.

5.2.2. Methods for quantification of individual carbohydrates

5.2.2.1. The enzymatic method. Analytical methods based on enzymes rely on their ability to catalyze specific reactions. These methods are rapid, highly specific, and sensitive. In addition, the sample preparation is less laborious. Therefore, this approach is suitable for carbohydrate quantification in royal jelly. If samples are not liquid, dissolve them in water.

Many enzyme assay kits for the analysis of specific carbohydrates are commercially available with detailed instructions on how to carry out the analysis. Two methods are commonly used: (i) allowing the reaction to proceed to completion and measuring the concentration of the product, which is proportional to the concentration of the initial substrate; (ii) measuring the initial rate of the enzyme-catalyzed reaction because the rate is proportional to the substrate concentration. Glucose, fructose, sucrose, and malic and citric acid have been quantitated in samples of honeydew, honey, and royal jelly by enzymatic UV tests. Preliminary qualitative analysis of carbohydrates present in the analyzed samples is performed by silica gel thin-layer chromatography.

The enzymatic method has shown appreciable advantages in specificity and ease of use. Main monosaccharides (fructose and glucose) and sucrose have been quantified by this method in royal jelly by Tourn, Lombard, Belliardo, and Buffa (1980).

5.2.2.2. The HPLC method. Binder (1980), Nikolov, Jakovljević, and Boškov (1984), Wong-Chong and Martin (1979), Takenaka and Echigo (1980) have shown good resolution of mono- and disaccharides by liquid chromatography with refractive index detection, using an amino-linked modified silica column (Binder, 1980; Nikolov et al., 1984; Takenaka & Echigo, 1980; Wong-Chong & Martin, 1979). In royal jelly, Bogdanov et al. (2004), Sesta (2006), and Serra Bonvehi (1992) have quantified carbohydrates using HPLC (Bogdanov et al., 2004; Serra Bonvehi, 1992; Sesta, 2006).

Using HPLC with Restek Pinnacle II amino column (250 × 3.2 5 μm), Sesta (2006) quantified fructose, glucose, sucrose, and maltose in 97 royal jelly samples. The refractive index detector is used for quantification. The sample preparation consisted of protein precipitation (Currez reagent) and removal of lipids. Maximum and minimum values have been presented for each individual sugar as well as the total sugar content. In another work, Sesta (2006) studied the influence of sugar feeding

by using honey, sucrose, Cerestar, and Apiinvert sources. Ninety-five royal jelly samples were analyzed by HPLC with refractive index detection. Maltose seemed to be present at higher concentrations when Cerestar syrup was used, while total sugar was found to be in agreement with previously published data. Thus, a sugar profile leads to important information regarding royal jelly quality control. The HPLC method, even with specific amino columns dedicated to sugar analysis, has some weaknesses: not enough sugars are separated during the analysis, refractive index detection is highly sensitive to temperature variations, and sensitivity is poor.

5.2.2.3. GC method. Carbohydrates are not usually analyzed by GC because of their low volatility and their thermal degradation. Therefore, they need to be transformed into volatile species prior to the injection step.

All methods using derivation of sugars have to take into account the moisture sensitivity of the derivation process. Preliminary drying or lyophilization of the sample is necessary (see Subsection 3.3).

Several derivatization pathways are available for the modification of the hydroxyl functions of the carbohydrates with silylating agents. Gee and Walker (1962), Bishop (1962), Fournet, Dhalliuin, Montreuil, Bosso, and Defaye (1980), Fournet, Strecker, Leroy, and Montreuil (1981) have analyzed methyl derivatives of carbohydrates (Bishop, 1962; Fournet et al., 1980, 1981; Gee & Walker, 1962). Acetylation derivatization has been applied to monosaccharide analysis by Lineback (1968) and Dutton (1973). Nevertheless, the use of trimethylsilyl derivatives is still the most popular pathway for sugar GC analysis, first introduced by Hedgley and Overend (1960) and Bayer and Witsch (1961) and broadly applied by Sweeley, Bentley, Makita, and Wells (1964) (Bayer & Witsch, 1961; Hedgley & Overend, 1960; Sweeley et al., 1964). Horváth and Molnár-Perl (1997) have achieved simultaneous GC-MS quantification of mono-, di-, and trisaccharides by means of their trimethylsilyl (TMS) ether oxime derivatives in honey samples (Horváth & Molnár-Perl, 1997).

This latter method is now widely employed for honey sugar analysis since the work of Pourtallier (1967). For royal jelly, the benchmark study was published by Lercker, Caboni, Sabatini, and Nanetti (1986). Numerous works using GC cite this study. Unlike honey, which is mainly composed of sugars (80%), royal jelly carbohydrates are present at lower concentrations (7.5–18%). The results indicate that the main carbohydrates are the monosaccharides fructose and glucose, constituting ~90% of total sugar (Bogdanov et al., 2004; Sabatini et al., 2009; Serra Bonvehi, 1992; Sesta, 2006). Between the two, fructose prevails. More carbohydrates have been identified and quantified using GC method; numerous disaccharides and trisaccharides as well as honey carbohydrate profiles have been successfully analyzed (Daniele & Casabianca, 2012; Sabatini et al., 2009; Wytrychowski, Daniele, & Casabianca, 2012; Wytrychowski et al., 2013).

Silylated derivatives of carbohydrates analyzed by GC are a powerful tool for resolution, identification, and quantification of sugars in royal jelly as described in numerous studies (Daniele & Casabianca, 2012; Lercker et al., 1986; Sabatini et al., 2009; Wytrychowski et al., 2012, 2013). In general, methyl-phenyl polysiloxane (HP 5 or DB 5) chromatographic capillary columns are used. An efficient resolution is obtained for mono-, di-, and trisaccharides. Due to calibration with a mixture of alkanes (C15–C40), retention indices were obtained (Table 7) (Cotte, 2003).

5.2.2.3.1. Sample preparation

(1) Lyophilise royal jelly (see Subsection 3.1.3).
(2) Weigh 40 mg of lyophilized royal jelly in a glass reactor.
(3) Add 1 mg of the internal standard (for example, sorbitol).
(4) Add 1 ml of anhydrous pyridine.
(5) Tightly close the reactor.
(6) Stir for 5 min.
(7) Add 200 µl of hexamethyldisilazane.
(8) Stir for 5 min.
(9) Add 100 µl of trimethylchlorosilane.
(10) Stir for 30 min.
(11) Leave the mixture for 20 h at room temperature with the reactor sealed.
(12) Analyze the derivatised sample by GC [see section 2.2.3 of the article by Torto et al. (2013)].

5.2.2.3.2. GC analytical conditions

(1) Connect the chromatograph with an HP5-MS column (30 m × 0.25 mm; 0.25 µm i.d.), a split-splitless injector, an autosampler, and a flame ionization detector (FID).
(2) Use helium (grade 5.0) as a carrier gas.
(3) Fix the injection volume at 1 µl in split mode with a ratio 1:20.
(4) Program the oven temperature as follows:
    - maintain the initial temperature (150 °C) for 5 min,
    - increase it to 325 °C at the rate of 3 °C/min,
    - maintain the final temperature for 10 min.
(5) Set the injector and detector temperatures to 280 °C.
(6) For the detector, set the hydrogen flow to 40 ml/min and the air flow to 450 ml/min.
(7) Maintain constant helium pressure at 22.04 psi.
(8) Inject 1 µl of a mixture of paraffins from C15 to C40 (at 0.2% in chloroform, w/v) prior to each batch of samples for calculation of retention indices (Cotte, 2003).
(9) Identify various sugars by means of Kovats indices instead of retention times.

Table 7. Retention indices of carbohydrates on HP5-MS.

Sugar	Anomer-1	Anomer-2
Monosaccharide		
Rhamnose	1653	
Arabinose	1640	
Xylose	1737	
Fructose	1843	1853
Mannose	1845	1942
Galactose	1900	1944
Glucose	1931	2030
Sorbitol	1980	
Disaccharide		
Lactulose	2674	2695
Saccharose	2707	
Maltose	2747	2792
Cellobiose	2756	2864
Maltulose	2773	2780
Nigerose	2784	2810
Turanose	2790	
Trehalose	2805	
Kojibiose	2814	
Palatinose	2824	
Neo-trehalose	2849	
Laminaribiose	2857	2889
Leucrose	2850	
Iso-Trehalose	2884	2941
Melibiose	2933	
Isomaltose	2952	3005
Gentiobiose	2978	
Trisaccharide		
Raffinose	3501	
Neo-Kestose	3512	
1-Kestose	3517	
Erlose	3549	
Melezitose	3585	
Maltotriose	3627	
Panose	3685	

Figure 32 shows a GC carbohydrate profile obtained for a royal jelly sample where 15 sugars are identified and quantified: fructose, glucose, sucrose, maltose, galactose, mannitol, trehalose, gentiobiose, isomaltose, turanose, erlose, maltulose, melezitose, and maltotriose (Daniele & Casabianca, 2012). The internal standard (IS) is sorbitol.

In recent years, an international working group (WG 13) elaborated a physicochemical standard for international business of royal jelly (Norm project ISO 12824 Royal-jelly: Specifications). Analysis of sugars is an important part of this project because they directly correlate with production pathways (environmental natural resources or sugar syrup bee feeding). Sesta (2006) and Daniele and Casabianca (2012) have shown important carbohydrate profile modifications (especially for di- and trisaccharides) when artificial feeding sugars were used instead of honey or natural resources (nectar). On the basis of the analysis of 800 royal jelly samples, Daniele and Casabianca (2012) and Wytrychowski et al. (2012, 2013) have proposed a discriminatory method combining the GC sugar profile with ^{13}C stable isotope measurements.

5.2.2.4. Ionic chromatography. With the development of polymeric stationary phases allowing for work with basic pH mobile phases (Dionex, 1993), important work has been done in food and agricultural chem0istry based on ionic chromatography of carbohydrates. Amperometric detection is typically used. In 1995, Goodall et al. classified honeys based on their floral origin by means of this technique (Goodall, Dennis, Parker, & Sharman, 1995). However, only a few research groups applied this method to the royal jelly matrix. In fact, unlike honey, royal jelly consists of a complex matrix containing fats and high-molecular-weight molecules (proteins) leading to difficulties with the development of robust methods. To study glucose, fructose, and sucrose in royal jelly, prior precipitation of proteins by trifluoroacetic acid is necessary, as described in the study by Lei, Peng, Ge, Mei, and Zhu (2013).

Ionic chromatography is highly sensitive to the royal jelly matrix, in contrast to routine control with honey. Right now, this method is not applicable to royal jelly due to pollution of the polymeric columns with fats and proteins and because of the amperometric detection.

5.2.2.5. Infrared spectroscopy. Infrared spectroscopy is a rapid, easy, and reliable method for quality analysis. In the case of honey, this method has shown good correlations among sugars, proline, free amino acids, invertase, moisture, HMF, pH, and electrical conductivity (Lichtenberg-Kraag, Hedtke, & Bienefeld, 2002; Ruoff et al., 2006a, 2006b, 2007). Using near-infrared transflectance spectroscopy, García-Alvarez, Huidobro, Hermida, and Rodríguez-Otero (2000) analyzed fructose, glucose, and moisture in 161 honey samples (García-Alvarez et al., 2000). Several studies have used this method for geographical classification and for adulteration control (Kelly, Downey, & Fouratier, 2004; Ruoff et al., 2006a, 2006b, 2007; Woodcock, Downey, Kelly, & O'Donnell, 2007). In general, an important part of the method is the statistical analysis of the numerous and multi-parametric results (principal component analysis). To our knowledge, this method has not yet been employed to study carbohydrates in royal jelly.

5.2.2.6. Nuclear magnetic resonance. Few studies are dealing with sugar analysis in bee products by nuclear magnetic resonance (NMR). Such a method requires considerable investment, advanced analytical skills, and time. Most of the studies involving NMR have been developed in ^{1}H mode especially for geographical characterization and adulteration control (Bertelli et al., 2010; Consonni & Cagliani, 2008). Specific magnetic fields are necessary to obtain higher ^{1}H resolution for chemical shifts because the range is smaller than that of ^{13}C (0–10 ppm), which represents an expensive investment and high-cost routine analysis.

Mazzoni, Bradesi, Tomi, and Casanova (1997) have conducted the first study on the qualitative and quantitative analysis of sugars in honey by ^{13}C NMR (40)

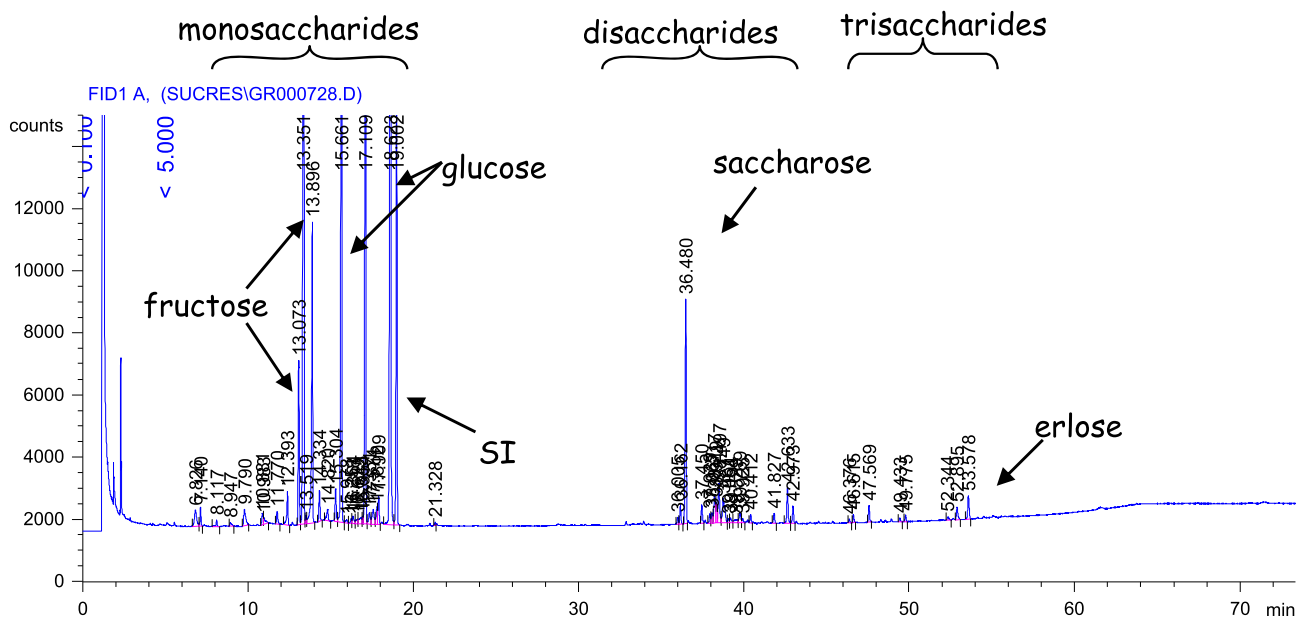

Figure 32. Royal jelly mono-, di- and trisaccharide profile obtained with GC.

(Mazzoni et al., 1997). The main advantage of ^{13}C NMR is that no separation is necessary for complex mixtures. ^{13}C chemical shifts offer an important working range allowing for identification even in complex mixtures (^{13}C chemical shift from 0 to 280 ppm).

5.3. *Discussion of methods for carbohydrate analysis as applied to royal jelly*

Only little information is available in the literature on carbohydrate analysis in royal jelly. Generally, 3 kinds of methods are described in the literature: a global one to determine the total sugar content of royal jelly, a method to quantify separately the 3 main sugars, and more specific procedures to quantify the maximal number of individual sugars.

All colourimetric methods have some drawbacks: the chemical reactivity of carbohydrates with the derivatization reagent (sulfuric acid) greatly depends on whether the carbohydrates are neutral or ionic. As a result, the molar absorption coefficients can greatly vary depending on the charge of the carbohydrates analyzed (Albalasmeh et al., 2013; Mecozzi, 2005). Quisumbing and Thomas (1921) have also discussed several parameters and conditions that can affect the Fehling solution as a sucrose-reducing action, and blank or reagent auto-reduction (Quisumbing & Thomas, 1921). In many cases with a colourimetric reaction, the other constituents of the matrix can react (e.g., polyphenols, proteins, or mineral salts), resulting in overestimation. Finally, the main disadvantage of these methods is the quantification of all carbohydrates without any information on target constituents. Indeed, production involving bee feeding with syrups (cane sugar, beet sugar, inverted sugars, or hydrolyzed starch) leads to modifications of the carbohydrate profile (Daniele & Casabianca, 2012; Sesta, 2006; Wytrychowski et al., 2012, 2013). As a consequence, to ensure quality of royal jelly, chromatographic resolution of sugars is necessary.

5.4. *Conclusion*

Quality control of royal jelly requires analysis of individual carbohydrates in order to determine a production pathway corresponding to future standard requirements. This information is available only via a separation method appropriate to the specific matrix under study. As discussed above, royal jelly complexity (higher water and lower sugar contents compared to honey, and the presence of proteins and lipids) does not match traditional quantification methods for sugars. Based on carbohydrate resolution power, only GC, LC, or IC methods offer chromatographic resolution of a sufficient number of components. From a robustness point of view, the best method seems to be GC with trimethylsilyl derivation of pre-lyophilized royal jelly (see Subsection 5.2.2.3). This method is not sensitive to protein and fat content of the matrix, while LC or IC cannot offer routine methodology in this case (pollution of chromatographic support and detector). GC with capillary columns offers enough resolution for the range of carbohydrates present in the royal jelly matrix (mono-, di-, and trisaccharides) and can be coupled to several detection systems [flame ionization or mass spectrometric detectors, see Torto et al. (2013)].

6. Research on royal jelly fatty acids

6.1. Introduction

Lipids in biological materials were described as lipid metabolites in the past but currently are known as the lipidome. The latter may be classified as a subset of metabolites, although it is typically regarded as distinct from other metabolites (Smith, Mathis, Ventura, & Prince, 2014). A new nomenclature system, Lipid Metabolites and Pathways Strategy Consortium, has been proposed for lipids in nature, which classifies them into 8 categories such as fatty acyls (i.e., free and esterified fatty acids), sterols, and glycerolipids (Fahy et al., 2005; Watson, 2006). Lipids in royal jelly, accounting for 3–8% of the fresh matter or 15–30% of the lyophilized product, are assumed to be synthesized by the mandibular glands of honey bee nurses (Li, Huang, & Xue, 2013). Although fatty acids and sterols (and even glycerolipids and glycerophospholipids) have been detected in lipid extracts from royal jelly, the lipids in royal jelly are mostly composed of (aliphatic) fatty acids (Townsend & Lucas, 1940a). Almost all of these acids are present as free fatty acids and barely any as esters (Li et al., 2013). Unlike fatty acids of most animal and plant materials, which consist mainly of triglyceride fatty acids having the main-chain length of 14–20 carbon atoms, most of fatty acids in royal jelly have a main-chain length of 6–12 carbon atoms and are therefore named as medium-chain fatty acids (MCFA) (Genç & Aslan, 1999). These fatty acids are hydroxylated at terminal and/or internal positions, terminated with mono- or dicarboxylic acid groups, and saturated or monounsaturated at the second position. Among them, the most abundant MCFA is 10-HDA, amounting to 70% of lipid extracts from royal jelly and more than 50% of free fatty acids in royal jelly (Li et al., 2013). This MCFA is considered a characteristic constituent of lipids in royal jelly and serves as an index for estimating quality of royal jelly (Chiron, 1982; Fray, Jaeger, Morgan, Robinson, & Sloan, 1961; Genç & Aslan, 1999).

