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Isolation and characterisation of substances from Royal Jelly

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Isolation and characterisation of substances from Royal Jelly

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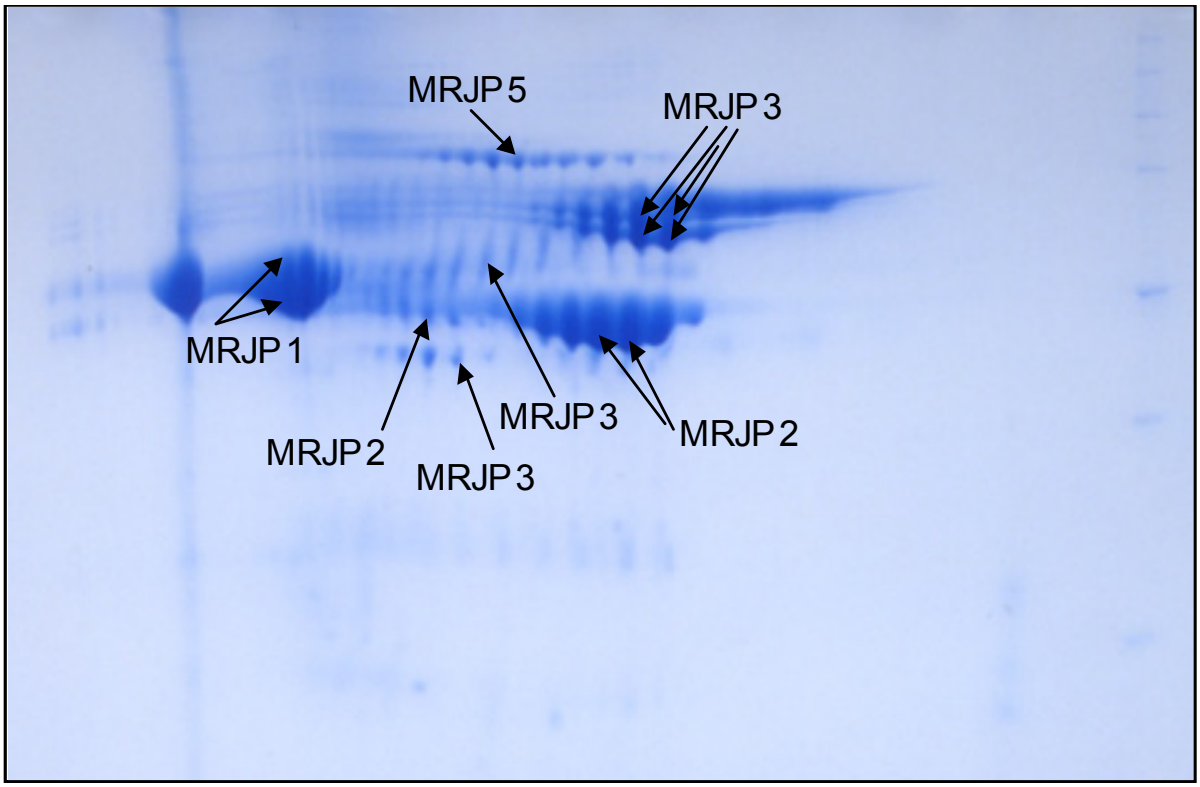

Central topic of this work:
Major royal jelly proteins (MRJP's), its polymorphism and proteolytic derivates (detailed information in fig. 6.5.1; page 147 and fig. 6.6.3; page 156).

Preface

This dissertation was completed as the first contracted German-French joint research project (Cotutelle de Thèse) of the Technische Universität München and the Centre de Biophysique Moléculaire, CNRS UPR 4301 affiliated to the University of Orléans. Substances and results were produced in about equal parts in Germany and in France. The multidisciplinary structure of the dissertation demanded that the dissertation was integrated in several institutes thereby enabling a multifaceted execution of the theme.

The joint research with the Centre de Biophysique Moléculaire, CNRS Orléans, permitted, among other procedures, the characterisation of peptides and proteins extracted from royal jelly by a multitude of physical-chemical and biochemical methods of separation. Particular peptides were produced by the Merryfield Synthesis for antibacterial tests and were characterised by NMR studies.

The dissertation originated from a Diplomarbeit at the TU München in Weihenstephan with the title: Antibacterial effects of proteins fractions extracted from Royal Jelly. This microbiological and protein-analytical research became the point of departure for the following dissertation.

At the TU München the research was mainly executed in the chairs of Technische Mikrobiologie, of Biologische Chemie and of Obstbau, and in the Institut für Mikrobiologie (FML), and with the laboratory of Proteomics.

At the GSF München, in the Institut für Ökologische Chemie numerous and different tests as the analysis of trace elements and the characterisation of sequences of peptides extracted from royal jelly were achieved with NMR spectroscopy.

In cooperation with the Universität Hohenheim, Stuttgart (Landesanstalt für Bienenkunde) the botanical origins of the royal jelly samples were determined by analysis of the pollen morphology.

In cooperation with the Max-Planck-Institut für Biochemie, Martinsried (laboratory of Proteinanalytik) proteins were characterised with MALDI-MS and Edman-degradation. Antiviral properties of royal jelly against Coxsackie viruses were tested in the laboratory of Virusforschung of the same institute.

The beginning phase of the Cotutelle de Thèse of the TU München was supported through organisation and financing by the Centre de Coopération Universitaire Franco-Bavarois (CCUFB), München.

The research was also supported financially by the Université Franco-Allemande (UFA), Sarrebruck, Allemagne (Robert-Bosch-Stiftung).

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Abbreviations

AU	Absorbance Units
APS	ammonium persulphate
cDNA	copy desoxiribonucleic acid
CF-IRMS	continuous flow isotope ratio mass spectrometry
CHAPS	cholamidopropyl-dimethylammonio-propanesulfonate
1D	one dimensional
2D	two dimensional
Da	Dalton
DMACA	p-dimethyl-aminocinnamaldehyd
DTT	Dithiothreitol
DSM	Deutsche Stammsammlung für Mikroorganismen und Zellkulturen
ESI-MS	electrospray ionization mass spectrometry
RJ	Royal Jelly
10-HDA	10-hydroxy-Δ2-decenoic acid
HPLC	High Performance Liquid Chromatography
ICP-MS	inductive coupled plasma mass spectrometry
IEF	isoelectric focussing
IPG	immobilized pH-gradients
IPG-Dalt	two dimensional electrophoresis with immobilized pH-gradients
kDa	kiloDalton
LMW	low molecular weight
MIC	minimal inhibitory concentration

MALDI-MS	matrix assisted laser desorption ionisation mass spectrometry
M_R	relative molecular weight
m/z	mass/charge ratio
NMR	nuclear magnetic resonance spectrometry
OES	optical emission spectrometry
pI	isoelectric point
ppb	parts per billion
ppm	parts per million
RT	room temperature
SDS	Sodium n-Dodecyl Sulphate
ssp.	subspecies
%T	proportion of total acrylamide
TEMED	N,N,N',N'-tetramethylendiamin
TI-MS	thermal ionisation mass spectrometry
TMW	Technische Mikrobiologie Weihenstephan
TOF	time of flight
Tris	Tris(hydroxymethyl)-aminomethan
w/v	mass per volume
v/v	volume per volume

1 General introduction and objectives

Royal jelly is a bee product from the hypopharyngeal, mandibular and postcerebral glands of young worker bees. It is produced under partial digestion of essentially pollen and nectar.

The worker bees receive this highly optimized nutrition only until the third day of larval stadium, and afterward they receive worker jelly. Worker jelly is a similar substance but has lower contents in some minor compounds. The queen bees develop from the same eggs as the worker bees. The only difference between queen bees and worker bees is the permanent, life span and exclusive nutrition of the queen with royal jelly. The nutrition with royal jelly for the complete development period from the larvae to the queen bee results without any condensed excrements. Royal jelly nutrition is the reason for the much larger size of the queen bee and the about 20-50 fold longer life span. The royal jelly enables the queen bee to produce every day a number of eggs with the total volume of the own body.

The worker bee cell contains only 2-4 mg of worker jelly whereas the queen bee cells contain 200-400 mg of royal jelly. For a long time the different amounts were regarded as the reason for the caste differentation (Haydak 1943). Comparisons of the jellies of worker bees, drones and queen bees showed that the main compounds are similar in nutrition of all three bee casts (Rembold 1987). Differences in the content of amino acids, nucleotides and vitamins in nutritions were assigned to play a role in the development of worker and queen bees (Rembold 1987). Royal jelly has higher contents of fructose and glucose as worker jelly (Ascenot and Lensky 1976). For the lipid fraction differences in the larval jellies of workers and queens are described (Lercker et al. 1984), in particular the relation between several fatty acids. In addition to the nutrition the juvenile hormone plays an important role (Rembold 1987; Ascenot and Lensky 1976).

In a fresh state, royal jelly has a gelatinous consistency and a white-yellowish opaque colour. Royal jelly is relatively acid (pH 3.9-4.1) and has a high buffer capacity in the pH region between 4 and 7 (Sauerwald 1997; Sauerwald et al.1998).

It consists of approximately 66% water, 15% sugars, 5% lipids and 13 % of proteins with a high content of essential amino acids (Lercker et al.1993, Pourtallier et al. 1987; Karaali et al.1988; Schmitzova et al. 1998). The concentration of vitamins is high (Serra-Bonvehi 1991), espacially the group of B vitamines B1, B2, B3, B6) as well as PP and E. Furthermore it contains mineral salts (K, Na, Mg, Ca, Zn, Fe, Cu, Cr, Pb) as well as a relatively high amount of organic acids (Lercker et al.1984; Pavesi 1984). 10-hydroxy-Δ2-decenoic acid (10-HDA) with a concentration of 1.4 to 6% has the highest concentration among the royal jelly specific lipid substances (Kim et al. 1980; Bloodworth et al. 1995). Amounts of 10-HDA have been used as a parameter for royal jelly quality.

Hormonlike substances are responsible for a favourable effect on the mammalian female sexual organs. Furthermore, there are reports about anti-tumoural effects (Townsend 1960), as well as favourable effects in cosmetics and dermal medicine (Debrovoda 1986). The potency of royal jelly against acne (*Propionibacterium subspecies*) indicates the antibacterial activity. For these reasons, royal jelly is appreciated for pharmaceutical application in humans (especially in Japan and China). Japan, for example, imported in the first half of the year 1990 more than 127 tons of royal jelly (Ianuzzi 1990). The main producer of royal jelly is China possessing more than 70 million hives.

The manifold effects of royal jelly offer a multitude of possible fields of application in medicine and pharmacy (Fuji et al. 1980) for new methods of alimentary conservation (Fujiwara et el. 1990; Krell 1996). Increasing levels erythrocytes (red blood cells), granulocytes (white blood cells) and thrombocytes (blood platelets) were observed in royal jelly treatments (Migdalska 1987). Generally stem cells are stimulated by royal jelly. Immunity regulating effects were reported (Matuszevsky et al. 1965). Endocrine effects on the levels of luteinizing hormone, progesterone and testosterone in the blood of rats were described (El-Banby et al. 1985). Several constitutional effects of royal jelly were described in mice and rats (Chauvin 1968; Chauvin 1987). The levels of the hormones thyroxin and cortisol increased. The ratio of the proteins albumin and globulin increased. Several further physiologic effects on body weight of the

animals were reported (Chauvin 1968; Chauvin 1987). Anti-fatigue effect of fresh royal jelly was described for a study in mice (Kamakura et al. 2001). Anti-oxidative activities of royal jelly samples were compared with propolis and honey (Nagai 2001). Due to the scavenging ability of superoxide anion radicals the royal jelly can be used for anti-oxydative protection.
Anti-inflammatory action and accelerated healing of skin lesions were described in animal tests with rats (Fujii et al. 1990). Inhibitory action of 10-hydroxy-Δ2-decenoic acid against tumor cell cultures was shown by Townsend et al. (1960). Anti-tumor effects of royal jelly in mice, after prophylactic-therapeutic or therapeutic oral administration was described by Tamura et al. (1987) for slowly growing solid tumors (Ehrlich and Sarcoma strains). Royal jelly was not effective against rapid growing tumors such as mouse advance leukemia L1210 and P388 strains (Tamura et al. 1987). Royal jelly is widely applied in apitherapy. The royal jelly should be taken orally. In-vitro, tolerata maxima for HeLa cells are about 500 ppm (Bengsch, unpublished). Therapeutic effects are not obligatory correlated to doses. Low quantities produce different effects than high quantities (Bengsch, unpublished). Positive effects in the immune response are described by Yonekura (1998), Krell (1996).

Antiviral effects were shown in vitro and better resistance to viral infections was found in mice (Derivici and Petrescu 1965). Fungicidal activity of royal jelly was described for several fungi, in particular *Fusarium subspecies* (Sauerwald 1997; Sauerwald et al. 1998). Antibacterial activity has been described for several bacteria, in particular for gram-positive bacteria (Stocker 1999, Sauerwald 1997; Yatsunami and Echigo 1985). The significance of particular substances in respect to their contribution to the antibacterial effect has to be specified for which this work presents a contribution. Highly active molecules as native and derivated proteins became now more and more evident (Fujiwara 1990; Sauerwald 1997).

The 10-hydroxy-Δ2-decenoic acid, long time considered as the main factor for the antibacterial activity of royal jelly is now questioned (Barker 1959). Cofactors of antibacterial and antiviral activities are a multitude of secondary metabolites, e.g. flavonoids. The flavonoids originate from the pollen and propolis. The pollen are used

for the preparation of the royal jelly. The propolis covers the honeybee combs and is in permanent contact with royal jelly.

Pollen shows an immense variety of active substances (proteins, glycosides and their derivates) and different kinds of secondary metabolites. Pollen is the essential precursor product of the royal jelly secretion. The composition of royal jelly is correlated significantly with the botanical origin of the natural raw product (Bonomi et al. 1986). The most evident biological properties are the anti-microbial activities of royal jelly samples, associated essentially to proteins and derivates. Research has to be done in the investigation of the correlation between botanical origin and the inhibitory properties.

For the present study we produced series of botanical defined royal jelly samples through the season in Vendée-Maine (France) and from different geographical regions. Commercially available royal jelly samples from other origins were investigated additionally. At least 200 bee hives of the race *Apis mellifera ligustica* were disposable for our procedure of royal jelly production in the main location St. André de la Marche, Vendée-Maine (France). Generally the hives were positioned in groups of 40 colonies at different botanical locations, selected after botanical standpoints. Samples were produced in the vegetation period between April and July. After putting the one day old larvae in the queen cells, 60 cells were inserted in a queenless colony. After 3 days the queen cells were truncated and the larvae removed mechanically. A detailed description of the method, perfected within decades from the apiarists is given e.g. by Chauvin (1968), Bengsch (1994), Krell (1996). The royal jelly was collected in a argon athmosphere. All the samples were pooled and stored until analysis at 5°Celsius. During one harvesting season approximately 400-600 g royal jelly can be produced with one colony.

For the extraction of royal jelly with high biological quality the choice of the hive location under the over-all aspect of optimal conditions (unspent acidic magmatic rocks, climate, plant stress-less environment, bee population dynamics) the dominating vegetation have a particular impact.

Pollen and nectar are essential sources of the biochemical composition of royal jelly. In a preliminary study different contents in proteins, lipids, amino acids and vitamins were observed depending on the basic plant material (Bonomi et al. 1986). Certain semi-artificial feeding of honeybees is reported for high-yielding royal jelly production (Chen 2002). For honeys adulterated production like additional feeding of corn sirup (sugars of C4 plants) were successfully detected with carbon isotope ratios (Roßmann 2001). A large data pool of stable isotope ratios of carbon, hydrogen and nitrogen exists for honey samples (Rossmann 2001). Multi-element stable isotope investigation of royal jelly samples and the associated honeys and bees were used for the assignment of authentic production methods and regional origin of royal jelly samples.

An optimal content of trace elements plays an important role. Trace elements are supplied from the crystalline (granite) basement. Biological assimilated boron compounds are involved in the protein composition of plants and pollen (Bengsch et al. 1989; 1996 and 2000). It is very likely that biological properties of royal jelly samples which derive from pollen, the precursor product are influenced from boron. The amounts of the most frequent elements K, Na, Mg, Ca as well as Fe, Cu and Zn are reported in several investigations (Serra Bonvehi 1991; Nation and Robinson 1971).
In the present study, we investigated the contents of 28 trace elements in botanical and geological defined royal jelly samples with atomic absorption spectroscopy (AAS, ICP-OES, ICP-MS). The trace element situation in the nectar and pollen, the biochemical precursor products of royal jelly depend on the chemistry and age of the rocks. We compared the trace element situations of royal jelly samples from different rocks and soils with trace element contents of honeys from the same sample origins (Bengsch 1992 and 1993).

Further optimal climatic conditions are important for the production of royal jelly: A high luminosity and the lack of stress conditions for plants like excessive dryness or pesticide burden stimulate the production of secondary metabolites.

All these favourable conditions are finally found together in the landscape we have chosen for our royal jelly production: Vendée-Maine in Western France is an optimal area. The region, a large hedge landscape without agricultural use of large surfaces, offers all the mentioned "optimal" conditions. The region contains the mainland basement of the Bretagne and the acidic crystalline rocks in a part of Vendée.

There are periodical rainfalls distributed over the whole year. Even the annual luminosity is high and comparable to the climate of the Mediterranean. The climate is mild and equilibrated. There are neither extreme heat nor extreme frost. The proximity of the Atlantic Ocean with the predominant west winds warrant a regular ventilation. The athmosphere is that of the Atlantic, and is not exposed to any local or cumulative environmental stress. The wild plant world is fully intact.

The microbiological properties of royal jelly were of particular interest in the present study. The role of proteins in the antibacterial activity of royal jelly was described for bacteria and fungi (Sauerwald 1997; Sauerwald et al. 1998; Stocker 1999). Antibacterial protein fractions were isolated using gelfiltration, RP-HPLC and poly-acrylamide gel assays (Stocker 1999). Further characterization of active proteins and peptides with bioanalytical investigation methods was therefore one of the aims of the present study.

The protein fraction of royal jelly consists of predominantly 5 proteins (MRJP 1-3 and 5) with a molecular mass from 49 to 87 kDa (Takenaka 1984; Sauerwald 1997; Stocker 1999; Schmitzova et al. 1998; Simuth 2001). These proteins play firstly an important role in the nutrition of bee larvae. The physiological function of these proteins in the larvae development is still unknown. A 55-kDa glycoprotein with an N-linked sugar chain was isolated from royal jelly which maintained the high viability of rat liver primary cultured cells (Kimura et al. 1996). A 350 kDa royal jelly glycoprotein from royal jelly stimulated the proliferation of human monocytes (Kimura 1995). Specific degradation of a 57-kDa royal jelly protein due to metalloproteinase activity was reported from Kamukara et al. (2002).
The enzymatic activity of the hypopharyngeal gland extracts from honeybees (Apis mellifera) were described by Costa et al. (2002). In newly emerged worker bees large

amounts were found of the N-acetyl-glucosaminidase which may digest the chitin in the ingested honeybee food. In forager bees the acid phosphatase was found which plays a role in authophagic processes, the α-glucosidase which is involved in the processing of nectar into honey, and the ß-glucosidase which acts in pollen digestion.
The large quantity of royal jelly proteins and peptides provide a substantial contribution for the antibacterial and fungicide activity (Stocker 1999; Bengsch et al. 2000; Sauerwald et al. 1998). Antibacterial proteins are known in many insects for the defense against pathogen microorganisms in their immune system (Nissen-Meyer 1996).

Peptides with antibacterial activity were already isolated from royal jelly. A strong inhibition of gram-positive bacteria has been observed of a peptide with a molecular mass of 2,3 kD (Jingwei 1996). The honeybee defensin Royalisin, a peptide with the molecular mass of 5523 Da (Fujiwara 1990) analysed from royal jelly inhibits also preferably grampositive bacteria. Inhibitory activity of Royalisin was found against grampositive bacteria *Paenibacillus larvae* which causes the American Foulbrood AFB (Bilikova et al. 2001). The fungus *Botrytis cinerea* was also inhibited from royalisin (Bilikova et al. 2001). A slightly modified isoform of royalisin was isolated from the hemolymph of the honeybee, the bee defensin has a single amino acid substitution from arginine (bee defensin) to tyrosine (royalisin) at position 50 (Casteels et al. 1993 and 1994). Further antibacterial active peptides of the humoral immunity system of the honeybee (hemolymph) are apidaecin, abaecin and hymenoptaecin with 18, 34 respectively 93 amino acids length (Casteels et al. 1993 and 1994).
Royal jelly plays certainly an important role in the defense against bacterial invasions of the honeybee and its offspring. For many royal jelly proteins the physiological function is so far unknown. One aim of the present study was to characterise the contribution of proteins and peptides for the microbial activity. The composition, structure and function of active proteins and peptides should be established. The potential of applications has to be developed for further pharmaceutical use of royal jelly and isolated substances.

The investigations within the scope of the present study of the system *Apis mellifera* with pharmaceutical orientation and biochemical, epidemiological and environmental aspects of the ecosystem plant / bee / parasites are based mainly on the identification and application of entomologic antiviral, bactericidal and fungicidal resistance mechanisms of the bee.

Firmly associated to its environment the system *Apis mellifera* with its herbal partners of the ecto-symbiosis constitutes the subject of the present investigation. The defense strategies against the parasites with active antiviral and immune stimulating substances together with associated parameters (trace elements) were investigated with a multitude of analytical methods. The subject had to be treated from different viewpoints to receive sufficient predications to the present objectives. The multidisciplinary components form a coherent thematic unit.

Our investigation target to the highly effective substances which ensure the working and the anti-parasitic defense of this system. The influence of the vegetation as the source material in the production of optimal bee products was investigated in consideration of their effects against viruses, micro-organisms and macro-parasites. Focusing points are the highly active substances which play a main role in the defense of the bee against its parasites.

2 Seasonal variation of microbiological activity and botanical origin

Summary

Royal jelly is produced in the hypopharyngeal, mandibular and postcerebral glands of nurse bees (Apis mellifera) under partial digestion of pollen and honey. Pollen is the main source of proteins, peptides and secondary metabolites in royal jelly. Thus, pollen is the essential precursor product of the resulting royal jellies and influences substantially the biological properties. The most evident biological properties are the antimicrobial properties. In the present study, royal jellies were produced within several harvesting years in systematic seasonal variation for microbiological and pollenmorphological analysis.

Antibacterial screening tests showed highly sensible indicator strains for the microbiological analysis of seasonal (i.e. botanical) varied royal jellies. *Micrococcus luteus* DSM 348 was the most sensitive of 12 tested commensal bacteria. Minimal Inhibitory Concentrations (MIC) against *Micrococcus luteus* of royal jellies from 3 consecutive harvesting years showed maxima of activity in the middle of the season and lower activities in the beginning and the end of the season. Activities (MIC) ranged from 40 µg/ml for highly active samples to 300 µg/ml for lower activities.

Pollenmorphological analysis of royal jellies from 3 consecutive harvesting years showed a yearly identic pollen pattern of seasonal changings. Royal jellies of every seasonal range have a characteristic botanical origin of the predominant pollen. Pollen patterns and microbiological activities correlate in yearly consistent trend lines. The most active royal jelly sorts in the middle of the season show respectively several pollen species. Activities can therefore not be assigned to only one pollen sort.

Analytical C8 RP-HPLC-analysis of highly active royal jellies from various botanical origin showed a constant HPLC basis profile and the variation of several protein peaks. Agar diffusion tests of protein peaks with *Micrococcus luteus* show the involvement of proteins and peptides in antibacterial activity. The activity of several protein peaks was seasonally relatively constant, others varied seasonally. The result suggests that different peptides respectively low molecular proteins are involved in activity. In accordance with the activity of royal jellies also the activity of the protein fractions varies with the seasonal (i.e. botanical) origin. Seasonal varying HPLC-peaks were sequenced N-terminally (Edman degradation) and showed slightly

varying peptide patterns. Certain homogeneities in the antimicrobial activities and HPLC profiles suggest, that de novo syntheses of the honeybee play a great role in royal jelly. Substantial correlation between botanical origin of the pollen species and biological activity of the resulting royal jellies could be shown.

2.1 Introduction

Royal jelly (RJ) is a bee product from the hypopharyngeal, mandibular and postcerebral glands of nurse bees (*Apis mellifera*). It is produced under partial digestion of honey and pollen. Pollen with its multitude of compounds (proteins, glycosides and their derivates) and its secondary metabolites is the essential precursor product of royal jelly.

Biological properties of royal jelly are therefore significantly correlated with the botanical origin. The systematical comparative investigation of botanical origin and biological activities is therefore of high interest.

The most evident biological activities are the microbial activities of royal jelly samples which are defined in this manner. Fatty acids especially the 10-hydroxy-decenoic acid as the main factor of antibacterial activity has been questioned (Barker 1959). Cofactors of antiviral and antibacterial activities are flavonoids which derive from pollen and propolis and can transfer into the royal jelly.

Proteins in royal jelly are found in fact in large abundance and diversity (Takenaka and Echigo 1980, 1984), being in permanent interaction, and providing a substantial contribution for the antibacterial and fungicide activity (Stocker 1999, Sauerwald et al. 1998). Proteins and peptides with antibacterial activity are largely used in the immune system of insects for the defense against pathogen microorganisms (Nissen-Meyer 1996). Proteins also exert a key-function in defense of *Apis mellifera* offspring.

Peptides with antibacterial activity have been already isolated from royal jelly. A strong inhibition of gram-positive bacteria was ascertained from a peptide with a molecular mass of 2.3 kD (Jingwei 1996). The defensin Royalisin, a peptide with the molecular mass of 5523 Da (Fujiwara 1990) analysed from royal jelly inhibits also preferably gram-positive bacteria and could play an important role in the defense

against bacterial invasions towards the honeybee. Pollen is the main source of proteins for the royal jelly producing nurse honey bee. In this study royal jelly samples from different pollen origin were investigated towards differences in their protein profile with HPLC separations. Antibacterial tests were made to characterise differences in the activity of the protein fractions derived therefrom. Protein peaks with differences in shape were further investigated by N-terminal sequencing (Edman degradation). Thus, taxonomy and function of royal jelly proteins and derivates are of fundamental interest.

Purpose of the present study was to investigate the correlation between biological activity and the botanical origin. Royal jelly samples were continuously harvested during 3 years to compare pollen analysis and microbiological activity.

Antibacterial activity was characterized with *Micrococcus luteus* DSM 348, the most sensitive indicator strain of a bacterial screening test serie.

2.2 Methods and Materials

Origin of royal jelly samples:

Botanical defined royal jelly samples were produced systematically and in large scale in cooperation with professional apiaries. Royal jelly samples were essentially produced in St. André de la Marche, Vendée-Maine (France). Samples were stored under refrigeration temperatures (4-7°C).

300 samples, obtained under defined conditions were provided from a wide hedge landscape without agriculture in large scale situated in the west of France, seated between Cholet, Nantes and La Roche sur Yon. The area covers the mainland basement of the Bretagne and the acidic magmatic rocks of the Vendée. The athmosphere is atlantic and has no local or cumulative environmental stress exposition. The world of wild plants is ecologically fully intact and offers optimal conditions to obtain efficient raw products, necessary for the present work.

Overall 200 bee hives of the race *Apis mellifera ligustica* were disposable for the royal jelly production. The hives were positioned in groups of 40 colonies at several locations. During the investigation period between 04/27/99 until 06/29/99 37 royal jelly samples were produced. For the period 2000 41 samples were harvested and

for the period 2001 35 samples were harvested. Beginning and end of the harvesting period depended on climatic conditions and apidologic factors. Locations of the hives were changed in dependance on optimal pollen supply, still situated in the same global environment. After putting the one day old larvae in the queen cells 60 cells were inserted in a queenless colony. After 3 days the queen cells were truncated, the larvae removed mechanically and the royal jelly was collected in a argon athmosphere. A detailed description of the method, perfected within decades from the apiarists is given e.g. by Chauvin (1968), Krell (1996) and Bengsch (1994). All the samples were pooled and stored until analysis at 5°Celsius.