10-HDA was shown to possess a variety of *in vitro* pharmacological effects such as anti-tumor, immunomodulatory, estrogen-like, collagen production-promoting, and neurogenesis-promoting effects (Li et al., 2013). These findings provide evidence that fatty acids in royal jelly make a contribution to unravelling some of the most basic processes of honey bee and offer the related knowledge on pharmacological significance of royal jelly in human health. In fact, free fatty acids in royal jelly are potential sources of bioactive compounds that function at least as nutrients required for both oogenesis in a virgin queen and early development of larvae (McFarlane, 1968; Prosser, 1978; Yanes-Roca, Rhody, Nystrom, & Main, 2009; Ziegler & Vanantwerpen, 2006), as nutritional factors in the queen-worker dimorphism (Kim, Friso, & Choi, 2009; Kucharski, Maleszka, Foret, & Maleszka, 2008; Spannhoff et al., 2011; Turner, 2000; Waterland & Rached, 2006; Zaina, 2010), hormone precursors of queen retinue pheromones (Kodai, Nakatani, & Noda, 2011), and as antimicrobial and mite-repellent agents for pathogen invasion of a queen host (Blum et al., 1959; Drijfhout, Kochansky, Lin, & Calderone, 2005; Iwanami, Okada, Iwamatsu, & Iwadare, 1979; Nazzi, Bortolomeazzi, Della Vedova, Del Piccolo, & Milani, 2009). Free acids in royal jelly are useful as preventive and supportive medicines and function, for example, as potential inhibitors of cancer growth, immune system modulators, as alternative therapies for menopause, skin-aging protectors, as neurogenesis inducers, and more (Li et al., 2013).

Just as the more established metabolomics, lipidomics is aimed at identifying and quantifying all endogenous and exogenous small metabolites with chromatography, spectrometry, and spectroscopy (Cerkowniak, Puckowski, Stepnowski, & Gołębiowski, 2013; Sandra & Sandra, 2013; Wang, Byun, & Pennathur, 2010). Methods of lipidomics are widely used for the quantification of fatty acids in royal jelly. Examples of methods are solvent extraction also known as liquid-liquid extraction (LLE), thin-layer chromatography (TLC), liquid chromatography (LC), GC, MS, optical activity (OA), attenuated total reflection-Fourier-transform infrared spectroscopy (ATR-FTIR), and nuclear magnetic resonance (NMR) as well as combined techniques. Extraction of the lipidome in royal jelly via LLE processes is usually the first step of lipidomics. Analysis of the separable fraction of lipids from royal jelly by TLC is intended to confirm the presence of the lipidome in royal jelly. Most of fatty acids in royal jelly are examined primarily by GC, in particular GC with a flame ionization detector (FID) because FID is used for analysis of organic compounds. GC-MS represents a powerful tool for the analyses of organic compounds and is a definite help in qualitative and quantitative assays of fatty acids in royal jelly. During the past decade, NMR, alone or in combination with OA and IR, has played an ever-increasing role in deciphering chemical structure of fatty acids and their derivatives and analogues in royal jelly.

6.2. Isolation of fatty acids from royal jelly

To ensure reliability of lipidomic analysis, fresh royal jelly is lyophilized into a powder (see Section 3.1.3). A discontinuous LLE method was initially used to extract the lipidome from royal jelly with diethyl ether (also referred to as ether in the literature) in a dilute sodium hydroxide solution or aqueous pyridine solution (Fevold, Hisaw, & Leoard, 1931; Lercker, Capella, Conte, & Ruini, 1981; Townsend & Lucas, 1940b). Besides diethyl ether, other organic solvents are suitable for preparation of the lipidome from royal jelly by LLE methods, including trichloromethane alone or trichloromethane followed by methanol or a mixture of trichloromethane and methanol (Kodai, Umebayashi, Nakatani, Ishiyama, & Noda, 2007; Noda, Umebayashi, Nakatani, Miyahara, & Ishiyama, 2005; Weaver, Johnston, Benjamin, & Law,

1968; Weaver & Law, 1960; Weaver, Law, & Johnston, 1964), a mixture of dichloromethane and methanol (Melliou & Chinou, 2005), a mixture of trichloromethane and acetone (Kodai et al., 2011), or hexane (Terada, Narukawa, & Watanabe, 2011). This exhaustive but requisite preparative stage for lipidomic analysis is the most common choice for efficient and cost-effective extraction of the lipidome from royal jelly. As an alternative method, a solid-liquid extraction with refluxing diethyl ether was suggested by Antinelli, Davico, Rognone, Faucon, and Lizzani-Cuvelier (2002). The presence of the lipidome in crude lipid extracts of royal jelly can be monitored by TLC (Lercker et al., 1981; Melliou & Chinou, 2005). Next in importance, preparative separation of various fatty acids from the extracts by classic LC is conducted by means of poly-silicic acids (also referred to as *silicic gel* in the literature and exemplified by Cosmosil 75C18-OPN), an ion exchange resin (such as Diaion HP-20), or hydroxypropyl Sephadex gel (such as SephadexLH-20) chromatography columns (Kodai et al., 2007; Melliou & Chinou, 2005; Noda et al., 2005; Weaver et al., 1968). In many cases, however, LC is modified into an advanced technique for faster preparation of fatty acids from royal jelly, including solid-phase extraction (SPE) in a cartridge (or filter) (Terada et al., 2011), medium-pressure liquid chromatography (Melliou & Chinou, 2005), and HPLC on a reversed-phase column (Kodai et al., 2011; Noda et al., 2005). Recently, isolation of volatile fatty acids from royal jelly was carried out with headspace solid phase micro-extraction on a specific device (Isidorov, Bakier, & Grzech, 2012).

6.2.1. *Preparation of fatty acids by LLE and SPE*

Requirements:

- A retort, test tube, or flask: e.g., a 25-mL volumetric type or bigger ones.
- Freeze dryer: e.g., FreezeDryer FDU-1100 model (Rikakikai Co., Ltd.).
- Paper filter: e.g., Whatman™ Grade 1 qualitative cellulose filter paper.
- Extraction reagents: diethyl ether or trichloromethane and methanol.

  Note: Both of them and other reagents to be stated in the following text are high-purity organic solvents suitable for LLE, LC, LC/MS, NMR, and other demanding analytical applications.
- Centrifugal vacuum evaporator: e.g., Centrifugal Evaporator CVE-3110 model (Rikakikai Co., Ltd.).
- Vacuum elution apparatus: e.g., 12-Port Visiprep DL Vacuum Manifold model (Sigma-Aldrich Co.). This disposable Liner type is equipped with an accessory collection rack with a group of receiving tubes plugged into the device.
- SPE cartridge: e.g., Ion Exchange Cartridge Bond Elut–DEA model (Agilent Technologies, Inc.). The stationary phase of the column in this cartridge is composed of an ion exchange resin with bonded aminopropyl moieties, activated by conditioning it with an appropriate amount of hexane and is fixed in a vacuum elution apparatus.

Procedure:

(1) Lyophilise a royal jelly sample (see Subsection 3.1.3).
(2) Put 500–1000 mg of lyophilized royal jelly into a retort.
(3) Add 10 ml of diethyl ether or trichloromethane.
(4) Vortex for 15 min to extract lipids.
(5) Pour the extract through a paper filter.
(6) Obtain as much fatty acids as possible from the same sample by extraction repeated 2 more times.
(7) Save combined filtrates.
(8) Transfer insoluble material into another retort.
(9) Add 10 ml of methanol.
(10) Vortex for 15 min to extract lipids.
(11) Pass the extract through a paper filter.
(12) Obtain as much fatty acids as possible from the same material by extraction repeated 2 more times.
(13) Save combined filtrates.
(14) Transfer joint filtrates obtained at step 7 in a plastic tube.
(15) Concentrate them with the help of a centrifugal vacuum evaporator.
(16) Transfer combined filtrates obtained at step 13 to a plastic tube.
(17) Concentrate them by means of a centrifugal vacuum evaporator.
(18) Add 2 ml of diethyl ether or trichloromethane to dissolve the oil-like residue (left on both the bottom and walls of the tube) obtained at step 15.
(19) Add 2 ml of methanol to dissolve the oil-like residue (left on both the bottom and walls of the tube) obtained at step 17.
(20) Load the solutions obtained after steps 18 and 19 into the top space of an SPE cartridge.
(21) Elute the column with 2% acetic acid in diethyl ether or trichloromethane and save the eluate.
(22) Concentrate the eluate by means of a centrifugal vacuum evaporator. Enriched material is labelled as "test sample" and is to be used for the following analyses.

6.2.2. *Monitoring of fatty acids in the presence of lipid extracts of royal jelly by TLC*

Requirements:

- TLC plate: e.g., a standard TLC plate (Sigma-Aldrich Co.) or self-made TLC plate cut from cleaned and dried glass (approximately with the

dimensions 20 × 20 cm or bigger). A uniform layer (approximately 0.2-mm thickness) of Silica Gel G slurry is spread on the plate by means of a spreader, dried at room temperature, and heated in an oven at 110 °C for 30 min.
- TLC tank: rectangular TLC development tank (Sigma–Aldrich Co.).
- Developing solvent: a solution mixed with hexane and diethyl ether (1.5:1.0 v/v).
- Detection reagent: 50% sulfuric acid.
- Reference standards: some lipid compounds from royal jelly, such as 10-HDA, dissolved in 2% acetic acid in diethyl ether or trichloromethane.

Procedure:

(1) Add the developing solvent beforehand to the TLC tank and close it with the lid, allowing it to saturate the chamber for 10 min at RT.
(2) Take a TLC plate and draw 2 straight lines: at the top and bottom, making the first one ~2 cm from the bottom and the second one ~1 cm from the top of the plate.
(3) Subdivide the bottom line into 2-cm gaps for spotting.
(4) Re-dissolve the test sample in 3–5 ml of 2% acetic acid in diethyl ether (the most effective solution for the extraction) or 2% acetic acid in trichloromethane (a comparable alternative to the extraction).
(5) Dissolve 20 mg of each reference standard in 1 ml of the solvent mentioned above.
(6) Pipette 10 μl or 20 μl of all reference standard solutions and of the test sample solution, and spot each of them on the spotting area on the plate.
(7) Dry the plate in ambient air for 5–10 min.
(8) Pick up the TLC plate holding the top by means of forceps.
(9) Place it in the TLC tank vertically.
Note: Ensure that the solvent phase moves uniformly along the plate.
(10) TLC is carried out in a laminar flow hood.
(11) Wait until the front of the solvent phase has moved approximately to the top of the line, allowing the mobile phase to evaporate completely.
(12) Remove the plate from the chamber and place it onto an experiment table surface in ambient air.
(13) Spray the detection reagent on the plate.
(14) Place the plate in an oven at 110 °C until areas containing the test sample get charred and appear as black spots.

6.3. Identification of fatty acids in royal jelly

Chromatographic analysis of 10-HDA and some other dominant fatty acids, e.g., 10-hydroxydecanoic acid, in royal jelly was first performed with reference to direct determination of these compounds by GC (Weaver et al., 1964). Currently, all dominant fatty acids in royal jelly and even those of low abundance, have been analyzed by means of an automated GC/MS apparatus, a high-screen capillary column coupled with a high-resolution mass spectrometer (Isidorov, Czyżewska, Isidorova, & Bakier, 2009; Isidorov et al., 2012; Li et al., 2013). Identification of a new fatty acid in royal jelly by GC-MS is dependent on the comparison of their respective mass spectra with the related data in the 2 databases, NIST (previously known as NBT) and Wiley (Online) library, and equivalents in the literature (Melliou & Chinou, 2005).

6.3.1. Quantitative determination of dominant fatty acids by GC/MS

Requirements:

- Derivatising reagents: pyridine, Bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS).
- Esterification reaction vial: e.g., a Reacti-Vial™ Small Reaction Vial (Thermo Fisher Scientific [China] Co., Ltd.).
- GC instrument: e.g., Agilent HP6890 GC model (Agilent Technologies, Inc.). This instrument is equipped with the split/splitless inlet.
- Chromatographic column: e.g., HP-1 ms fused with silica model (Agilent Technologies, Inc.). This non-polar capillary column has 30-m length, 0.25-mm inner diameter, and 0.25-μm film thickness.
- MS instrument: e.g., Agilent 5973 MSD model (Agilent Technologies, Inc.). This instrument is equipped with an independently heated electron-ionization source.
- Liquid sampler: e.g., Agilent HP 7673 autosampler model (Agilent Technologies, Inc.).

Procedure:

(1) Prepare a range of 6 application solutions, covering the range 20–2000 mg/mL of 10-HDA, by diluting each standard stock solution of dominant fatty acids with methanol.
(2) Re-dissolve the test sample in a reaction vial with 2 ml of methanol.
(3) Into a separate reaction vial, add 0.5 ml of each application solution and an equal amount of the test sample solution.
(4) After solvent evaporation, combine each of them with 80 μl of BSTFA with 1% TMCS, and 220 μl of pyridine.
(5) Seal the cap.
(6) Mix thoroughly.
(7) Heat at 60 °C for 30 min.
(8) Cool to RT.

(9) Keep a trimethylsilyl (TMS) derivative to be used for GC-MS analysis as follows.
(10) Typical GC conditions:
- Inlet temperature: 250 °C.
- Inlet mode: splitless.
- Sample value: 1 μl.
- Carrier gas: helium.
- Column flow: 1 ml/min of constant flow.
- Initial column temperature: 122 °F.
- Oven ramp: at 5 °C per min to 300 °C.

(11) Typical MS conditions:
- Ionization source: 70 eV electron ionization.
- Scan mode: full scan.
- Scan range: 41–600 atomic mass units.

(12) The TMS derivative is subjected to GC-MS analysis.
(13) A regression equation is calculated on the basis of analysis of the results.

6.3.2. *Qualitative identification of fatty acids by GC-MS*

Requirements:

- Derivatising reagents: the same as in Subsection 6.3.1.
- Esterification reaction vial: the same as in Subsection 6.3.1.
- GC instrument: the same as in Subsection 6.3.1.
- Chromatographic columns: e.g., HP-1 ms fused silica model (as a non-polar capillary column) and HP-5 ms fused silica model (as a low-polarity capillary column) (Agilent Technologies, Inc.). They all have 30-m length, 0.25-mm inner diameter, and 0.25-μm film thickness.
- MS instrument: e.g., an Agilent 5973 MSD model (Agilent Technologies, Inc.). This instrument has an independently heated electron ionization source.
- Liquid sampler: the same as in Subsection 6.3.1.

Procedure:

(1) Re-dissolve a test sample in a reaction vial with 2 ml of methanol.
(2) Add 0.5 ml of the test sample solution into the reaction vial.
(3) Allow the solvent to evaporate.
(4) Combine the residue with 80 μl of BSTFA with 1% TMCS, and 220 μl of pyridine.
(5) Seal the cap.
(6) Mix thoroughly.
(7) Heat at 60 °C for 30 min.
(8) Cool to RT.
(9) Keep the TMS derivative for GC-MS analysis as follows.
(10) Typical GC conditions: the same as in Subsection 6.3.1.
(11) Typical MS conditions: the same as in Subsection 6.3.1.
(12) The TMS derivative is subjected to GC-MS analysis.
(13) The fatty acid ester is compared with data registered in NIST, Willy libraries, and previously published data.

6.4. *Structural characterization of fatty acids in royal jelly*

By comparing infrared absorption spectra data to those of authentic standards from the literature, it was initially confirmed that the there is a *trans* configuration of the double bond in the main chain of fatty acids in royal jelly rather than at its specific position (Brown & Freure, 1959; Melliou & Chinou, 2005). The configuration at the anomeric carbon in fatty acids in royal jelly has been assigned on the basis of specific optical rotation from empirical data (Kodai et al., 2007, 2011; Melliou & Chinou, 2005; Noda et al., 2005). NMR spectral data on the free fatty acids in royal jelly are produced to directly determine the relative amounts and partial assignment of test compounds, including ^{1}C spectra and ^{13}C spectra (Kodai et al., 2007; Noda et al., 2005). Nevertheless, the similar magnetic resonance of MCFA in royal jelly suggests that this technique has limitations for characterization of a complete pattern of fatty acids in royal jelly. In contrast, MS approaches with electrospray ionization, chemical ionization, and fast atom bombardment have proven to be the most inclusive for lipid molecular species (Kodai et al., 2007). To date, it has been well established that a combination of MS and NMR can be used in lipidomics for quantification of a wide range of fatty acids in various biofluids (Gürdeniz et al., 2013). In this section, 3 methods are presented below for quantification of fatty acids in royal jelly, including optical rotation, infrared absorption, and nuclear magnetic absorption.

6.4.1. *Infrared absorption measurement of fatty acids by ATR-FTIR*

Requirements:

- Infrared spectrometer: Perkin-Elmer 500 spectrum model (Perkin-Elmer, Inc.).
- Sample slot: an ATR accessory.
- Solvent: methanol (to dissolve hydrophilic or polar fatty acids) or dichloromethane (to dissolve lipophilic or non-polar fatty acids) or trichloromethane (instead of trichloromethane under the same experimental conditions).

Procedure:

(1) Adjust spectrometer/ATR accessory settings as follows:
- Temperature during measurement: 65 °C.
- Resolution: 4 cm^{-1}.

- Wavelength range: 4000–400 cm^{-1}.

(2) Deposit uniformly a drop or several drops of a test sample solution on the crystal surface of the ATR accessory until transparent detection window in the crystal surface is fully filled.
(3) Collect infrared absorption spectra of the fatty acids versus a dry air background of an empty ATR well.

6.4.2. *Optical rotation measurement of fatty acids by OA*

Requirements:

- Polarimeter: e.g., Perkin-Elmer 341 model (Perkin-Elmer, Inc.) or JASCO DIP-140 model (JASCO China (Shanghai) Co., Ltd.).
- Solvent: methanol.

Procedure:

(1) Re-dissolve the test sample in methanol until the solution is saturated. Note: It is important for the clear solution to be saturated before researchers attempt to measure its OA.
(2) Adjust polarimeter settings as follows:
   - Detection mode: optical rotary dispersion.
   - Wavelength range: 250–660 nm.
   - Temperature during measurement: 25 °C or at RT.
(3) Use a solvent blank after each run to determine the baseline.
(4) Convert the observed value to the degree of specific rotation, $[\alpha]^{25}$, using the following formula:
   $[\alpha]^{25} = \alpha/b \quad (l \times c)$, where α = measured value, l = sample path length in decimetres, and c = concentration of a sample in grams per ml.

6.4.3. *Nuclear magnetic absorption measurement of fatty acids*

Requirements:

- NMR instruments: e.g., JMN GX400 model (for ^{1}H spectra) and ECA 600SN model (for ^{13}C spectra) (JEOL Ltd.), or DRX 400 model (for ^{1}H spectra) and Bruker AC 200 model (^{13}C spectra) (Bruker Biospin Co.).
- Solvent: d4-methanol (CD3OD), trichloromethane-d ($CDCl_3$).
- Internal standard: tetramethylsilane.

Procedure:

(1) Re-dissolve the test sample in the matrix.
(2) Transfer to NMR tubes.
(3) Typical NMR conditions:
   - Probe temperature: 25–35 °C.
   - Acquisition of ^{1}H spectra: obtained at 600 or 400 MHz.
   - Acquisition of ^{13}C spectra: obtained at 50, 100, or 150 MHz.
   - Chemical shifts: on the δ scale (ppm) with tetramethylsilane or solvent signals.
   - Coupling constant: given as the J value.
(4) Record ^{1}H and ^{13}C NMR spectra on the NMR spectrometer.

7. Residue analysis of main veterinary drugs and acaricides in royal jelly

7.1. *Introduction*

Bee products can be polluted by different sources of contamination, including environmental and apicultural sources. The most important contaminants in royal jelly are veterinary drugs used against bee diseases or for prevention of outbreaks of diseases. Acaricides that are used for *Varroa* control are also important contaminants of bee products.

Although most of veterinary drugs are not authorised for the treatment of honey bees in the EU or strictly limited in other countries, veterinary-drug residues can be found in some royal jelly samples. The most important and harmful veterinary-drug residues in royal jelly are chloramphenicol, nitroimidazole, sulphonamides, fluoroquinolone, macrolides, and tetracyclines. Fluvalinate and amitraz are the main acaricides used in apiculture and are often retained in bee products.

To ensure the quality of bee products, sensitive methods for residue determination are necessary. In this section, residue analysis of the veterinary drugs and acaricides mentioned above in royal jelly is addressed, and 1 or 2 classical analytical methods for each target compound are described in detail.