Palynological Analysis:

In cooperation with H. Horn, who is the leading scientist in this field, I have shown the botanical origin of royal jelly samples. Botanical determination of the samples was done according to the standard methods of palynology (Louveaux et al., 1978; Ricciardelli d´Àlbore, 1978). Samples of exactly 1 g royal jelly were dissolved in 8 ml of distilled water, neutralised and clarified by using potassium hydroxide (5 %) and centrifuged for 10 minutes by 3.000 rpm. After removing the supernatant, the procedure was repeated by using 10 ml of 0.1 N sulfuric acid. Then, the sediment was transferred to a slide and spread out over an area of about 15x15 mm. After drying on a heating plate at 40°C, the sediment was sealed with a cover slide using glycerine gelatin. All pollen grains, including species from entomophilous and anemophilous plants were recorded by evaluating the whole sediment by microscopic examination (x 400 magnification). In total, at least 500 pollen grains were counted. The frequency classes were calculated according the international methods of melissopalynology (Louveaux et al. 1978) as follows:

- very frequent: > 45 %
- frequent: > 15 - 45 %
- rare: 3 - 15 %
- sporadic: < 3 %

For the study of pollen spectra obtained for the 3 years (table 2.4) only the classes „frequent“ and „very frequent“ were taken into account. The reproducibility of seasonal variation of the main pollen for the three royal jelly years 1999, 2000 and

2001 allowed to display the results in a concise diagram (fig. 2.6). A precision analysis was made for royal jelly samples 1999 and show all pollen, including rare and sporadic forms (table 2.5). Microscopical preparations (magnification from 400 to 1200-fold) of pollen in royal jelly samples 1999 are shown in figure 2.7.

Separation of water-soluble and water-insoluble royal jelly components:
For screening tests of 12 bacterial strains (table 2.1, fig. 2.1): To separate the water-soluble supernatant from the insoluble pellet, the royal jelly sample was diluted threefold with distilled water (4°C) and centrifugated at 27 000×g, 15 minutes at 4°C. The supernatant was concentrated in a vacuum dryer, to one third to obtain roughly the original concentration before dilution of royal jelly (Stocker 1999; Sauerwald et al. 1998, Sauerwald 1997). The water-insoluble pellet was threefold washed with cold (4°C), distilled water and centrifugated. For agar diffusion tests one drop of pure royal jelly, approx. 100 mg of the water-insoluble wet pellet and 40 µl the water-soluble supernatant were separately applied on agarplates (table1).

Purification of Royal jelly proteins and their sequence analysis:
Royal jelly was diluted 3 fold with water, vortexed to homogenity and solids removed by centrifugation at 18 000 x g, 4°C, 15 minutes. Injections of 20 µl supernatant were purified by HPLC on a aquapore RP-300 column, C8, 7 microns, 2.1 x 220 mm, with the Applied Biosystems µHPLC system 140 B. Absorbance was monitored at 220 nm, and the elution solvents consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). Samples were eluted at a flow rate of 120 µl/min using the following solvent gradient: 0':0%B; 60':10%B; 155':30%B; 185':45%B. Fractions were collected and dried under vacuum for further analysis by Edman degradation and agar diffusion test.
Up to 40 fractions were collected based on chromatograms which were 5 fold more sensitive than chromatograms as shown in fig. 2.3 and fig 2.4. The smallest peaks on the presented chromatogram are hardly visible due to the smaller sensitivity. Volumes were collected between 100 µl and 1400 µl. About 30 peaks were detected. There was no antibacterial activity without peak. The peaks with activity against *Micrococcus luteus* were marked with arrows in the chromatogram (fig. 2.3). Very small peaks, visible in chromatograms with five-fold higher sensitivity, indicate that the peptides have high antibacterial activity.

Sequence Analysis: The sequence of purified peptides was determined by automatic Edman degradation using an Applied Biosystems Procise 492 microsequencer. N-terminal sequences of royal jelly proteins are given in table 2.3.

Organisms and media:
Bacterial growth conditions: Bacterial strains from table 2.1 with the numbers 1, 4, 6, 7, 10 and 12 were cultivated aerobically in Standard-I nutrient broth and incubated at 30°C. Bacterial strains with the numbers 2, 3 and 9 were cultivated anaerobically in MRS nutrient broth and incubated at 30°C. Bacterial strains with numbers 5, 8 and 11 were cultivated aerobically in Standard-I nutrient broth and incubated at 37°C.

Standard-I nutrient broth contained the following components per liter: 15 g peptone from caseine, 3 g yeast extract, 6 g NaCl, 1 g glucose; pH was adjusted at 7.5.
MRS Broth was prepared acc. to (De Man, J.C., Rogorosa, M. and M.E. Sharpe 1960); pH was adjusted at 5.0.
Solid media contained 15 g/l agar agar. Soft agar contained 7g/l agar agar and 2g/l glucose; pH was adjusted to the used nutrient agar before autoclaving.
All media were autoclaved at 121°C.

Screening of 12 bacterial strains with agar diffusion tests on sensitivity of royal jelly:
To obtain a highly sensitive indicator organism and to investigate the specifity of bacterial inhibition, agar diffusion tests with 12 bacterial strains (table 21) were carried out.
Agar diffusion tests: Petri dishes with nutrient agar were covered with a thin layer of bacteria in soft agar. 100 µl of bacterial overnight culture, corresponding to approximately 10^8 cells, were mixed with 7 ml soft agar at 50°C and then applied on the petri dishes.
Pure royal jelly and its water-soluble and water-insoluble fractions were prepared as described and applied on agar plates. After 24 hours incubation the inhibition zones were measured in millimeter (table 2.1). The inhibition zones were measured in millimeter after 24 hours of incubation. The twofold distance of the inhibition zone from the solid sample was measured for the pure royal jelly drop and the water-insoluble pellet by subtraction of the solid diameter from the inhibition diameter. For

the supernatants the diameters of the inhibition zones were measured. The correlation between antibacterial activity and diameter of the inhibition zone is log-linear (Parente et al. 1995).

Determination of inhibitory activity of purified HPLC fractions of royal jelly towards Micrococcus luteus:
Dried royal jelly peptides fractioned by HPLC were dissolved in 10 µl H_2O. Inhibitory activities were measured by agar diffusion tests using *Micrococcus luteus*, Standard 1 media as described. Aqueous solution of peptides (10 µl) were put onto the agar plates before incubation. The inhibition zones (diameter) were measured in millimeter after 24 hours of incubation (table 2.2, fig. 2.5).

Determination of Minimal Inhibitory Concentrations (MIC) of seasonal royal jelly samples towards Micrococcus luteus DSM 348:
Stock solutions of royal jelly [conc. 124 mg/ml H_2O] were prepared by dilution in H_2O and vigorous mixing. The MIC's were determined in Standard-I Nutrient broth containing royal jelly stock solutions. Serial two fold dilutions of the solutions of royal jelly were prepared to obtain concentrations of royal jelly ranging from 8.3 mg/ml to 8.1×10^{-3} mg/ml. (For royal jelly samples of season 2000 continous refrigeration at 4-7 °C could not be guaranted since antibacterial activity of all samples were reduced. Concentrations of royal jelly in the microdilution plates were doubled to have a good measurement range for the curve fit routines). Dilutions were inoculated with *Micrococcus luteus* DSM 348 to a cell density of 10^5 cells/ml and were incubated at 30°C for 16 h. Thereafter, the growth was judged by measuring the optical density at 600 nm. MIC was calculated from the growth curve where royal jelly concentrations caused 50% growth inhibition (fig. 2.2). Sigma Plot 1.02 software was used for all curve fit routines (Gänzle et al. 2000).

2.3 Results:
Despite intensive studies, the role of proteins in the antibacterial defense of the bee product royal jelly is not fully solved. In this investigation, biologically optimized samples of royal jelly were separated into a water-soluble supernatant and a water-insoluble pellet. Pure royal jelly and the above mentioned two fractions were used to

investigate the role of proteins in the antibacterial action and to search for highly sensible bacterial test strains (table 2.1).

Table 2.1: inhibitory properties of royal jelly samples

Two-fold tests with Royal Jelly samples of 14./15. May 2001 from Vendée-Maine / France

bacteria	Royal Jelly (RJ) Twofold distance [mm]	RJ supernatant diameter [mm]	RJ pellet Twofold distance [mm]
1. Micrococcus luteus DSM 348	12±0	23.5±0.5	10 ±0
2. Leuconostoc mesenteroides DSM 20193	11±1	18±0	6±0
3. Lactobacillus plantarum CTC 305	12±0	17±0	4±0
4. Enterococcus faecalis WS 1028	10±0	17±0	4 ±0
5. Bacillus subtilis DSM 347	16±0	16±0	18±2
6. Erwinia carotovora TMW 2.20	17±1	12.5±1.5	8±0
7. Micrococcus varians TMW 2.121	10±2	13.5±0.5	7±1
8. Bacillus licheniformis DSM 13	18±0	13±0	13±1
9. Lactococcus lactis ssp. diacetylactis LTH 2034	13±1	11.5±0.5	6±0
10. Alcaligenes eutrophus DSM 531T	6±0	12±0	6±0
11. Escherichia coli K12JM83	6 ±0	12±0	3±1
12. Pseudomonas fluorescens DSM 50106	6±0	11 ±0	2±0

abbreviations:

TMW Technische Mikrobiologie Weihenstephan, TU München, Germany

DSM Deutsche Stammsammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany

CTC Centre de Tecnologia de la Carn, IRTA, Monells, Spain

LTH Lebensmittel-Technologie Hohenheim, Germany

WS Weihenstephaner Stammsammlung, Institute of Microbiology, TU München, Germany

12 different bacteria were tested and very specific inhibitory activities of the royal jelly samples were found. Table 2.1 was sorted according to the inhibiton effect of water-soluble fraction of royal jelly. The water-soluble supernatant was used for analytical C8 RP-HPLC-purification of protein fractions combined with antibacterial tests as described. *Micrococcus luteus* was found as being the most sensitive against the water-soluble royal jelly fraction which contains most of the proteins of royal jelly.
Primarily the antibacterial effects of three different fractions of royal jelly against 12 different bacterial strains were tested. The 12 used bacterial strains are different respectively the media requirements, the oxygen requirements and their food microbial or medical importance.

Although the antibacterial effect of royal jelly is selective, all 12 investigated bacteria showed a significant growth inhibition (table 2.1, fig. 2.1) against the tested royal jelly samples. *Micrococcus luteus* was inhibited most strongly. As further seen in table 2.1 especially gram-positive bacteria are inhibited by preference from the royal jelly samples. Strongly inhibited gram-positive bacteria are *Erwinia carotovora*, *Micrococcus varians*, and *Bacillus licheniformis.* Gram-negative bacteria are also inhibited but noticeable less.

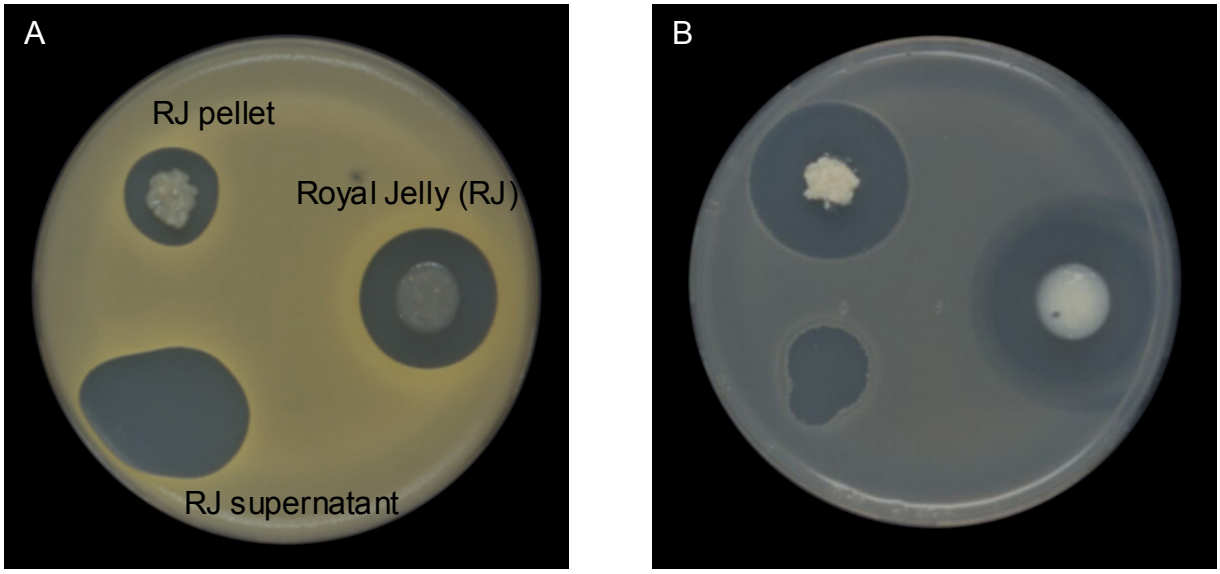

Figure 2.1A: Inhibitory activity of Royal Jelly (RJ), its water-soluble RJ supernatant and its water-insoluble RJ pellet against *Micrococcus luteus* DSM 348 (A) and *Bacillus subtilis* DSM 347 (B).

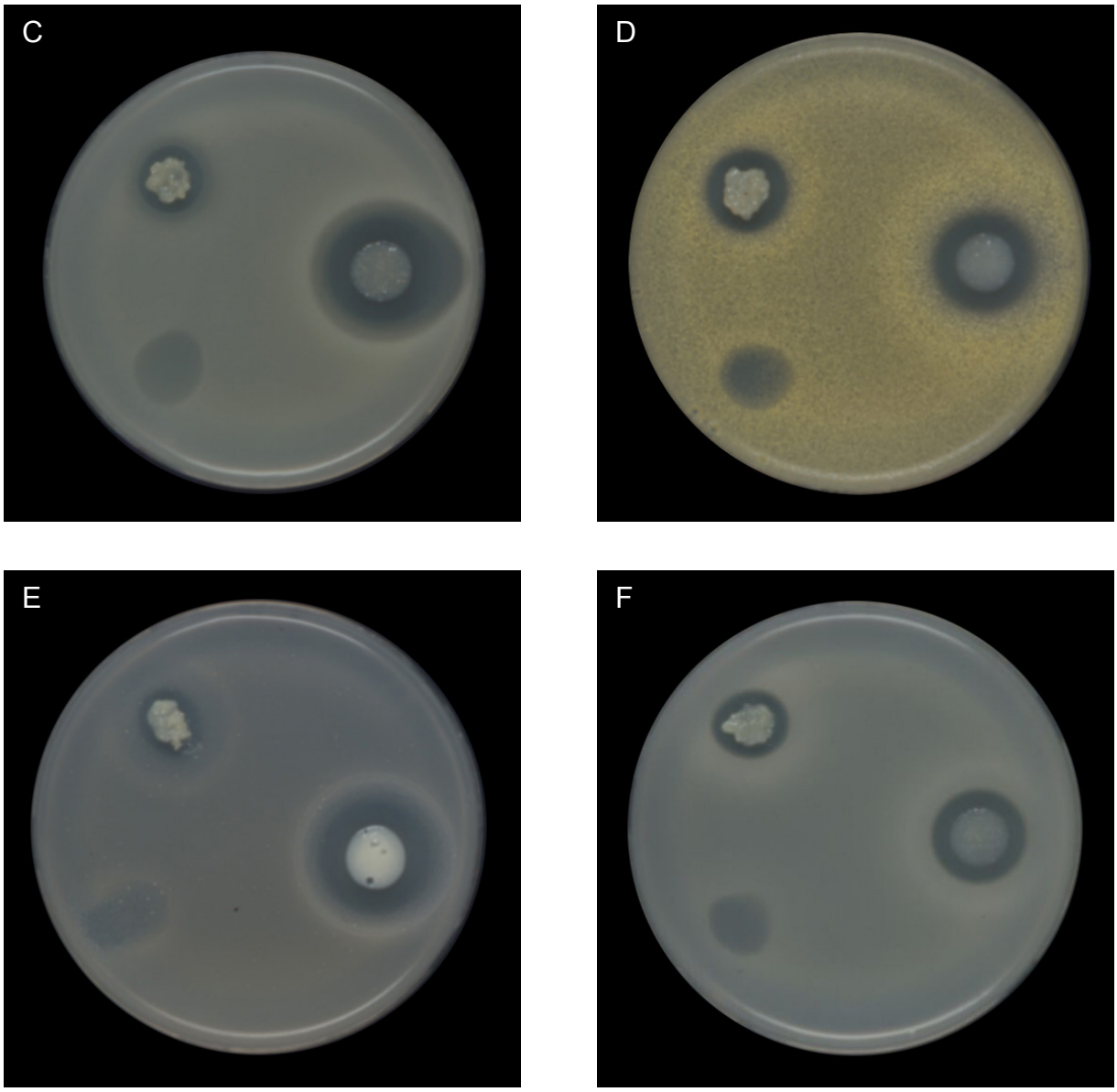

Figure 2.1B: Inhibitory activity of Royal Jelly (RJ), its water-soluble RJ supernatant and its water-insoluble RJ pellet against *Erwinia carotovora* TMW 2.20 (C), *Micrococcus varians* TMW 2.121 (D), *Bacillus licheniformis* DSM 13 (E) and *Alcaligenes eutrophus* DSM 531 T (F).

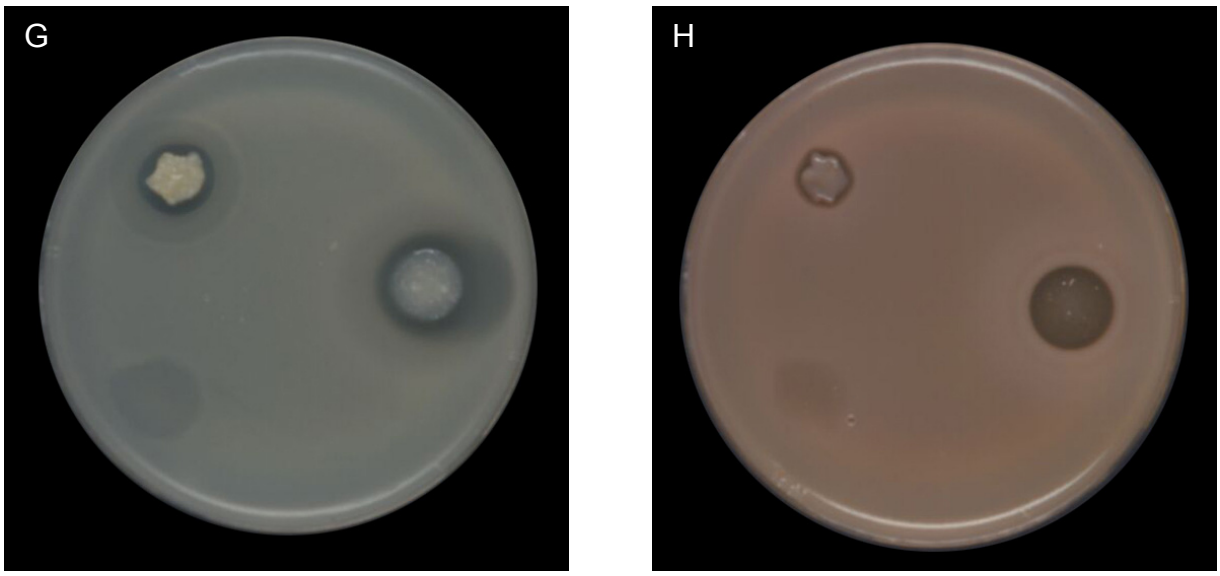

Figure 2.1C: Inhibitory activity of Royal Jelly (RJ), its water-soluble RJ supernatant and its water-insoluble RJ pellet against *Escherichia coli* K12JM83 (G) and *Pseudomonas fluorescens* DSM 50106 (H).

Gram-negative bacteria are positioned at the end of the table. Among the gram-negative bacteria only *Erwinia carotovora* was inhibited even stronger than three of the gram-positive bacteria. The stronger antibacterial activity of royal jelly against gram-positive bacteria than against gram-negative bacteria were already described in further studies (McCleskey et al. 1939, Sauerwald et al. 1998). The most sensitive bacteria strains in this screening tests are also five gram-positive bacteria.

An antibacterial activity of the water-insoluble wet pellet of royal jelly could be observed at all investigated bacteria. Only a few bacteria like *Micrococcus luteus* and *Bacillus licheniformis* are inhibited beyond the pellet. Thus, also the pellet contains also antibacterial components.

Minimal Inhibitory Concentrations (MIC) of royal jelly samples from 3 harvesting years 1999, 2000 and 2001 (fig. 2.2) were analysed with *Micrococcus luteus* DSM 348 as indicator strain. Between 35 and 41 samples were harvested between end of April and beginning of July in each year investigated. For every royal jelly season in 1999, 2000 and 2001 the MIC values are presented (fig. 2.2). Remember, low MIC-values correspond to high activity. MIC values were reproducible with a coefficient of variation of 30% or less (sum of dilution and culture error). The trend line represents

the fit of the data to a second order polynomial equation to indicate a trend of activity over time.

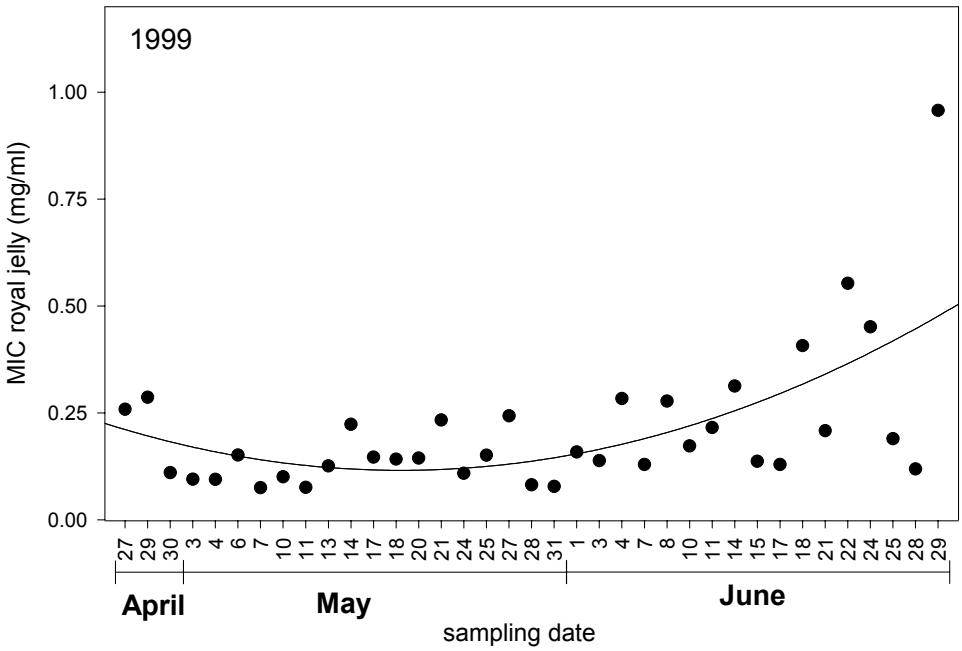

Figure 2.2.A: inhibitory activities of royal jelly samples 1999 from Vendée-Maine against *Micrococcus luteus* DSM 348

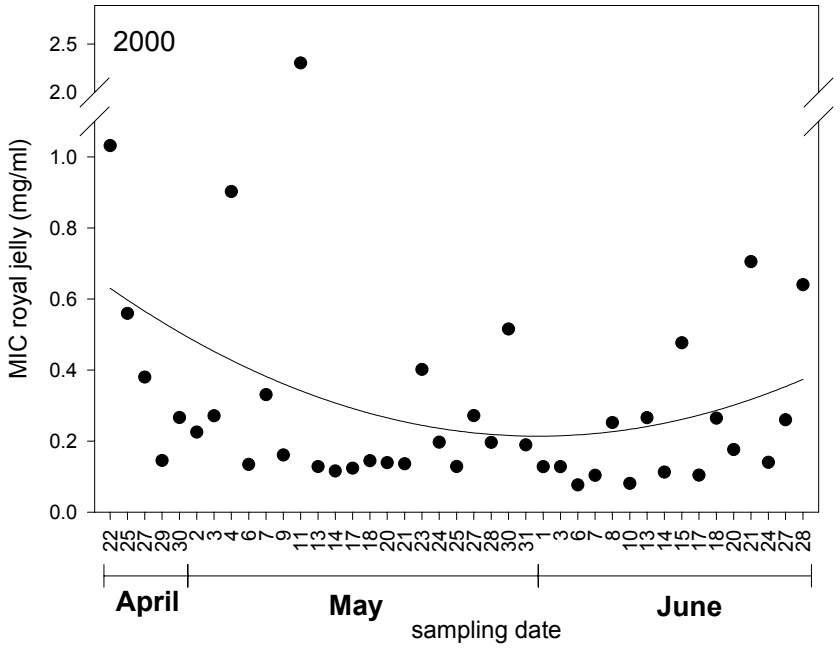

Figure 2.2.B: inhibitory activities of royal jelly samples 2000 (*Micrococcus luteus*)

Activities (Minimal Inhibitory Concentrations) ranged from about 40 µg/ml for highly active samples to 300 µg/ml for lower activities. In all three seasons antibacterial activity against *Micrococcus luteus* was lower at the beginning of the season and was

highest in the middle of the collecting season. Substances from periods with highest activity are between middle and end of May. The end of the season showed lower antibacterial activity for the seasons 1999 and 2000. Season 2001 showed no significant decrease of antibacterial activity at the end of the season. The period of the highest activity in royal jelly season 1999 (fig. 2.2) shows a shift to the left, nearer to the beginning of the season compared with the inhibitory activities of the seasons 2000 and 2001 (fig. 2.2).

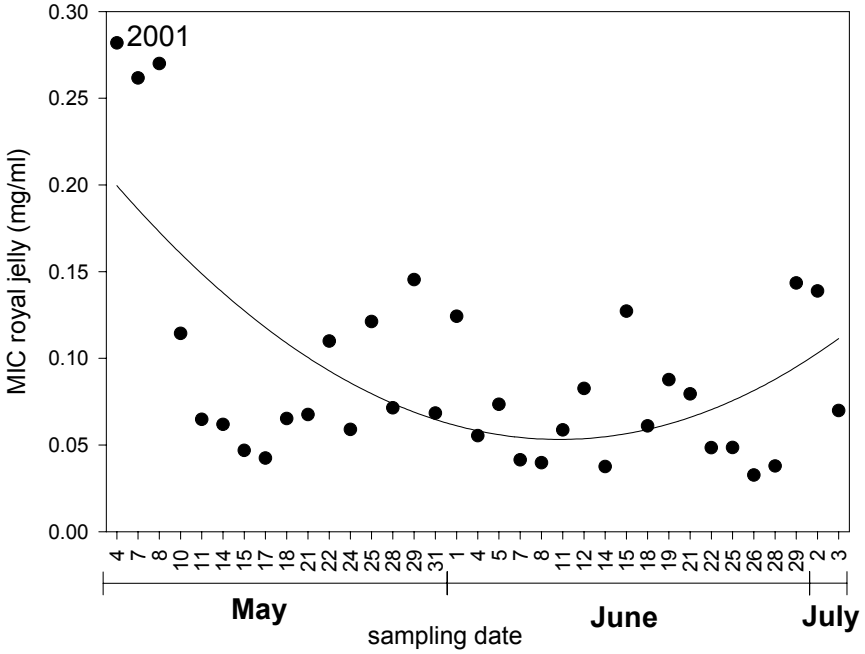

Figure 2.2.C: inhibitory activities of royal jelly samples 2001 (*Micrococcus luteus*)

Identic tests were carried out with royal jelly samples of the seasons 2000 and 2001 from Picardie, Northern France. The same trendlines were found with highest activities against *Micrococcus luteus* in May and decreasing antibacterial activities to the end of the season.

Antibacterial activity of the samples against *Micrococcus luteus* shows the same trendline for the investigated 3 years (fig. 2.2). The results of palynological analysis was also an identic pollen pattern for all 3 years (fig. 2.6, table 2.4). In the most active period in the middle of the season several main pollen could be found in the royal jelly samples. These comparisons showed that botanical origin and antibacterial activity of royal jelly have reproducible trend lines.

Analytical RP-HPLC comparison of 5 representative royal jelly-samples from the begin, middle and end of the harvesting season (1999) were carried out to investigate differences in the protein profiles. The 5 royal jelly samples are representative for different botanical origins. Antibacterial tests against *Micrococcus luteus* were made twofold with the collected peaks to investigate differences in the activity of HPLC separated protein peaks of these samples.
The 5 selected royal jelly samples from 04/27/99, 05/07/99, 05/17/99, 06/03/99 and 06/29/99 represent 5 different main pollen species (fig. 2.6) of the royal jelly harvesting season 1999. As expected the differences in the activity level (MIC-values of fig. 2.2) were enormous.

Between 6 and 8 antibiotic active HPLC-peaks were found for the samples 04/27, 05/07, 05/17 and 06/03/1999. Sample 04/27 referring to the beginning of the season showed the greatest number of active peaks but inhibition zones from the sampled peaks of 05/07 and 05/17 led to greater diameters (table 2.2, fig. 2.5). The higher activity of samples in the middle of the season is therefore in accordance with the results of Minimal Inhibitory Concentrations (MIC) from fig. 2.2. Only 2 active HPLC peaks were found with this last sample from 06/29/1999 at the end of the collecting season. This sample was also found as the least active against *Micrococcus luteus* with the critical dilution assay (MIC values).

The antibacterial activity of HPLC-fractions is also higher for sample 05/17/99 than for 06/29/99 (table 2.2). This confirms also the trend of the MIC results of royal jelly during the season. Whereas the chromatogram of sample 04/27/99 with lower MIC values had one active peak more than chromatogram of sample 05/17/99. Some of the active peaks were found at the same places (retention time). Other active peaks showed differences between the samples from different botanical origin (table 2.2).

RP-HPLC-chromatograms showed a nearly identical peak profile for most of the peaks (fig. 2.3). However, three peaks with a retention time between 30-40 minutes (gradient 5-7% acetonitrile) were significantly different for the 5 samples. The shape of the three peaks is shown in fig. 2.4.

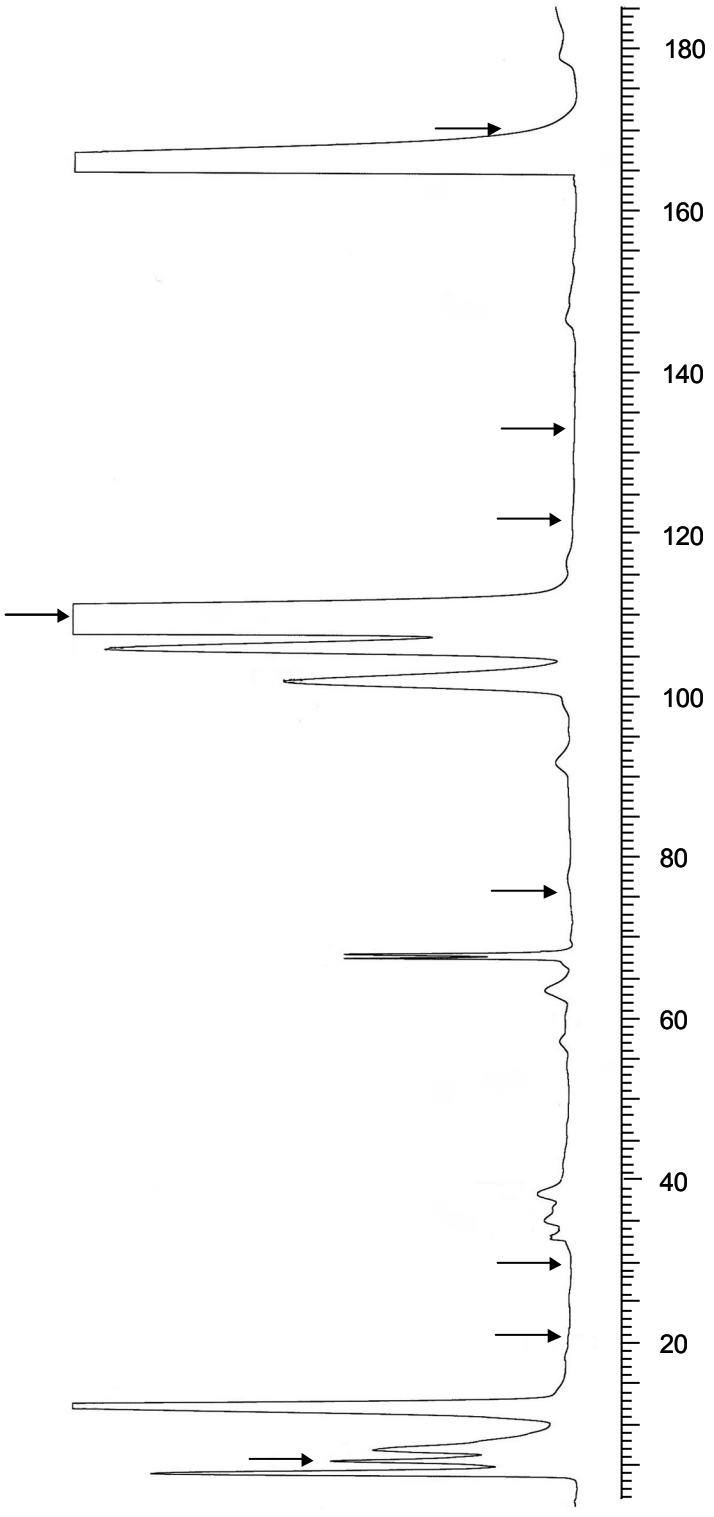

Figure 2.3: Analytical C8 RP-HPLC-chromatogram of royal jelly (sample 04/27/1999) Antibacterial active peaks against *Micrococcus luteus* DSM 348 are signed with arrows. Peaks were collected from a chromatogram with fivefold higher sensitivity (λ= 220 nm). Very small peaks, visible in the chromatograms with five-fold higher sensitivity, indicate the high antibacterial activity of the peptides. There was no activity without peak.

Table 2.2: Inhibition of analytical C8 RP-HPLC-purified royal jelly protein samples against *Micrococcus luteus* DSM 348 (agar diffusion tests)

- (HPLC-fractions derived from chromatogram shown in fig 2.3);
- the inhibiton zones of the agar diffusion test of royal jelly HPLC sample 05/17/1999 are shown in fig 2.5
- the inhibition zones of the very small peaks indicate the high activity of the protein and peptide fractions

sample 04/27/99		sample 05/07/99		sample 05/17/99	
retention time [min]	inhibition diameter [mm]	retention time [min]	inhibition diameter [mm]	retention time [min]	inhibition diameter [mm]
6	7	115	8	97	8
21	7	128	11	130	9
30	7	135	14	142	9
76	5	139	7	148	8
110	8	169	9	154	8
122	5	181	7	171	6
133	8			183	7
170	9				

sample 06/03/99		sample 06/29/99	
retention time [min]	inhibition diameter [mm]	retention time [min]	inhibition diameter [mm]
74	6	42	8
110	8	171	6
135	7		
144	5		
153	6		
170	6		

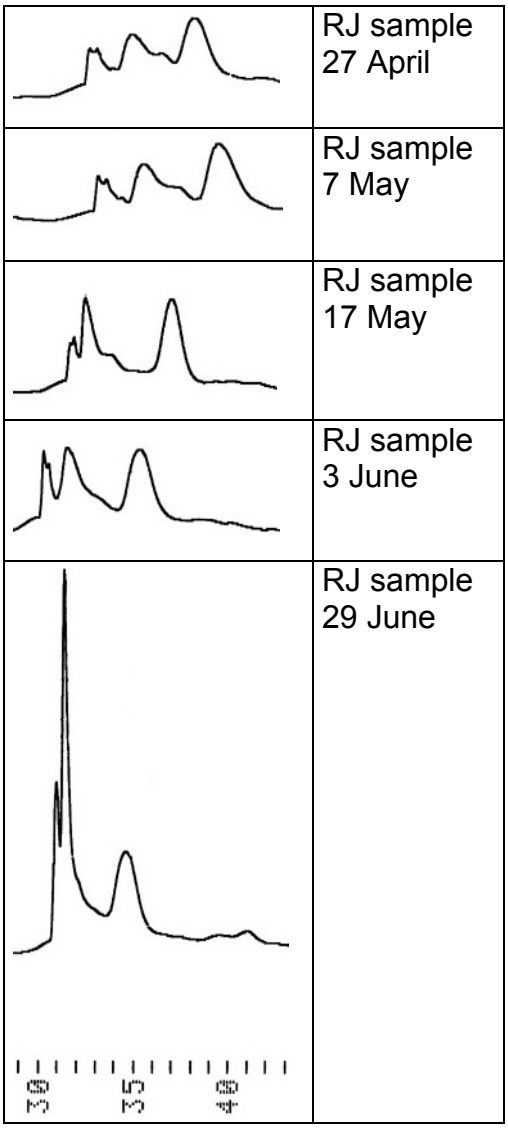

Figure 2.4: seasonal shapes of an analytical C8 RP-HPLC triple peak from royal jelly samples 1999

Samples like 05/17/99 and 06/29/99 showed a great difference in antibacterial activity of pure royal jelly and also in the HPLC-profile of the above mentioned triple peak (recording of the three peaks were carried out with a fivefold more sensitive UV-signal which showed even more the differences in the three peaks). There was no activity without peak.

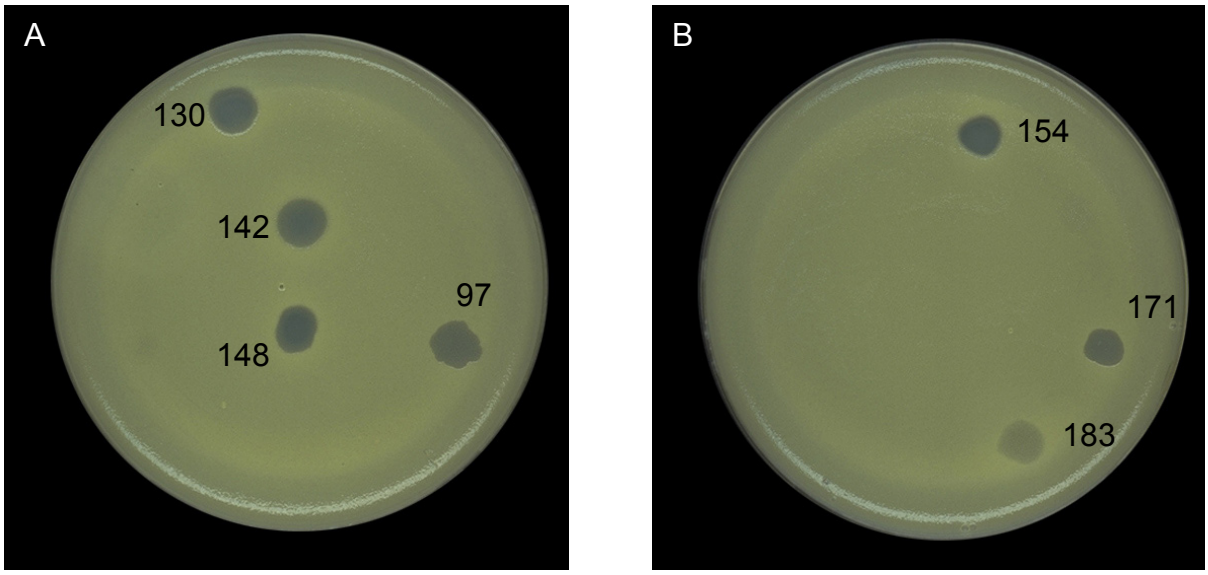

Figure 2.5: Agar diffusion tests (inhibition zone assays) of royal jelly protein fractions from analytical C8 RP-HPLC; sample 05/17/1999 (tab. 2.2). Retention time of the microbiological tested HPLC samples are given as numbers beside the inhibition zone.

The three peaks of the samples 05/17/99 and 06/29/99 which showed the greatest difference in reverse phase HPLC-profiles were sequenced N-terminally by Edman degradation. The sequences (multi-sequences) are shown in table 2.3. The Edman-sequencing of protein peaks which contain several proteins shows several amino acids at each step of degradation respectively. The amino acids are ordered according to decreasing signal intensity. The first amino acid position respectively can therefore be assumed as the sequence of the most abundant protein of the HPLC peak. The sequences shown in table 2.3 show in sections homology to the N-terminal ends of Major Royal Jelly Proteins (MRJP's). The sequence of MRJP2: 1 Ala- 2 Ile- 3 Val- 4 Arg- 5 Glu- 6 Asn- 7 Ser- 8 Pro- 9 Arg- 10 Asn- 11 Leu- 12 Glu- 13 Lys- 14 Ser- 15 Leu- 16 Asn- 17 Val- 18 Ile- can be found at several positions of peak 2 (sample 05/17/99). Database searches of the multi-sequences showed no homology to known proteins, presumably due to the high number of combination possibilities. The similarity of several sequences (e.g. peak 2 and 3 of sample 06/29/99) in adjoining peaks of table 2.3 give important information with regard to the separativity of the HPLC runs and the protein compounds in the collected peaks.

Table 2.3: N-terminal sequences of royal jelly HPLC-fractions

N-terminal sequences (Edman degradation) of three HPLC-peaks (fig. 2.3, fig. 2.4) with a retention time between 30 and 40 minutes (gradient of 5 to 7% of acetonitrile), which showed different shapes in royal jelly samples of different main pollen. The peaks from the samples 05/17/99 and 06/29/99 were sequenced:

sample 06/29/99 (main pollen of the sample: Brassica group, Sambucus spec., Pyrus group)

peak 1
1 Ile, Ala, Asn- 2 Ile, Gln, Asn, Lys- 3 Gln, Arg, Asn, Met- 4 Arg, Gln, Ala- 5 Asp, Met, Ile- 6 Asp- 7 Gln, Arg- 8 Gln, Lys, Phe- 9 Gln, Asp, Ala- 10 Asp, Gly- 11 Asp, Gly, Arg- 12 Gln, Phe, Lys- 13 Gln, Arg-

peak 2
1 Asp, Asn, Ala, Val- 2 Lys, Leu, Met- 3 Val, Arg, Gln- 4 Arg, Gln, Ala- 5 Gly, Met, Pro- 6 Lys, Pro- 7 Arg, Lys, Gln- 8 Lys, Gln, Met- 9 Gln, Ala- 10 Gly- 11 Gly- 12 Lys- 13 Gln, Arg- 14 X-15 Gly

peak 3
1 Asp, Asn, Ala, Glu- 2 Gln, Thr, Leu, Lys- 3 Gln, Val, Arg- 4 Arg, Gln, Glu, Ala- 5 Ala, His, Gly, Met- 6 Ala- 7 Arg- 8 Gln, Met- 9 X- 10 Ala- 11 Val, Ala, Gly, Leu- 12 Gln, Lys- 13 Arg- 14 Ala- 15 Ala, Gly, Val- 16 Ala, Val- 17 Arg- 18 Lys- 19 Ala

sample 05/17/99 (main pollen of the sample: Castanea sativa)

peak 1
1 Asn, Ala, Ile- 2 Gln, Ile, Lys- 3 Arg, Val, Asn- 4 Ala- 5 Gly, Asp, Met- 6 Asp, Ser- 7 Gln, Arg- 8 Gln, Lys- 9 Ala, Asp, Gly- 10 Asp, Gly- 11 Asp

peak 2
1 Ala, Asp, Asn- 2 Asn, Ile, Leu, Met- 3 Val, Gln, Arg- 4 Arg, Gln, Ala- 5 Gly, Asp, Met- 6 Asp- 7 Arg, Ser- 8 Gln, Lys, Leu 9 X- 10 Asp- 11 Gly, 12 Leu, Lys

peak 3
1 Phe, Lys- 2 Ile- 3 Gln, Val, Arg- 4 Lys- 5 Glu, Met- 6 X- 7 Arg, Ala- 8 Lys, Leu

The three peaks (triple peak) of the samples 05/17/99 and 06/29/99 which showed the greatest difference in reverse phase HPLC-profiles were sequenced N-terminally by Edman degradation. The sequences (multisequences) are shown above. Amino acid positions with no UV-signal were signed with X. Several amino acids after one number indicate different peptides in the HPLC peak.

The three peaks were different in size for the 5 chromatographed samples. The shape of the triple peak is shown in fig 2.4. Samples 05/17/99 and 06/29/99 showed the greatest differences. N-terminal sequencing (Edman degradation) showed multi-sequences of peptides (table 2.3) with similarities to the major royal jelly proteins (MRJP's). The sequences are different in some amino acid positions in the N-terminal ends and other positions are equal. Several amino acids at one position indicate that the HPLC peak contain several potentially similar peptides.

Palynological analysis were made to investigate a correlation between botanical origin and biological activity. Characterization of the botanical origin of the investigated royal jelly samples and variations in botanical compositions in different years were also purpose of this study.
9-15 days old nurse bees produce royal jelly under partial digestion of pollen (protein source) and nectar (glycoside source). Honey is a precursor product with a very low protein content. Therefore the pollen is the main source of proteins in royal jelly.
One part of the pollen grains arrives via proventriculus into the midgut of the honey bee and is widely digested. Especially larger and more ornamented pollen arrive to a great extent in the midgut of the honey bee. Proteins of these pollen are digested in the midgut and the amino acids arrive with the hemolymph system in the royal jelly producing glands of the worker bees. The other part of the pollen grains, especially small pollen forms like *Castanea sativa* (14 µm) and pollen forms like *Pyrus species* (34 µm) with a smooth surface morphology only hardly pass the proventriculus. These smaller pollen grains are partially digested together with honey and return directly from the honey stomach back into the royal jelly. Biochemical substances as proteins, peptides, flavonoids, lipids and carbohydrates of the pollen migrate directly into the royal jelly.
Pollen is therefore the essential preliminary product and main source of proteins and peptides in royal jelly. For this reason partially filled pollen grains respectively empty pollen exines allow to conclude the botanical origin of the royal jelly samples. The biological properties of royal jelly samples depend to a great extent on proteins, peptides and secondary metabolites e.g. flavonoids of the predominant pollen forms.

Royal jelly samples harvested during 3 years 1999, 2000 and 2001 were analyzed with palynological methods (Louveaux et al. 1978) and the main pollen was shown in

table 2.4. A mathematical convention allows to order the counted pollen schemata into frequent forms and rare forms. Frequent forms can be expected to influence the biological properties of royal jelly samples to a much more greater extent than rare pollen grains. Pollen forms of all royal jelly samples were taken into account which were found "very frequent" (>45%) and "frequent" (16-45%).

Table 2.4: Melissopalynological determination of royal jelly samples, harvested in France during 1999, 2000 and 2001. Only those pollen are given which were found "very frequent" (>45%) and "frequent" (16-45%).

Date of harvesting	Year 1999	Year 2000	Year 2001
Species			
22.04.	no sample	Brassica group, Asteraceae T, Pyrus group, Genista spec.	no sample
25.04.	no sample	Brassica group, Asteraceae T, Pyrus group, Genista spec.	no sample
27.04.	Brassica group, Pyrus group, Genista ssp.	Brassica group, Asteraceae T, Pyrus group, Salix spec.	no sample
29.04.	Brassica group, Pyrus group, Genista ssp.	Brassica group, Pyrus group, Salix spec., Genista spec.	no sample
30.04.	Brassica group, Pyrus group, Genista ssp.	Brassica group, Pyrus group, Salix spec., Genista spec.	no sample
02.05.	no sample	Brassica group, Pyrus group, Salix spec.	no sample
03.05.	Brassica group, Pyrus group, Genista ssp.	Brassica group, Pyrus group, Prunus group, Salix spec.	no sample
04.05.	Brassica group, Pyrus group, Apiaceae A	Brassica group, Pyrus group, Prunus group, Salix spec.	Pyrus group, Brassica group
06.05.	Brassica group, Pyrus group, Heracleum sphondylium	Brassica group, Pyrus group, Genista spec.	no sample
07.05.	Brassica group, Pyrus group, Heracleum sphondylium	Brassica group, Pyrus group, Prunus group	Pyrus group, Brassica group
08.05.	no sample	no sample	Brassica group, Pyrus group
09.05.	no sample	Brassica group, Pyrus group, Prunus group	no sample
10.05.	Ilex aquifolium, Pyrus group, Brassica group	no sample	Brassica group, Pyrus group
11.05.	no sample	Brassica group, Pyrus group	Brassica group, Pyrus group
13.05.	no sample	Brassica group, Pyrus group	no sample
14.05.	Sambucus spec., Pyrus group, Heracleum sphondylium, Ilex aquifolium	Brassica group, Pyrus group	Brassica group, Pyrus group
15.05.	no sample	no sample	Brassica group, Apiaceae A, Pyrus group
17.05.	Brassica group, Sambucus spec., Pyrus group	Brassica group, Pyrus group	Brassica group, Pyrus group
18.05.	Brassica group, Sambucus spec., Pyrus group	Brassica group, Pyrus group	Brassica group, Pyrus group
20.05.	Brassica group, Pyrus group,	Brassica group, Pyrus group	Brassica group, Apiaceae

	Sambucus spec.		A, Pyrus group,
21.05.	Brassica group, Pyrus group, Apiaceae A	Brassica group, Pyrus group, Apiaceae A	Brassica group, Pyrus group, Apiaceae A
22.05.	no sample	no sample	Brassica group, Apiaceae A, Pyrus group
23.05.	no sample	Brassica group, Asteraceae T, Rhamnus frangula	no sample
24.05.	Brassica group, Apiaceae A	Brassica group, Asteraceae T, Rhamnus spec.	Brassica gruop, Apiaceae A, Pyrus group
25.05.	no sample	Brassica group, Asteraceae T, Apiaceae A	Apiaceae A, Asteraceae T, Pyrus group
27.05.	no sample	Brassica group; Asteraceae T, Rhamnus spec.	no sample
28.05.	Apiaceae A, Brassica group	Asteraceae T, Ilex aquifolium	Apiaceae A, Pyrus group
29.05.	no sample	no sample	Apiaceae A, Pyrus group
30.05.	no sample	Asteraceae T, Rhamnus spec.	no sample
31.05.	Heracleum sphondylium, Pyrus group, Trifolium repens	Asteraceae T, Rhamnus spec.,	no sample
01.06.	Heracleum sphondylium, Pyrus group, Trifolium repens	Asteraceae T; Trifolium repens, Trifolium pratense	Apiaceae A, Heracleum sphondylium, Pyrus group
03.06.	Heracleum sphondylium, Sambucus spec., Pyrus group	Heracleum sphondylium, Trifolium repens, Rhamnus spec.	no sample
04.06.	Heracleum sphondylium	no sample	Apiaceae A, Heracleum sphondylium, Pyrus group
05.06.	no sample	no sample	Heracleum sphondylium, Apiaceae A, Pyrus group
06.06.	no sample	Heracleum sphondylium, Trifolium repens, Rhamnus spec.	no sample
07.06.	no sample	Heracleum sphondylium, Trifolium repens, Rhamnus spec.	Heracleum sphondylium, Apiaceae A, Pyrus group
08.06.	Heracleum sphondylium, Pyrus group, Sambucus spec.	Heracleum sphondylium, Pyrus group, Trifolium repens	Heracleum sphondylium, Apiaceae A, Pyrus group
10.06.	Poaceae, Heracleum sphondylium, Apiaceae A, Pyrus group	Apiaceae A, Rhamnus spec. Heracleum sphondylium	no sample
11.06.	Heracleum sphondylium, Apiaceae A, Trifolium repens	no sample	Heracleum sphondylium, Apiaceae A, Pyrus group
12.06.	no sample	no sample	Apiaceae A, Pyrus group, Sambucus spec.
13.06.	no sample	Apiaceae A, Trifolium repens	no sample
14.06.	Apiaceae A	Apiaceae A	Apiaceae A, Pyrus group
15.06.	Castanea sativa, Apiaceae A, Pyrus group	Apiaceae A, Trifolium repens	Apiaceae A, Pyrus group, Trifolium repens
17.06.	Castanea sativa	Apiaceae A, Trifolium repens	no sample
18.06.	Castanea sativa	Castanea sativa,	Pyrus group, Trifolium repens, Apiaceae A
19.06.	no sample	no sample	Castanea sativa, Apiaceae A, Trifolium repens
20.06.	no sample	Castanea sativa	no sample
21.06.	Castanea sativa	Castanea sativa,	Castanea sativa, Trifolium repens group, Rubus group
22.06.	Castanea sativa	no sample	Castanea sativa, Trifolium repens, Rubus group

24.06.	Castanea sativa	Castanea sativa	no sample
25.06.	Castanea sativa	no sample	Castanea sativa
26.06.	no sample	no sample	Castanea sativa
27.06.	Castanea sativa	Castanea sativa	no sample
28.06.	no sample	Castanea sativa	Castanea sativa
29.06.	Castanea sativa	no sample	Castanea sativa
02.07.	no sample	no sample	Castanea sativa
03.07.	no sample	no sample	Castanea sativa

Apiaceae A = Umbelliferae, Anthriscus-type
Asteraceae T = Compositae, Taraxacum-type

A steady seasonal characteristic of the predominant forms, the main pollen was found for the three royal jelly seasons 1999, 2000 and 2001. Therefore the characteristical pattern of the botanical origin of the royal jelly samples could be transformed into a concise diagram (fig. 2.6).

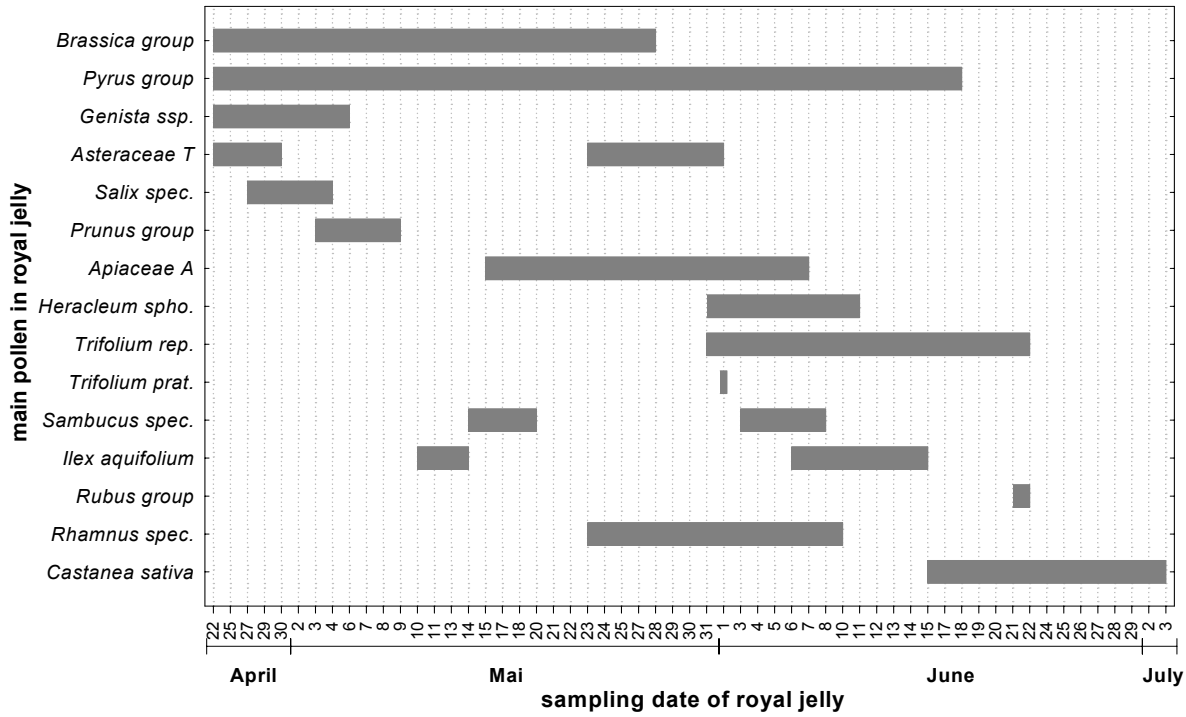

Figure 2.6: diagram of the main pollen forms in royal jelly seasons, Vendée, France

The main pollen yield in the investigated region is *Castanea sativa* (sweet chestnut), followed by *Heracleum sphondylium* (cow parsnip), *Apiaceae A* and different *Rosaceae species* (*Pyrus spec.*, *Prunus spec.*, *Rosa spec.*).