7.2. *The LC-MS/MS method for chloramphenicol analysis in royal jelly*

Chloramphenicol (CAP) is a broad-spectrum antibiotic, showing activity against a variety of aerobic and anaerobic microorganisms. Its protein synthesis-inhibiting properties make it effective in the treatment of several infectious diseases (Forti, Campana, Simonella, Multari, & Scortichini, 2005). It is often used in beekeeping to control European and American foulbrood (Ortelli, Edder, & Corvi, 2004). Because severe side effects such as aplastic anaemia and hypersensitivity have been demonstrated in humans (Allen, 1985), the European Community banned CAP use in food-producing animals since 1994, in order to protect consumers' health. Consequently, CAP was listed in Group A of the Council Directive 96/23/EC, including those substances for which a "zero tolerance residue limit" has been established in edible tissues. Nonetheless, this drug is still

illicitly used in animal farming because of its ready availability and low cost.

A method for detection of CAP in honey has been developed by Robert Sheridan and contains an acid hydrolysis step to liberate the sugar-bound sulphonamides followed by solid-phase extraction to remove possible interfering substances. Analysis was based on liquid chromatography–electrospray ionization–tandem mass spectrometry in negative mode for all 15 analytes. This MRM method generated 2 structurally significant transitions per compound, and it was designed to conform to U.S. Food and Drug Administration MS confirmation guidelines. One hundred sixteen samples from 25 countries were analyzed, and 38% were found to contain at least 1 target antimicrobial agent. Five target compounds were found in honey from 13 countries (Sheridan, Policastro, Thomas, & Rice, 2008).

Ishii, Horie, Murayama, and Maitani (2006) developed a method for detection of CAP in royal jelly samples. The quantification limit of CAP in royal jelly was 1.5 ng/g. The recovery rates of CAP from both honey and royal jelly at the quantification limits were over 92% (Ishii et al., 2006). Moreover, a liquid chromatographic/tandem mass spectrometric method was developed and validated for quantification of CAP in royal jelly. Royal jelly samples were first denatured with lead acetate solution, and the CAP was extracted with solid-phase extraction before separation by liquid chromatography. A triple-quadrupole mass spectrometer operated in the negative electrospray ionization and selected-reaction monitoring mode was used for the detection of CAP. For method validation, royal jelly samples were spiked with CAP between 0.1 and 10.0 μg/kg; at these levels, recovery values (internal standard-corrected) ranged from 93.3 to 105.0%, and the within-laboratory reproducibility (relative standard deviation) was 9.1%. The decision limit was 0.07 μg/kg, and the detection capability was 0.1 μg/kg.

7.2.1. *Sample preparation*

(1) Lyophilise royal jelly (see Subsection 3.1.3).
(2) Weigh 2.00 g of lyophilized royal jelly in a centrifuge tube (50 ml).
(3) Mix the sample.
(4) Leave the mixture at room temperature for 30 min.
(5) Add 10 ml of a $Pb(AcO)_2$ solution (200 g/l).
(6) Homogenize it for 40 s using Ultra-Turrax (Suzhou, PRC).
(7) Centrifuge the mixture for 5 min at 4000*g*.
(8) Use a Millex filter to filter the supernatant in another centrifuge tube (50 ml).
(9) Condition the Oasis HLB 60 mg SPE cartridge with 3 ml of methanol and 5 ml of water.
(10) Add the filtered supernatant to the cartridge and elute it by gravity or vacuum (1 drop/s).
(11) Wash the cartridge twice using 3 ml of the methanol–water mixture (1:4, v/v).
(12) Elute the CAP using 5 ml of methanol.
(13) Evaporate eluate under a nitrogen stream (40 °C).
(14) Reconstitute the residue in 1.0 ml methanol–water (3:7, v/v).
(15) Filter the solution using a 0.2-μm nylon filter before injection into the LC-MS/MS apparatus.

7.2.2. *LC-MS/MS analytical conditions*

(1) Connect the chromatograph with a LUNA ODS C_{18} column (7.5 × 4.6 mm; Phenomenex, Torrance, CA, USA).
(2) Use isocratic mobile phase of methanol: 5 mM ammonium acetate (60:40, v/v).
(3) Set the flow rate to 0.2 ml min^{-1}.
(4) Fix the injection volume to 5 μl.
(5) Set the column temperature to 40 °C.
(6) Set the MS detector to negative ion mode.
(7) Heat the TurboIon Spray source to 450 °C.
(8) Set the capillary voltage to 4.5 kV with an orifice potential of 20 V.
(9) Use nitrogen as a curtain and a collision gas.

Optimize the collision energies separately for the two selected ion transitions of CAP (321→152; 321→121). Typical LC-MS/MS analysis of CAP standard, negative sample, and spiked sample is shown in Figure 33.

7.3. *The LC-MS/MS method for nitroimidazole analysis in royal jelly*

Dimetridazole (DMZ), metronidazole (MNZ), and ronidazole (RNZ) are 5-nitroimidazole-based drugs and have antibacterial and anticoccidial properties. These drugs are widely used for the treatment of infections in poultry, cattle, swine, and farmed fish (Sakamoto et al., 2011). Unfortunately, these compounds have been suspected of being human carcinogens and mutagens. The Ministry of Health, Labor and Welfare in many countries including China set "not detected" as the standard for DMZ, MNZ, and RNZ in foods, and the detection limits of DMZ, MNZ, and RNZ are 0.2, 0.1, and 0.2 μg/kg, respectively .

A method for detection of traces of MTZ, DMZ, and RNZ residues in royal jelly was developed on the basis of HPLC with tandem mass spectrometry (HPLC-MS/MS). After samples were dissolved in a sodium hydroxide solution to disassociate target analytes from the matrix, liquid-liquid extraction methods by ethyl acetate solvent were used. Matrix effects were minimized, and good quantitation results were obtained using the highly selective reaction monitoring (H-SRM)

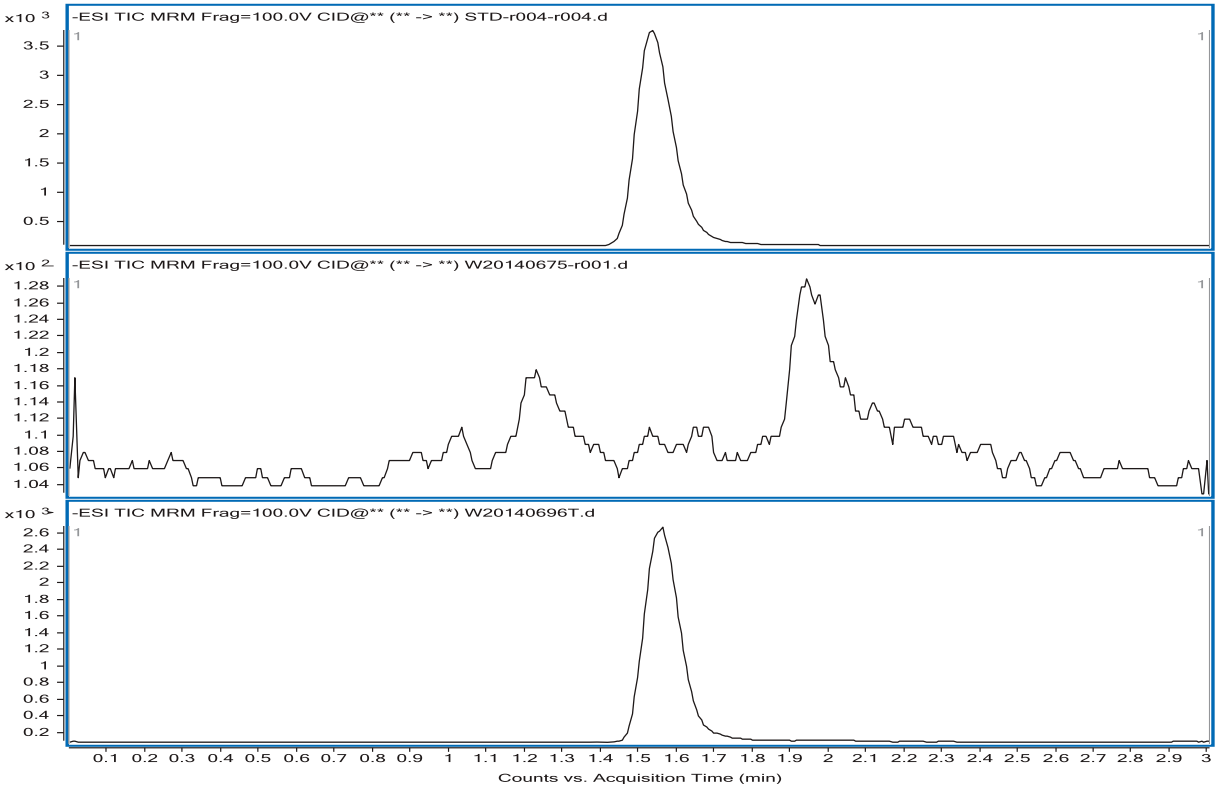

Figure 33. LC/MS/MS chromatography of chloramphenicol standard (top), spiked sample (bottom) and negative sample (middle).

technology. Limits of detection (LODs) were 1.0 mg/kg for DMZ and 0.5 mg/kg for MTZ and RNZ (Signal/Noise > 5). Limits of quantitation (LOQs) were 2.0 mg/kg for DMZ and 1.0 mg/kg for MTZ and RNZ (S/N > 10) (Ding et al., 2006).

7.3.1. Sample preparation

(1) Lyophilise royal jelly (see Subsection 3.1.3).
(2) Add 5 g of royal jelly to a centrifuge tube (50 mL).
(3) Add 10 ml of 0.5 M NaOH.
(4) Mix for 15 s to dissolve.
(5) Add 10 ml of EtOAc.
(6) Mix for 30 s.
(7) Centrifuge the mixture for 3 min at 545*g*.
(8) Transfer the supernatant to a test tube (50 ml).
(9) Repeat extraction steps 5–8.
(10) Combine EtOAc layers obtained at step 8.
(11) Dry in a water bath at 40 °C.
(12) Dissolve the residue in 5 ml of ACN containing 10% formic acid.
(13) Pour 3 ml of methanol on top of the column for conditioning.
(14) Pour 3 ml of water on top of the column for conditioning.
(15) Load the mixture at 1–2 ml/min onto the column.
(16) Wash the column with 3 ml of water.
(17) Dry it for 5 min.
(18) Elute the column using 3 ml of methanol.
(19) Collect the eluate at 1–2 ml/min.
(20) Evaporate it.
(21) Filter the solution using a 0.2-μm nylon filter before injection into the LC-MS/MS apparatus.

7.3.2. LC-MS/MS analytical conditions

(1) Set the flow rate to 0.4 ml/min, with flow ramp 2.00.
(2) Set the column temperature to 25 °C.
(3) Set the mobile phase to the 40/60 ratio (0.1% formic acid in water/0.1% formic acid in ACN).
(4) Set the injection volume to 5 μl.
(5) Set the ESI polarity to positive.
(6) Set the capillary voltage to 3 kV; set the RF lens to 0.1.
(7) Set the source temperature to 140 °C.
(8) Set the desolvation temperature to 450 °C, and desolvation gas flow to 650 l/h.
(9) Use the cone gas flow 150 l/h.
(10) Set the ion energy to 10.5.
(11) Set the entrance lens to 5.
(12) Set the collision gas flow to 18.
(13) Set the MS2 low-mass resolution to 14.5.

(14) Use the high-mass resolution of 14.5.
(15) Set the multiplier voltage to 650 V.

A typical LC-MS/MS chromatogram of a nitroimidazole standard, negative sample, and spiked sample is shown in Figure 34.

7.4. The LC-MS/MS method for analysis of sulphonamides in royal jelly

The sulphonamide family of antibiotics includes a large spectrum of synthetic bacteriostatics used against most gram-positive and many gram-negative microorganisms and protozoa. In the past decade, the irresponsible use of sulphonamide drugs in the veterinary field, for therapeutic and prophylactic purposes, as well as for treatment of human infectious diseases, has favored the development of bacterial resistance (Wegener et al., 2003), which makes it difficult to efficiently treat infections with the presently known antibiotics (Furusawa & Kishida, 2001). In bees, these antimicrobials are used to prevent and treat bacterial diseases such as American foulbrood caused by *Paenibacillus larvae* and European foulbrood caused by *Melissococcus plutonius* (Bogdanov, 2006). The method

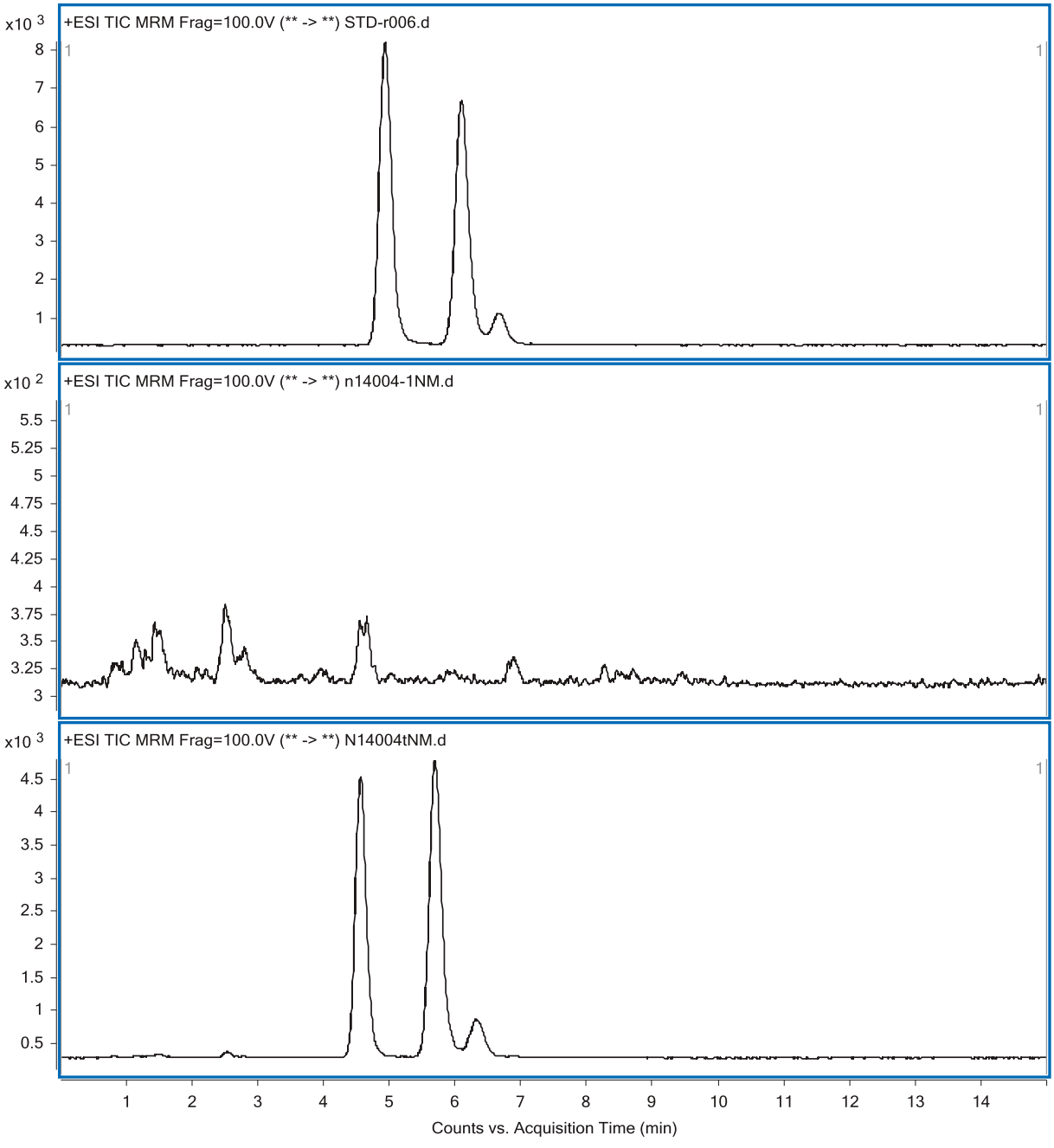

Figure 34. LC/MS/MS chromatography of nitroimidazole standard (top), negative sample (middle) and spiked sample (bottom).

commonly used for prevention is to feed the bees with a certain amount of sulphonamides in winter or early spring to improve their immunity. On the other hand, contamination of food with sulphonamide residues poses risks to human health, including an increased resistance of bacteria to antimicrobial agents, allergic reactions, and possible carcinogenicity (Enne, Livermore, Stephens, & Hall, 2001). According to European Commission (EC) Directive 2377/90, the rehabilitation of honey bees with sulphonamide antibiotics was banned in the European Union. Switzerland, Belgium, and the United Kingdom have chosen 0, 20, and 30 ng/g, respectively, as maximum residue limits (MRLs) for sulphonamide antibiotics in honey. A recent study on antibiotic residues (from sulphonamide and other antibiotic groups) in honey revealed test-positive samples exported to Europe from India, China, and Argentine that are above the MRLs (Dubreil-Chéneau, Pirotais, Verdon, & Hurtaud-Pessel, 2014).

The liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the simultaneous confirmation of 13 sulphonamides in honey was developed and fully validated by Dubreil-Chéneau in accordance with the European Commission Decision No. 2002/657/EC (Dubreil-Chéneau et al., 2014). The validation scheme was built in accordance with the target level of 50 μg kg^{-1} for all analytes. The sulphonamides analyzed were the following: sulfaguanidine (SGN), sulfanilamide (SNL), sulfadiazine (SDZ), sulfathiazole (STZ), sulfamerazine (SMR), sulfamethizole (SMZ), sulfadimerazine (SDM), sulfamonomethoxine (SMNM), sulfamethoxypyridazine (SMP), sulfadoxine (SDX), sulfamethoxazole (SMX), sulfaquinoxaline (SQX), and sulfadimethoxine (SDT). Several extraction procedures were tested during the development phase. Finally, the best results were obtained with a procedure involving acidic hydrolysis and cation exchange purification. Chromatographic separation was achieved on a C18 analytical column. Matrix effects were also studied. Data acquisition implemented for the confirmatory purpose was performed by monitoring 2 MRM transitions per analyte in positive electrospray mode. Mean relative recovery ranged from 85.8 to 110.2%, and relative standard deviations were between 2.6 and 19.8% under intra-laboratory reproducibility conditions. The decision limits ranged from 1.8 to 15.5 μg/kg.

7.4.1. *Sample preparation for royal jelly*

(1) Heat the fresh royal jelly in a water bath at 50 °C for 5 min.
(2) Transfer 2 g of heated royal jelly (or 0.5 g of royal jelly powder) to a test tube.
(3) Mix the sample.
(4) Leave the mixture in darkness at RT for 10 min.
(5) Condition the SCX cartridges with 4 ml of MeOH.
(6) Condition the SCX cartridges with 4 ml of ultra-pure water.
(7) Acidify the samples using 10 ml of citric acid solution (0.3 M).
(8) Mix by vortexing for 10 s.
(9) Shake the mixture immediately for 10 min.
(10) Centrifuge the mixture at 14,000× *g* and 4 °C.
(11) Transfer the upper phase to the cartridges.
(12) Elute the cartridges at a flow rate of 1 drop/s.
(13) Wash the cartridge with 4 ml of ultra-pure water.
(14) Wash the cartridge twice with 4 ml of a MeOH–ACN mixture (50:50; v/v).
(15) Elute the sulphonamides residues into a clean tube twice with 0.6 mL of a 2% ammonium hydroxide solution in methanol.
(16) Dry the extract at 40 °C under a gentle stream of N_2.
(17) Reconstitute the residue in 400 μl of ultra-pure water.
(18) Mix the solution.
(19) Centrifuge for 5 min at 2,500× *g* at 4 °C.
(20) Transfer the solution to auto-sampler vials for LC-MS/MS analysis.