The pollen spectrum of the investigated royal jelly samples is widely concordant with the pollen spectrum in honeys from the same pollen yield period. The observed pollen accord with the botanical investigation on site and the pollen yield period acquainted for the beekeepers. The pollen supply is reflected in royal jelly samples.

Selected examples of pollen exines are shown exemplarily to visualize morphology of pollen species in royal jelly:

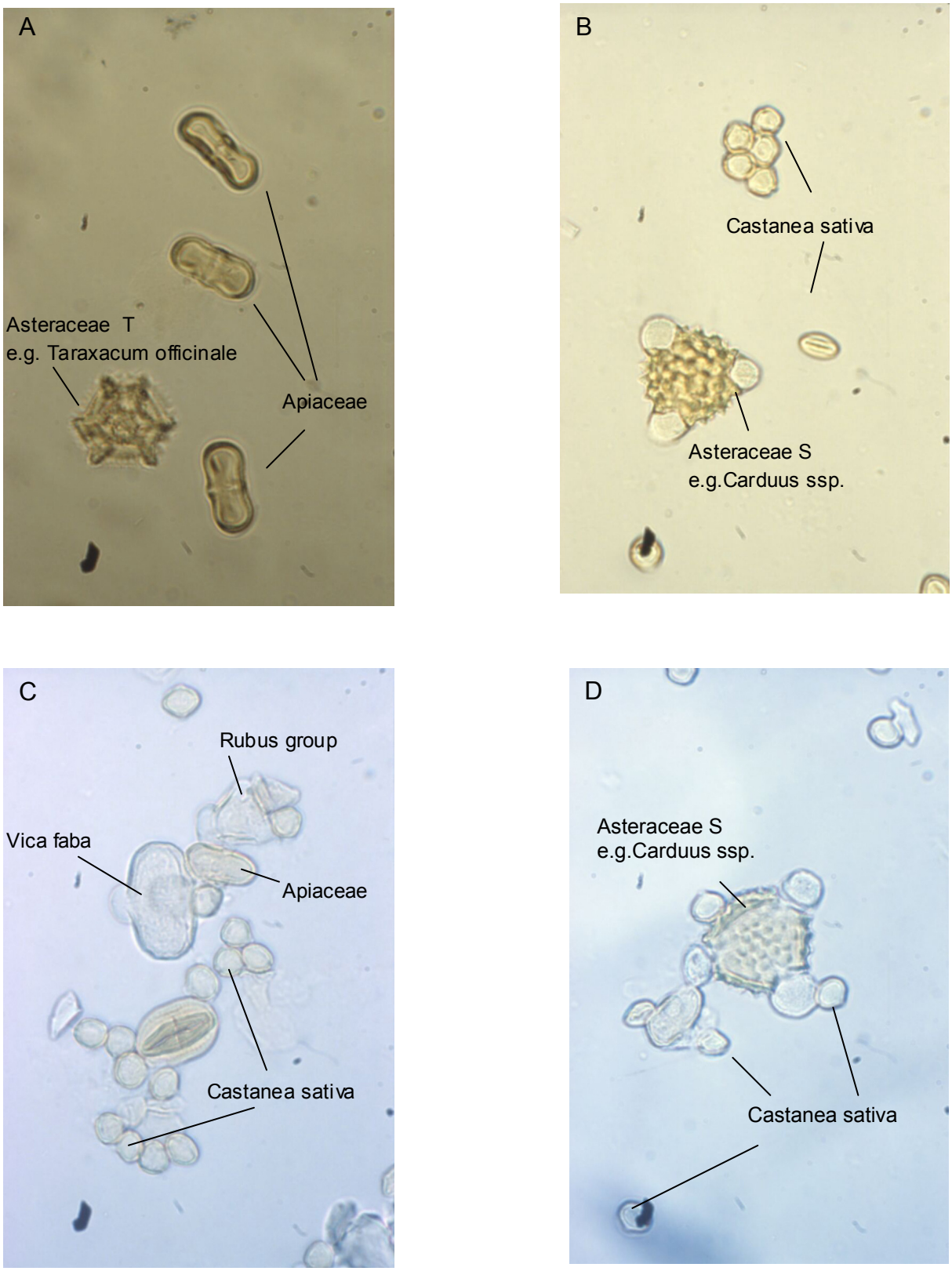

Figure 2.7 A-D: Pollen species in Royal Jelly from Vendée-Maine 1999

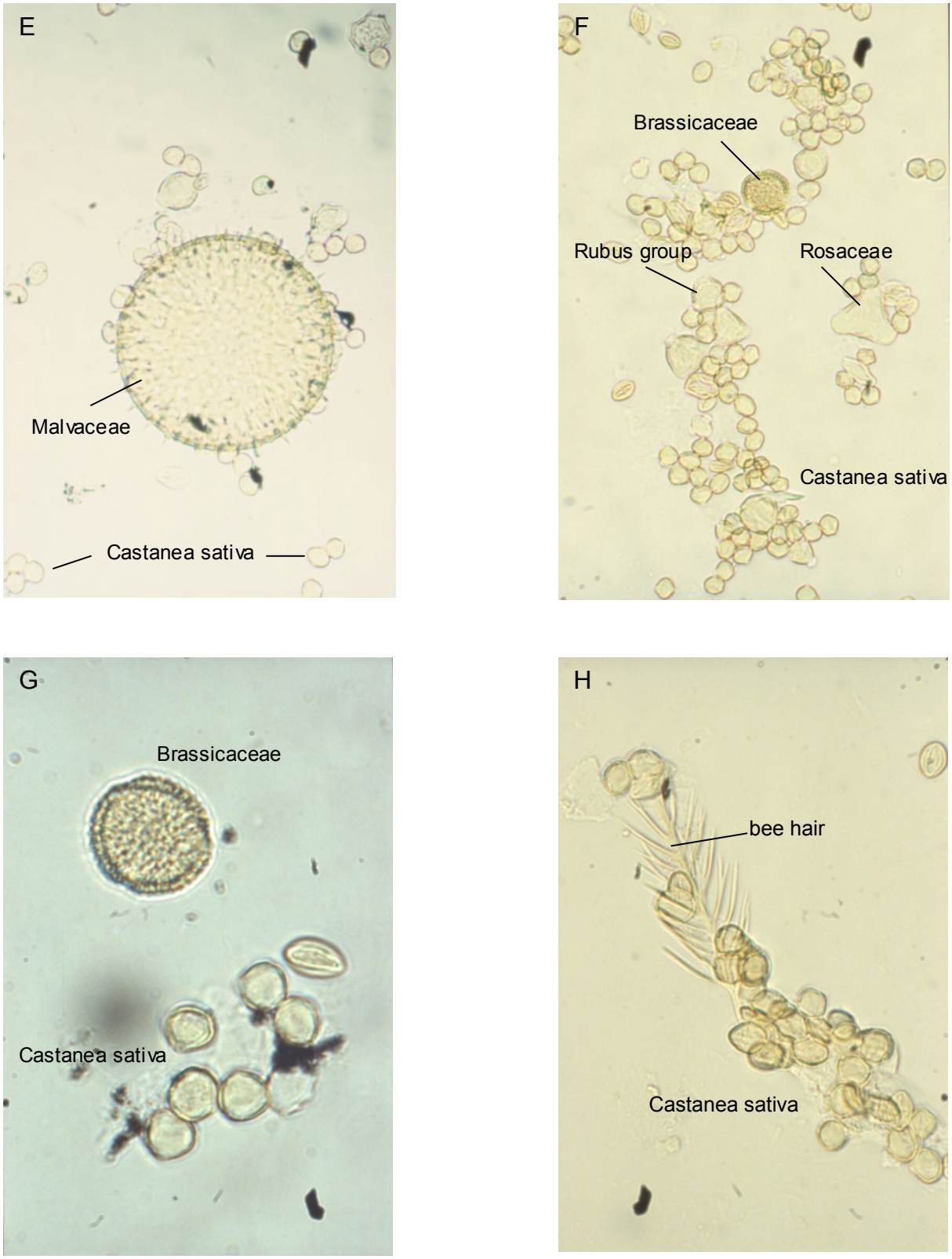

Figure 2.7 E-H: Pollen species in Royal Jelly from Vendée-Maine 1999

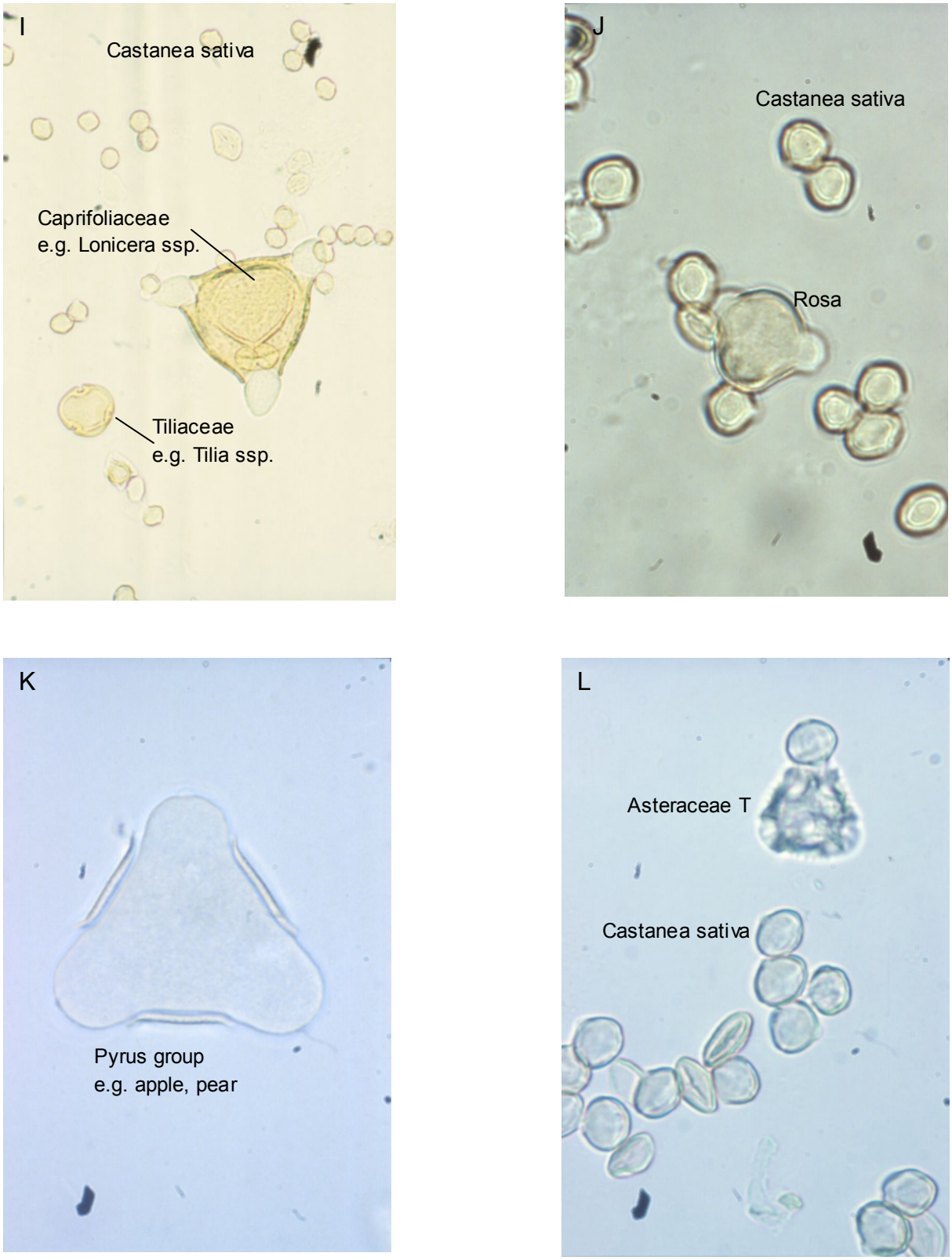

Figure 2.7 I-L: Pollen species in Royal Jelly from Vendée-Maine 1999

For the daily royal jelly production the worker bees uses preferentially the freshly collected pollen. The use of storaged pollen (bee bread) with its darker colour, assumed to be a consequence of Maillard browning reactions during lactic acid fermentation, can be identified during palynological analysis with the light-optical microscope.

120 different pollen species were identified in the examined royal jelly samples (table 2.5) which characterise the intact wild plant world of the investigated region. About 100 species are assigned essentially to the entomophilic species. 20 pollen species derived from the remaining anemophilic plants. The very frequent and frequent pollen forms which contribute highly to the biological activities of the royal jelly samples are given in fig. 2.6 and table 2.4.

Table 2.5: 120 pollen forms of 37 royal jelly samples from season 1999, Vendée-Maine, France (precision analysis). All pollen species are shown including "sporadic" (3-15%) and "rare" (<3%) forms. The table characterises the intact melliferous wild plant world of the investigated region.

Family	**Species**	**German name**
Aceraceae	Acer ssp.	Ahorn ssp.
Apiaceae	Anthriscus type	Kerbel-Typ
	Heracleum sphondylium	Bärenklau
Aquifoliaceae	Ilex aquifolium	Stechpalme
Asteraceae	Taraxacum officinale	Löwenzahn
	Centaurea jacea	Flockenblume
	Centaurea cyanus	Kornblume
	Bidens group	Zweizahn-Gruppe
	Achillea ssp.	Garbe ssp.
	Senecio group	Greiskraut-Gruppe
	Helianthus annuus	Sonnenblume
	Cirsium ssp./Carduus ssp.	Kratzdistel ssp./Distel ssp.
	Artemisia ssp.	Beifuß ssp.
Balsaminaceae	Impatiens ssp.	Springkraut ssp.
Begoniaceae	Begonia ssp.	Begonie ssp.
Betulaceae	Betula ssp.	Birke ssp.
	Alnus ssp.	Erle ssp.
	Carpinus betulus	Hainbuche
	Corylus avellana	Haselnuss
Boraginaceae	Myosotis ssp.	Vergissmeinnicht ssp.
	Echium vulgare	Natternkopf

Brassicaceae	Brassica group	Raps-Gruppe
	Sinaps group	Senf-Gruppe
Buxaceae	Buxus sempervirens	Buchsbaum
Campanulaceae	Campanula ssp.	Glockenblumen ssp.
Caprifoliaceae	Sambucus ssp.	Holunder ssp.
	Viburnum ssp.	Schneeball ssp.
	Lonicera ssp.	Heckenkirsche ssp.
	Symphoricarpos ssp.	Schneebeere ssp.
Caryophyllaceae	Agrostemma githago	Kornrade
Chenopodiaceae	Chenopodium ssp.	Gänsefuss ssp.
Cistaceae	Helianthemum ssp.	Sonnenröschen ssp.
	Cistus ssp.	Zistrosen ssp.
Convolvulaceae	Convolvulus ssp.	Ackerwinde ssp.
	Calystegia sepium	Zaunwinde
Cornaceae	Cornus sanguinea	Hartriegel
Cucurbitaceae	Bryonia dioica	Zaunrübe
Cupressaceae	Juniperus ssp.	Wacholder ssp.
Cyperaceae	Carex ssp.	Seggen ssp.
Dipsacaceae	Knautia arvensis	Acker-Witwenblume
Elaeagnaceae	Elaeagnus ssp.	Ölweide ssp.
Ericaceae	Vaccinium ssp.	Heidel-, Preiselbeere
Euphorbiaceae	Mercurialis ssp.	Bingelkraut ssp.
	Euphorbia ssp.	Wolfsmilch ssp.
Fabaceae	Vicia faba	Ackerbohne
	Robinia pseudoacacia	Scheinakazie
	Onobrychis viciifolia	Esparsette
	Trifolium repens group	Weißklee-Gruppe
	Trifolium pratense group	Rotklee-Gruppe
	Genista ssp.	Ginster ssp.
	Lotus corniculatus	Hornklee
	Lupinus ssp.	Lupine ssp.
	Gleditsia triacanthos	Lederhülsenbaum
Fagaceae	Fagus ssp.	Buche ssp.
	Quercus ssp.	Eichen ssp.
	Castanea sativa	Edelkastanie
Geraniaceae	Geranium pratense	Wiesenstorchschnabel
	Erodium cicutarium	Reiherschnabel
Hippocastanaceae	Aesculus hippocastanum	Rosskastanie
Hydrophyllaceae	Phacelia tanacetifolia	Büschelschön
Hypericaceae	Hypericum perforatum	Johanniskraut
Juglandaceae	Juglans regia	Walnussbaum
Lamiaceae	Labiatae S (Salvia type)	Salbei–Typ
	Labiate M (Mentha type)	Minzen–Typ
	Labiate L (Lamium-Type)	Taubnessel-Typ
Liliaceae	Lilium ssp.	Lilien ssp.
	Asparagus officinalis	Spargel
	Allium ursinumn	Bärlauch
Lythraceae	Lythrum salicaria	Blutweiderich

Magnoliaceae	Liriodendron tulipifera	Tulpenbaum
Malvaceae	Malva ssp.	Malven ssp.
Oleaceae	Ligustrum ssp.	Liguster ssp.
	Fraxinus excelsior	Esche
Oenotheraceae	Epilobium angustifolium	Weidenröschen
Papaveraceae	Papaver ssp.	Mohn ssp.
	Chelidonium majus	Schöllkraut
Pinaceae	Pinus ssp.	Kiefer ssp.
	Abies ssp.	Tanne ssp.
Plantaginaceae	Plantago ssp.	Wegerich ssp.
Poaceae	Poa ssp.	Gräser ssp.
	Secale cereale-typ	Getreide
	Zea mays	Mais
Polemoniaceae	Phlox ssp.	Phlox ssp.
Polygonaceae	Polygonum bistorta	Wiesenköterich
	Rumex ssp.	Ampfer ssp.
	Polygonum persicaria	Flohknöterich
Ranunculaceae	Ranunculus ssp.	Hahnenfuss ssp.
	Anemone nemorosa	Buschwindröschen
	Clematis ssp.	Waldrebe ssp.
Resedaceae	Reseda ssp.	Reseda ssp.
Rhamnaceae	Rhamnus frangula	Faulbaum
	Rhamnus catharticus	Kreuzdorn
Rosaceae	Pyrus group.	Kernobst-Gruppe
	Prunus group	Steinobst-Gruppe
	Rubus group	Himbeeren-, Brombeeren-Gruppe
	Potentilla ssp.	Fingerkraut ssp.
	Aruncus dioicus	Geißbart
	Rosa ssp.	Rosen ssp.
	Crataegus ssp.	Weissdorn ssp.
	Sanguisorba officinalis	Grosser Wiesenknopf
	Sanguisorba minor	Pimpernell
Rubiaceae	Galium ssp.	Labkraut ssp.
Salicaceae	Salix ssp.	Weiden ssp.
	Populus ssp.	Pappel ssp.
Saxifragaceae	Ribes ssp.	Johannisbeere, Stachelbeere
Scrophulariaceae	Verbascum ssp.	Königskerze ssp.
	Rhinanthus ssp.	Klappertopf ssp.
	Veronica ssp.	Ehrenpreis ssp.
Simarubaceae	Ailanthus altissima	Götterbaum
Solanaceae	Solanum spp.	Nachtschatten ssp.
	Datura ssp.	Stechapfel ssp.
	Atropa bella-donna	Tollkirsche
Taxaceae	Taxus baccata	Eibe
Tiliaceae	Tilia ssp.	Linden ssp.
Tropaeolaceae	Tropaeolum ssp.	Kapuzinerkresse ssp.
Typhaceae	Typha ssp.	Rohrkolben ssp.

Ulmaceae	Ulmus ssp.	Ulmen ssp.
Urticaceae	Urtica ssp.	Brennessel ssp.
Valerianaceae	Valeriana ssp.	Baldrian ssp.
Vitaceae	Vitis vinifera	Weinrebe
Violaceae	Viola tricolor	Ackerstiefmütterchen

2.4 Various seasonal and geograpical origins

Royal jelly samples from St. André de la Marche, Vendée-Maine, France from Lugny, Picardie, France, from Chézelles, Centre, France, from Weilheim, Bavaria, Syria, Thailand and China were examined in order to investigate activities and protein profiles (HPLC) in samples from various origins. The royal jellies were examined with identic methods as seasonal royal jelly samples from Vendée-Maine described in chapter 2.2. Microbiological activity against *Micrococcus luteus* was measured by microdilution assay (fig. 2.8). Analytical C8 RP-HPLC-investigations were carried out for the microbiological analysis of purified HPLC-samples. Palynological analysis was made to improve and characterize the botanical origin of the samples.

Analytical C8 RP-HPLC-analysis of the above mentioned samples under conditions described in chapter 2.2 showed a similar basic HPLC profile for all analysed royal jellies with variation of the shape of some peaks. These results are in accordance with the HPLC-results of seasonal royal jelly samples from our results.

Microdilution assays of royal jelly samples with *Micrococcus luteus* as indicator strain showed seasonal variations of antibacterial activity of several geographical origins (fig. 2.8A), given as Minimal Inhibitory Concentrations (MIC). Seasonal effects of antibacterial activity of royal jelly are shown for royal jelly samples from Picardie (fig. 2.8A). The results of chapter 2.2 are confirmed for a geographical origin with similar climatic conditions.

Several activity tested seasonal samples from Picardie, Centre and Bavaria shown in figure 2.8 indicate the same characteristic as found for royal jelly seasonal samples 1999, 2000 and 2001: Samples harvested in the middle of the season (May) showed the highest activity and samples from the later season (July and August) were less active. Samples from Asian provenance showed high antimicrobial inhibitory property.

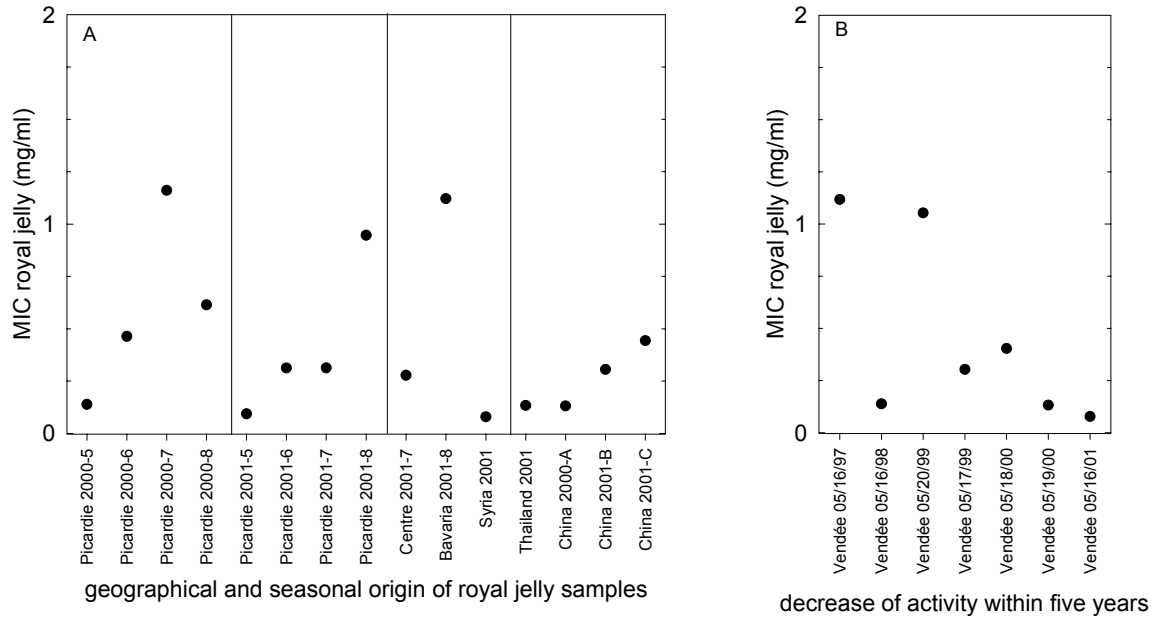

Figure 2.8: inhibitory activities of royal jelly samples from various seasonal and geographical origins against *Micrococcus luteus* DSM 348.

There seem to be a minimum of activity under these storage and test conditions. Royal jelly samples from less active periods and also storage dependent alterated samples (fig. 2.8B) showed an activity of approximately 1.2 mg/ml.

2.5 Discussion

Royal jelly is a highly active, polyvalent natural product and can be produced in larger quantities. The biological activities are selective to some degree.

Antibacterial screening tests with royal jelly samples showed a favoured inhibition of gram-positive bacteria (table 2.1, fig. 2.1). *Micrococcus luteus* was the most sensitive indicator strain of the test serie. Other gram-positive bacteria like *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Enterococcus faecalis*, *Bacillus subtilis*, *Micrococcus varians* and *Bacillus licheniformis* are inhibited distinctly. The gram-negative bacteria *Alcaligenes eutrophus*, *Escherichia coli* and *Pseudomonas fluorescens* were the least active strains of the investigation serie. The gram-negative strain *Erwinia carotovora* was found more sensitive as the gram-positive strains *Micrococcus varians*, *Bacillus licheniformis* and *Lactococcus lactis*. These results are in accordance with further investigations (Sauerwald et al. 1998, Stocker 1999). Antibactbacterial activity, preferably against gram-positive bacteria were confirmed

for *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus*, *Salmonella* and *Escherichia coli* (McCleskey and Melampy 1938 and 1939; Lavie 1968; Yatsunami and Echigo 1985). Due to the above mentioned antibiotic properties the natural product royal jelly can be widely used in apitherapy (Matzke and Bogdanov 2003; Fuji 1995).

The results obtained with anaerobic growing bacteria like lactic acid bacteria were different to earlier investigations (Sauerwald 1997). *Lactobacillus plantarum* and *Leconostoc mesenteroides* were both noticeable inhibited by pure royal jelly and its water-soluble supernatant. The question if oxygen influence for the inhibition (Sauerwald 1997) can obviously be negated.

The organic acid the 10-hydroxy-Δ2-decenoic acid (10-HDA) was isolated from royal jelly and a slight antibacterial and fungicide acitvity was reported (Barker et al. 1959). A comparison of the antibacterial activity showed that the organic acid had less than one fourth of activity of penicillin against *Micrococcus pyogenes* (Blum et al. 1959). Against *Escherichia coli* the acitivity of 10-hydroxy-Δ2-decenoic acid (10-HDA) was less than one fifth of chlortetracyclin. A study of Korean royal jelly samples reported high 10-HDA contents from 2 to 3 % (Kim et al. 1989). Further studies (Bloodworth et al. 1995) reported 10-HDA contents from 2 to 6.4 percent and regarded the fatty acid as the main active compound of royal jelly. Variations in 10-HDA content due to the origin of the royal jelly and characterisics of the bee were taken in account.

The poorly water-soluble 10-hydroxy-Δ2-decenoic acid is among the pellet compounds of the antibacterial screening tests (table 2.1). The pellet contains also high-molecular protein polymers (Simuth 2001). For the inhibitory effects of fractionated royal jelly shown in table 2.1 solubility and diffusivity of the active substances play an important role and have to be considered. In the water-insoluble wet pellet, with low solubility and hence slow diffusion lead to a total inhibition of bacteria around the pellet (respectively also around royal jelly). Active substances in the water-soluble supernatant are well soluble and form therefore larger inhibition zones with sporadic appearing resistances. These effects have to be considered for the comparison of inhibitory effects from royal jelly, the water-soluble supernatant and the insoluble wet pellet thereof.

In the present study *Micrococcus luteus* was distinctly inhibited from the pellet, however, more inhibited from the water-soluble supernatant. Thereby, the different diffusion of the substances from the different viscous royal jelly samples plays probably also a role. Evidently, different components are involved in the antibacterial action of royal jelly, both water-soluble components in the supernatant and water-insoluble in the pellet.

Earlier studies (Stocker 1999, Sauerwald et al. 1998) confirmed the role of the proteins in the antibacterial activity of royal jelly. A peptide from royal jelly with a molecular mass of 5523 Da, the defensin royalisin was isolated and acitivity against gram-positive bacteria was confirmed (Fujiwara 1990). Royalisin-fragments, isolated with dialysation membranes (Bilikova et al. 2001) were found to be active against the gram-positive honeybee pathogen bacteria *Paenibacillus larvae* and the fungus *Botrytis cinerea*. *Paenibacillus larvae larvae* causes American foulbrood (AFB) the most serious larval honeybee disease. Inhibition of the *Paenibacillus larvae* was also confirmed with growth-inhibition assay on polyacrylamide gel (Bachanova et al. 2002).

A study on the antimicrobial activity of royal jelly samples from different regional origins in Turkey (Nazime Mercan et al. 2002) confirmed variations in antibacterial activity. Royal jelly samples from three provinces were tested towards antibacterial activity against 9 bacteria strains. *Micrococcus flavus*, *Bacillus subtilis*, *Micrococcus luteus* and *Bacillus megaterium* were the most inhibited strains. Gram-negative bacteria strains were not inhibited. From 9 tested royal jelly samples 3 samples were ineffective. Different storage conditions of the samples and the shelf-life were given as the explanation for the different activities. The other 6 royal jelly samples were even more effective than some tested antibiotics.

Micrococcus luteus DSM 348 was found to be a highly sensitive test strain and for this reason selected for the systematic investigation of the antibacterial activity of royal jelly samples from different botanical origins. Minimal Inhibitory Concentrations established similar trend lines for 3 tested royal jelly harvesting years 1999, 2000 and 2001 (fig. 2.2). The highest antibacterial activities were found in the middle of the season whereas at the beginning and the end of the season lower activities were

found. The seasonal influence on the biological activity is unambiguous. The pollen situation should play a great role in the variability of microbial activity. A certain homogeneity of the activity recommends also that substances from de novo synthesis of the honeybee plays a great role.

Palynological analysis of the royal jelly samples showed a identic main pollen collecting in royal jelly samples in the three consecutive examined years. This indicates that forager bees prefer every time the most interesting harvest plant to produce royal jelly. If available the nurse bees prefers the fresh pollen. The pollen is the predominant source of proteins and peptides and affects decisively the biological properties of the resulting royal jelly samples.

The larger the pollen spectrum the more balanced is the amino acid spectrum in the bee nutrition, as a result of varieties in the content of essential amino acids in different pollen forms. Bonomi et al. (1986) described variable amounts of proteins, lipids, vitamins and amino acids in royal jelly samples from different botanical origins. The most complete royal jelly samples derived from a large spectrum of different pollen species. The honey bee tries therefore to use preferred pollen species. There is however a clear trend to use predominantly the most attractive pollen resource. The highest attractivity of a pollen source are plants which are good suppliers of both pollen as nectar. Thus the sweet chestnut (*Castanea sativa*) which supplies both much pollen and nectar is the dominant pollen source in the end of the season (fig. 2.6, table 2.4). This means that attractivity and presence of the pollen resource is decisive for the botanical origin of the royal jelly samples.

Among the *pyrus species* (*Rosaceae*) apple trees are good but pears are rather moderate nectar suppliers. Therefore bees fly additionally to *Asteraceae T* (dandelion) immediately after appearance. *Asteraceae T* appears at the beginning of the season and comes back after some weeks of absence. *Asteraceae T* do not tolerate humid weather conditions and also agricultural production of grass silage can be the reason for the temporary disappearance. Also for *Ilex aquifolium* (holly) and *Sambucus species* (elder) this behaviour of temporary disappearance was found (fig. 2.6).

Sambucus spec. (elder) is a nectarless plant therefore additional pollen sources are required during this pollen period. The *Rhamnus spec*. (buckthorn, common buckthorn) are nectar suppliers. The pollen disposability is highly weather dependent. Factors like temperature, ground humidity and luminosity play a role.
Agricultural influences like the massive appearance and the sudden disappearance of pollen sources like *Trifolium pratense* (red clover) play also a great role. The smaller *Trifolium repens* (white clover) is too small for the mowing-maschine and stays therefore longer and is largely used as pollen source in a certain period (fig. 2.6).
The rubus group (bramble) which was expected more often appeared only shortly at the end of the season. *Apiaceae* (*Umbelliferae*) of the A-form (*Anthriscus*) and the H-form (*Heracleum*) play a very great role. Crops like the *Brassica napus* (rape), *Helianthus annuus* (sunflower) or *Trifolium pratense* (red clover) play a secondary role as nectar source at least in the regions observed.
In the investigated periods we found pollen situations (fig. 2.6) and microbiological activities (fig. 2.2) as displayed. The comparison of palynological analysis of the royal jelly samples and the trend lines of antibacterial activity indicates a correlation between botanical origin and biological activities. The most active period of royal jelly samples is in the middle of the season, at the beginning and the end of the season lower activities were found. However in the most active period pollen of several plants were found and it is not possible to indicate one pollen form as the unique reason for the most active samples.

The great influence of botanical origin towards the biological activity is evident. All substance classes of the pollen like proteins, flavonoids and lipids can contribute to the biological activities.

Analytical C8 RP-HPLC-analysis together with agar diffusion tests (zone inhibition assays) shows that in fact proteins, peptides and derivates are the main origin of the observed biological activity. HPLC-chromatography of samples from different botanical origin showed similar basic chromatograms (fig. 2.3) with only a few peaks which were different in shape (fig. 2.4). This indicates a highly constant protein profile of royal jelly samples with some seasonal variations. Seasonal variations of protein profiles have already been observed (Bengsch et al. 2000; Sauerwald 1997; Bonomi

et al. 1986) before the large scale and systematic investigation of the correlations in the present study. The antibacterial tests of the reverse phase HPLC peaks (table 2.2, fig. 2.5) showed constant activity of some peaks. The activity of some other peaks however varied as a result of different botanical origins over the season.

3 Microbiological Effects

Summary

Royal jelly has antibacterial, antifungal and antiviral properties. Antibiotic screening tests with royal jelly and protein fractions thereof showed strong inhibitory antimicrobial activities against nosocomial pathogenic bacteria, fungi and viruses. Altogether more than 150 bacteria, several fungi and virus strains were tested. Activities against antibiotic resistant bacteria offer a great potential of applications. Antibacterial screening tests with catechins were used in the search of bacteria strains with high sensitivity for subsequent histochemical detection reactions and microscopic investigation. Accumulation of catechins in bacteria correlated with inhibitory activity. Plaque assays showed the antiviral potential of royal jelly against Coxsackie B3 virus.

3.1 Inhibitory activities of royal jelly against pathogen, nosocomial bacteria

Introduction:

Apis mellifera and its plant environment constitutes a remarkable example of a co-evolution. It stands in the forefront of the biological systems and is capable to provide a multitude of active substances with pharmaceutical applicability. These molecules were widespread conserved in the evolution due to their fundamental importance. The impressive mechanic, metabolic and reproductive capacities of the bee and its anti-parasitic resistance in an environment of permanent overpopulation (50 000 bees are densely packed in a volume of 60 l) are the substantial evidence therefore.

Highly developed glands of the bee constitute 50 % of its total weight and establishes so a record in the living world. In this flying enzyme factory the collected plant substances receive immediately transformations with which they become adapted to the needs of the insect metabolism.

Firmly associated to its environment the system *Apis mellifera* with its vegetal partners of the ecto-symbiosis, the parasites and defense strategies of active antiviral

and immune stimulating substances constitutes the theme of the present investigations.

Among all investigated and disposal natural materials royal jelly offers probably the greatest potential to isolate new antibiotic substances: An enormous long evolution time of the system *Apis mellifera* lead to an evolutionary optimization. Highly active compounds are disposal in large quantities of the natural raw material.

It was the aim of this work to develop and select highly optimised royal jelly samples. Approximately 150 bacteria and fungi strains, thereof 70 human-pathogen strains were tested with soluble and insoluble protein fractions from royal jelly and the natural raw material itself. Some of the tested bacteria cause particular problems in view of resistance against classic antibiotics. These bacteria are therefore under especial monitoring in the public health system. The present 25 families of clinical active antibiotic agents lose increasingly their activity (Levy 1998; Jungkind 1996). This is caused by the increasing development of multi-resistances in bacteria and fungi (hospital, animal mast and human therapy effects). Since 25 years no basically new antibiotic came to a widespread commercial application (Levy 1998).

A further field of potential applications is the food preservation. Royal jelly is antifungal (Sauerwald 1997) and active compounds of royal jelly can be used to prevent intoxications with mycotoxins. Antimicrobial active substances from royal jelly can contribute to the substitution of classic chemical food preservatives with new unproblematic substances. Purpose of this study was to characterize the antibiotic potential of royal jelly and its protein fractions.

Methods and Materials

Origin of royal jelly samples (described in details in chapter 2.2):
Botanical defined royal jelly samples were produced systematically and in large scale in cooperation with professional apiaries. The royal jelly samples were essentially produced in St. André de la Marche, Vendée-Maine (France). Samples were stored under refrigeration temperatures (4-7°C). Two samples harvested 05/20/99 and 05/18/2000 were selected for this investigation.

The samples, obtained under defined conditions were provided from a wide hedge landscape without agriculture in large scale situated in the west of France, seated between Cholet, Nantes and La Roche sur Yon. The area covers the mainland basement of the Bretagne and the acidic magmatic rocks of the Vendée. The athmosphere is atlantic and has no local or cumulative environmental stress exposition. The world of wild plants is ecologically fully intact and offers optimal conditions to obtain efficient raw products, necessary for the present work.

Separation of water-soluble and water-insoluble royal jelly components (according to chapt.2.2) for screening tests of pathogen, nosocomial bacterial strains (tables 3.1, 3.2):

To separate the water-soluble supernatant from the insoluble pellet, the royal jelly sample was diluted threefold with distilled water (4°C) and centrifugated at 27 000×g, 15 minutes at 4°C. Afterwards the pellet was threefold washed with cold (4°C), distilled water to remove the water-soluble fractions. The supernatant was concentrated in a vacuum dryer to obtain roughly the original concentration (Sauerwald 1997; Sauerwald et al. 1998).

Agar diffusion tests (according to chapter 2.2):

For agar diffusion tests (inhibition zone assays) one drop of pure royal jelly, approx. 100 mg of the water-insoluble wet pellet and 40 µl the water-soluble supernatant were applied on agar plates. After 24 hours of incubation at 37°C the inhibition zones were measured in millimeter. For the pure royal jelly drop and the water-insoluble pellet the twofold distance of the inhibition zone from the solid sample was measured in millimeter by subtraction of the solid sample diameter from the inhibition diameter. For the supernatant the diameter was measured in millimeter (Sauerwald 1997; Stocker 1999). Several bacteria showed a durable, clear inner inhibition zone with no bacterial growth and a second outer inhibition zone with small growth of bacteria with slight growth of resitant bacteria (fig. 3.1). Inhibition zones were measured using transmitted light and zones with slight growth of several resistant bacteria as well as zones with total inhibition were taken into account for measurement of inhibitory diameters. Different diffusion capability of the active substances groups consitute also a reason for sporadic growth of bacteria in predominantly inhibited zones.

Organisms and media:

Bacteria growth conditions: bacteria were cultivated aerobically in Brain-Heart-Infusion (BHI) nutrient broth from Merck, Darmstadt, Germany (pH was adjusted at 7.4) and incubated at 37°C for 16 hours.

7 *Staphylococcus aureus subsp. aureus* strains (WS 2438, WS 2609, DSM 46320, CIP 100922; CIP 103514, CIP 103594 CIP 1038 II) were tested towards antibacterial activity of 3 royal jelly fractions:

-royal jelly,
-its water-soluble supernatant
-and its insoluble pellet (according to chapter 2.2)

with agar diffusion tests. Strains were cultivated aerobically in BHI nutrient broth at 37°C for 24 hours.

40 *Listeria monocytogenes* (table 3.3) reference strains were tested with two RJ samples from 05/20/99 and 05/18/00. Agar diffusion tests were carried out with one drop of two royal jelly samples. Most of the strains are medical isolates from foodborne epidemic diseases. Strains were cultivated aerobically in BHI nutrient broth at 37°C for 16 hours. Solid media contained 15 g/l agar agar. Soft agar contained 7 g/l agar agar and 2 g/l glucose; pH was adjusted to 7.4 for the used nutrient agar (BHI) before autoclaving. All media were autoclaved at 121°C.

Microbiological and rheological stability of royal jelly:

Royal jelly is a natural substance which has a shelf-life. The biologic activity decreases with the time depending on the storage conditions (temperature, oxygen). The temporal stability of the antibiotic activity against *Micrococcus luteus* DSM 348 was investigated within 6 months with varied temperature conditions under argon atmosphere.

The temporal trend of the microbiological stability of royal jelly samples was measured for the storage temperatures -80°C, -20°C, +4°C, +30°C as raw substance and lyophilized (-80°C) over a period of 6 months with agar diffusions tests und micro dilution assays (according chapter 2.2).

For the storage at +4°C within 6 months a decrease of activity could be observed. The storage at +30°C lead within few weeks to a substantial decrease of the activity, after 2 months storage the activity was completely disappeared. The deep-frozen, –20°C resp. –80°C stored samples showed roughly constant inhibition zones even after 6 months.

In addition to the antibacterial tests changes in rheological parameters were measured as a function of storage time. A royal jelly sample (05/17/1999) from Vendée-Maine (France) was stored at +4°C for rheological measurements. The used rotational rheometer was the shear stress controlled Rheometer SR 5000 from Rheometric Scientific, Munich (Germany). The tests carried out were an oscillation test to measure viscoelastic properties and a stress sweep test to measure viscous properties. A cone and plate geometry (40 mm Ø) was used for the shear rate test and oscillation test (according to Först 2001). The Cone Angle was 0.0394 Radians, the Gap 0.045 mm and the measurement temperature was held at 20°C with a peltier element. The volume of 0.670 cm^3 was filled with royal jelly and surplus material was removed. After a delay time before test of 120 seconds the measurement was started with an initial stress of 0.1 Pa and a final stress of 100 Pa. The oscillation test as a non-destructive measurement procedure was carried out immediately before the stress sweep test. For that measurement a constant strain of 0.2 % was applied in a frequency range between 10 Hz and 0.01 Hz.

The time and shear stress dependent viscosity was determined within 6 months with stress sweep test. The time dependent viscoelastic properties were determined with an oscillation test. Both are well established techniques (Senge and Annemüller 1995; Först 2001). The shear rate test gives the dependence of viscosity on the flow condition of the measured fluid as described by Weipert et al. 1993, Först 2001. The oscillation test gives information to the ratio of elastic to viscous characteristics of the fluid.

Results

a) Antibacterial tests:

Antibacterial tests of royal jelly and its protein fractions against a selected number of pathogen bacteria (Tables 3.1, 3.2 and 3.3) show the inhibitory potential of the protein fractions.

Table 3.1: Inhibitory properties of royal jelly samples on 28 selected pathogen bacteria: Royal jelly sample collected 05/20/1999 was investigated in two independent analyses.

Royal Jelly samples	05/20/ 1999	05/20/ 1999	05/18/ 2000	05/20/ 1999	05/20/ 1999	05/18/ 2000	05/20/ 1999	05/20/ 1999	05/18/ 2000
Bacteria	Royal Jelly (RJ) Twofold distance [mm]			RJ-supernatant diameter [mm]			RJ pellet Twofold distance [mm]		
Bacillus cereus WSBC 10028 (gram+)	12	8	4	12	12.5	12	2	0	2
Bacillus cereus T WS 1537 (gram+)	15	14	2	17	12	10.5	2	0	2
Corynebacterium diphteriae T; WS 3021 (gram+)	19	16	12	12	12	15.5	2	0	2
Enterococcus faecalis T WS 2331 (gram+)	3	2	2	11	11	11	4	2	2
Enterococcus faecium T WS 1052 (gram+)	3	4	2	14	11	12	3	0	2
Listeria momonocytogenes ScottA WSLC 1685 (gram+)	16	14	8	11	9	9	6	6	4
Listeria monocytogenes WSLC 1364 Vacherin Mont d'Or (gram+)	20	8	8	12	10	11	7	12	10
Staphylococcus aureus WS 2898 (gram+)	17	10	10	10	10	10	5	8	8
Staphylococcus epidermidis WS 2894 (gram+)	11	12	12	8.5	10	11	11	10	12
Streptococcus bovis WS 1050 (gram+)	4	8	6	10	9	16	4	0	0
Streptococcus pyogenes T WS 3154 (gram+)	8	8	8	25	8	15	6	4	0
Escherichia coli EHEC WS 2810 (gram-)	4	4	0	11	10	10	0	0	1
Escherichia coli EIEC WS 2585 (gram-)	11	4	0	11	10.5	10	6	0	4
Escherichia coli EPEC WS 2572 (gram-)	11	8	0	11	11	10	1	0	1
Escherichia coli ETEC WS 2581 (gram-)	8	6	0	11	11	10	1	0	1
Klebsiella pneumoniae T WS 1342 (gram-)	9	8	6	10	10	11	0	0	0

Klebsiella pneumoniae WS 2900 (gram-)	1	4	0	10	10	10	0	0	0
Pseudmonas aeruginosa T WS 2062 (gram-)	1	0	0	9.5	10	10	0	0	0
Pseudmonas aeruginosa WS 2457 (gram-)	4	2	0	8.5	10	9.5	0	0	0
Salmonella infantis WS 2700 (gram-)	18	14	0	11	10	10	1	1	2
Salmonella indiana WS 2695 (gram-)	10	8	0	1	10	10	9	0	0
Shigella flexneri T WS 2853 (gram-)	2	10	1	10	10	10	3	2	2
Shigella sonnei T WS 2854 (gram-)	2	10	2	9.5	10	12	2	0	0
Yersinia enterocolitica T WS 2860 (gram-)	23	28	8	12	10	11	17	10	0
Yersinia enterocolitica WS 2589 (gram-)	25	26	30	12	11	11	8	4	6

Table 3.2: Inhibitory properties of royal jelly samples on 7 *Staphylococcus aureus subsp. aureus* strains; Tests with 3 royal jelly fractions: royal jelly, the water-soluble supernatant and the insoluble pellet (sample 05/18/2000)

strain-number	Royal Jelly (RJ) Twofold distance [mm]	RJ supernatant diameter [mm]	RJ pellet Twofold distance [mm]
WS 2438 T	4	11	4
WS 2609	4	11	6
DSM 46320	8	14	8
CIP 100922	0*	11	6
CIP 103514	9	20	16
CIP 103594	8	12	2
CIP 103811	9	13	10

* inhibitory activity could only be observed below the sample

Table 3.3 Inhibitory effects of 2 royal jelly samples on 40 *Listeria monocytogenes* reference strains. Agar diffusion tests were carried out with one drop of royal jelly of each sample. Twofold distance of the inhibitory effect from the royal jelly drop was measured in millimeter [mm] by subtraction of the sample diameter

Origin (remark)	serotype	RJ 05/20/1999 Twofold distance [mm]	RJ 05/18/2000 Twofold distance [mm]
SLCC 2372 / ATCC 19112 / WS 2247	1/ 2c	12	6
SLCC2373 / ATCC 19113 / WS2248	3a (4b)	12	6
SLCC 2375 / ATCC 19115 / WS2249	4b (1/2b)	14	6

SLCC 5633 / WS 2250	4b	10	6
SLCC 5634 / WS 2251	1/ 2a	12	9
NCTC 7973 / WS 2252	1/2a	12	6
SLCC 5635 / WS 2261	1/2a	8	4
SLCC 5778 (R-Form) / WS 2263	4b	8	5
SLCC 2378 / ATCC 19118 /WS 2264	4e	10	3
SLCC 2376 / ATCC 19116 / WS 2265	4a	8	4
SLCC 2374 / ATCC 19114 / WS 2266	4a (4c)	10	4
SLCC 5758 / WS 2274	1/ 2a	9	10
SLCC 5782 (R-Form) /WS 2275	1/ 2a	8	2
SLCC 2755 / WS 2276	1/2b	9	4
SLCC 1694 / WS 2277	3b	9	6
SLCC 2479 / WS 2278	3c	8	4
SLCC 2377/ ATCC 19117/ WS 2279	4d	8	2
SLCC 2482 / WS 2280	7	8	6
ATTC 13932 /WS 2300	4b	4	5
ATTC 15313 (TS)/SLCC 53/ WS 2301	1/ 2a	9	4
ATTC 19111/ WS 2302	1/2a	10	6
ATTC 23074 / WS 2303	4b	8	6
ATTC 35152 (propsd. TS) / WS 2304	1/ 2a	10	10
SLCC 5764/(MACK) / WS 2308	1/2a	11	10
SLCC 1806 (in Rocourt Set) / WS 2309	4d	6	4
SLCC 1807 (in Rocourt Set)/ WS 2310	1/ 2c	12	10
SLCC 1090 (in Rocourt Set)/ WS 2311	4d	3	4
SLCC 1652 (in Rocourt Set) / WS 2312	4d	4	4
SLCC 2671 (in Rocourt Set) /WS 2313	4a	12	12
SLCC 8793 / WS 2332	4b	6	6
SLCC 8795 /WS 2333	4b	6	5
SLCC 8797 / WS 2334	1/ 2b	6	6
SLCC 8800 / WS 2335	1/2b	6	6
SLCC 8802 /WS 2336	4b	4	4
SLCC 8794 /WS 2337	1/ 2a	4	4
SLCC 8798 /WS 2338	1/ 2b	6	8
SLCC 8806 /WS 2339	1/ 2b	4	4

SLCC 8792 /WS 2340	4b	9	10
SLCC 8807 / WS 2341	4b	4	4
SLCC 8796 /WS 2342	1/ 2b	6	6

Altogether for the major part of the investigated 70 human pathogen bacteria strains of tables. 3.1-3.3 a massive antibiotic effect could be observed. As expected the effect profile, already obtained for non-pathogen bacteria (chapter 2), is confirmed: gram-positive bacteria strains are inhibited preferably and almost without expception (Sauerwald et al. 1998; Stocker 1999). Surprisingly also some gram-negative bacteria show clear inhibition, e.g. *Yersinia enterocholitica* Fig.3.1E, Tab 3.1)

Different test strains of the same microorganism are inhibited differently on subspecies level (Tab.3.1; 3.2, 3.3). Even for different serovar types clearly different inhibitons were observed as shown for *Listeria monocytogenes*.

Variously purified protein fractions (royal jelly raw material, water-soluble supernatant and insoluble pellet, RP-HPLC-fractions) have different effects. For the inhibitory effects of protein fractions shown in Table 3.1 and 3.2 solubility and diffusion of the active substances play a role and have to be considered. In the water-insoluble wet pellet high molecular protein polymers with poor solubility and hence slow diffusion capability lead to a total inhibition around the pellet (respectively around royal jelly complete material). The active substances in the water-soluble supernatant are well soluble and form therefore larger inhibition zones with sporadic appearing resistance development.

These effects have to be considered for the comparison of inhibitory effects from royal jelly, the water-soluble supernatant and the insoluble wet pellet thereof. The twofold distance of the inhibition zone from the solid sample was measured for the pure royal jelly drop and the water-insoluble pellet by subtraction of the solid diameter from the inhibition diameter. The active substances are diffusing from the gelatinous complete material and the wet pellet into the agar. For the supernatants the diameters of the inhibition zones were measured. The correlation between antibacterial activity and diameter of the inhibition zone is log-linear (Parente et al. 1995; Kayser et al. 1998). This is accordant to our observations.

In particular the residual water-insoluble protein pellet in the royal jelly separation has distinct and selective effects. The rigid protein polymers is effective in suspension and remains active over long time. The simultaneous effect of different royal jelly fractions indicate synergy between substances in the water-soluble supernatant and the insoluble pellet: single effects become potentiated and not only added together. So is for *Corynebacterium diphteriae* T (fig. 3.1; Tab. 3.1) the inhibition zone of royal jelly larger as the sum of soluble supernatant and insoluble pellet.

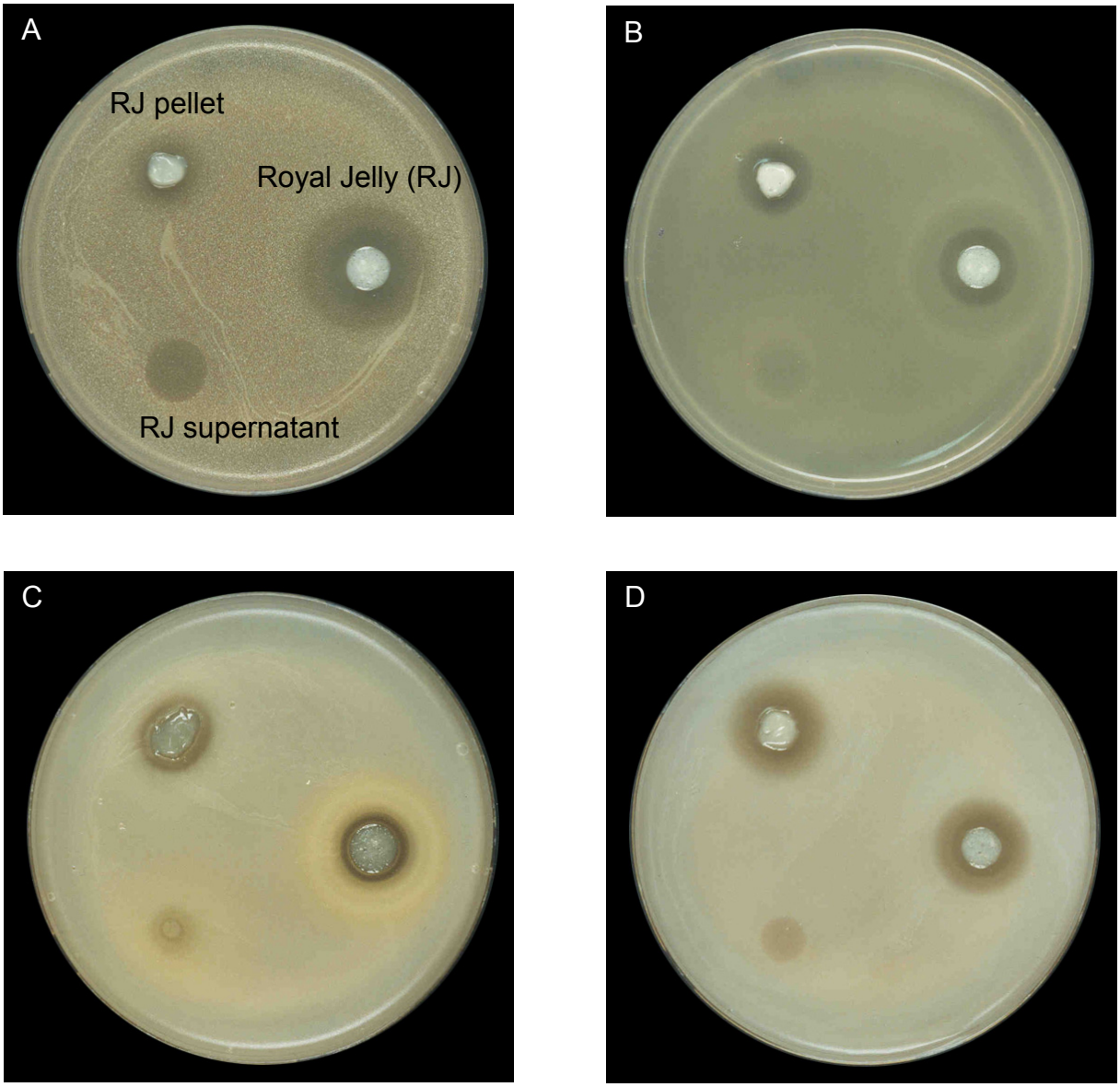

Figure 3.1A: Inhibitory activity of Royal Jelly (RJ), its water-soluble RJ supernatant and its water-insoluble RJ pellet against *Corynebacterium diphteriae* T WS3021 (A), *Listeria monocytogenes* WSLC 1364 Vacherin Mont d'Or (B), *Staphylococcus aureus* WS 2898 (C), *Staphylococcus epidermidis* WS 2894 (D).