7.4.2. *LC-MS/MS analytical conditions*

(1) Use distilled water with 0.2% formic acid as mobile phase A, and mobile phase B is pure analytical-grade ACN.
(2) Use the following gradient conditions:
  - from 0 to 0.1 min, ramp linearly from 98% to 70% of mobile phase A,
  - ramp over 2.9 min to 40% of A and hold for 5 min,
  - return to initial conditions in 2 min,
  - hold for 7 min to re-equilibrate the system.
(3) Set the flow rate to 0.25 ml/min.
(4) Set the oven temperature to 25 °C.
(5) Use the injection volume of 5 μl.
(6) Operate the instrument using electrospray ionization (ESI) in positive mode.
(7) Collect the data using Xcalibur software.
(8) Set the sample tube or desolvation temperature to 350 °C.
(9) Set the spray voltage to 4,500 V.
(10) Set the sheath gas (air) to 55 arb.
(11) Set the Aux gas (air) to 20 arb.
(12) Set the Ion sweep gas pressure (air) to 10 arb.
(13) Set the Collision gas (argon) to 1.5 mTorr.
(14) Use the dwell time of 20 ms.
(15) See Table 8 for the specific MRM parameters (two transitions) for each sulphonamide.

A typical LC-MS/MS chromatogram of a sulphonamide standard, negative sample, and spiked sample is shown in Figure 35.

7.5. The LC-MS/MS method for fluoroquinolone analysis in royal jelly

Quinolones belong to a family of synthetic antibiotics structurally related to nalidixic acid, itself being the first quinolone used clinically in animals in the early 1960s. Because of their narrow spectrum of activity and bacterial resistance issues, the original class of quinolones was supplanted in the mid-1980s by a new generation of drugs, still structurally related to nalidixic acid, containing a fluorine covalently bound to the carbon at position 6 and a piperazine ring at carbon 7. These 6-fluoroquinolones (FQs) were shown to have a much broader spectrum of activity because they were more effective against gram-negative bacteria and moderately effective against gram-positive bacteria. In apiculture, FQs are used for the prevention and treatment of American foulbrood.

A method for detection of fluoroquinolone residues in royal jelly was developed by Zhou et al. (2009). Sample preparation includes deproteination, ultrasonication-assisted extraction with a mixed inorganic solution of monopotassium phosphate (KH_2PO_4) and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), and clean-up on a solid-phase extraction cartridge. The extraction procedure was optimized regarding the amount of an inorganic solvent and the duration of sonication for royal jelly as a complicated matrix.

7.5.1. Sample preparation for royal jelly

(1) Weigh 5 g of fresh royal jelly in a centrifuge tube (50 ml).
(2) Add 10 ml of 0.1 M NaOH.
(3) Mix for 1 min.
(4) Centrifuge the mixture for 5 min at 5581× *g*.
(5) Transfer the supernatant to a clean test tube (50 ml).
(6) Condition the SPE (C_{18} or OASIS HLB) column with 6 mL of MeOH.
(7) Condition the SPE column with 6 ml of water.
(8) Load the mixture at 1–2 ml/min.
(9) Wash the column with 6 ml of water.
(10) Dry it for 5 min.
(11) Elute the analytes using 6 ml of formic acid: methanol (1:1).
(12) Collect them at 1–2 ml/min.
(13) Evaporate at 40 °C.
(14) Dissolve the residue in 1 ml of methanol:water (1:1).
(15) Pass the solution through a 0.2-μm nylon filter before injection into the LC-MS/MS apparatus.

7.5.2. LC-MS/MS analytical conditions

(1) Use a C18 column (3 μm, 150 × 2.1 mm) or other similar columns.
(2) Use distilled water with 0.1% formic acid as the mobile phase A and pure analytical grade MeOH as mobile phase B.
(3) Use the following gradient program:
  • from 0 to 2 min, 90% of mobile phase A,
  • from 2 to 4 min, ramp linearly from 90% to 10% of mobile phase A,
  • from 4 to 8 min, 10% of mobile phase A,
  • return to initial conditions in 1 min,
  • hold for 3 min to re-equilibrate the system.
(4) Set the flow rate to 0.2 ml/min.
(5) Set the oven temperature to 30 °C.
(6) Use the injection volume of 25 μl.
(7) Operate the instrument using electrospray ionization (ESI) in positive mode.
(8) Collect the data using the Xcalibur software.
(9) Set the sample tube or desolvation temperature to 350 °C.
(10) Use the spray voltage of 4,100 V.
(11) See Table 9 for the specific MRM parameters (2 transitions).

A typical LC-MS/MS chromatogram of a fluoroquinolone standard, negative sample, and spiked sample is shown in Figure 36.

Table 8. LC-MS/MS parameters for the sulphonamide analytes.

Analyte	*m/z* Precursor ion	Tube lens offset	Quantification ion *m/z* (collision energy-V)	Confirmation ion *m/z* (collision energy-V)	Ion ratio (%)
SGN	215.0	68	156.0 (18)	108.0 (30)	32.7
SNL	173.1	39	93.2 (22)	76.2 (42)	24.2
SDZ	251.0	78	156.0 (18)	92.0 (26)	54.5
STZ	256.0	76	156.0 (19)	92.0 (34)	39.4
SMR	265.1	89	172.1 (16)	108.0 (25)	67.1
SMZ	271.0	141	108.0 (24)	92.0 (28)	133.2
SDM	279.1	78	186.0 (20)	156.0 (19)	34.0
SMNM	281.0	77	156.0 (20)	108.0 (35)	41.3
SMP	281.1	85	156.0 (18)	108.0 (28)	42.2
SDX	311.0	79	156.0 (18)	108.0 (30)	31.2
SMX	254.1	82	156.0 (16)	108.0 (26)	49.4
SQX	301.1	73	156.0 (17)	108.0 (36)	26.3
SDT	311.1	83	156.0 (23)	108.0 (35)	25.1

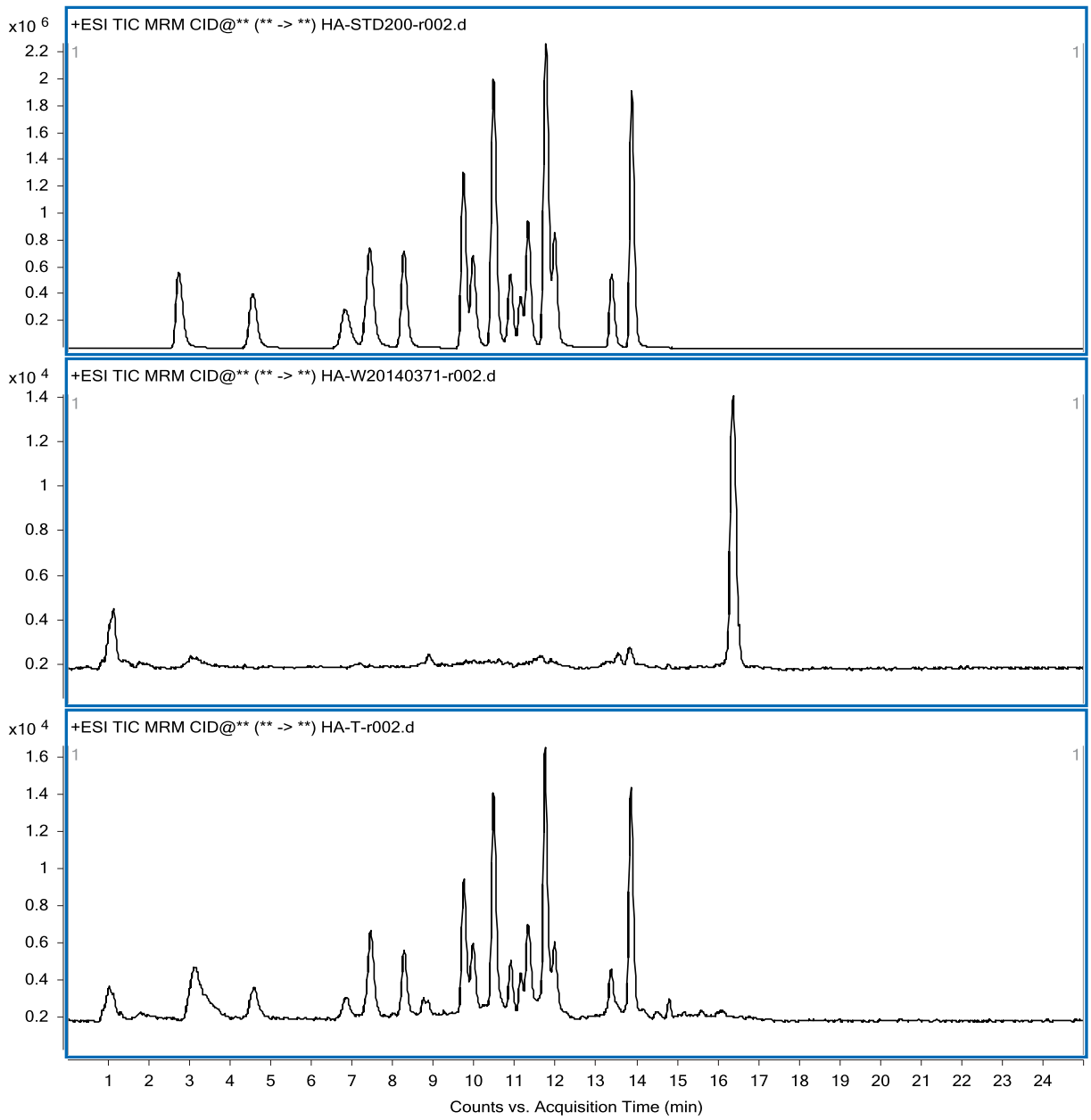

Figure 35. LC/MS/MS chromatography of sulfonamides standard (top), negative sample (middle) and spiked sample (bottom).

7.6. The LC-MS/MS method for tetracycline analysis in royal jelly

Tetracyclines (TCs) are broad-spectrum antibiotics and show a strong activity against a variety of gram-positive and gram-negative microorganisms. Due to their broad spectrum of activity and cost-effectiveness, TCs are widely used in animal husbandry as veterinary drugs. The mechanism of action of TCs is inhibition of protein synthesis by binding to the small ribosomal sub-unit at the A site, which binds to the RNA. In beekeeping, TC antibiotics are used to treat bacterial brood diseases such as American Foulbrood and European Foulbrood. Because these drugs have been widely used for prevention and treatment of diseases, and often, the label directions for their use have not been followed, the resulting residues often remain in food. These antibiotic residues can lead to increased drug resistance of microbial strains in consumers and can cause allergic or toxic reactions in some hypersensitive individuals.

LC-MS/MS is the most recently adopted technique for quantification of TC residues in honey. It is a quantitative and a multi-residue method that is also characterized by increased sensitivity and accuracy. This is why LC-MS/MS is used in both screening and confirmatory tests. Almost all the TCs, including tetracycline (TET), oxytetracycline (OTC), chlortetracycline (CTC), and doxycycline (DOX), can be quantified by this method (Tarapoulouzi, Papachrysostomou, Constantinou, Kanari,

Table 9. LC-MS/MS parameters for the fluoroquinolones.

Analyte	*m/z* Precursor ion	Quantification ion *m/z* (collision energy-V)	Confirmation ion *m/z* (collision energy-V)
ENR	360	316 (17) 245 (25)	316 (17)
CIP	332	231 (16) 288 (34)	231 (16)
NOR	320	276 (22) 302 (16)	276 (22)
OFL	362	261 (35) 318 (26)	261 (35)
DIF	400	299 (31) 382 (26)	299 (31)
SAR	386	299 (31) 368 (23)	299 (31)
SPA	393	292 (24) 375 (22)	292 (24)
DAN	358	340 (23) 283 (27)	340 (23)
FLU	262	202 (29) 244 (18)	202 (29)
FLE	370	326 (21) 332 (16)	326 (21)
MAR	363	72 (21) 345 (14)	72 (21)
ENO	321	232 (35) 303 (18)	232 (35)
ORB	396	352 (19) 295 (25)	352 (19)
PIP	304	217 (30) 189 (22)	217 (30)
PEF	334	233 (25) 290 (20)	233 (25)
LOM	352	308 (30) 334 (215)	308 (30)
NAL	233	187 (28) 215 (24)	187 (28)

& Hadjigeorgiou, 2013). Xu et al. (2008) analyzed tetracycline residues in royal jelly by LC-MS/MS. The overall recovery of spiked royal jelly at the levels of 5.0, 10.0, and 40.0 μg/kg ranged from 62% to 115%, and the coefficients of variation ranged from 3.4 to 16.3% ($n = 6$). The detection limits for TCs are under 1.0 μg/kg (Xu et al., 2008). The LC-MS/MS method is a simple, quick, and reliable assay with high sensitivity and selectivity. Furthermore, it can detect TCs in foods with good reproducibility. Thus, the LC-MS/MS method was selected for presentation here.

7.6.1. Sample preparation for royal jelly

(1) Weigh 2.0 g of royal jelly into a polypropylene centrifuge tube (50 ml).
(2) Add 20 ml of 1% trichloroacetic acid extraction solution to royal jelly.
(3) Vortex for 2 min.
(4) Centrifuge the mixture at 3684× *g* for 5 min.
(5) Transfer the supernatant to polypropylene centrifuge tube (50 ml).
(6) Add 1.5 ml of a 1.0 mol/l Na_2HPO_4 solution to adjust pH to 6.0–7.0.
(7) Centrifuge the mixture at 3,684*g* for 5 min.
(8) Pass the supernatant through a filter paper.
(9) Precondition the Oasis HLB cartridges with 5 ml of methanol followed by 5 ml of Milli-Q water.
(10) Pass the supernatant through the cartridge in vacuum, at a flow rate of 1–2 ml/min.
(11) Wash the cartridge with 5 ml of Milli-Q water.
(12) Wash the cartridge with 5 ml of methanol:water mixture (2:8, v/v).
(13) Elute the TCs with 5 ml of methanol.
(14) Dry the extract under a stream of nitrogen at 45 °C.
(15) Reconstitute it in 1 ml of methanol:water solution (3:7, v/v).
(16) Pass the solution through a 0.45-μm nylon filter directly into HPLC vials for LC-MS/MS analysis.

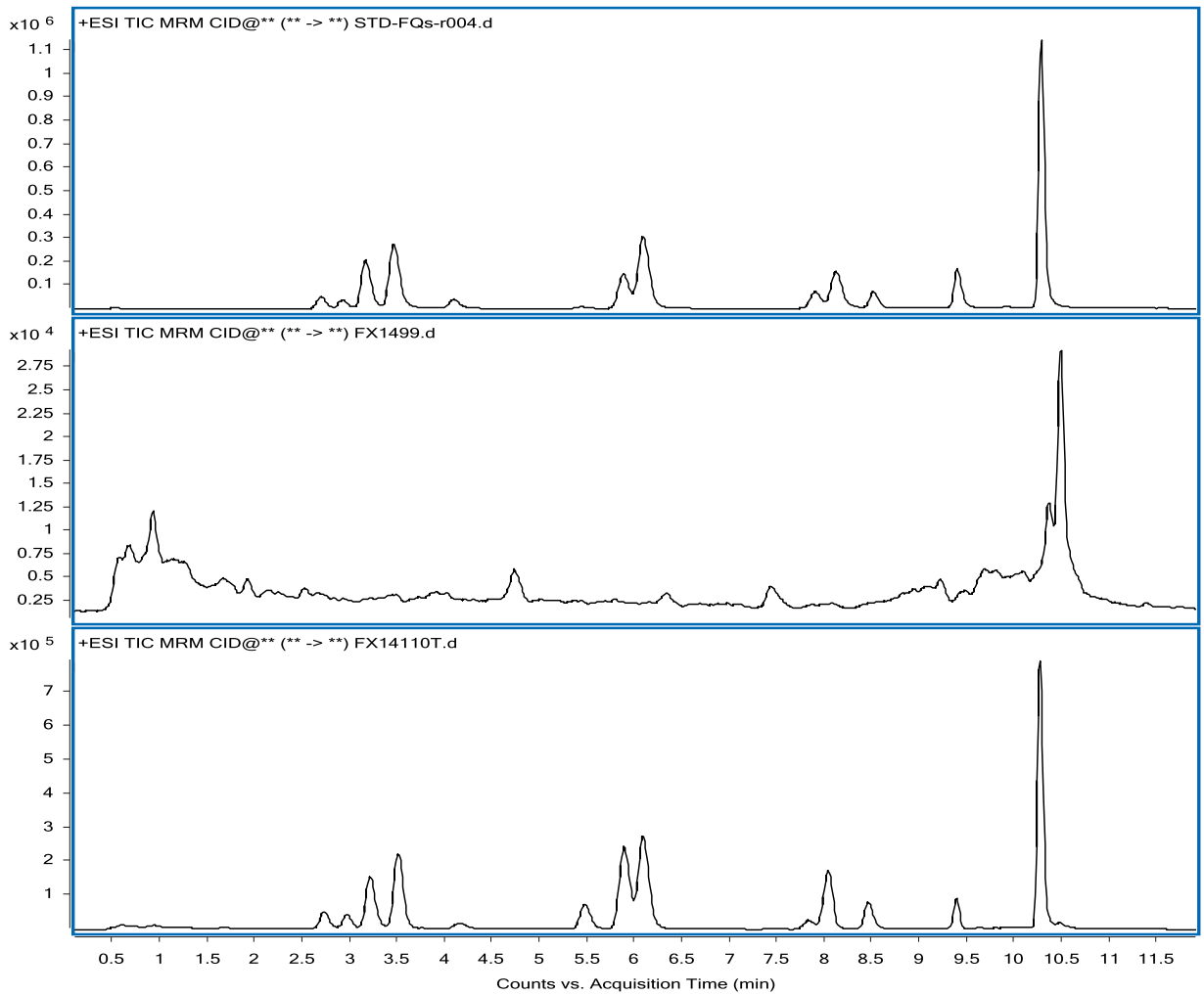

Figure 36. LC/MS/MS chromatography of fluoroquinolone standard (top), negative sample (middle) and spiked sample (bottom).

7.6.2. LC-MS/MS analytical conditions

(1) Connect the chromatograph with a Sunfire C18 column (150 × 2.1 mm i.d., 5.0-μm particle size) (Waters, Milford, MA, USA).
(2) Use distilled water with 0.1% formic acid as mobile phase A, and methanol as mobile phase B.
(3) Set the mobile phase gradient as follows: 0.0–5.0 min, 20–95% of mobile phase B; 5.10–7.00 min 95% of B; and 7.1–9.0 min 20% of B.
(4) Set the flow rate to 0.25 ml/min.
(5) Keep the column at room temperature.
(6) Set the injection volume to 25 μl.
(7) Detect the TC-positive electrospray ionization in multiple reaction-monitoring mode with a capillary voltage at 4.5 kV and a source temperature at 350 °C.
(8) See Table 10 for the specific MRM parameters (2 transitions).

A typical LC-MS/MS chromatogram of a TC standard, negative sample, and spiked sample is shown in Figure 37.

7.7. The LC-MS/MS method for macrolide analysis in royal jelly

The macrolides are lipophilic molecules having a central lactone ring bearing 12–16 atoms, to which several amino and/or neutral sugars are bound. They are broad-spectrum antibiotics active against gram-positive bacteria and mycoplasmas, as well as some gram-negative bacteria and members of the chlamydia group. Macrolides are widely used in veterinary medicine to treat respiratory diseases and enteric infections.

It is believed that macrolide residues in food may pose a risk to consumers because of allergic reactions of individuals to the antibiotics and/or their metabolites. Incorrect use of these drugs or insufficient withdrawal time after treatment can possibly lead to the presence

Table 10. ESI MS/MS conditions of tetracycline drugs.

Analyte	*m/z* Precursor ion	Quantification ion *m/z* (collision energy-V)	Confirmation ion *m/z* (collision energy-V)
TET	445	410 (18) 427 (16)	427 (16)
OTC	461	426 (18) 443 (14) 337 (25)	426 (18)
CTC	479	444 (20) 462 (18)	462 (20)
DOX	445	428 (15) 410 (27)	428 (15)

of macrolide residues in bee products, and increases the risk to consumers in terms of allergic reactions among people sensitive to the antibiotics (Draisci, Palleschi, Ferretti, Achene, & Cecilia, 2001). Macrolides, for example, tylosin, can potentially be used for prevention and treatment of American Foulbrood disease. Nevertheless, the presence of macrolides is not allowed in honey in some countries.