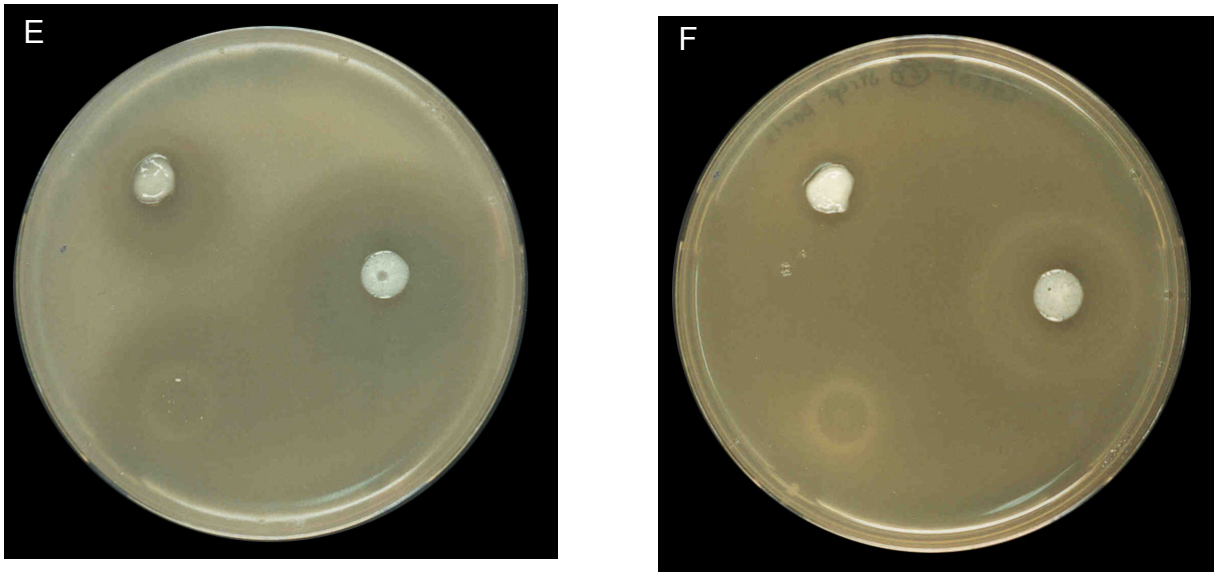

Figure 3.1B: Inhibitory activity of Royal Jelly (RJ), its water-soluble RJ supernatant and its water-insoluble RJ pellet against *Yersinia enterocolitica* T WS 2860 (E) and *Streptococcus bovis* WS 1050 (F).

Particular attention was given to the resistance development of commensal and pathogen bacteria, the potential nosocomial pathogen and here above all the septicaemic bacterial strains. Results with particular pharmaceutical relevance:

Corynebacterium diphteriae (gram+) (Fig. 3.1A; Table 3.1): the pure royal jelly raw material creates an inner inhibition zone, in which no resistance occurs. The concentric secondary inhibition zone derives from recovered resistance.

Listeria monocytogenes (gram+) (Fig. 3.1B; Tab. 3.1 and Tab 3.3): the poorly soluble pellet (protein polymer) shows here a particular high inhibitiory potential, for single fractions sharp concentration limits could be observed between enduring inhibition (primary inhibition zone) and recovered resistance (secondary inhibition zone; Fig 3.1B, water-insoluble pellet, pure royal jelly).

Staphylococcus aureus (gram+) (Fig. 3.1A; Tab. 3.1, Tab 3.3): the inhibitory action of the royal jelly components derive predominantly from water-insoluble substances: The protein-polymers in the pellets constitutes the main part of the activity.

Staphylococcus epidermidis (gram+) (Fig. 3.1D; Tab 3.1): the water-insoluble pellet (protein-polymer, above left) and pure royal jelly (right) show a distinct inhibition; the pure royal jelly shows a durable inner inhibition zone which is not found for the pellet. A synergy effect with the water-soluble proteins exists. The pellet should contain the poorly soluble 10-hydroxy-Δ2-decenoic acid (10-HDA) in addition to water-insoluble protein polymers. For this reason we have shown that in addition to the 10-HDA also proteins are involved in the antimicrobial effect.

Yersinia enterocolitica T (gram-) (Fig. 3.1E): the water-soluble protein fraction (below left) and the royal jelly raw substance (right) form large inhibition zones in the dimension of commercialised antibiotics. Thus, *Yersinia enterocolitica* is the most sensitive bacteria of the series, tested here.

Streptococcus bovis (gram+) (Fig. 3.1F): Pure royal jelly (right) shows the sum of the single effect of the water-soluble protein fraction (below left) and the insoluble pellet (above left): a narrow zone of durable transparent inhibition and a large secondary inhibition corona with recovered resistance.

b) Sugar plates of royal jelly:

Microbiological activity and composition of antibacterial active sugar plates in royal jelly: Royal jelly samples with stronger affinity to granulation and formation of plate-shaped sugarlike plates count at each case to the biological most active samples (Stocker 1999). Agar diffusion tests (according to chapter 3.2) with *Micrococcus luteus* as indicator-strain showed a high antibiotic activity of both the granulating royal jelly samples as well as the sugar plates. The poorly soluble sugar plates were two-fold washed with distilled water. Subsequent antibiotic tests showed a high antibacterial activity (Stocker 1999).

The washed sugar plates were analysed with thin layer chromatography using Merck silica gel 60 HPTLC-plates (glass, 10x10 cm) and silica gel 60 TC-plates (plastic, 20x10 cm). As elution liquid butanol (10)/ ethanol (100)/ water (3)/ acetic acid (10)/ pyridine (30) was used in the given volume proportions. The poorly soluble sugar plates were dissolved in bi-distilled water (concentration 5 mg/ml) and 50 respectively 150 µg of the sugar plate material was applied on the TC-plates. The staining with

orcinol / H_2SO_4 (20/80) showed a higher amount of glucose and a smaller amount of a fatty acid.

A second TC-plate was stained with ninhydrin respectively viewed under UV-light and proteins respectively peptides could be observed at the place of application. A subsequent analytical C8 RP-HPLC-analysis of the sugar plates (according to chapter 2.2) showed a still greater number of proteins in the sugar plates.

The melting point of the sugar plates was determined after intensive washing with ethanol with a heating-light microscopical element E 347 (E. Leitz-Wetzlar). A melting interval was observed and therefore a conglomerate of various substances (lipids, sugars, proteins) in the plates is also indicated. In our observations royal jelly samples from semi-artificial production (adulteration by additional feeding of corn sirup and proteins in opposite to natural, authenic bee products) showed only scarcely granulation respectively no sugar-like plates.

c) *Microbiological and rheological stability of royal jelly:*

From our results the oscillation characteristic shows that royal jelly is an elastic substance because the storage modulus is always higher as the loss modulus. The storage modulus describes the elastic properties and was roughly independent from the frequency although slightly increasing with the frequency. This indicates a disperse structure. The loss modulus runs a minimum in the investigated frequency range.

The stationary flow behaviour shows that the behaviour of royal jelly is strongly non-newtonian, this means that the viscosity decreases strongly with increasing shear rate. The viscosity changes in the investigated shear rate range at 5 orders of magnitude.

The characteristic over the storage time shows that the consistency factor, an admeasurement for the viscosity, increases with the storage time; this means that royal jelly becomes more viscous. The flow index, an admeasurement for the divergence of newtonian characteristic, decreases with the storage time. This means the royal jelly becomes more shear thinning. The storage modulus, which describes the elastic characteristic (aroused from polymeric macro-molecules), decreases with storage time.

3.1.3 Discussion:

Antibacterial tests:

Due to the actual problematic of therapy resistant human pathogen bacteria screening tests with such bacteria were started to lead research also in this direction. A strong inhibition of pathogen bacteria could be shown. The results constitute a perspective to the potential to develop antibiotics against the medical relevant bacteria with the aid of the natural raw substance royal jelly.

As we have pointed out, the partly highly selective inhibition (e.g. for *Yersinia enterocolitica*, *Staphylococcus aureus*, Tab. 3.1 and 3.2) is in some cases in the dimension of therapeutic antibiotics. Therefore this generation of antimicrobially active substances have potential applications where classic antibiotics are ineffective in consequence of resistance developments.

The septicaemic bacterial strains are responsible for more than 50 % of the cases of death in the intensive care of the hospitals e.g. *Staphylococcus aureus*, *Streptococcus ssp.* (Tab. 2.1, 2.3). A further field of application are commensal bacteria, which became pathogen after phage infection: killer bacteria.

pH-effects can not be fully excluded. Royal jelly is relatively acid (pH 3.9-4.1) and has a high buffer capacity in the pH region between 4 and 7 (Sauerwald 1997; Sauerwald et al.1998). As previous investigations have shown (Stocker 1999) these effects were negligible small for individual exemplarily investigated gram-positive bacteria (*Micrococcus luteus*) as well as also for gram-negative bacteria (*Escherichia coli*). Agar diffusion tests (inhibition zone assays) with pH-adjusted water-soluble royal jelly supernatants from pH 3 to pH 8 showed constant inhibition of *Micrococcus luteus* and *Escherichia coli*. As shown for *Pseudomonas fluorescens* (Stocker 1999) for several bacteria a pH-dependence has to be considered. *Pseudomonas fluorescens* was not inhibited from water-soluble royal jelly supernatants from pH 5 to pH 8.
A further indication in this direction are the test results of table 3.3 where royal jelly samples from the same origin and identical pH-value have very different inhibitory properties although the pH-values are identical. The royal jelly samples from different harvesting date (05/20/1999 and 05/18/2000) but the same pH-value show different activities on one and the same agar test plate.

The distinct antibacterial activity of the water-soluble fractions and derivates (HPLC-fractions) show the involvement of peptides and proteins. The effects of peptides and proteins occur independent from the poorly soluble 10-hydroxy-Δ2-decenoic acid (10-HDA) whose antibacterial and antifungal effects were described (Takenaka 1987; Bloodworth et al. 1995). Takenaka (1987) identified thirteen carboxylic acids in royal jelly. The bacteriostatic activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* was greater for 10-HDA as for the other C_{10} carboxylic acids (Takenaka 1987). A contribution of the 10-HDA which is precipitated in the water-insoluble pellet to the activity of royal jelly complete material and its pellet is therewith not questioned. However, the contribution should be less important as expected.

Earlier investigations restricted the antibacterial activity of royal jelly exclusively to the 10-hydroxy-Δ2-decenoic acid (Bogdanov 1999). Newer investigations (chapter 2; Stocker 1999; Sauerwald et al. 1998) show however, that also proteins play a role in the antimicrobial activities. The investigations of the present work (chapter 2 and 6) show, that peptides as proteolytic fragments of royal jelly proteins contribute to the antibacterial activity of royal jelly.

For fungi also highly selective inhibitions are described. For example *Fusarium species* are totally inhibited from royal jelly protein fractions, *Aspergillus niger* however is stimulated (Sauerwald 1997; Sauerwald et al. 1998). From our results tests of water-soluble protein fractions of royal jelly against *Aspergillus niger* showed no inhibitory activity. As fungi release mycotoxins in foodstuffs, the present investigations interfere also in the food technological sector (food hygiene, intoxic food preservatives).

Further investigations (Bachanova et al. 2001) describe the antibacterial activity of a peptide fragment from the insect defensin Royalisin against 2 strains of the gram-positive bee-pathogen bacteria *Paenibacillus larvae* and 3 further gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus* and *Sarcina lutea*) and tests without effect with two gram-negative bacteria (*Escherichia coli* and *Serratia marcescens*). *Paenibacillus larvae* causes the larval honeybee disease American Foulbrood (AFB). These results are in accordance with our investigations (chapter 2 and 6). N-terminal sequencing (Edman degradation) of several antibacterial protein peaks in the

backward sector of the RP-HPLC-chromatogram (acetonitrile gradient 21-45%; 174 - 264 min in fig. 2.4, chapter 2) show the N-termini of Royalisin in different fractions isolated or as concomitant peptide with further proteins. The partition of this active peptide in several places suggest chemical modifications in the peptide royalisin like proteolytic cleavage, glycolysation and further modifications.

Bilikova et al. (2001) described that royalisin fractions in the concentration 180 µg/ml showed a clearly weaker inhibition against *Bacillus subtilis* as tetracyclin in the concentration 50 µg/ml. Additionally antifungal activity of a royalisin-fragment isolated with dialysis membranes against the fungus *Botrytis cinerea* was described (Bilikova et al. 2001). With agar diffusion tests the antibacterial activity also for this royalisin-fragment against the gram-positive bacteria like *Bacillus subilis, Sarcina lutea* and 2 *Paenibacillus larvae* strains were described. The tested gram-negative strains *Escherichia coli* and *Serratia marcescens* were not inhibited.

Royal jelly with its manifold inhibitory poperties offers a great potential of applications. A substantial contribution to the activity provide the proteins and their derivates, as shown in details in chapter 6.

Microbiological and rheological stability of royal jelly:
Royal jelly is a natural substance which has a shelf-life. From our results the biologic activity decreased with storage time and viscosity increased. The correlation of the increasing aggregation of proteins to macro-molecules with increasing storage time and the decrease of antibacterial activity, deriving from peptides and proteins is evident.
The decrease of the antibacterial activity dependent on the storage temperature is accordant to the results of an earlier study. Royal jelly was stored at +5°C, +37°C and –40°C for antibiotic tests with *Bacillus subtilis* and *Staphylococcus aureus* (Yatsunami and Echigo 1985) and decreasing activity could be observed for storage at +5°C. Antinelli et al. (2003) described diminishing amounts of 10-hydroxy-Δ2-decenoic acid depending on the storage temperatures. Chinshuh and Soe-Yen (1995) reported changing relative amounts and molecular weight distributions of water-soluble protein components during storage. Browning intensity in royal jelly during storage is ascribed to the Maillard browning reaction. The degradation of a 57-

kDa protein in proportion to both storage temperature and storage period was described by Kamakura et al. (2001). The mitogenic activity of the 57-kDa protein on rat hepatocytes is lost after royal jelly storage at 40°C for 7 days.

3.2 Visualisation of flavan-3-ols in bacteria and lymphocytes - inhibitory effects of flavan-3-ols in bacteria and accumulation in lymphocytes

Introduction

Flavanoids are secondary metabolites in the phenylpropanoid-pathway. They consitute a main substance group in the infection control in biological systems. Flavonoids play a key role on different levels of plant-microbe interactions. These pathogen and symbiontic interactions lead to specific host reactions of the plant. Their genetic regulation in cell compartiments, affected by infection (Casuarina glauca / Frankia symbiosis) was described for flavan-3-ols (Laplaze et al. 1999). Activities of flavan-3-ols against fungi causing diseases in fruit crops (*Prunus domestica* and *Pyrus avium*) due to accumulation of catechins in the affected plant cells are reported by Feucht and Treutter (1999). As antibacterial and antiviral protective mechanism of the plant 5000 flavonoids are already detected and offer a great potential of application. The flavonoids play an important role in the disease resistance and have manifold own activities against micro-organisms. New molecules of the flavonoid-class are permanently discovered. These protection principle migrate in the food chain and plays a great role for vertebrates (human) and invertebrates (e.g. insects). For the health of social insects with high population density like *Apis mellifera* the flavonoids from bee products like propolis, honey, wax and royal jelly play a decisive role (König and Dustmann 1985 and 1989).

In order to assess the potential role of bee products also other flavononoid classes can be detected with histochemical detection reactions. The field of investigations can be extended using further staining methods e.g. Neu's reagent for flavones and their glycosides (Laplace et al. 1999, Neu 1956) with subsequent epifluorescence observations. Wilson reagent can be applied for the detection of various flavonoid compounds by histofluorescence (Hariri et al. 1991). Sarkar and Howarth (1976) reported the employment of a vanillin-HCl reagent for analysis of catechins and condensed tannins. Application of the above mentioned further histochemical staining reactions with the described combination of antibacterial tests and stainings can offer a great potential in the characterisation of the biological role of further flavanoid compounds.

The different inhibitory sensitivity of micro-organisms to the different flavanoids can be investigated specifically with selective staining. The substance group of the flavan-3-ols (catechins) play a great role in the antibacterial defense of the plant and transmit these activities also to the human nutrition. These catechins can be detected by a highly specific staining with p-dimethyl-aminocinnamaldehyd, the DMACA-staining (Feucht and Treutter 1989 and 1999; Treutter and Feucht 1990) and subsequent light-microscopical analysis. The staining sensitivity is high and indicates of the amounts of absorbed flavan-3-ols in bacteria. The field of pharmaceutical applications of this investigation method can be extended to further stainable cells like lymphocytes (fig.3.1C).

Methods and Materials

Agar diffusion tests and micro dilution assay were used for the determination of antibacterial activities as described in chapter 2.2. Histochemical stainings of bacteria and lymphocytes with 4-dimethylaminocinnamaldehyde (DMACA) after incubation with catechins were used as visualization method according to Feucht and Treutter 1989 and 1999; Treutter and Feucht 1990.

Commercially disposable flavan-3-ols (catechins) were exemplarily used as model substances for antibacterial tests and histochemical stainings because of their specific blue colour reaction. (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechingallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin-gallate (EGCG) were received from Roth, Karlsruhe, Germany.

Agar diffusions tests with catechins in aqueous solutions (in 17-33% ethanol) were used according to chapter 2.2 to detect highly sensitive bacterial test strains for subsequent histochemical staining (tab. 3.4 and 3.5). The catechins were dissolved and applied immediately. Aqueous solutions (17% resp. 33% ethanol) without catechins showed no inhibitory effects. Bacteria of table 3.4 were incubated in Standard1-medium, the bacteria of table 3.5 in BHI-medium.

Incubation of bacteria in flavan-3-ol solutions before chemical reaction detection with 4-dimethylaminocinnamaldehyde (DMACA):

500 µl overnight culture of *Micrococcus varians* TMW 1.121 was centrifugated at 2200 x g for 5 minutes and the pellet was washed twice with 1 ml 0.89% NaCl-solution to remove extracellular proteins of the nutrition broth. Then, bacteria were kept in 500 µl 0.89% NaCl-solution with 1.1 mg EGCG respectively 10 mg/ml EC in (50% ethanol/NaCl-solution) and shaken 5 hours (30°C; 130 RPM) with a Braun Certomat® H shaker . Bacterial cells were washed twice with 1 ml NaCl-solution to remove extracellular catechins. Preliminary tests with 12 fungi and yeasts, incubated with EGCG, were carried out in order to develop the DMACA detection reaction also in this direction.

Mononuclear human lymphocytes were also incubated with EGCG and washed four times with 1 ml NaCl-soution after EGCG-incubation. Additionally to EGCG, also samples of red wine were used for incubations. The lymphocytes were centrifugated at 90 x g for 10 minutes.

Source of mononuclear lymphocytes
Lymphocytes (peripheral blood mononuclear cells, PBMC) were isolated from blood by density gradient centrifugation using Ficoll and heparin. The lymphocytes were resuspended in RPMI 1640/10% FCS. The isolate was produced by the institute of immunology of the Ludwig-Maximilians-Universität München (head: Prof. Dr. G. Riethmüller) in cooperation with the Connex GmbH (München-Martinsried, Germany) and put at our free disposal. The isolate contained 1.0 x 10^7 cells/ml and was immediately used after reception.

Results and Discussion:
Investigations concerning the antibacterial activity of the flavan-3-ols (-)-epigallocatechin-3 gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (+)-catechin (C), and (-)-epicatechin (EC) showed a high substance specific antibacterial activity of several catechins on various bacteria (table 3.4 and 3.5). The antibacterial activity of the catechins, tested with agar diffusion tests (inhibition zone assays) and micro dilution assays was furthermore very strain specific and ranged from strong inhibition to complete resistance of the bacteria. In accordance of agar diffusion tests and microdilution assays *Micrococcus varians* was the most sensitive strain of the test series (Minimal Inhibitory Concentration, MIC= 10 mg/l).

Micrococcus varians was approximately 10-fold more sensitive towards EGCG as *Micrococcus luteus*.

Table 3.4: Inhibitory properties of flavan-3-ols (catechins)

bacteria	**EGCG** Diameter [mm]	**EGC** Diameter [mm]	**C** Diameter [mm]
Micrococcus varians TMW 2.121	20	4	11
Micrococcus luteus DSM 348	11	3	3
Alcaligenes eutrophus DSM 531T	12	4	7
Escherichia coli K12JM83	12	0	0
Enterococcus faecalis WS 1028	8	0	7
Bacillus licheniformis DSM 13	6	0	0
Bacillus subtilis DSM 347	6	0	6
Pseudomonas fluorescens DSM 50106	4	4	7
Lactobacillus plantarum CTC 305	5	0	0
Leuconostoc mesenteroides DSM 20193	3.5	0	0
Erwinia carotovora TMW 2.20	3.5	4	7
Lactococcus lactis ssp. diacetylactis LTH 2034	0	0	0

Below-mentioned amounts of flavan-3-ols were tested in aqueous solutions (17-33% ethanol): (+)-catechin (C) 300 µg; (-)-epigallocatechin (EGC) 60 µg; (-)-epigallocatechin gallate (EGCG) 113 µg.

Table 3.5: Inhibitory properties of flavan-3-ols (catechins)

Bacteria	**EGCG** Diameter [mm]	**ECG** Diameter [mm]	**C** Diameter [mm]
Bacillus cereus	9	12.5	6
Bacillus cereus T	9.5	12	0
Corynebacterium diphteriae T	7	10	0
Corynebacterium urealyticum T	0	0	0
Enterococcus faecalis T	6	10	9
Enterococcus faecium T	3	9	9
Escherichia coli EHEC	3	6	4
Escherichia coli EIEC	4	8	4
Escherichia coli EPEC	2.5	6.5	4
Escherichia coli ETEC	4.5	8	7

Klebsiella pneumoniae T	3.5	5.5	6.5
Klebsiella pneumoniae	3	0	5.5
Listeria momonocytogenes Scott A	4	7.5	0
Listeria monocytogenes	5	9	7
Pseudmonas aeruginosa T	4	7.5	3.5
Pseudmonas aeruginosa	4	0	5
Salmonella infantis	4	8	7
Salmonella indiana	3.5	7	5
Serratia marcescens T	4	7.5	5
Shigella flexneri T	4	9	0
Shigella sonnei T	4	10	5
Staphylococcus aureus	5.5	7	0
Staphylococcus epidermidis	3	6	0
Streptococcus bovis	0	0	0
Streptococcus pyogenes T	10	12	0
Yersinia enterocolitica T	5	8	0
Yersinia enterocolitica	5	8	6

Below-mentioned amounts of flavan-3-ols were tested in aqueous solutions (17-33% ethanol): (-)-epigallocatechin gallate (EGCG) (93.8 µg); (-)-epicatechin gallate (ECG) (127.5 µg); (+)-catechin (C) (327 µg). Amounts of 70 µg (-)-epicatechin (EC) showed no inhibitory effects.

The histochemical determination of the catechins in bacteria showed the accumulation of the catechins in bacteria. The intensity of the staining, associated to the catechin absorption is just as specific as the antibacterial activity. *Micrococcus varians* was the most sensitive indicator strain of the test series and also the most coloured. *Micrococcus varians* constitutes in the DMACA-staining reactions an excellent detection system for the absorption and accumulation of catechins. Figure 3.1A and fig. 3.1B show DMACA-stainings of *Micrococcus varians* after EC-incubation (fig. 3.1A) respectively after EGCG-incubation (fig. 3.1B).

The absorption of catechins (EGCG) was also successfully applied on the visualisation of flavanols in human lymphocytes (fig. 3.1C). For catechin (C) immunoenhancing effects on macrophages and on the T-cell system are described (Rauch 1986). For epigallocatechin gallate (EGCG) mitogenic activity is described for B cells (Zenda et al. 1997; Hu et al. 1992). This combination of antibacterial tests and

specific histochemical stainings could also be used for the detection of other substance-classes of the flavonoid-pathway. As shown for the stained lymphocytes (fig. 3.1C), these visualisation reactions offer a multitude of applications as selective and diagnostic detections of substance classes involved in different biologic processes.