Only limited data are available in the literature on the macrolide quantification in honey and royal jelly. Generally, only 2 kinds of methods are available: PremiTest method and LC-MS/MS method. Premi Test method is very simple to operate and inexpensive for analytical laboratories. However, it is a screening method without exact qualitative analysis of analytes. The LC-MS/MS method has been extensively used to quantify macrolides in honey and royal jelly, partly due to its simplicity and rapidity as well as its high sensitivity and selectivity.

7.7.1. *Sample preparation for royal jelly*

(1) Weigh 2.0 g of fresh royal jelly (or 0.5 g of dried royal jelly powder) in a polypropylene centrifuge tube (50 ml) with a screw cap.
(2) Add 10 ml of a Tris solution (1 M, Ph = 6.8).
(3) Stir for 10 min.
(4) Centrifuge the mixture for 10 min at 18,000× *g*.
(5) Use Millex filter (0.2 μm) to filter the supernatant in another centrifuge tube (50 ml).
(6) Precondition the HLB 500 mg 60 ml SPE cartridge with 10 ml of methanol followed by 10 ml of water.
(7) Add the filtered supernatant to cartridge.
(8) Elute by gravity or vacuum (1 ml/min).
(9) Wash the cartridge with 5 ml of water.
(10) Wash with 10 ml of methanol:water (4:6, v/v).
(11) Elute the macrolides with 10 ml of methanol.
(12) Decrease the volume to 1 ml under a stream of nitrogen at 50 °C.
(13) Reconstitute the residue in 1.0 ml of ammonium acetate:water (17:3, v/v).
(14) Pass it through the Mini-UmiPrep syringeless filter vials (PVDF 0.2 μm; Whatman Inc., Clifton, NI, USA) for LC-MS/MS analysis.

7.7.2. *LC-MS/MS analytical conditions*

(1) LC-MS/MS system: Alliance 2695 HPLC coupled with a MicromassOuattro Ultima Pt tandem mass spectrometer with an ESI interface and MassLynx 4.0 software (Waters Corp).
(2) Connect the chromatograph with an Atlantis C18 column (3 μm, 150 × 2.1 mm) and set the column oven temperature to 30 °C.
(3) Use distilled water with 0.1% formic acid as mobile phase A, and methanol as mobile phase B.
(4) Set the mobile phase gradient as follows: 0.0–7.0 min, 20–95% of mobile phase B; 7.10–9.00 min 95% of B; and 9.1–15.0 min 20% of B.
(5) Program flow at 0.2 ml/min.
(6) Fix the injection volume to 20 μl.
(7) Program MS conditions as follows:
- ionization mode, electrospray positive ion mode;
- capillary voltage, 5.5 kV;
- source temperature, 550 °C;
- nebuliser nitrogen flow rate, 0.4 l/min;
- collision gas argon pressure, 0.24 MPa;

(8) See Table 11 for the specific MRM parameters (2 transitions).

A typical LC-MS/MS chromatogram of a macrolide standard, negative sample, and spiked sample is shown in Figure 38.

7.8. *General LC-MS/MS methods for analysis of nitrofurans in royal jelly*

Furaltadone (FTD), furazolidone (FZD), nitrofurazone (NZF), and nitrofurantoin (NFT) are parent drugs of nitrofurans that contain the characteristic 5-nitrofuran ring group. For many years, this class of broad-spectrum antibacterial drugs has been widely used as additives in livestock and aquaculture feed for treatment of certain bacterial infections. In addition, nitrofurans have been used as a growth promoter in farm animal feeds and aquaculture. Nonetheless, FTD, FZD, NZF, and their metabolites have proven to have carcinogenic and muta-

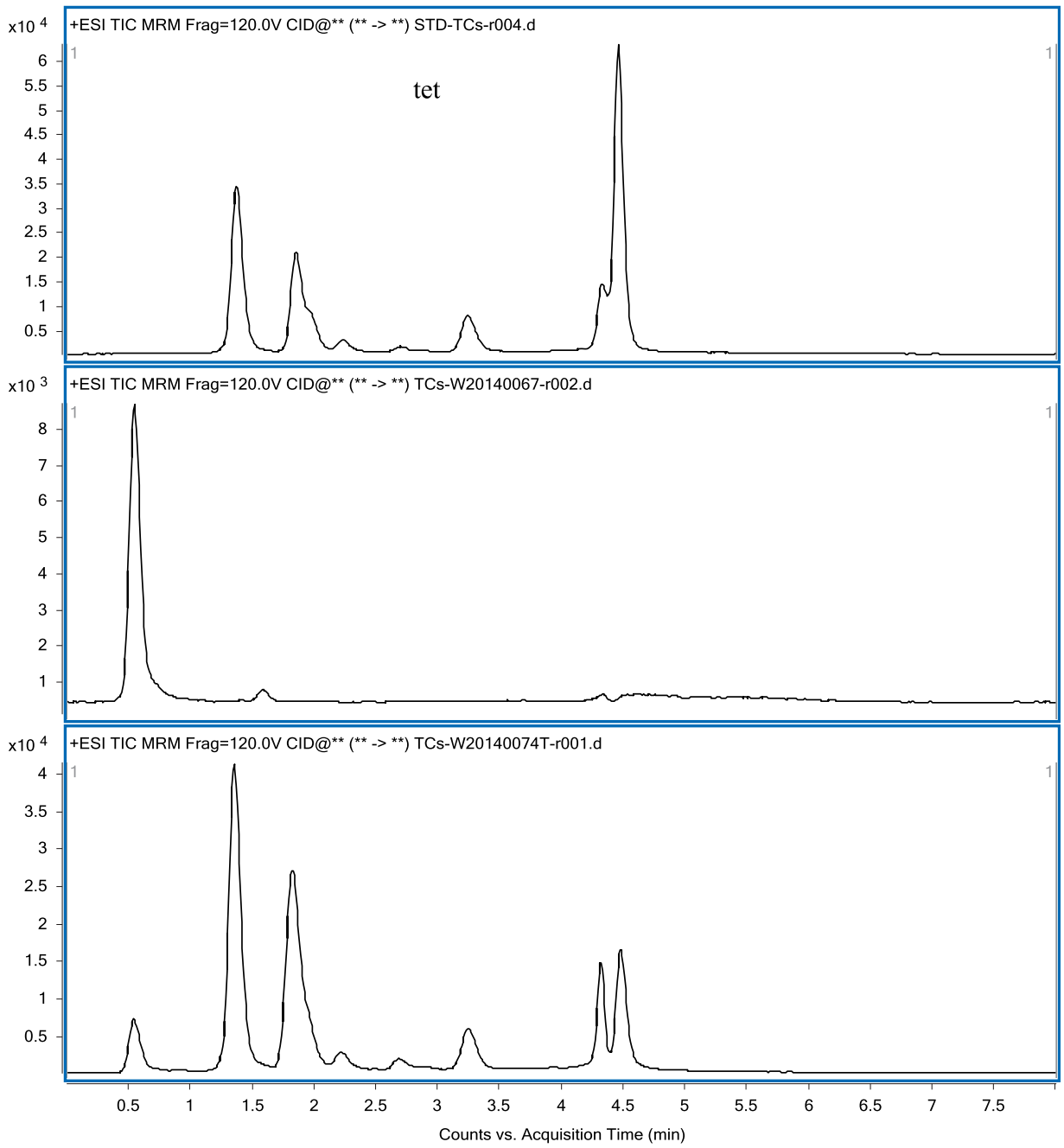

Figure 37. LC/MS/MS chromatography of tetracyclines standard (top), negative sample (middle) and spiked sample (bottom).

genic effects. As a consequence, nitrofurans have been banned from use in animal husbandry in the European Union since 1995. Because beekeepers use these nitrofurans to treat bees with bacterial infections, residues of these compounds have already been found in bee products including honey and royal jelly (Bargańska, Namieśnik, & Ślebioda, 2011; Cooper & Kennedy, 2005).

Only a few reports are available in the literature about nitrofuran detection in honey and royal jelly. Generally, two methods are available: the method of enzyme-linked immunosorbent assay (ELISA) with some modifications, and the method of LC-MS/MS. The immunoassay method has a broad detection range and high sensitivity and is a valid and cost-effective means for high-throughput monitoring of residual nitrofuran levels in many food matrices. Yet a drawback in analysis of all 4 metabolites of nitrofuran using the ELISA approach is that this approach would require 4 separate plates for testing due to the limited cross-reactivity of the antibodies. The biochip array technology is a modification of an immunochemical detection platform that offers the advantage of multiplexing several specific antibodies on a single biochip to increase the number of analytes covered. Nevertheless, the biochip method is

Table 11. ESI MS/MS conditions for macrolide drugs.

Analyte	*m/z* Precursor ion	Quantification ion *m/z* (collision energy-V)	Confirmation ion *m/z* (collision energy-V)	Cone voltage V
Lincomycin	407	126 (37) 359 (24)	126 (37)	50
Erythromycin	734	158 (42) 576 (28)	158 (42)	50
Tilmicosin	869	174 (62) 132 (70)	174 (62)	90
Tylosin	916	174 (54) 132 (70)	174 (54)	80
Clindamycin	425	126 (45) 377 (28)	126 (45)	53
Spiramycin	843	142 (48) 174 (50)	142 (48)	60
Kitasamycin	772	215 (43) 109 (42)	215 (43)	70
Josamycin	828	174 (45) 109 (45)	174 (45)	80

only a screening method. LC-MS/MS is a confirmatory and quantitative method for quantification of 4 metabolites of nitrofuran antibiotics in honey and royal jelly. Compared with other analytical methods, the sensitivity, selectivity, and specificity of the LC-MS/MS method are considerably better. In addition, LC-MS/MS provides precise results in accordance with EU requirements (2002/756/EC, 2002). Consequently, the LC-MS/MS method has been applied extensively to detect nitrofuran antibiotics in honey and royal jelly and is described below.

7.8.1. Sample preparation for royal jelly

This method is from Chinese Commodity Inspection Standards/The industry standards of entry-exit inspection and quarantine of the People's Republic of China: SN/T 2061–2008).

(1) Weigh 2.0 g of fresh royal jelly in a 50 ml centrifuge tube with a screw cap.
(2) Add 25 ml of HCl (2.0 M).
(3) Add 100 μl of 2-nitrobenzaldehyde (2-NBA).
(4) Incubate the mixture overnight at 37 °C in an oven.
(5) Add 1 ml of trichloroacetic acid.
(6) Mix and centrifuge at 1395× *g* for 5 min.
(7) Collect the supernatant.
(8) Add 1 M NaOH to adjust pH to 7.5 in the filtered supernatant.
(9) Precondition Oasis HLB solid-phase extraction (SPE) cartridge with 5 mL of methanol followed by 5 ml of deionized water.
(10) Add the adjusted supernatant mixture onto the cartridge.
(11) Add 10 ml of deionized water to clean up the cartridge.
(12) Elute the nitrofurans with 10 ml of ethyl acetate.
(13) Dry the eluate under a stream of nitrogen at 40 °C in vacuum.
(14) Reconstitute the extract with 1 ml of 0.2% acetic acid:acetonitrile (7:3, v/v).
(15) Pass the solution through a 0.45-μm filter membrane for LC-MS/MS analysis.

7.8.2. LC-MS/MS analytical conditions

(1) LC-MS/MS system: Agilent 6460 LC-MS/MS system (Santa Clara, CA, USA).
(2) Connect the chromatograph to an Agilent SB C8 column (5×150 mm, 5 μm).
(3) Set the column temperature to 30 °C.
(4) Use acetonitrile as mobile phase A; use deionized water with 5 mM ammonium acetate as mobile phase B.
(5) Program a linear gradient profile of mobile phase as follows:
  • from 0 to 7 min, ramp linearly from 30% to 90% of mobile phase A,
  • from 7 to 12 min, 90% of mobile phase A,
  • return to initial conditions in 0.1 min.
(6) Hold for 4.9 min to re-equilibrate the system (the total run time is 17 min).
(7) Set the sample injection volume to 30 μl.
(8) Use the flow rate of 0.3 ml/min.
(9) Operate the instrument using electrospray ionization (ESI) in positive mode.
(10) Use nitrogen for nebulization (42 Psi) and as a cone gas (25 Psi).
(11) Set the source temperature to 540 °C.
(12) Set the spray voltage to 4.1 kV.
(13) Use high-purity nitrogen as the collision gas.
(14) See Table 12 for the specific MRM parameters (2 transitions).

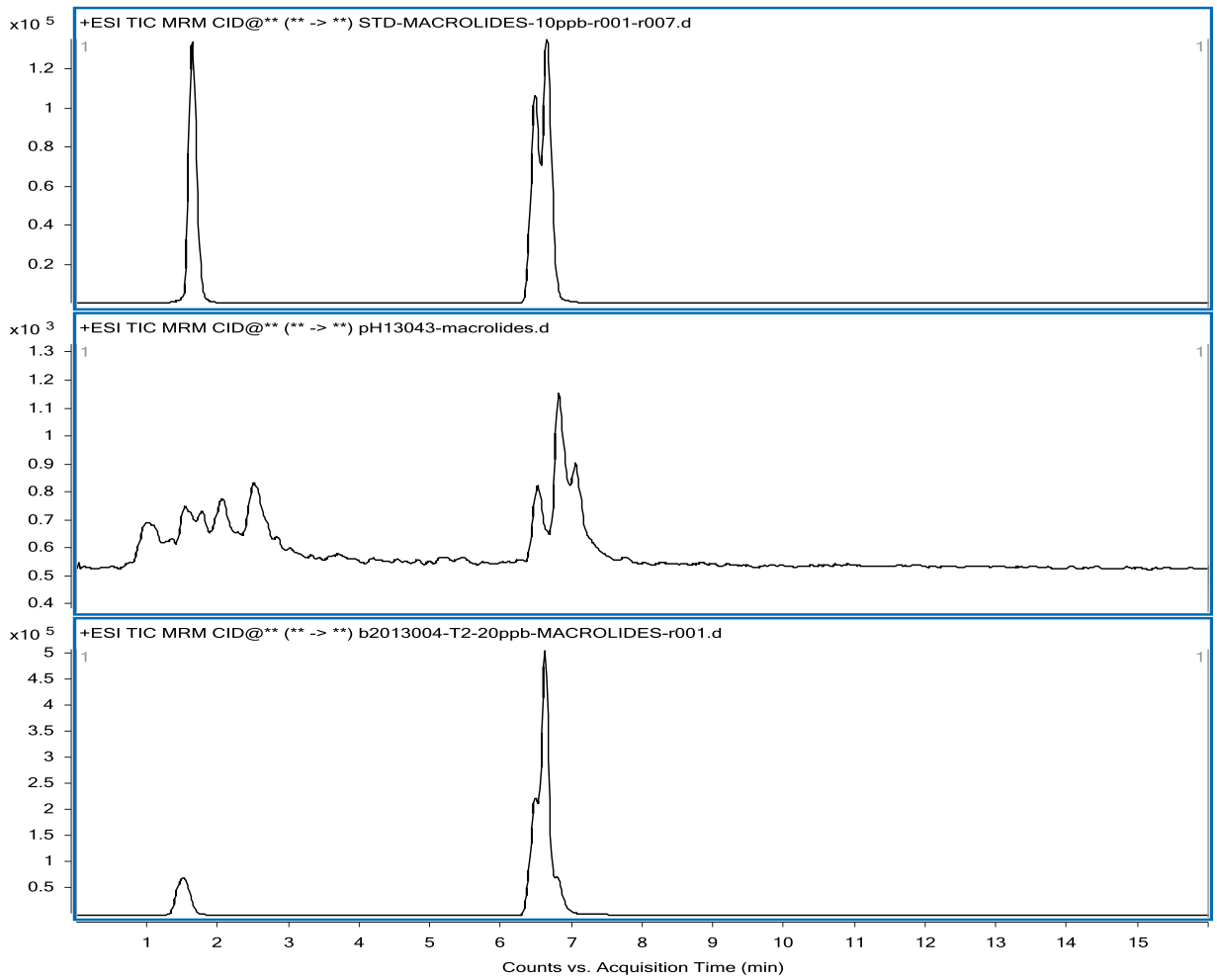

Figure 38. LC/MS/MS chromatography of macrolides standard (top), negative sample (middle) and spiked sample (bottom).

A typical LC-MS/MS chromatogram of a nitrofuran standard, negative sample, and spiked sample is shown in Figure 39.

7.9. GC-ECD method for fluvalinate analysis in royal jelly

Fluvalinate has been extensively used worldwide by beekeepers since 1988 to prevent varroatosis. Lately, it became one of the most widely used acaricides. Fluvalinate residues can cause genetic mutations and cellular degradation in addition to several public health problems. These problems may occur through direct contamination as a result of beekeeping practices as well as via indirect contamination from environmental sources. The indirect contamination from the environment takes place because of the widespread use and extensive distribution of pesticides, which helped to introduce their residues into royal jelly by bees that have been consuming contaminated blossoms. This assay protocol is applicable to monitoring of fluvalinate residue in royal jelly according to national regulatory authorities and accredited labs.

7.9.1. Sample preparation for royal jelly

(1) Weigh 0.5 g of fresh royal jelly (0.15 g of royal jelly powder) in a tube.
(2) Dissolve royal jelly in 10 ml of acetonitrile:water (1:1, v/v).
(3) Homogenize the mixture in an ultrasonic bath for 15 min at 40 °C.
(4) Centrifuge the mixture for 10 min at 893× *g*.
(5) Precondition a C_{18} cartridge with 5 mL of methanol followed by 5 ml of water.
(6) Pass the supernatant at a constant flow rate (1 ml/min) through the C_{18} cartridge.
(7) Dry the cartridge in vacuum for 15 min.
(8) Elute the analytes with 2 mL of ethyl acetate and 2 ml of n-hexane.
(9) Evaporate the eluate in vacuum at RT.
(10) Dissolve the residue in 1 ml of isooctane.
(11) Pass it through a 0.45-μm nylon filter directly into vials for GC-ECD analysis.

Table 12. LC-MS/MS parameters for the nitrofurans.

Analyte	*m/z* Precursor ion	Ion *m/z* (collision energy-V)	Cone energy (V)
SEM-NBA	209.2	166.2 (15) 192.2 (17)	60
AHD-NBA	249.2	134.1 (18) 104.1 (32)	66
AMOZ-NBA	335.2	262.3 (25) 291.3 (17.7)	66
AOZ-NBA	236.2	134.2 (18.3) 104.1 (32)	66

7.9.2. Primary parameters of GC-ECD instruments

(1) Connect the chromatograph to an HP-1 (30 × 0.25 mm × 0.25 μm).
(2) Set the injector temperature to 270 °C.
(3) Set the detector temperature to 300 °C.
(4) Use the nitrogen carrier gas rate of 10 ml/min.
(5) Set the make-up to 20 ml/min.
(6) Set the initial column temperature to 250 °C (3 °C/min) to 270 °C and hold it for 30 min.
(7) Use the injection volume of 1 μl.

A typical GC-ECD chromatography of fluvalinate standard is shown in Figure 40.

7.10. The GC-MS method for amitraz and 2,4-DMA analysis in royal jelly

Amitraz [N-methylbis(2,4-xylyliminomethyl)amine] is widely applied to beehives for control of *V. destructor*. Amitraz is a labile pesticide whose degradation products include 2,4-dimethylaniline (2,4-DMA).

Sample treatment is the key step for acaricide analysis to reduce the matrix interference and increase the sensitivity. This treatment often includes 2 main steps: extraction and clean-up. Amitraz in honey bee products is commonly extracted with an organic solvent, after diluting the samples with a buffer at different pH values because amitraz is labile in acidic media. Then, SPE cartridges with different absorbents are used to clean-up the extract. Finally, the eluent is dried under a nitrogen stream and redissolved in a suitable organic solvent for quantification. In the last decades, many new sample-processing methods were developed to shorten the running time and to automate the treatment. Solid-phase microextraction (SPME) (Blasco, Fernández, Picó, & Font, 2004; Rialotero, Gaspar, Moura, & Capelo, 2007), accelerated solvent extraction (ASE) (Korta, Bakkali, Berrueta, Gallo, & Vicente, 2002), and headspace solvent microextraction have also been used to detect amitraz and 2,4-dimethylaniline (2,4-DMA) in bee products (Shamsipur, Hassan, Salar-Amoli, & Yamini, 2008). These technologies are not easy to implement and require expensive facilities.

In contrast, GC with an electronic capture detector (ECD) is a low-cost, practical method for analysis of amitraz and its main metabolites. The required equipment is cheap and common in analytical laboratories (Amoli, Hasan, & Hejazy, 2009; Yu, Tao et al., 2010). It shows good performance, with an LOD of 5 μg/kg and LOQ of 10 μg/kg, but ECD is a specific detector that is used only for compounds with electronegative atoms. Thus, amitraz can be analyzed by GC-ECD, but the latter requires hydrolysis of amitraz to 2,4-DMA and derivatization with hepta-fluorobutyric acid anhydride. These steps make the method time consuming and problematic.

For some pesticides, analysis by GC is impossible due to their low thermal stability or insufficient volatility without further chemical derivatization. The most widely used HPLC detectors for acaricide analysis are diode array (DAD) and UV (Çobanoğlu & Tüze, 2008). HPLC-DAD is a simple, quick, and effective method for quantification of amitraz and its main metabolite in bee products. The sensitivity and stability of the method are suitable in most cases.

GC-MS is the most widely used method for analysis of amitraz and its metabolites, given the simple sample processing and the satisfying analytical performance (Mărghita, Bonta, Mihai, & Dezmirean, 2012; Notardonato, Avino, Cinelli, & Russo, 2014). Additionally, the HPLC tandem with MSD detection employing atmospheric pressure ionization (API), in positive and negative modes, is becoming the detection system of choice for more researchers (Gómez-Pérez, Plaza-Bolaños, Romero-González, Martínez-Vidal, & Garrido-Frenich, 2012). Nonetheless, the high price of MSD along with the expensive maintenance considerably impedes dissemination.

The GC-MS analysis for amitraz and 2,4-DMA in royal jelly is introduced in this section.

7.10.1. Sample preparation for royal jelly

(1) Weigh 0.5 g of fresh royal jelly (0.15 g of royal jelly powder) accurately in a tube.
(2) Dissolve royal jelly in 10 ml of acetonitrile:water (1:1, v/v).
(3) Homogenize it in an ultrasonic bath for 15 min at 40 °C.

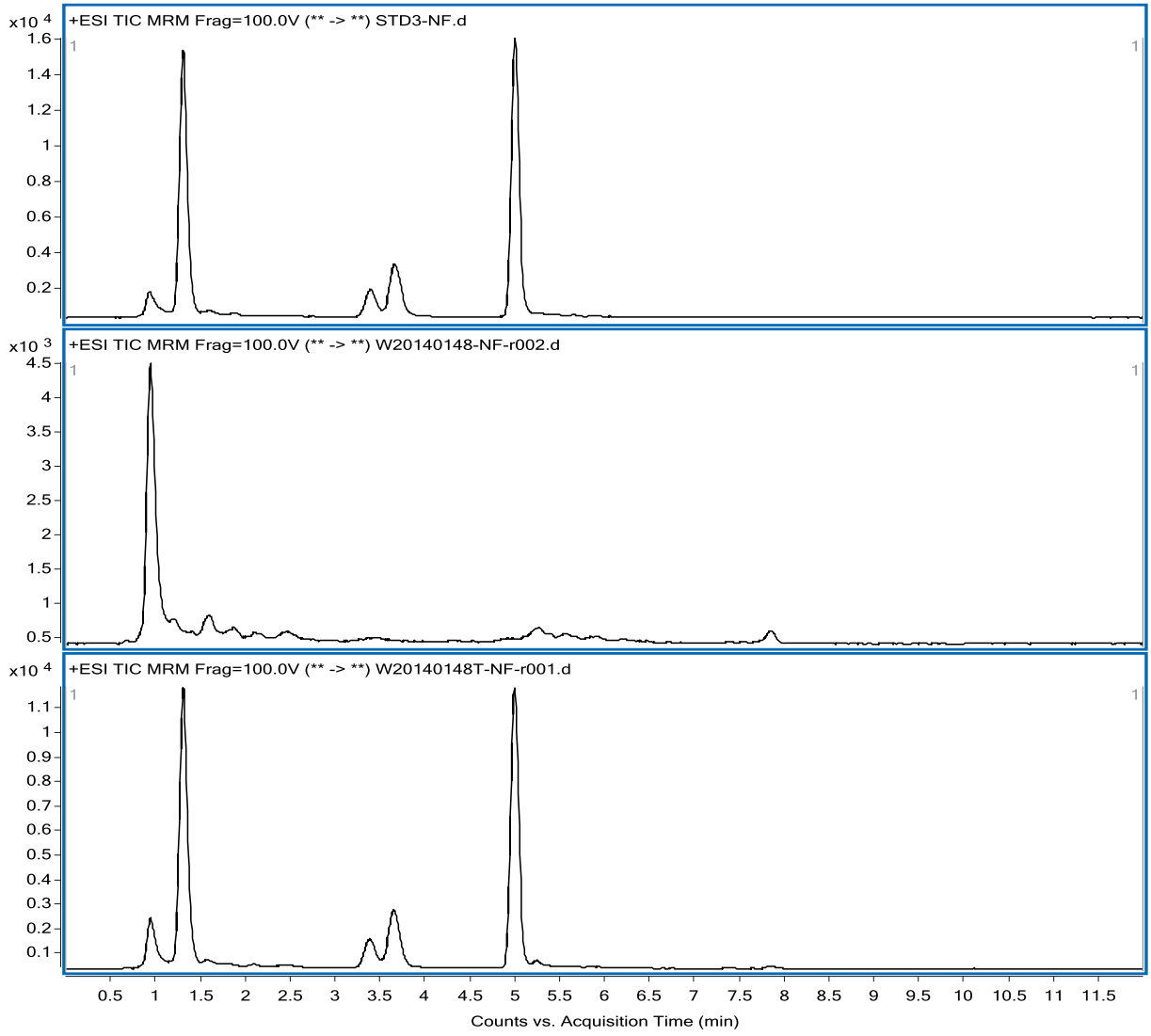

Figure 39. LC/MS/MS chromatography of nitrofuran standard (top), negative sample (middle) and spiked sample (bottom).

(4) Centrifuge the extract for 10 min at 893× *g*.
(5) Precondition a C18 cartridge with 5 ml of methanol followed by 5 ml of water.
(6) Pass the supernatant at a constant flow rate (1 ml/min) through the C18 cartridge.
(7) Dry the cartridge in vacuum for 15 min.
(8) Elute analytes with 2 ml of ethyl acetate.
(9) Elute again with 2 ml of n-hexane.
(10) Evaporate the eluate in vacuum at RT.
(11) Dissolve the residue in 1 ml of isooctane.
(12) Pass it through a 0.45-μm nylon filter directly into vials for GC-MS analysis.

7.10.2. The primary GC-MS instrument parameters

(1) Connect the chromatograph to CP-Sil 8, or SE-54, or HP-5MS, or HP-1MS (30 m × 0.25 mm × 0.25 μm).
(2) Set the injector temperature to 250 °C.
(3) Use helium as the carrier gas.
(4) Set the ion source EI to 230 °C.
(5) Set the transfer line to 270 °C.
(6) Manage the oven temperature as follows
  • start at 120 °C,
  • increase to 270 °C (20 °C/min),
  • hold at 270 °C for 7 min.
(7) Use the injection volume of 1 μl.
(8) Set the scan mode to Selected Ion Monitoring (SIM), *m/z* 293, 147, 121, 120, and 106.

7.11. Conclusion and outlook

Methods such as HPLC, GC, GC/MS, and LC-MS/MS can be used for the detection of various drugs in bee products. LC-MS/MS and GC-MS presented here are so far the most sensitive, effective, and reliable assays (with good reproducibility), and have been extensively used for quantification of antibiotics and acaricides in honey (Paradis, Bérail, Bonmatin, & Belzunces, 2014; see the

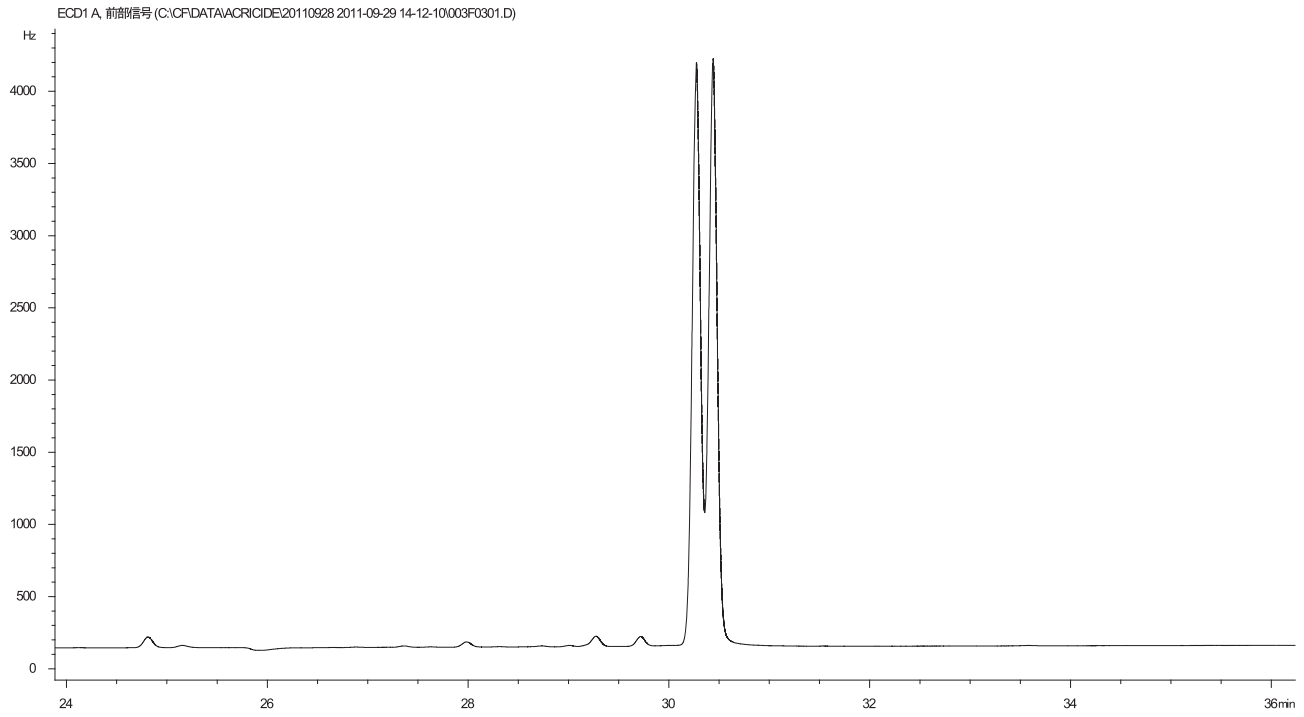

Figure 40. A typical GC-ECD chromatography of fluvalinate standard.

BEEBOOK paper on honey (in prep) and royal jelly. Although many new analytical techniques have been used for quality control of bee products, there is still some need for development of more advanced methods (urgent tasks are listed):

(1) To improve inadequate LC separations, as encountered for analyte classes of widely varying polarity.
(2) To develop analytical approaches for the "multi-compound class", including antibiotics and acaricides, in bee products.
(3) To find a way to adequately deal with ion-suppression effects on quantification.
(4) To enhance analyte detectability to reach LODs approximately 0.1–0.5 μg/kg for prohibited substances and drugs.
(5) To improve strategies for confirmation of analyte identity by careful perusal of IP-derived guidelines and protocols, and via evaluation of the potential of Q-TOF-MS-based detection.

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References

Albalasmeh, A.A., Berhe, A.A., & Ghezzehei, T.A. (2013). A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry. *Carbohydrate Polymers, 97*, 253–261.

Allen, E.H. (1985). Review of chromatographic methods for chloramphenicol residues in milk, eggs, and tissues from food-producing animals. *Journal - Association of Official Analytical Chemists, 68*, 990–999.

Amoli, J.S., Hasan, J., & Hejazy, M. (2009). Determination of amitraz residue by headspace gas chromatography in honey and beeswax samples from Iran. *American Journal of Food Technology, 4*, 56–59.

Anonymous. (1989). Turkish Standards for Royal Jelly (TS 6666).

Antinelli, J.F., Davico, R., Rognone, C., Faucon, J.P., & Lizzani-Cuvelier, L. (2002). Application of solid/liquid extraction for the gravimetric determination of lipids in royal jelly. *Journal of Agricultural and Food Chemistry, 50*, 2227–2230. doi:10.1021/jf0112466

Antinelli, J.F., Zeggane, S., Davico, R., Rognone, C., Faucon, J.P., & Lizzani, L. (2003). Evaluation of (E)-10-hydroxydec-2-enoic acid as a freshness parameter for royal jelly. *Food Chemistry, 80*, 85–89. doi:10.1016/S0308-8146(02)00243-1

Bargańska, Ż., Namieśnik, J., & Ślebioda, M. (2011). Determination of antibiotic residues in honey. *TrAC Trends in Analytical Chemistry, 30*, 1035–1041.

Bayer, E., & Witsch, H.G. (1961). *Gas chromatography*. New York, NY: Elsevier.

Bertelli, D., Lolli, M., Papotti, G., Bortolotti, L., Serra, G., & Plessi, M. (2010). Detection of honey adulteration by sugar syrups using one-dimensional and two-dimentional high-resolution nuclear magnetic resonance. *Journal of Agricultural and Food Chemistry, 58*, 8495–8501. doi:10.1021/jf101460t

Bíliková, K., & Šimúth, J. (2010). New criterion for evaluation of honey: Quantification of royal jelly protein apalbumin 1 in honey by ELISA. *Journal of Agricultural and Food Chemistry, 58*, 8776–8781. doi:10.1021/jf101583s

Bíliková, K., Klaudiny, J., & Šimúth, J. (1999). Characterization of the basic major royal jelly protein MRJP2 of honey bee (*Apis mellifera* L.) and its preparation by heterologous expression in *E.coli*. *Biologia, 54*, 733–739.

Bíliková, K., Wu, G., & Šimúth, J. (2001). Isolation of a peptide fraction from honey bee royal jelly as a potential antifoulbrood factor. *Apidologie, 32*, 275–283.

Bíliková, K., Hanes, J., Nordhoff, E., Saenger, W., Klaudiny, J., & Šimúth, J. (2002). Apisimin, a new serine-valine-rich peptide of honey bee (*Apis mellifera* L.) royal jelly: Purification and molecular characterization. *Febs Letters, 528*, 25–129.doi:10.16/S0014-5793(02)03272-6

Bíliková, K., Mirgorodskaya, E., Bukovská, G., Gobom, J., Lehrach, H., & Šimúth, J. (2009). Towards functional proteomics of minority component of honey bee royal jelly: The effect of post-translational modifications on the antimicrobial activity of apalbumin2. *Proteomics, 9*, 2131–2138. doi:10.1002/pmic.200800705

Binder, H. (1980). Separation of monosaccharides by high-performance liquid chromatography: Comparison of ultraviolet and refractive index detection. *Journal of Chromatography A, 189*, 414. doi:10.1016/S0021-9673(00)80322-2

Bishop, C.T. (1962). Separation of carbohydrate derivates by gas-liquid partition chromatography. *Methods in Biochemical Analysis, 10*, 1–42. doi:10.1002/9780470110270.ch1

Blank, S., Bantleon, F.I., McIntyre, M., Ollert, M., & Spillner, E. (2012). The major royal jelly proteins 8 and 9 (Api m 11) are glycosylated components of *Apis mellifera* venom with allergenic potential beyond carbohydrate-based reactivity. *Clinical and Experimental Allergy, 42*, 976–985. doi:10.1111/j.1365-2222.2012.03966.x

Blasco, C., Fernández, M., Picó, Y., & Font, G. (2004). Comparison of solid-phase microextraction and stir bar sorptive extraction for determining six organophosphorus insecticides in honey by liquid chromatography-mass spectrometry. *Journal of Chromatography A, 1030*, 77–85. doi:10.1016/j.chroma.2003.11.037

Bloodworth, B.C., Harn, C.S., & Hock, C.T. (1995). Liquid chromatographic determination of trans-10-hydroxy-2-decenoic acid content of commercial products containing royal jelly. *Journal of AOAC International, 78*, 1019–1023.

Blum, M.S., Novak, A.F., & Taber, S. (1959). 10-Hydroxy-delta 2-decenoic acid, an antibiotic found in royal jelly. *Science, 130*, 452–453. doi:10.1126/science.130.3373.452

Bogdanov, S. (2006). Contaminants of bee products. *Apidologie, 37*, 1–18.

Bogdanov, S., Bieri, K., Gremaud, G., Iff, D., Kanzig, A., Seiler, K., … Zurcher, K. (2004). Swiss Food Manual: Gelée royale, Chap. 23C, Bienenprodukte, BAG (Swiss Federal Office for Public Health), Berne.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254. doi:10.1016/0003-2697(76)90527-3

Brown, W.H., & Freure, R.J. (1959). Some carboxylic acids present in royal jelly. *Canadian Journal of Chemistry, 37*, 2042–2046. doi:10.1139/v59-296

Büchler, R., Andonov, S., Bienefeld, K., Costa, C., Hatjina, F., Kezic, N., … Wilde, J. (2013). Standard methods for rearing and selection of *Apis mellifera* queens. In V. Dietemann, J.D. Ellis, & P. Neumann (Eds.), *The COLOSS* BEEBOOK, *Volume I: Standard methods for* Apis mellifera *research. Journal of Apicultural Research, 51*(5). doi:10.3896/IBRA.1.52.1.07

Buttstedt, A., Moritz, R.F.A., & Erler, S. (2014). Origin and function of the major royal jelly proteins of the honey bee (*Apis mellifera*) as members of the yellow gene family. *Biological Reviews, 89*, 255–269. doi:10.1111/brv.12052

Cao, L.F., Zheng, H.Q., Pirk, C.W.W., Hu, F.L., & Xu, Z.W. (2016). High royal jelly-producing honey bees (*Apis mellifera ligustica*) (Hymenoptera: Apidae) in China. *Journal of Economic Entomology, 109*, 510–514.

Cerkowniak, M., Puckowski, A., Stepnowski, P., & Gołębiowski, M. (2013). The use of chromatographic techniques for the separation and the identification of insect lipids. *Journal of Chromatography B, 937*, 67–78. doi:10.1016/j.jchromb.2013.08.023

Chen, Y.C. (1993). *Apiculture in China* (61–63 pp.). Beijing: Agriculture Press.

Chen, C., & Chen, S. (1995). Changes in protein components and storage stability of Royal Jelly under various conditions. *Food Chemistry, 54*, 195–200.

Chiron, R. (1982). New synthesis of royal jelly acid. *Journal of Chemical Ecology, 8*, 709–713. doi:10.1007/BF00988312

Ciulu, M., Floris, I., Nurchi, V.M., Panzanelli, A., Pilo, M.I., Spano, N., & Sanna, G. (2015). A possible freshness marker for royal jelly: Formation of 5-hydroxymethyl-2-furaldehyde as a function of storage temperature and time. *Journal of Agricultural and Food Chemistry, 63*, 4190–4195.

Çobanoğlu, S., & Tüze, Ş. (2008). Determination of amitraz (Varroaset) residue in honey by high performance liquid chromatography (HPLC). *Tarim Bilimleri Dergisi, 14*, 169–174.

Consonni, R., & Cagliani, L.R. (2008). Geographical characterization of polyfloral and acacia honeys by nuclear magnetic resonance and chemometrics. *Journal of Agricultural and Food Chemistry, 56*, 6873–6880. doi:10.1021/jf801332r

Cooper, K.M., & Kennedy, D.G. (2005). Nitrofuran antibiotic metabolites detected at parts per million concentrations in retina of pigs—A new matrix for enhanced monitoring of nitrofuran abuse. *The Analyst, 130*, 466–468.

Cotte, J.F. (2003). *Development of method and databank applied to naturalness control of monofloral honeys* (Thesis N 33-2003). Claude Bernard University – LYON-1 France.