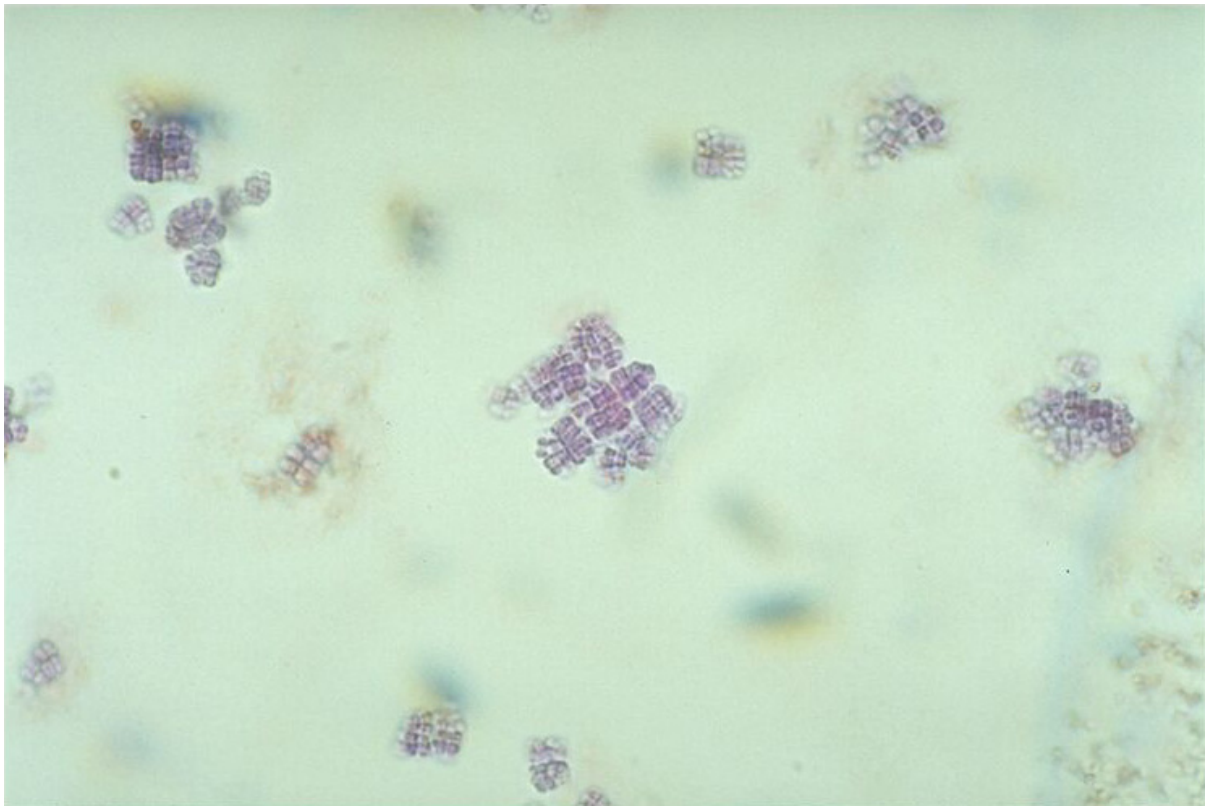

Figure 3.2A: Histochemical stainings of flavanols (catechins) with p-dimethylaminocinnamaldehyde (DMACA) according to Feucht and Treutter (1989 and 1999): *Micrococcus varians* incubated with (-)-epicatechin (EC); (photographed by W. Feucht)

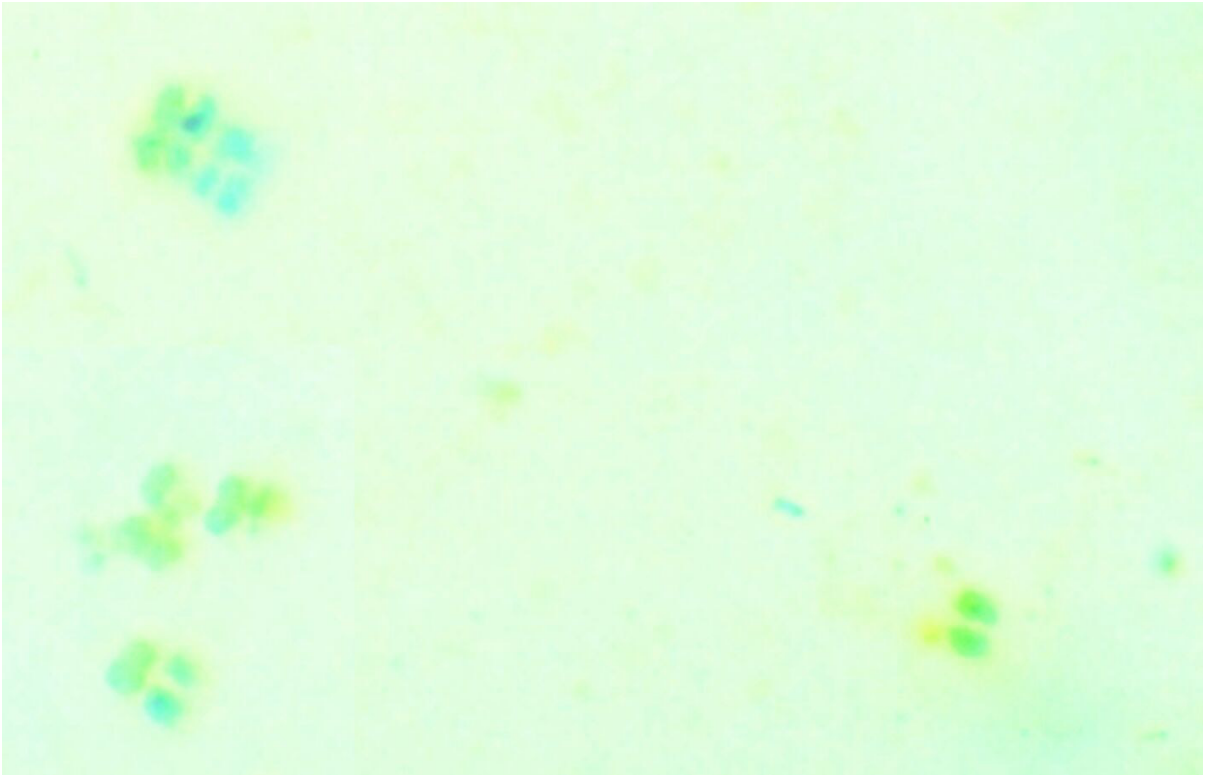

Figure 3.2B: DMACA-staining of *Micrococcus varians* incubated with (-)-epigallocatechin gallate (EGCG); (photographed by W. Feucht)

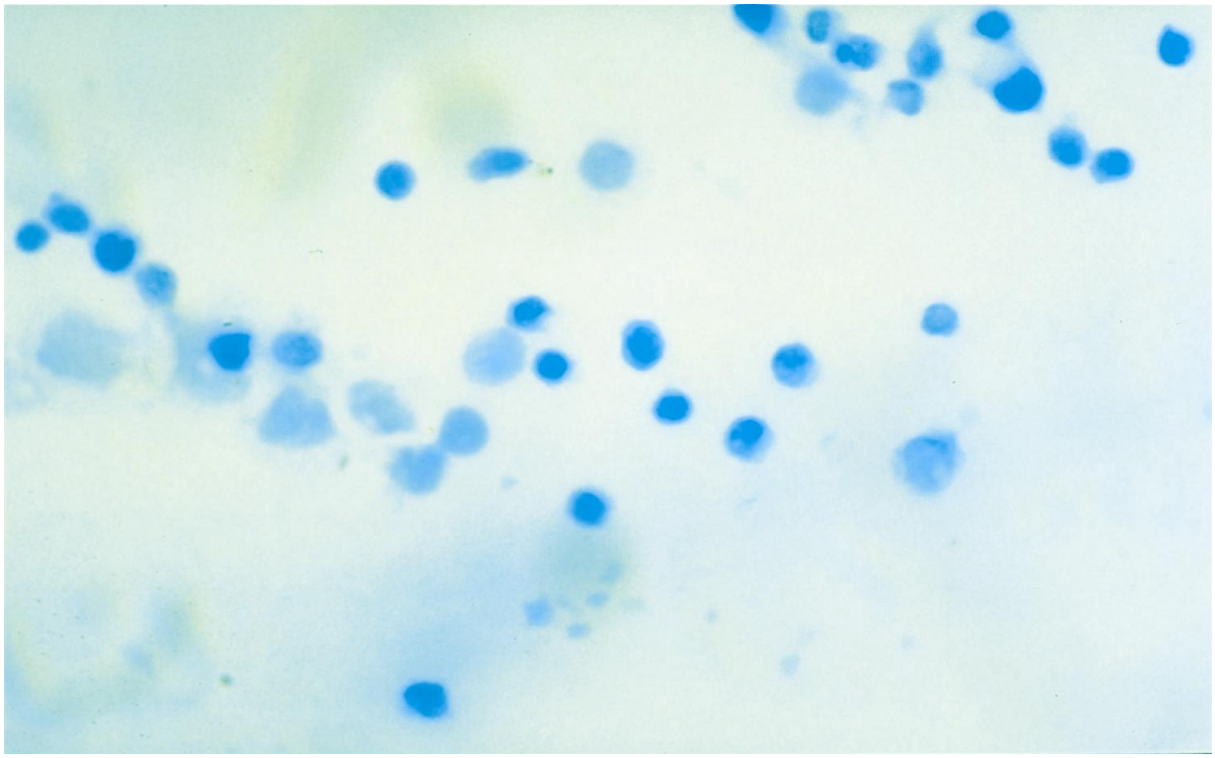

Figure 3.2C: DMACA-staining of mononuclear human lymphocytes incubated with (-)-epigallocatechin gallate (EGCG); (photographed by W. Feucht)

3.3 Extension of the investigated antimicrobial activities on viral infections

Introduction

The manifold biological activity of royal jelly (RJ) is not restricted to gram-positive and gram-negative bacteria but also active in the domain of fungi and virus. As at the moment few antiviral drugs without cell toxic adverse effects exists, the extension of the investigations to the virology is of particular interest. Supplementary to the above mentioned studies these aspects were investigated selectively on viral infections in vitro and in vivo.

Apis mellifera produces royal jelly (RJ) which is an approved system for the control of viruses. For therapeutic use of royal jelly no adverse effects are known. With RJ at least the development of the symptoms is suppressed. Thus, the affected host can realise a normal life and reproduction cycle in presence of the virus which is disposed of its virulence.

Royal jelly takes care that the infective agent cannot recapture the terrain with development of resistances and resurgence of virulent strains. The development of resistance can be prevented with a kind of poly-therapy with a multitude of related molecules. RJ is the key substance in the antimicrobial function of the system *Apis mellifera*. Royal jelly is the nutrition of all bee larvae within the first days of larval stage. For the queen bee royal jelly is the exclusive nutrition during their whole lifespan. The proteinaceous jelly is also transferred to other adult worker bees and drones (Crailsheim 1991 and 1992).

Influenza-virus A2 (orthomyxo-virus) and the rhabdo-virus (bullous stomatitis) were significantly controlled with the described royal jelly sorts in the animal (mouse) experiment (Bengsch; unpublished).

Suggestively following preliminary experiments in the human domain should be indicated: influenza B (orthomyxo-virus, non-pandemic influenza), herpes-2 virus (herpes-virus), hepatitis B virus (hepadna-Virus), as well as rudimentary certain retro viruses and HTLV-2-virus (sickle cell anaemia).

Virus and antiviral substances can transit between plants and insects: Secondary metabolites which perform the antiviral defense of the plant are transferred to the insects. The antiviral protection crosses the interface plant / insect: it is particularly referred to insects which collect the pollen, together with the stigma surface the boron-richest component of the plant.

The single bee is in the most cases permanently unapparent cerebrally infected with numerous virus, particularly the ubiquitary prevalent paralysis-virus (APV), without any appearance of symptom expressions under normal conditions. If the bee colony gets a pollen deficiency, that means the precursor product to obtain the antiviral protein molecules is no longer disposable, or they are drawn from parasites (the mite *Varroa jacobsoni*) from the hemolymph of the normally protected bee, the activated virus penetrates the brain-gland barrier and infects the larvae over the protein deficient breeding jellies. The whole bee colony gets gripped by the epidemic.
New pollen supply or where applicable the elimination of the hemolymph-drawing parasite lead to the breakdown of the epidemic and the rapid regeneration of the bee colony. Thereby certain pollen species are more efficient as others. The specific effect of certain pollen in the bee colony correlates with the achieved effects in-vitro and in-vivo of the resulting royal jellies.

Methods and Materials

Virus/host systems were used for investigations in-vitro as well as in-vivo, a selectively high anti-viral activity was detected for royal jelly samples effectuated on the base of *Crategus oxycantha* and *Genista tinctoria* pollen. Plaque assays for plaque purification of CB3 virus (Nancy strain) and virus titration were carried out in petri dishes with transfected Vero cells and HeLa cells as described by Kandolf et al. 1985, Kandolf and Hofschneider 1985, according to established techniques (Melnick et al. 1979).
Plaque assays of Coxsackie B3 virus on confluent monolayer of HeLa cells and Vero cells were applied with 200 ppm of royal jelly in the overlaying complete medium with 1% agarose (according to Kandolf et al. 1985) respectively without royal jelly as a control (Bengsch, Kandolf, unpublished).

Selection of the royal jelly raw material:

Floral selective royal jelly samples were produced based on more as 60 different melliferous plant species. Their effects were observed in the bee hive in which permanent unapparent viral infections occur without or with only minor symptom formation. Subsequently the antiviral activity from the royal jelly samples, deriving from floral dominated sources were tested in vitro and in vivo and selectively in the human domain.

Results and Discussion

Using the concentration of 200 ppm royal jelly in the DME medium (Dulbecco's modified Eagle's minimal medium), a total reduction of virus titer was observed for the HeLa cells (no infection plaques on the test plate). In the royal jelly treated Vero cells a visible but incomplete reduction of the CB3 virus titer was found.

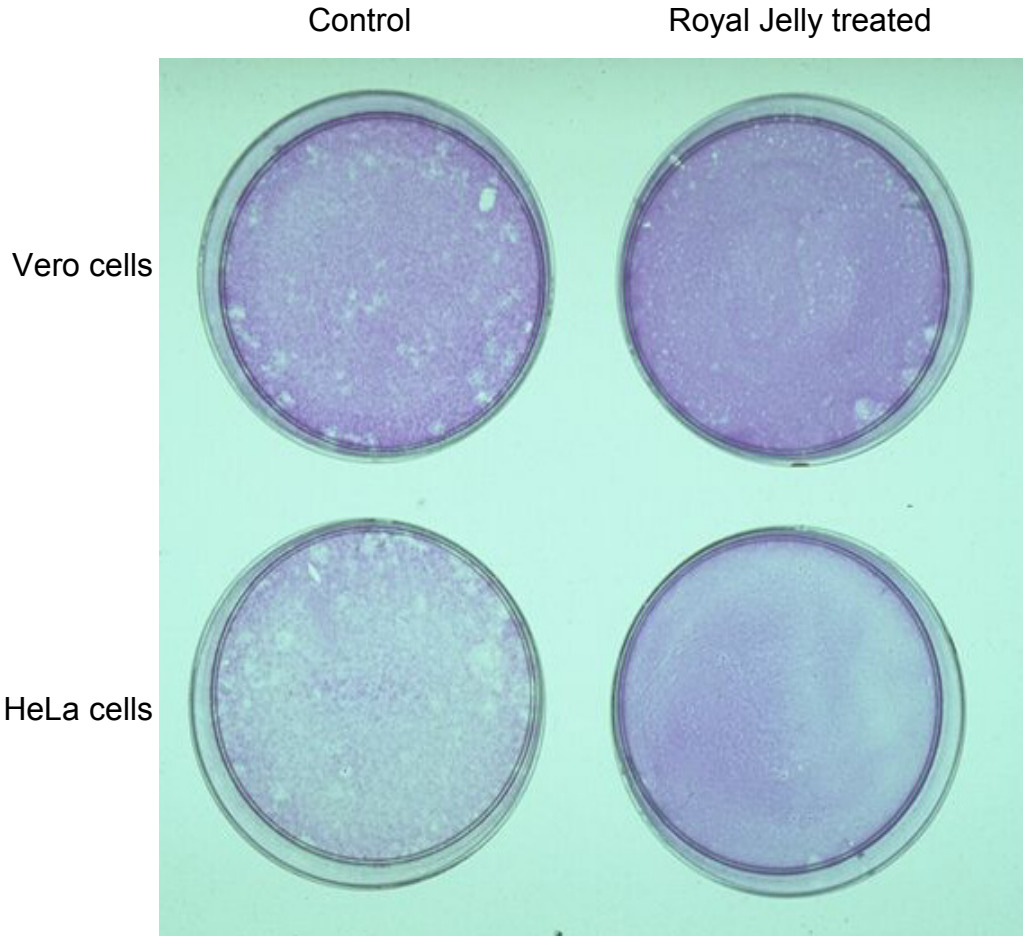

Figure 3.3: Plaque assay of Coxsackie B3 virus on confluent monolayers of Vero cells and HeLa cells. Inhibitory activity of royal jelly in the concentration 200 ppm and control plates without royal jelly are given (image from E. Bengsch and R. Kandolf).

In-vitro essays showed that the protein fractions of certain royal jelly sorts are active against the heart virus coxsackie B3 virus (CVB3) the herpes 2 virus and certain rhabdo and influenza virus. Numerous floral selective royal jellies show a enormous and very selective potential against such micro-organisms.

Finally also the bee hive itself was used as a virological test and experimental system. Among the floral dominated royal jelly samples several showed a surprisingly high antiviral activity: such with *Crategus oxycantha* (whitethorn) and *Genista tinctoria* (dyer's broom) as pollen and therefore as protein source. A serie of further plant species followed in a certain distance: *Brassica napus*, *Taraxacum officinale*, especially numerous *Raphanus species* and certain *Brassica species*, nearly exclusively wild plants. These phenomenons were already described in chapter 2, related to the antibacterial activities. For the antiviral activity the same trend of activity could be observed.

Based on a selected vegetation according to above mentioned criteria (chapter 2, optimal locations) that means growing on a soil which is rich in trace elements (chapter 4 trace elements, e.g. boron), we attained bee products which showed the investigated biological activities.

As for bacteria, the here investigated active molecules are also for the defense against viral infections of high interest. For the control of viral infections currently no satisfactory solutions are disposable. In the future a further aggravation of the problematic of viral infections can be attended. They are of particular interest in the perspective of permanently appearing new viruses, mutants and serotypes and their changeover between species, even between plant and animal (tospo viruses, filo viruses).

4 Contents of trace elements – homeostatic effects

Summary

Concentrations of inorganic compounds in royal jelly are of considerable interest from various perspectives. Trace elements have a multitude of known and unknown biological functions. Trace element concentrations of 28 elements in botanically and geographically defined royal jelly samples were analysed. Elements K, Na, Mg, Ca, P, S, Cu, Fe, Zn, Al, Ba and Sr in royal jelly were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES). Elements Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Mn, Ni, Ti, V, Co and Mo in royal jelly were determined by sectorfield ICP-MS. Concentrations of 14 trace elements were measured in the associated honey samples. In honey samples trace element concentrations of several elements (P, S, Ca, Mg, K, Zn, Fe, Cu, Mn, Si, B, Al, Ba, Sr) were strongly depending on botanical and geographical origin. The concentrations of several trace elements (P, S, Ca, Mg, K, Na, Zn, Fe, Cu, Mn) were highly constant in the associated royal jelly samples. Homeostatic adjustments of several main and trace element concentrations in royal jelly secreted by nurse bees for needs of bee larvae are evident.

4.1 Introduction

Royal jelly has a gelatinous consistency, a white-yellowish colour, is opaque and relatively acidic (pH 3.9-4.1) (Sauerwald 1997). It consists of approximately 66 % water, 15% sugars and 5 % lipids and 13 % proteins with a high content of essential amino acids (Lercker et al. 1993; Karaali et al. 1988).

The concentration of vitamins is high especially for the group of B vitamins B1, B2, B3 and B6 as well as PP and E (Lercker et al. 1984; Serra Bonvehi 1991). The 10-hydroxy-Δ2-decenoic acid concentrations of 1.4 to 6 % are the highest concentration among the royal jelly specific lipid substances (Bloodworth et al. 1995).

Furthermore, it contains minerals in the concentration range of 0.7 - 1.2% (Lercker et al 1984). Several elements (K, Na, Mg, Ca, Zn, Fe, Cu, Cr, Pb) were compared with the content of 10-hydroxy-Δ2-decenoic acid (10-HDA) in a study of 15 royal jelly samples from China (Serra Bonvehi 1991). Comparison of Ca and 10-HDA contents

in several royal jelly samples were useful to decide on question the authenticity of several samples (Serra Bonvehi 1991).

Elemental concentrations of K, Na, Ca, Mg, Cu, Fe, Mn, Co and Zn were investigated in pollen, nectar and royal jelly of the honey bee (Rudolf 1956, Nation et al. 1971, Matsuka 1980, Takenaka and Takashi 1980; Ivanov and Chervenakova 1983). Preliminary studies investigated K, Na, Ca, Mg, Cu, Fe, Mn, Zn and Cr in royal jelly samples from different regions in Italy with regard to the alimentation of the honey bee (Benfenati et al. 1986; Bonomi et al. 1986).

Trace elemental analysis of 94 honey samples from several defined botanical and geographical origins in France showed highly varying element concentrations depending on the geochemical character of (underlying) rocks in the region (i.e. rock type) and plant material (Bengsch 1992 and 1993). Investigations of trace element contents in multi-floral honey samples from 4 different regional origins in Northern Italy were described by Bontempo (2000). The contents of Al, B, Ba, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Rb, Sr and Zn in honey samples from four mountain valleys were given and significant differences in the concentrations of several elements depending on prevailing rock types were found (Bontempo 2000). Trace elements in honey samples from a large area in Hungary were useful as environmental indicators (Fodor and Molnar 1993). Higher levels of trace elements in pollen, honey and bee tissues were described for soils on volcanic rocks in Kenya compared to samples deriving from metamorphic rocks (Wamwangi et al. 2000). Trace elements Pb, Cd and Mn were analysed in honey samples of various seasonal origins - higher Pb contents were found for the summer months (Stein and Umland 1986).

The purpose of the present study was to investigate a greater number of main and trace elements in royal jelly samples by considerating botanical (seasonal) and geographical sample origins. We produced and investigated royal jelly samples from crystalline rocks, carbonate rocks and loess from several regions of France. A commercial royal jelly sample from semi-artificial production was analysed additionally. Comparisons of main and trace elements of royal jelly and associated honey samples was a further focus of the work as it has never been performed before.

Several trace elements are essential for nutrition and metabolism e.g. Fe, Mn, Zn, Cu, Mo, Co, as well as F, I, Se and Si for humans and animals and Cl and B for plants. Trace elements are integral part of enzymes, vitamins and hormones. Certain trace elements e.g. B and Si can protect the plant against viral infections without celltoxic effects (Bengsch et al. 1989a,b). Trace elements situations in royal jelly samples and associated honey samples can therefore be regarded as parameters of biological activities.

4.2 Methods and Materials:

Samples

Botanically and geographically defined royal jelly samples were produced 2001 in cooperation with apiaries in St. André de la Marche, Vendée-Maine (Bretagne), France, in Lugny, Picardie, Northeast-France, in Chézelles, Centre, France. A commercial royal jelly sample from China was investigated additionally. In cooperation with our apiarists in Europe we produced geologically (crystalline rocks, carbonates and loess) and botanically defined royal jellies. We produced seasonal royal jellies from Vendée-Maine from the same honeybee colonies with following botanical origins: *Castanea sativa, Trifolium repens, Rubus group*, *Brassica group, Pyrus group* (according to chapter 2). Royal jellies were kept at refrigeration temperature (4-7°C) until analysis. The sample from China was a commercial sample from high-yielding royal jelly production.

Botanically defined honey samples were produced in cooperation with apiaries in several regions of France. Provenances of the honey samples are equivalent to above mentioned royal jelly samples. Altogether 94 honeys were harvested and rationed into 16 different groups of botanical origin: garigue (i.e. scrubland), chestnut, forest, fir, calluna vulgaris L., basswood, multi-floral, alpine flora, erica, lavender, sunflower, thyme, rhododendron, rape, rosemary, lucerne. Honeys were kept under room temperature conditions until analysis.

Sample treatment

Royal jelly and honey samples were wet ashed with HNO_3 in pressurized PTFE bombs (Schramel et al. 1980). The samples were ashed in quartz tubes. The whole block is heated in a oven at 170°C. Approximately 500 mg of royal jelly respectively

1000 mg of honey were wet ashed with 1 mL HNO_3 (subboiling dest., Merck, Germany). The acids were subsequently diluted with approximately 10 ml Milli-Q H_2O (Millipore) to adequate sample solutions and analysed. In some cases further dilution was necessary.

Analytical instruments

Determinations of several elements (table 4.1) in royal jelly were carried out by double focusing magnetic sector field inductively coupled plasma mass spectrometry (SF-ICP-MS according to Schramel and Wendler 1998), using the ELEMENT1 (Thermo Finnigan MAT GmbH, Bremen, Germany) Pneumatic nebulization (Meinhard), a water cooled spray chamber (Scott type), a peristaltic pump (0.9 mL/min) and a sample changer ASX-400 (CETAC, USA) were used for sample introduction. The commercially available Ni-cones were used in the interface.

In cooperation with P. Schramel, who is the leading scientist in this field, I have shown concentrations of 28 trace elements in royal jellies. The elements Bi, Cd, Hg, Pb, Sn, Te, Tl, W and Sb in royal jelly (bulk material) were measured with SF-ICP-MS as described above at a resolution of 300 (i.e. performance of the separation). The elements Cr, Mn, Ni, Ti, V, Co and Mo were also measured with SF-ICP-MS at a resolution of 5500 (table 4.1). Error of a determination from a sample was less than 5 % (accuracy and precision).

Table 4.1: Elements in royal jelly and honey and methods used for analyses:

Samples	Methods	Elements
Royal jelly	ICP-OES	K, Na, Mg, Ca, P, S, Cu, Fe, Zn, Al, Ba, Sr
Royal jelly	ICP-MS Resolution of 300	Bi, Cd, Hg, Pb, Sn, Te, Tl, W , Sb
Royal jelly	ICP-MS Resolution of 5500	Cr, Mn, Ni, Ti, V, Co, Mo
Honey	ICP-OES	Al, B, Ba, Ca, Cu, Fe, K, Mg, Mn, P, S, Si, Zn, Sr

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used for measurement of the elements K, Na, Mg, Ca, P, S, Cu, Fe, Zn, Al, Ba and Sr in royal jelly samples (bulk material). Combined sequential/simultaneous spectrometers (JY70 and JY 66 ICP OES from Jobin Yvon S.A.S., Longjumeau cedex, France), 1.5

kW generator (Plasma-Therm), and computer system (DEC PDP 11/03) were used with a concentric pneumatic nebulizer for routine analysis (Schramel 1983). Error of a determination from a sample was less than 5 % (accuracy and precision).

All elements from honey samples Al, B, Ba, Ca, Cu, Fe, K, Mg, Mn, P, S, Si, Zn and Sr (Bengsch 1992 and 1993) were analyzed with ICP-OES as described above. Error of a determination from a sample was less than 5 % (accuracy and precision).
Analytical accuracy and precision in the analyses of several bio-medical reference materials as well as chemical and procedural blank values are described by Schramel et al. 1980, Schramel 1983, Schramel and Wendler 1998.

4.3 Results

Trace elements of 7 royal jelly samples from defined botanical and geological origins were analysed. Concentrations of 22 main and trace elements are given in tables 4.2 and 4.3. Concentrations of 6 ultratrace elements in royal jelly are given in the text. Concentrations of 14 trace elements of 94 honey samples from the same origins (Bengsch 1992 and 1993) were compared with the concentrations of the royal jelly samples. Elementary concentrations of royal jellies and associated honeys are graphically represented (figures 4.1 to 4.8).

The royal jelly sample 1 from Vendée-Maine (06/22/01) was analysed in solutions from two independent digestion of aliquots and trace element values are given as mean of two results ± difference to the mean value (table 4.2 and 4.3).

Concentrations of the main elements phosphorus (P), sulphur (S), calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) are roughly identical in the royal jelly samples (table 4.2, fig. 4.1 and 4.4). No significant difference could be found for main element concentrations in royal jellies from different botanical and geographical origins.

Concentration of the main elements P, S, Ca, Mg and K in the associated honey samples showed significant variability. Main element concentrations in honeys are varying within approximately one decimal power and have significant lower concentrations as the royal jelly samples. For the trace element concentrations of Si,

Al, Ba, and Sr in honey samples (fig 4.3) the very same high variations could be found, only boron showed small variations.

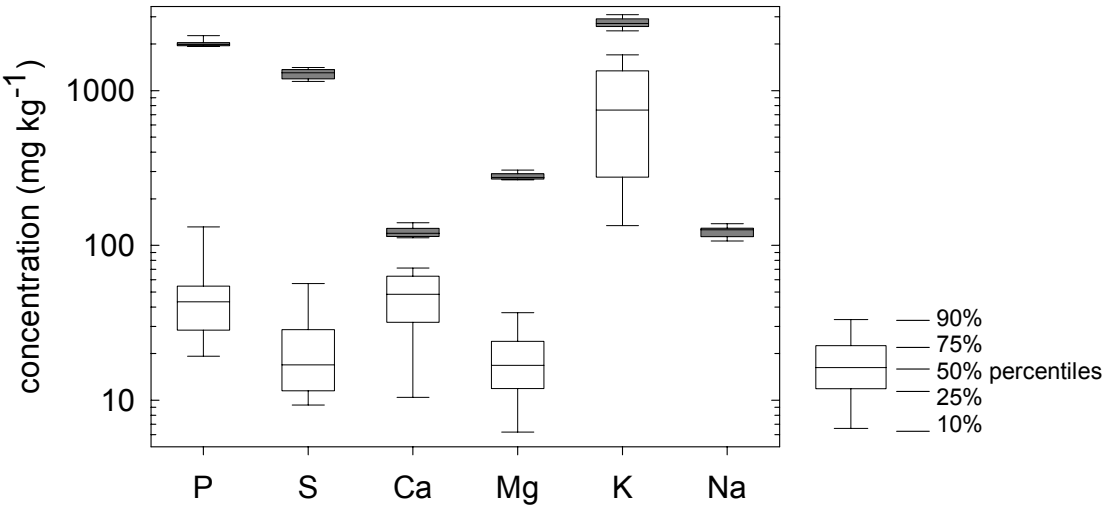

Figure 4.1A: Concentrations of main elements in royal jelly and honey samples of various seasonal and geographical origins. Shown are the 10 %, 25 %, 50 %, 75 %, and 90 % percentiles of P, S, Ca, Mg, K and Na concentrations in 7 royal jelly samples (grey bars), and in 94 honey samples (white bars). Na was only measured in royal jelly samples.