Daniele, G., & Casabianca, H. (2012). Sugar composition of French royal jelly for comparison with commercial and artificial sugar samples. *Food Chemistry, 134*, 1025–1029. doi:10.1016/j.foodchem.2012.03.008

Di Girolamo, F., D'Amato, A., & Righetti, P.G. (2012). Assessment of the floral origin of honey via proteomic tools. *Journal of Proteomics, 75*, 3688–3693. doi:10.1016/j.jprot.2012.04.029

Ding, T., Xu, J., Shen, C., Jiang, Y., Chen, H., Wu, B., & Liu, F. (2006). Determination of three nitroimidazole residues in royal jelly by high performance liquid chromatography-tandem mass spectrometry. *Chinese Journal of Chromatography, 24*, 331–334.

Dionex. (1993). *Technical note*. Sunnyvale, CA.

Draisci, R., Palleschi, L., Ferretti, E., Achene, L., & Cecilia, A. (2001). Confirmatory method for macrolide residues in bovine tissues by micro-liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A, 926*, 97–104.

Dreywood, R. (1946). Qualitative test for carbohydrate material. *Industrial & Engineering Chemistry Analytical Edition, 18*, 499–499. doi:10.1021/i560156a015

Drijfhout, F.P., Kochansky, J., Lin, S., & Calderone, N.W. (2005). Components of honey bee royal jelly as deterrents of the parasitic varroa mite, *Varroa destructor*. *Journal of Chemical Ecology, 31*, 1747–1764. doi:10.1007/s10886-005-5925-6

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356. doi:10.1021/ac60111a017

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.

Dutton, G.G.S. (1973). Application of gas-liquid chromatography to carbohydrates. *Advances in Carbohydrate Chemistry and Biochemistry, 28*, 11–160. doi:10.1016/S0065-2318(08)60382-0

Englis, D., & Becker, H. (1943). Sugar analysis by alkaline ferricyanide method—Determination of ferrocyanide by iodometric and other procedures. *Industrial & Engineering Chemistry Analytical Edition, 15*, 262–264. doi:10.1021/i560116a011

Enne, V.I., Livermore, D.M., Stephens, P., & Hall, L.M. (2001). Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. *The Lancet, 357*, 1325–1328. doi:10.1016/s0140-6736(00)04519-0

Fahy, E., Subramanlam, S., Brown, H.A., Glass, C.K., Merrill, A.H., Murphy, R.C., … Dennis, E.A. (2005). A comprehensive classification system for lipids. *The Journal of Lipid Research, 46*, 839–862. doi:10.1194/jlr.E400004-JLR200

Fevold, H.L., Hisaw, F.L., & Leoard, S.L. (1931). The gonad stimulating and the iuteinizing hormones of the anterior lobe of the hypophysis. *American Journal of Physiology, 97*, 291–301. Retrieved from http://ajplegacy.physiology.org/content/97/2/291.full-text.pdf+html

Fontana, R., Mendes, M.A., Souza, B.M., & Konno, K. (2004). Jelleines: A family of antimicrobial peptides from the Royal Jelly of honey bees (*Apis mellifera*). *Peptides, 25*, 919–928. doi:10.1016/j.peptides.2004.03.016

Forti, A.F., Campana, G., Simonella, A., Multari, M., & Scortichini, G. (2005). Determination of chloramphenicol in honey by liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta, 529*, 257–263.

Fournet, B., Dhalliuin, J.M., Montreuil, J., Bosso, C., & Defaye, J. (1980). Gas-liquid chromatography and mass spectrometry of oligosaccharides obtained by partial acetolysis of glycans of glycoproteins. *Analytical Biochemistry, 108*, 35–56. doi:10.1016/0003-2697(80)90690-9

Fournet, B., Strecker, G., Leroy, Y., & Montreuil, J. (1981). Gas-liquid chromatography and mass spectrometry of methylated and acetylated methyl glycosides. Application to the structural analysis of glycoprotein glycans. *Analytical Biochemistry, 116*, 489–502. doi:10.1016/0003-2697(81)90393-6

Franks, F. (1998). Freeze-drying of bioproducts: Putting principles into practice. *European Journal of Pharmaceutics and Biopharmaceutics, 45*, 221–229. doi:10.1016/S0939-6411(98)00004-6

Fray, G.I., Jaeger, R.H., Morgan, E.D., Robinson, R., & Sloan, A.D.B. (1961). Synthesis of trans-10-hydroxydec-2-enoic acid and related compounds. *Tetrahedron, 15*, 18–25. doi:10.1016/0040-4020(61)80003-3

Fujita, T., Kozuka-Hata, H., Ao-Kondo, H., Kunieda, T., Oyama, M., & Kubo, T. (2013). Proteomic analysis of the royal jelly and characterization of the functions of its derivation glands in the honey bee. *Journal of Proteome Research, 12*, 404–411. doi:10.1021/pr300700e

Furusawa, N., & Kishida, K. (2001). High-performance liquid chromatographic procedure for routine residue monitoring of seven sulfonamides in milk. *Fresenius Journal of Analytical Chemistry, 371*, 1031–1033.

Furusawa, T., Rakwal, R., Nam, H.W., Shibato, J., Agrawal, G.K., Kim, Y.S., & Yonekura, M. (2008). Comprehensive royal jelly (RJ) proteomics using one- and two-dimensional proteomics platforms reveals novel RJ proteins and potential phospho/glycoproteins. *Journal of Proteome Research, 7*, 3194–3229. doi:10.1021/pr800061j

Garcia, L., Carlos, H., Garcia, S., Calabria, K., da Cruz, G., Puentes, A., & de Sousa, M. (2009). Proteomic analysis of honey bee brain upon ontogenetic and behavioral development. *Journal of Proteome Research, 8*, 1464–1473. doi:10.1021/pr800823r

García-Alvarez, M., Huidobro, J.F., Hermida, M., & Rodríguez-Otero, J.L. (2000). Major components of honey analysis by Nearinfraed transflectance spectroscopy. *Journal of Agricultural and Food Chemistry, 48*, 5154–5158. doi:10.1021/jf000170v

Gee, M., & Walker, H.G. (1962). Gas-liquid chromatography of some methylated mono-, di-, and trisaccharides. *Analytical Chemistry, 34*, 650–653. doi:10.1021/ac60186a020

Genç, M., & Aslan, A. (1999). Determination of trans-10-hydroxy-2-decenoic acid content in pure royal jelly and royal jelly products by column liquid chromatography. *Journal of Chromatography A, 839*, 265–268. doi:10.1016/S0021-9673(99)00151-X

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 Chromatography A, 1248*, 130–138. doi:10.1016/j.chroma.2012.05.088

Goodall, I., Dennis, M.J., Parker, I., & Sharman, M. (1995). Contribution of high-performance liquid chromatographic analysis of carbohydrates to authenticity testing of honey. *Journal of Chromatography A, 706*, 353–359. doi:10.1016/0021-9673(94)01074-O

Gürdeniz, G., Rago, D., Bendsen, N.T., Savorani, F., Astrup, A., & Dragsted, L.O. (2013). Effect of trans fatty acid intake on LC-MS and NMR plasma Profiles. *PLoS One, 8*, e69589. doi:10.1371/journal.pone.0069589

Han, B., Fang, Y., Feng, M., Lu, X., Huo, X., Meng, L., & Li, J.K. (2014). In-depth phosphoproteomic analysis of royal jelly derived from western and eastern honey bee species. *Journal of Proteome Research, 13*, 5928–5943. doi:10.1021/pr500843j

Hanes, J., & Simuth, J. (1992). Identification and partial characterization of the major royal jelly protein of the honey bee (*Apis mellifera* L.). *Journal of Apicultural Research, 31*, 22–26. doi:10.1080/00218839.1992.11101256

Hartfelder, K., Bitondi, M.M.G., Brent, C., Guidugli-Lazzarini, K.R., SimÕes, Z.L.P., Stabentheiner, A., … Wang, Y. (2013). Standard methods for physiology and biochemistry research in *Apis mellifera*. In V. Dietemann, J.D. Ellis, & P. Neumann (Eds.), *The COLOSS* BEEBOOK, *Volume I: Standard methods for* Apis mellifera *research*. Journal of Apicultural Research, *52*(1). doi:10.3896/IBRA.1.52.1.06

Hedgley, E.J., & Overend, W.G. (1960). Trimethylsily derivates of carbohydrates. *Chemistry and Industry,* 378–380.

Hojo, M., Kagami, T., Sasaki, T., Nakamura, J., & Sasaki, M. (2010). Reduced expression of major royal jelly protein 1 gene in the mushroom bodies of worker honey bees with reduced learning ability. *Apidologie, 41*, 194–202. doi:10.1051/apido/2009075

Horváth, K., & Molnár-Perl, I. (1997). Simultaneous quantitation of mono-, di-and trisaccharides by GC-MS of their TMS ether oxime derivatives: II. In honey. *Chromatographia, 45*, 328–335. doi:10.1007/BF02505579

Human, H., Brodschneider, R., Dietemann, V., Dively, G., Ellts, J., Forsgren, E., … Zheng, H.Q. (2013). Miscellaneous standard methods for *Apis mellifera* research. In V. Dietemann, J.D. Ellis, & P. Neumann (Eds.), *The COLOSS* BEEBOOK, *Volume I: Standard methods for* Apis mellifera *research. Journal of Apicultural Research, 52*(4). doi:10.3896/IBRA.1.52.4.10

Ishii, R., Horie, M., Murayama, M., & Maitani, T. (2006). Analysis of chloramphenicol in honey and royal jelly by LC/MS/MS. *Journal of the Food Hygienic Society of Japan (Shokuhin Eiseigaku Zasshi), 47*, 58–65.

Isidorov, V.A., Czyżewska, U., Isidorova, A.G., & Bakier, S. (2009). Gas chromatographic and mass spectrometric characterization of the organic acids extracted from some preparations containing lyophilized royal jelly. *Journal of Chromatography B, 877*, 3776–3780. doi:10.1016/j.jchromb.2009.09.016

Isidorov, V.A., Bakier, S., & Grzech, I. (2012). Gas chromatographic-mass spectrometric investigation of volatile and extractable compounds of crude royal jelly. *Journal of Chromatography B, 885–886*, 109–116. doi:10.1016/j.jchromb.2011.12.025

ISO 12824:2106. (2016). Royal jelly—Specifications.

Iwanami, Y., Okada, I., Iwamatsu, M., & Iwadare, T. (1979). Inhibitory effects of royal jelly acid, myrmicacin, and their analogous compounds on pollen germination, pollen tube elongation, and pollen tube mitosis. *Cell Structure and Function, 4*, 135–143.

Kamakura, M., Fukuda, T., Fukushima, M., & Yonekura, M. (2001). Storage-dependent degradation of 57-kDa Protein in royal jelly: A possible marker for freshness. *Bioscience, Biotechnology, and Biochemistry, 65*, 277–284. doi:10.1271/bbb.65.277

Karaali, A., Meydanoğlu, F., & Eke, D. (1988). Studies on composition, freeze-drying and storage of Turkish royal jell. *Journal of Apicultural Research, 27*, 182–185. doi:10.1080/00218839.1988.11100799

Kayashima, Y., Yamanashi, K., Sato, A., Kumazawa, S., & Yamakawa-Kobayashi, K. (2012). Freeze-dried royal jelly maintains its developmental and physiological bioactivity in *Drosophila melanogaster*. *Bioscience Biotechnology and Biochemistry, 76*, 2107–2111. doi:10.1271/bbb.120496

Kelly, J.F.D., Downey, G., & Fouratier, V. (2004). Initial study of honey adulteration by sugar solutions using midinfrared (MIR) spectroscopy and chemometrics. *Journal of Agricultural and Food Chemistry, 52*, 33–39. doi:10.1021/jf034985q

Kelly, S.M., Jess, T.J., & Price, N.C. (2005). How to study proteins by circular dichroism. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1751*, 119–139. doi:10.1016/j.bbapap.2005.06.005

Kim, K.C., Friso, S., & Choi, S.W. (2009). DNA methylation, an epigenetic mechanism connecting folate to healthy embryonic development and aging. *The Journal of Nutritional Biochemistry, 20*, 917–926. doi:10.1016/j.jnutbio.2009.06.008

Kimura, Y., Washino, N., & Yonekura, M. (1995). N -linked sugar chains of 350-kDa royal jelly glycoprotein. *Bioscience, Biotechnology, and Biochemistry, 59*, 507–509. doi:10.1271/bbb.59.507

Kimura, Y., Kajiyama, S.I., Kanaeda, J., Izukawa, T., & Yonekura, M. (1996). N -linked sugar chain of 55-kDa royal jelly glycoprotein. *Bioscience, Biotechnology, and Biochemistry, 60*, 2099–2102. doi:10.1271/bbb.60.2099

Kimura, M., Kimura, Y., Tsumura, K., Okihara, K., Sugimoto, H., Yamada, H., & Yonekura, M. (2003). 350-kDa royal jelly glycoprotein (apisin), which stimulates proliferation of human monocytes, bears the. BETA. 1-3galactosylated N-glycan: Analysis of the N-glycosylation site. *Bioscience, Biotechnology, and Biochemistry, 67*, 2055–2058. doi:10.1271/bbb.67.2055

Kodai, T., Umebayashi, K., Nakatani, T., Ishiyama, K., & Noda, N. (2007). Compositions of royal jelly II. Organic acid Glycosides and Sterols of the royal jelly of honey bees (*Apis mellifera*). *Chemical & Pharmaceutical Bulletin, 55*(10), 1528–1531. doi:10.1248/cpb.55.1528

Kodai, T., Nakatani, T., & Noda, N. (2011). figThe absolute configurations of hydroxy fatty acids from the royal jelly of honey bees (*Apis mellifera*). *Lipids, 46*(3), 263–270. doi:10.1007/s11745-010-3497-x

Korta, E., Bakkali, A., Berrueta, L.A., Gallo, B., & Vicente, F. (2002). Study of an accelerated solvent extraction procedure for the determination of acaricide residues in honey by high-performance liquid chromatography–diode array detector. *Journal of Food Protection, 65*, 161–166.

Koya-Miyata, S., Okamoto, I., Ushio, S., Iwaki, K., Ikeda, M., & Kurimoto, M. (2004). Identification of a collagen production-promoting factor from an extract of royal jelly and its possible mechanism. *Bioscience, Biotechnology, and Biochemistry, 68*, 767–773.

Kucharski, R., Maleszka, R., Hayward, D.C., & Ball, E.E. (1998). A royal jelly protein is expressed in a subset of kenyon cells in the mushroom bodies of the honey bee brain. *Naturwissenschaften, 85*, 343–346. Retrieved from http://link.springer.com/article/10.1007%2Fs001140050512#page-1

Kucharski, R., Maleszka, J., Foret, S., & Maleszka, R. (2008). Nutritional control of reproductive status in honey bees via DNA methylation. *Science, 319*, 1827–1830. doi:10.1126/science.1153069

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685. doi:10.1038/227680a0

Lei, M.K., Peng, F., Ge, X.H., Mei, K., & Zhu, Z.T. (2013). Determination of Glucose, Fructose and Sucrose in royal jelly by ion chromatography coupled with dialysis as an online sample pretreatment technique. *Food Science, 34*, 231–233. doi:10.7506/spkx1002-6630-201316046

Lercker, G., Capella, P., Conte, L.S., & Ruini, F. (1981). Components of royal jelly: I. Identification of the organic acids. *Lipids, 16*, 912–919.

Lercker, G., Caboni, M.F., Sabatini, A.G., & Nanetti, A. (1986). Carbohydrate determination of Royal Jelly by high resolution gas chromatography (HRGC). *Food Chemistry, 19*, 255–264. doi:10.1016/0308-8146(86)90049-X

Li, J.K., Feng, M., Zhang, L., Zhang, Z.H., & Pan, Y.H. (2008). Proteomics analysis of major royal jelly protein changes under different storage conditions. *Journal of Proteome Research, 7*, 3339–3353. doi:10.1021/pr8002276

Li, X., Huang, C., & Xue, Y. (2013). Contribution of lipids in honey bee (*Apis mellifera*) royal jelly to health. *Journal of Medicinal Food, 16*, 96–102. doi:10.1089/jmf.2012.2425

Lichtenberg-Kraag, B., Hedtke, C., & Bienefeld, K. (2002). Infrared spectroscopy in routine quality analysis of honey. *Apidologie, 33*, 327–337. doi:10.1051/apido:2002010

Lineback, D.R. (1968). The carbohydrate-polypeptide linkages in a fungal glucoamylase. *Carbohydrate Research, 7*, 106–108. doi:10.1016/S0008-6215(00)81445-7

Lis, H., & Sharon, N. (1993). Protein glycosylation. Structural and functional aspects. *European Journal of Biochemistry, 218* (1), 1–27. doi:10.1111/j.1432-1033.1993.tb18347.x

Maeda, T., Kuroda, H., & Motoyoshi, K. (1987). Effects of royal jelly and 10-hydroxy decenoic acid on the sebaceous glands of hamster ear. *The Japanese Journal of Dermatology, 98*, 469–475.

Malecová, B., Ramser, J., O'Brien, J.K., Janitz, M., Júdová, J., Lehrach, H., & Šimúth, J. (2003). Honey bee (*Apis mellifera* L.) mrjp gene family: Computational analysis of putative promoters and genomic structure of mrjp1, the gene coding for the most abundant protein of larval food. *Gene, 303*, 165–175. doi:10.1016/S0378-1119(02)01174-5

Marconi, E., Caboni, M.F., Messia, M.C., & Panfili, G. (2002). Furosine: A suitable marker for assessing the freshness of royal jelly. *Journal of Agricultural and Food Chemistry, 50*, 2825–2829. doi:10.1021/jf0114987

Mărghita, L.A., Bonta, V., Mihai, C.M., & Dezmirean, D.S. (2012). Acaricides determination from honey through GC-MS technique using different extraction methods. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, 69*, 19–23.

Mazzoni, V., Bradesi, P., Tomi, F., & Casanova, J. (1997). Direct qualitative and quantitative analysis of carbohydrate mixtures using13C NMR spectroscopy: Application to honey. *Magnetic Resonance in Chemistry, 35*, S81–S90. doi:10.1002/(SICI)1097-458X(199712)35:13<S81::AID-OMR202>3.0.CO;2-E

McFarlane, J.E. (1968). Fatty acids, methyl esters and insect growth. *Comparative Biochemistry and Physiology, 24*, 377–384. doi:10.1016/0010-406X(68)90989-4

Mecozzi, M. (2005). Estimation of total carbohydrate amount in environmental samples by the phenol–sulphuric acid method assisted by multivariate calibration. *Chemometrics and Intelligent Laboratory Systems, 79*, 84–90. doi:10.1016/j.chemolab.2005.04.005

Melliou, E., & Chinou, I. (2005). Chemistry and bioactivity of royal jelly from Greece. *Journal of Agricultural and Food Chemistry, 53*, 8987–8992. doi:10.1021/jf051550p

Munson, L.S., & Walker, P.H. (1906). The unification of reducing sugar methods. *Journal of the American Chemical Society, 28*, 663–686. doi:10.1021/ja01972a001

Nazzi, F., Bortolomeazzi, R., Della Vedova, G., Del Piccolo, F., & Milani, N. (2009). Octanoic acid confers to royal jelly varroa-repellent properties. *Naturwissenschaften, 96*, 309–314. doi:10.1007/s00114-008-0470-0

Neilson, K.A., Ali, N.A., Muralidharan, S., Mirzaei, M., Mariani, M., Assadourian, G., … Haynes, P.A. (2011). Less label, more free: Approaches in label-free quantitative mass spectrometry. *PROTEOMICS, 11*, 535–553. doi:10.1002/pmic.201000553

Nikolov, Ž.L., Jakovljević, J.B., & Boškov, Ž.M. (1984). High performance liquid chromatographic separation of oligosaccharides using amine modified silica columns. *Starch - Stärke, 36*, 97–100. doi:10.1002/star.19840360307

Noda, N., Umebayashi, K., Nakatani, T., Miyahara, K., & Ishiyama, K. (2005). Isolation and characterization of some hydroxy fatty and phosphoric acid esters of 10-hydroxy-2-decenoic acid from the royal jelly of honey bees (*Apis mellifera*). *Lipids, 40*, 833–838.

Notardonato, I., Avino, P., Cinelli, G., & Russo, M.V. (2014). Trace determination of acaricides in honey samples using XAD-2 adsorbent and gas chromatography coupled with an ion trap mass spectrometer detector. *RSC Adv, 4*, 42424–42431. doi:10.1039/C4RA06822J

Ohashi, K., Sawata, M., Takeuchi, H., Natori, S., & Kubo, T. (1996). Molecular cloning of cDNA and analysis of expression of the gene for α-glucosidase from the hypopharyngeal gland of the honey bee *Apis mellifera* L. *Biochemical and Biophysical Research Communications, 221*, 380–385. doi:10.1006/bbrc.1996.0604

Ohashi, K., Natori, S., & Kubo, T. (1997). Change in the mode of gene expression of the hypopharyngeal gland cells with an age-dependent role change of the worker honey bee *Apis mellifera* L. *European Journal of Biochemistry, 249*, 797–802. doi:10.1111/j.1432-1033.1997.t01-1-00797.x

Ong, S.E., & Pandey, A. (2001). An evaluation of the use of two-dimensional gel electrophoresis in proteomics. *Biomolecular Engineering, 18*, 195–205. doi:10.1016/S1389-0344(01)00095-8

Ortelli, D., Edder, P., & Corvi, C. (2004). Analysis of chloramphenicol residues in honey by liquid chromatography-tandem mass spectrometry. *Chromatographia, 59*, 61–64.

Pan, Q.Z., Wu, X.B., Guan, C., & Zeng, Z.J. (2013). A new method of queen rearing without grafting larvae. *American Bee Journal, 153*, 1279–1280.

Paradis, D., Bérail, G., Bonmatin, J.M., & Belzunces, L.P. (2014). Sensitive analytical methods for 22 relevant insecticides of 3 chemical families in honey by GC-MS/MS and LC-MS/MS. *Analytical and Bioanalytical Chemistry, 406*, 621–633. doi:10.1007/s00216-013-7483-z

Patel, V.J., Thalassinos, K., Slade, S.E., Connolly, J.B., Crombie, A., Murrell, J.C., & Scrivens, J.H. (2009). A comparison of labeling and label-free mass spectrometry-based proteomics approaches. *Journal of Proteome Research, 8*, 3752–3759. doi:10.1021/pr900080y

Pourtallier, J. (1967). Quantitative determination of honey sugars with gas chromatography. *Bulletin Apicole, 10*, 209–212.

Prosser, C.L. (1978). *Comparative animal physiology, environmental and metabolic animal physiology*. Hoboken, NJ: John Wiley & Sons.

Quisumbing, F.A., & Thomas, A.W. (1921). Conditions affecting the quantitative determination of reducing sugars by fehling solution. Elimination of certain errors involved current in methods. *Journal of the American Chemical Society, 43*, 1503–1526. doi:10.1021/ja01440a012

Ramadan, M.F., & Al-Ghamdi, A. (2012). Bioactive compounds and health-promoting properties of royal jelly: A review. *Journal of Functional Foods, 4*, 39–52. doi:10.1016/j.jff.2011.12.007

Ramadan, M., & Al-Ghamdi, A. (2012). Bioactive compounds and health-promoting properties of royal jelly: A review. *Journal of Functional Foods, 4*, 39–52. doi:10.1016/j.jff.2011.12.007

Rialotero, R., Gaspar, E.M., Moura, I., & Capelo, J.L. (2007). Gas chromatography mass spectrometry determination of acaricides from honey after a new fast ultrasonic-based solid phase micro-extraction sample treatment. *Talanta, 71*, 1906–1914.

Rudd, P.M., Elliott, T., Cresswell, P., Wilson, I.A., & Dwek, R.A. (2001). Glycosylation and the immune system. *Science, 291*, 2370–2376. doi:10.1126/science.291.5512.2370

Ruoff, K., Luginbühl, W., Bogdanov, S., Bosset, J.O., Estermann, B., Ziolko, T., & Amado, R. (2006a). Authentication of the botanical origin of honey by near-infrared spectroscopy. *Journal of Agricultural and Food Chemistry, 54*, 6867–6872. doi:10.1021/jf060770f

Ruoff, K., Luginbühl, W., Künzli, R., Iglesias, M.T., Bogdanov, S., Bosset, J.O., … Amado, R. (2006b). Authentication of the botanical and geographical origin of honey by mid-infrared spectroscopy. *Journal of Agricultural and Food Chemistry, 54*, 6873–6880. doi:10.1021/jf060838r

Ruoff, K., Luginbühl, W., Bogdanov, S., Bosset, J.O., Estermann, B., Ziolko, T., … Amado, R. (2007). Quantitative determination of physical and chemical measurands in honey by near-infrared spectrometry. *European Food Research Technology, 225*, 415–423. doi:10.1007/s00217-006-0432-8

Sabatini, A.G., Marcazzan, G.L., Caboni, M.F., Bogdanov, S., & De Almeida-Muradian, L.B. (2009). Quality and standardisation of royal jelly. *Journal of ApiProduct and ApiMedical Science, 1*(1), 1–6. doi:10.3896/IBRA.4.1.01.04

Sakamoto, M., Takeba, K., Sasamoto, T., Kusano, T., Hayashi, H., Kanai, S., … Nagayama, T. (2011). Determination of dimetridazole, metronidazole and ronidazole in salmon and honey by liquid chromatography coupled with tandem mass spectrometry. *Food Hygiene and Safety Science (Shokuhin Eiseigaku Zasshi), 52*, 51–58.

Sandra, K., & Sandra, P. (2013). Lipidomics from an analytical perspective. *Current Opinion in Chemical Biology, 17*, 847–853. doi:10.1016/j.cbpa.2013.06.010

Schägger, H., & von Jagow G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry, 166*, 368–379. Retrieved from http://molbiol.ru/forums/index.php?act=Attach&type=post&id=92824

Scheparts, A. (1965). The glucose oxidase of honey. II. Stereochemical substrate specificity. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis, 96*, 334–336. doi:10.1016/0005-2787(65)90597-6

Schmitzová, J., Klaudiny, J., Albert, Š., Schröder, W., Schreckengost, W., Hanes, J., & Šimúth, J. (1998). A family of major royal jelly proteins of the honey bee *Apis mellifera* L. *Cellular and Molecular Life Sciences CMLS, 54*, 1020–1030.

Serra Bonvehi, J. (1992). Sugar, acidity and pH royal jelly. *Anales de Bromatologia, 44*, 65–69.

Sesta, G. (2006). Determination of sugars in royal jelly by HPLC. *Apidologie, 37*, 84–90. doi:10.1051/apido:2005061

Sesta, G., & Lusco, L. (2008). Refractometric determination of water content in royal jelly. *Apidologie, 39*, 225–232. doi:10.1051/apido:2007053

Shamsipur, M., Hassan, J., Salar-Amoli, J., & Yamini, Y. (2008). Headspace solvent microextraction-gas chromatographic thermionic specific detector determination of amitraz in honey after hydrolysis to 2,4-dimethylaniline. *Journal of Food Composition and Analysis, 21*, 264–270. doi:10.1016/j.jfca.2007.10.004

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 Agricultural and Food Chemistry, 56*, 3509–3516.

Šimúth, J. (2001). Some properties of the main protein of honey bee (*Apis mellifera* L.) royal jelly. *Apidologie, 32*, 69–80. doi:10.1051/apido:2001112

Šimúth, J., Bíliková, K., Kováčová, E., Kuzmová, Z., & Schroeder, W. (2004). Immunochemical approach to detection of adulteration in honey: Physiologically active royal jelly protein stimulating TNF-alpha release is a regular component of honey. *Journal of Agricultural and Food Chemistry, 52*, 2154–2158. doi:10.1021/jf034777y

Smith, A.D. (1997). *Oxford dictionary of bichemistry and molecular biology* (p. 23). Oxford: Oxford University.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., … Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*, 76–85. doi:10.1016/0003-2697(85)90442-7

Smith, R., Mathis, A.D., Ventura, D., & Prince, J.T. (2014). Proteomics, lipidomics, metabolomics: A mass spectrometry tutorial from a computer scientist's point of view. *BMC Bioinformatics, 15*, S9. doi:10.1186/1471-2105-15-S7-S9

Spannhoff, A., Kim, Y.K., Raynal, N.J., Gharibyan, V., Su, M.B., Zhou, Y.Y., … Bedford, M.T. (2011). Histone deacetylase inhibitor activity in royal jelly might facilitate caste switching in bees. *EMBO reports, 12*, 238–243. doi:10.1038/embor.2011.9

Sweeley, C.C., Bentley, R., Makita, M., & Wells, W.W. (1964). Gas-liquid chromatography of trimethysilyl derivates of sugars and related substances. *Journal of the American Chemical Society, 85*, 2497–2507. doi:10.1021/ja00899a032

Takenaka, T., & Echigo, T. (1980). General chemical composition of the royal jelly. *Bulletin of the Faculty of Pharmacy, Tamagawa University, 20*, 71–78.

Takenaka, T., Yatsunami, K., & Echigo, T. (1986). Changes in quality of royal jelly during storage. *Journal of Japanese Society of Food Science and Technology (Japan), 33*, 1–7. doi:10.3136/nskkk1962.33.1

Tamura, S., Amano, S., Kono, T., Kondoh, J., Yamaguchi, K., Kobayashi, S., … Moriyama, T. (2009). Molecular characteristics and physiological functions of major royal jelly protein 1 oligomer. *Proteomics, 9*, 5534–5543. doi:10.1002/pmic.200900541

Tang, C.Z., & Yuan, Y.L. (1999). Effect of temperature on activity of superoxide dismutase in royal jelly. *Apiculture of China, 50*, 10–13.

Tarapoulouzi, M., Papachrysostomou, C., Constantinou, S., Kanari, P., & Hadjigeorgiou, M. (2013). Determinative and confirmatory method for residues of tetracyclines in honey by LC-MS/MS. *Food Additives & Contaminants: Part A, 30*, 1728–1732. doi:10.1080/19440049.2013.814075

Terada, Y., Narukawa, M., & Watanabe, T. (2011). Specific hydroxyl fatty acids in royal jelly activate TRPA1. *Journal of Agricultural and Food Chemistry, 59*, 2627–2635. doi:10.1021/jf1041646

Teresa, S., Helena, R.-C., Ewa, W., & Piotr, S. (2009). Water determination in bee products using the Karl Fischer titration method. *Journal of Apicultural Science, 53*, 49–56.

The Honey bee Genome Sequencing Consortium. (2006). Insights into social insects from the genome of the honey bee *Apis mellifera*. *Nature, 443*, 931–947. doi:10.1038/nature05260

Torto, B., Carroll, M.J., Duehl, A., Fombong, A.T., Gozansky, K.T., Nazzt, F., … Teal, P.E.A. (2013). Standard methods for chemical ecology research in *Apis mellifera*. In V. Dietemann, J.D. Ellis, & P. Neumann (Eds.), *The COLOSS BEEBOOK, Volume I: Standard methods for* Apis mellifera *research. Journal of Apicultural Research, 52*(4). doi:10.3896/IBRA.1.52.4.06

Tourn, M.L., Lombard, A., Belliardo, F., & Buffa, M. (1980). Quantitative analysis of carbohydrates and organic acids in honeydew, honey and royal jelly by enzymatic methods. *Journal of Apicultural Research, 19*, 144–146. doi:10.1080/00218839.1980.11100013

Townsend, G.F., & Lucas, C.C. (1940a). The chemical examination of the lipid fraction of royal jelly. *Science, 92*, 43. doi:10.1126/science.92.2376.43

Townsend, G.F., & Lucas, C.C. (1940b). The chemical nature of royal jelly. *Biochemical Journal, 34*, 1155–1162.

Townsend, G.F., Brown, W.H., Felauer, E.E., & Hazlett, B. (1961). Studies on the *in vitro* antitumor activity of fatty acids. The esters of acids closely related to 10-hydroxy-2-decenoic acid from royal jelly against transplantable mouse leukemia. *Biochemistry and Cell Biology, 39*, 1765–1770.

Turner, B.M. (2000). Histone acetylation and an epigenetic code. *BioEssays, 22*, 836–845. doi:10.1002/1521-1878(200009)22:9<836::AID-BIES9>3.0.CO;2-X

Wang, J.H., Byun, J., & Pennathur, S. (2010). Analytical approaches to metabolomics and application to systems biology. *Seminars in Nephrology, 30*, 500–511. doi:10.1016/j.semnephrol.2010.07.007

Watanabe, K., Shinmoto, H., Kobori, M., Tsushida, T., Shinohara, K., Kanaeda, J., & Yonekura, M. (1998). Stimulation of cell growth in the U-937 human myeloid cell line by honey royal jelly protein. *Cytotechnology, 26*, 23–27. doi:10.1023/A:1007928408128

Waterland, R.A., & Rached, M.T. (2006). Developmental establishment of epigenotype: A role for dietary fatty acids? *Scandinavian Journal of Food and Nutrition, 50*, 21–26. doi:10.1080/17482970601066488

Watson, A.D. (2006). Thematic review series: Systems biology approaches to metabolic and cardiovascular disorders. lipidomics: A global approach to lipid analysis in biological systems. *The Journal of Lipid Research, 47*, 2101–2111. doi:10.1194/jlr.R600022-JLR200

Weaver, N., & Law, J. (1960). Heterogeneity of fatty acids from royal jelly. *Nature, 188*, 938–939. doi:10.1038/188938b0

Weaver, N., Law, J., & Johnston, N.C. (1964). Studies on the lipids of royal jelly. *Biochimica et Biophysica Acta (BBA) - Specialized Section on Lipids and Related Subjects, 84*, 305–315. doi:10.1016/0926-6542(64)90058-7

Weaver, N., Johnston, N.C., Benjamin, R., & Law, J.H. (1968). Novel fatty acids from the royal jelly of honey bees (*Apis mellifera*, L.). *Lipids, 3*, 535–538.

Wegener, H.C., Hald, T., Wong, D.L.F., Madsen, M., Korsgaard, H., Bager, F., … Mølbak, K. (2003). Salmonella control programs in Denmark. *Emerging Infectious Diseases, 9*, 774–780. doi:10.3201/eid0907.030024

Wei, W.T., Hu, Y.Q., Zheng, H.Q., Cao, L.F., Hu, F.L., & Hepburn, H.R. (2013). Geographical influences on content of 10-hydroxy-trans-2-decenoic acid in royal jelly in China. *Journal of Economic Entomology, 106*, 1958–1963. doi:10.1603/EC13035

Wisniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods, 6*, 359–362. doi:10.1038/Nmeth.1322

Wong-Chong, J., & Martin, F.A. (1979). Analysis of sugar cane disaccharides by liquid chromatography.1. Adsorption chromatography with flow programming. *Plant Physiology, 27*, 927–929.

Woodcock, T., Downey, G., Kelly, J.D., & O'Donnell, C. (2007). Geographical classification of honey samples by near-infrared spectroscopy: A feasibility study. *Journal of Agricultural and Food Chemistry, 55*, 9128–9134. doi:10.1021/jf072010q

Wu, C.W., & Zhang, F.X. (1990). Effect of temperature and duration on the activity of glucose oxidase in royal jelly. *Apiculture of China, 41*, 4–6.

Wu, X.B., Zhang, F., Guan, C., Pan, Q.Z., Zhou, L.B., Yan, W.Y., & Zeng, Z.J. (2015). A new method of royal jelly harvesting without grafting larvae. *Entomological News, 124*, 277–281. doi:10.3157/021.124.0405

Wytrychowski, M., Daniele, G., & Casabianca, H. (2012). Combination of sugar analysis and stable isotope ratio mass spectrometry to detect the use of artificial sugars in royal jelly production. *Analytical and Bioanalytical Chemistry, 403*, 1451–1456. doi:10.1007/s00216-012-5934-6

Wytrychowski, M., Chenavas, S., Daniele, G., Casabianca, H., Batteau, M., Guibert, S., & Brion, B. (2013). Physicochemical characterisation of French royal jelly: Comparison with commercial royal jellies and royal jellies produced through artificial bee-feeding. *Journal of Food Composition and Analysis, 29*, 126–133. doi:10.1016/j.jfca.2012.12.002

Xu, J.Z., Ding, T., Wu, B., Yang, W.Q., Zhang, X.Y., Liu, Y., … Jiang, Y. (2008). Analysis of tetracycline residues in royal jelly by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B, 868*, 42–48. doi:10.1016/j.jchromb.2008.04.027

Yanes-Roca, C., Rhody, N., Nystrom, M., & Main, K.L. (2009). Effects of fatty acid composition and spawning season patterns on egg quality and larval survival in common snook (Centropomus undecimalis). *Aquaculture, 287*, 335–340. doi:10.1016/j.aquaculture.2008.10.043

Yu, F., Mao, F., & Li, J. (2010). Royal Jelly Proteome Comparison between *A. mellifera* ligustica and A. cerana cerana. *Journal of Proteome Research, 9*, 2207.

Yu, H., Tao, Y., Le, T., Chen, D., Ishsan, A., Liu, Y., … Yuan, Z. (2010). Simultaneous determination of amitraz and its metabolite residue in food animal tissues by gas chromatography-electron capture detector and gas chromatography-mass spectrometry with accelerated solvent extraction. *Journal of Chromatography B, 878*, 1746–1752. doi:10.1016/j.jchromb.2010.04.034

Zaina, S. (2010). Tune into fatty acids for epigenetic programming news. *Current Opinion in Lipidology, 21*, 469–470. doi:10.1097/MOL.0b013e32833e4f00

Zeng, Z.J. (2013). *Technique for mechanized production of royal jelly* (pp. 2–46). Beijing: China Agriculture Press.

Zhang, L., Fang, Y., Li, R., Feng, M., Han, B., Zhou, T., & Li, J. (2012). Towards posttranslational modification proteome of royal jelly. *Journal of Proteomics, 75*, 5327–5341. doi:10.1016/j.jprot.2012.06.008

Zhang, L., Han, B., Li, R., Lu, X., Nie, A., Guo, L., … Li, J. (2014). Comprehensive identification of novel proteins and N-glycosylation sites in royal jelly. *BMC Genomics, 15*, 135. doi:10.1186/1471-2164-15-135

Zhao, F.Y., Wu, Y.J., Guo, L.L., Li, X.S., Han, J.X., Chen, Y., & Ge, Y.Q. (2013). Using proteomics platform to develop a potential immunoassay method of royal jelly freshness. *European Food Research and Technology, 236*, 799–815. doi:10.1007/s00217-013-1939-4

Zheng, H.Q., Wei, W.T., Wu, L.M., Hu, F.L., & Dietemann, V. (2012). Fast determination of royal jelly freshness by a chromogenic reaction. *Journal of Food Science, 77*(6), S247–S252. doi:10.1111/j.1750-3841.2012.02726.x

Zhou, J., Zhao, J., Yuan, H., Meng, Y., Li, Y., Wu, L., & Xue, X. (2007). Comparision of UPLC and HPLC for determination of trans-10-Hydroxy-2-Decenoic acid content in royal jelly by ultrasound-assisted extraction with internal standard. *Chromatographia, 66*, 185–190.

Zhou, J., Xue, X., Chen, F., Zhang, J., Li, Y., Wu, L., … Zhao, J. (2009). Simultaneous determination of seven fluoroquinolones in royal jelly by ultrasonic-assisted extraction and liquid chromatography with fluorescence detection. *Journal of Separation Science, 32*, 955–964. doi:10.1002/jssc.200800545

Ziegler, R., & Vanantwerpen, R. (2006). Lipid uptake by insect oocytes. *Insect Biochemistry and Molecular Biology, 36*, 264–272. doi:10.1016/j.ibmb.2006.01.014

Zielinska, D.F., Gnad, F., Wiśniewski, J.R., & Mann, M. (2010). Precision mapping of an in vivo N-glycoproteome reveals rigid topological and sequence constraints. *Cell, 141*, 897–907. doi:10.1016/j.cell.2010.04.012