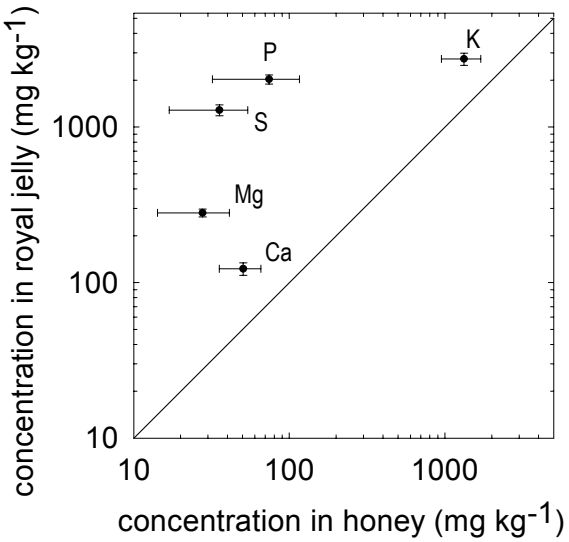

Figure 4.1B: Concentrations of main elements in royal jelly samples compared to the concentration of these elements in honey samples. Shown are means ± standard deviation of 7 royal jelly samples and 94 honey samples.

Thus concentrations of main elements in royal jellies are accumulated compared to the associated honeys. Factors of accumulation are approximately 20-fold for P, 20-fold for S, 5-fold for Ca, 10-fold for Mg and 1-fold for K (fig.4.1).

Aluminium (Al) was found in highly differing concentrations. As Al and Si are elements which are well known for exogenous contaminations with dust aerosols concentrations of Al and Si have to be regarded carefully. Values in the royal jelly samples between 54.7 mg/kg (tab. 4.2; sample 1) and 27.64 (sample 4) are most likely aerosol contaminations from former uranium mines in Vendée-Maine. Al concentrations of 8.49 mg/kg, 4.78 mg/kg and 1.62 are more reliable (table 4.2).

For the trace elements zinc (Zn), iron (Fe), copper (Cu) and manganese (Mn) a similar situation could be found as for the main elements (fig. 4.2). Zn concentrations in royal jelly are highly adjusted as observed for main elements and Fe, Cu and Mn show only a small range of variation. Mn is the only element with smaller concentrations in royal jelly samples as compared to the associated honeys. Factors of elementary accumulation in royal jelly are approximately 30-fold for Zn, 20-fold for Fe and 15-fold for Cu.

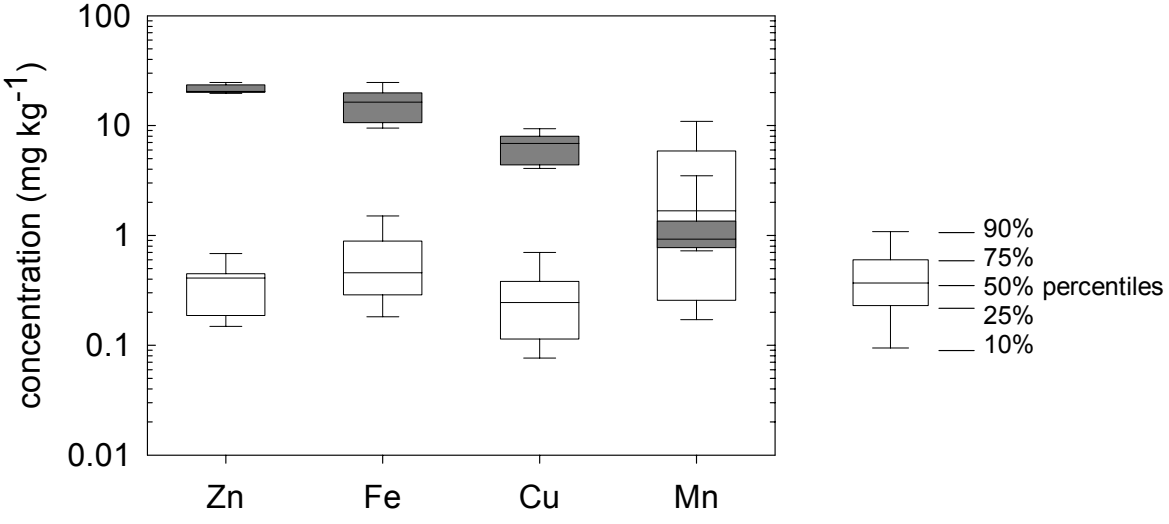

Figure 4.2A: Concentrations of trace elements in royal jelly and honey samples of various seasonal and geographical origins. Shown are the 10 %, 25 %, 50 %, 75 %, and 90 % percentiles of Zn, Fe, Cu and Mn concentrations in 7 royal jelly samples (grey bars), and in 94 honey samples (white bars).

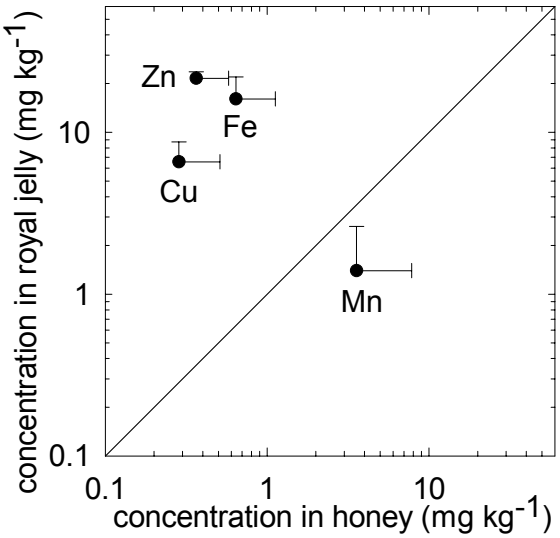

Figure 4.2B: Concentrations of trace elements in royal jelly samples compared to the concentration of these elements in honey samples. Shown are means ± standard deviation of 7 royal jelly samples and 94 honey samples.

In honey samples boron concentrations showed small variations (fig. 4.3) compared to concentrations of silicon, aluminium, barium and strontium:

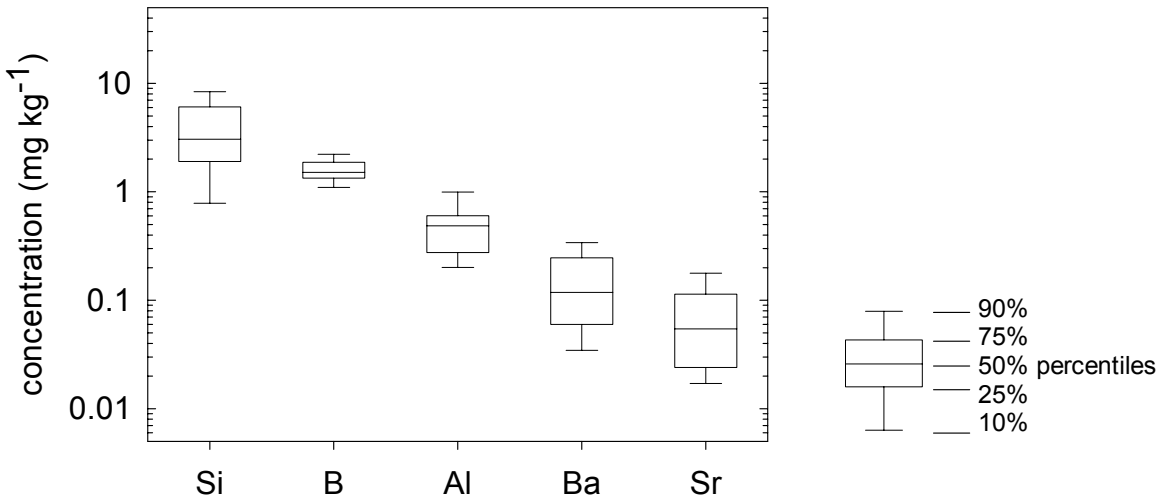

Figure 4.3: Concentrations of trace elements in honey samples of various seasonal and geographical origins. Shown are the 10 %, 25 %, 50 %, 75 %, and 90 % percentiles of Si, B, Al, Ba and Sr concentrations in 94 honey samples.

For chromium (Cr) and nickel (Ni) concentrations in royal jelly samples higher variations were observed (table 4.2, fig. 4.4) . For inconstancy of concentrations and reproducibility of measurement also contamination with the extraction tool which is well-known for Cr and Ni can play a role.

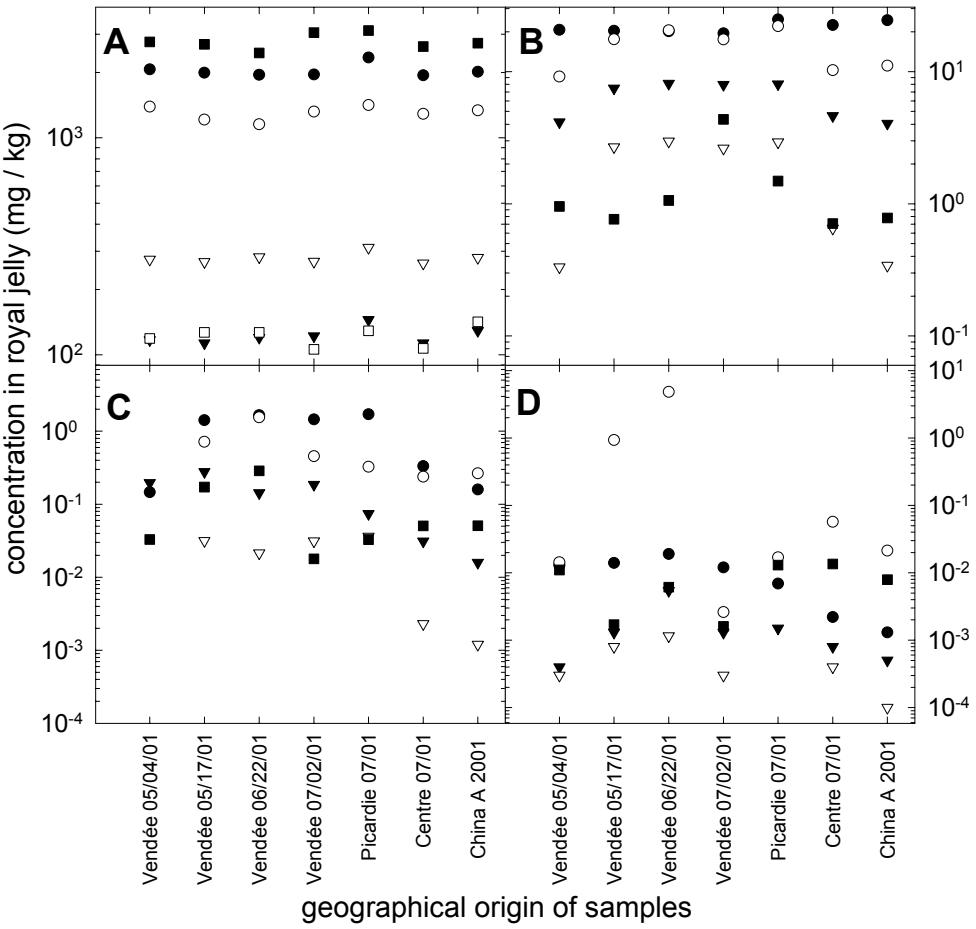

Figure 4.4: Concentrations of main and trace elements in 7 royal jelly samples of different geographical origins. The royal jelly samples from Vendée (France) had the very same regional origin but had different seasonal harvesting dates (i.e. botanical origin). Concentrations are given in section

A: elements P ● , S ❍ , Ca ▼ , Mg ▽ , K ■ and Na ❑

B: elements Zn ● , Fe ❍ , Cu ▼ , Cr ▽ and Mn ■

C: elements Ni ● , Ti ❍ , Sn ▼ , Hg ▽ and Pb ■

D: elements Cd ● , W ❍ , Sb ▼ , Tl ▽ and Bi ■

Lead (Pb), (Hg) mercury and stannous (Sn) concentrations have parallels: royal jelly samples from Vendée-Maine show seasonal (i.e. botanical) differences and samples produced on crystalline rocks are higher in Pb, Hg and Sn compared to samples from

carbonates and loess. The element Pb is biologically coupled with S. Ti, Cd and W concentrations have analogy to the other heavy metals Pb, Sn and Hg and show varying concentrations depending on seasonal effects and geographical origin (fig. 4.4).

Wolfram (W) showed highly varying concentrations. Highest values are found in crystalline basements of the Vendée (table 4.2, fig. 4.4). Antimony (Sb), thallium (Tl) and bismuth (Bi) accord to the schema of the other heavy metals. Concentrations in royal jelly samples show significant differences depending on seasonal and geographical origin.

Concentration of 6 trace elements Ba, V, Co, Te, Mo and Sr in royal jelly were analysed and traces were partly found below the limit of determination: Barium (Ba) could be detected in royal jelly sample 4 (07/02/01; crystalline basement) with 0.07 µg/kg and sample 6 (carbonates) with 0.114 µg/kg. Ba concentrations of the other royal jelly samples were below the limit of determination. Vanadium (V) concentrations were not detectable in all samples (below the limit of determination). Cobalt (Co) was analysed in royal jelly sample 1 in two digestions and was below the limit of determination. Tellurium (Te) was found in the concentrations 3 µg/kg (sample 2), 4.9 µg/kg (sample 3), 7.8 µg/kg (sample 4), 9.1 µg/kg (sample 5), 8 µg/kg (sample 6) and 7 µg/kg (sample 7). Te was not analysed in sample 1. Strontium (Sr) was analysed in all royal jelly samples and concentrations were found below the limit of determination (not detectable). Molybdenum (Mo) was analysed in two digestions in royal jelly sample 1 and found with 0.523 mg/kg and below the limit of determination (<0.407 mg/kg).

Concentrations of main and trace element in honey samples showed significant differences. In figures 4.5 and 4.6 elementary concentrations of 94 honey samples were rationed into 16 groups of botanical origin and sorted according to decreasing potassium levels. Mg and S levels correlated unambiguously to the K levels with an r^2 (i.e. correlation coefficient) of 0.59 for Mg and 0.35 for S. Ca and P showed no correlation to K (fig 4.5).

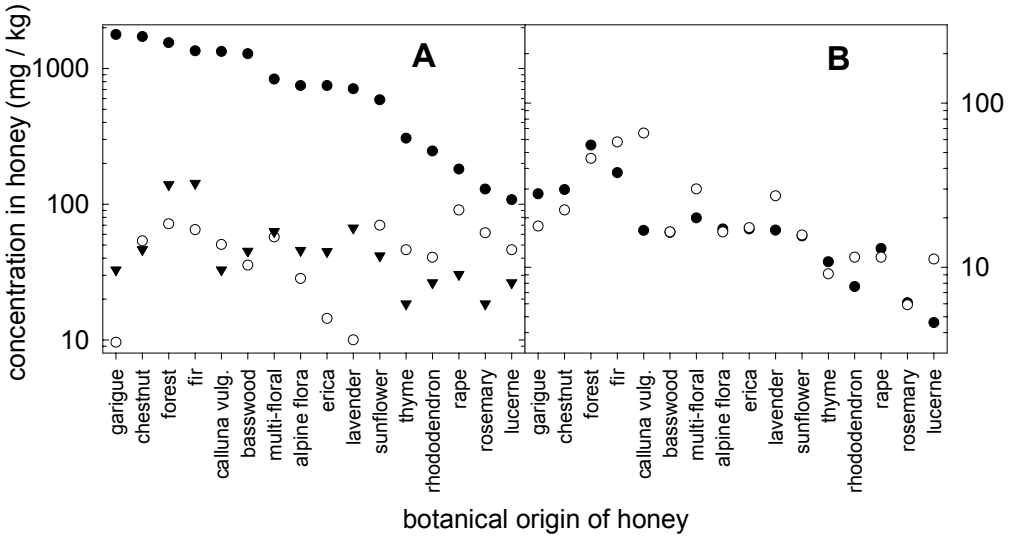

Figure 4.5: Concentrations of main elements in 94 honey samples of different botanical origins. Concentrations are given in section

A: elements K ●, Ca ❍, and P ▼

B: elements Mg ● and S ❍ .

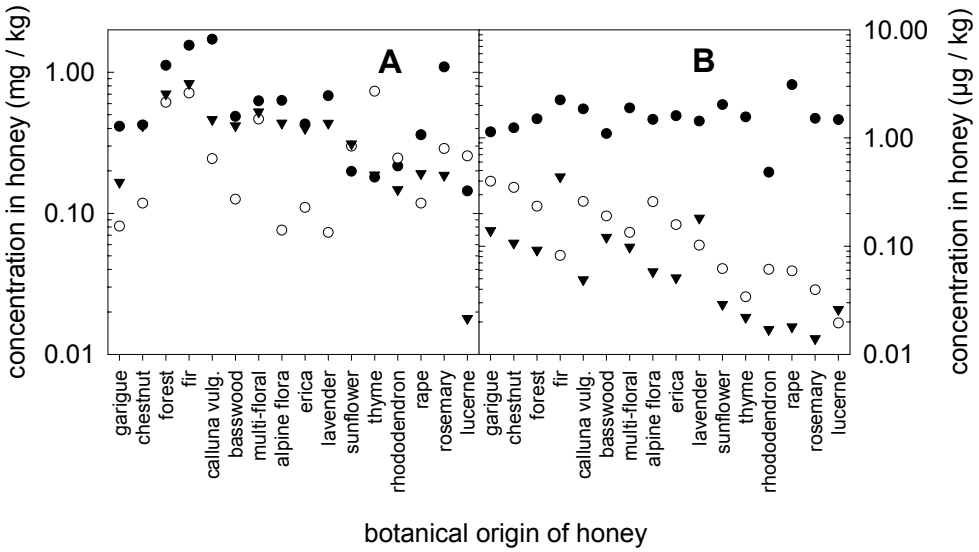

Figure 4.6: Concentrations of trace and ultratrace elements in 94 honey samples of different botanical origins. Concentrations are given in section

A: elements Fe ●, Cu ❍, and Zn ▼

B: elements B ●, Ba ❍ and Sr ▼.

The trace elements Zn, Ba and Sr showed clear correlations to K whereas for Fe, Cu and B no correlation was found (fig 4.6). The correlation of the trace elements Sr and Ba to K in 94 honey samples is graphically presented in figures 4.7 and 4.8.

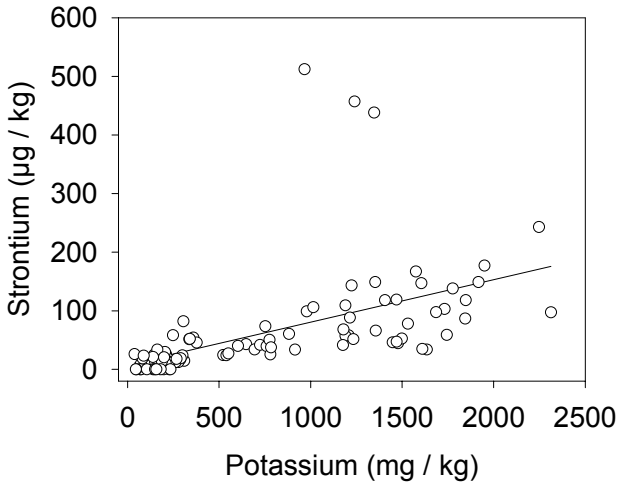

Figure 4.7: Concentrations of potassium and strontium in 94 honey samples from various botanical and geographical origins in France. In 13 samples, Sr-levels were below the limit of determination (10.3 to 23.3 µg/kg depending on the sample weight), the Sr-levels of these samples are graphically represented as 0 µg Sr / kg. Potassium levels correlate to Sr levels with an r^2 of 0.28, not taking into account the three outliers the r^2 is 0.63.

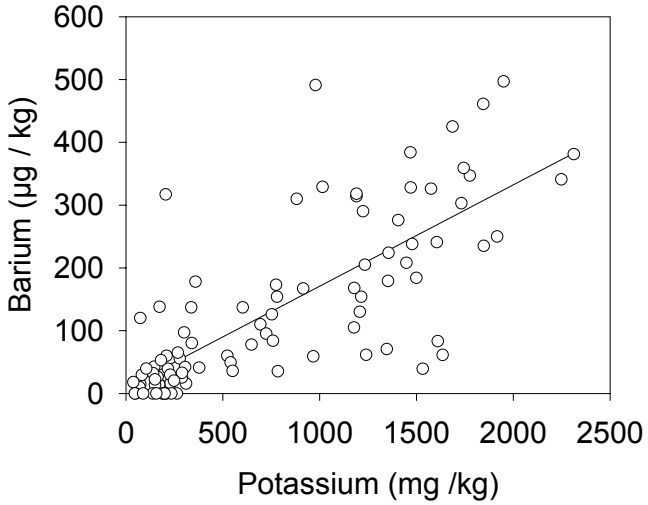

Figure 4.8: Concentrations of potassium and barium in 94 honey samples from various botanical and geographical origins in France. In 11 samples, Ba-levels were below the limit of determination (0.9 to 20.7 µg/kg depending on the sample weight), the Ba-levels of these samples are graphically represented as 0 µg Ba / kg. Potassium levels correlate to Ba levels with an r^2 of 0.58.

Table 4.2: Main elements (mg/kg) in royal jelly samples from different botanical and geographical origins:
The sample from Vendée-Maine (06/22/01) was analysed for 2 independent sample digestions of aliquots. Results are given as mean ± deviations.

Source rocks	Crystalline rocks	Crystalline rocks	Crystalline rocks	Crystalline rocks	Loess	Carbo-nates	Loess
Sample [mg/kg]	1a,b) 6/22/2001 Vendée	2) 5/4/2001 Vendée	3) 5/17/2001 Vendée	4) 7/2/2001 Vendée	5) 7/2001 Picardie	6) 7/2001 Centre	7) 2001 China A
P	1949±26	2064	1996	1957	2345	1942	2014
S	1154±16	1391	1212	1320	1415	1288	1338
Ca	120±9	117	113	122	145	113	129
Mg	282±7	275	268	269	312	264	280
K	2462±82	2765	2697	3051	3117	2634	2728
Na	127±2	119	127	106	129	107	142

Table 4.3: Trace elements (mg/kg) in royal jelly samples from different botanical and geographical origins:

Source rocks Sample [mg/kg]	Crystalline rocks 1a,b) 6/22/2001 Vendée	Crystalline rocks 2) 5/4/2001 Vendée	Crystalline rock 3) 5/17/2001 Vendée	Crystalline rocks 4) 7/2/2001 Vendée
Al	54.7±21.6	0.8	40.2	27.6
Zn	20.1±0.1	20.6	20.3	19.4
Fe	20.5±5.3	9.1	17.5	17.5
Cu	8.1±1.8	4.1	7.4	7.9
Cr	2.9±1.3	0.33	2.69	2.62
Mn	1.06±0.16	0.95	0.76	4.35
Ni	1.65±1.05	0.14	1.41	1.45
Ti	1.55±0.70	(< 0.136)**	0.71	0.45
Sn	0.143±0.013	0.196	0.277	0.185
Hg	0.021 (0.3144)*	0	0.031	0.031
Pb	0.287±0.085	0.033	0.172	0.017
Cd	0.019±0.003	0.013	0.014	0.012
W	4.817±1.6	0.014	0.933	0.0026
Sb	0.0054±0.0023	0.0004	0.0013	0.0013
Tl	0.00115±0.00005	0.0003	0.0008	0.0003
Bi	0.0061±0.0009	0.011	0.0017	0.0016

Source rocks Sample [mg/kg]	Loess 5) 7/2001 Picardie	Carbonates 6) 7/2001 Centre	Loess 7) 2001 China A
Al	8.4	4.7	1.6
Zn	24.8	22.4	24.4
Fe	22.1	10.2	11.0
Cu	8.0	4.6	4.0
Cr	2.93	0.65	0.34
Mn	1.48	0.70	0.78
Ni	1.70	0.33	0.15
Ti	0.32	0.23	0.26
Sn	0.073	0.031	0.015
Hg	0.036	0.002	0.001
Pb	0.032	0.050	0.051
Cd	0.0069	0.0022	0.0013
W	0.016	0.056	0.021
Sb	0.0015	0.0008	0.0005
Tl	0	0.0004	0.0001
Bi	0.013	0.0135	0.0079

(0.3144)* mg/kg of Hg in the second measurement of royal jelly from Vendée-Maine (sample 6/22/2001) seem to be a local contamination. The sample from Vendée-

Maine (6/22/2001) was analysed for 2 independent sample digestions of aliquots. Results are given as mean with deviations. (< 0.136)** for a Ti value (sample 2) signifies that the measured value is not detectable, below the limit of determination of 136 mg/kg. Data to the concentrations of the elements Ba, V, Co, Te, Mo and Sr in royal jelly samples are given in the text.

4.4. Discussion

The most abundant main elements in royal jelly samples are K, Na, Mg, Ca, P, and S. Zn, Fe, Cu, Al and Mn are abundant trace elements in royal jelly (Tab. 4.2 and 4.3; Fig. 4.1, 4.2).

The concentrations of phosphorus (P) and sulphur (S) in royal jelly are adjusted for the needs of the bee larvae. P is a structure element in the nucleic acids of royal jelly and construction element for the larvae. S concentrations in the royal jelly are likely to be related with the disulphur bridges of proteins. Homeostatic adjustments of P and S in royal jelly samples are likely related to high and constant amounts of nucleic acids and phospho-proteins.

Ca concentrations and K concentrations in honey depend on the bioavailability in the soil and therefore on the chemistry of the source rocks. Highly variable Ca and K concentrations are therefore found in honey samples (fig 4.1, fig 4.5). The different chemistry and geological age of source rocks and resulting soils e.g. crystalline rocks, carbonates and loess lead to a different bioavailability of the elements for the plants (Horn el al. 1993). Further on a element fractionation of the plants depending on the deepness of the roots plays a role. The variability of main elements like Ca and K depending on the bioavailability can be found as a consequence in the honeys from different floral origins (fig. 4.1 to 4.3, 4.5 to 4.8). Highly varying concentrations of main elements are also described for pollen of different plant species (Dedic and Koch 1957; Serra-Bonvehi 1991; Nation and Robinson 1971, Echigo et al. 1986). K, Na, Mg and Ca are the predominant minerals in pollen. Honey and pollen are the biochemical precursor substances of royal jelly. Royal jelly is much more processed and modified as honey. In bee tissue K was also found in high concentrations of 11 180 mg/kg (Kump et al. 1996) Homeostatic adjustment of potassium in royal jelly corresponding to the needs of the bee larvae is therefore very likely.

Sodium (Na) concentrations in royal jelly samples were also very adjusted. (Sodium was not measured in honeys). Royal jelly sample 4 from Vendée-Maine from the end of the royal jelly harvesting season showed a lower Na value of 106 mg/kg. A elevated Na concentration of 142 mg/kg was found in a commercial royal jelly sample (China 2001 A) which derived from semi-artificial production with additional sugar feeding (according to chapter 5). Serra-Bonvehi (1991) described that Na concentrations of the larval food showed no significant difference in the worker jelly samples and authentic royal jelly. Whereas increased Ca concentrations were described by Serra-Bonvehi (1991) as a parameter for not authentic royal jelly samples (worker jelly).

The ratios [K]/[Na] ranged from approximately 19 to 28 in our investigation of 7 royal jelly samples. Thus only low seasonal and geographical variation could be observed . The ratios [Mg/Ca] in the present investigation were found very similar from 2.2 to 2.3. In royal jelly seasonal and geographical variation change more the [K]/[Na] ratios as [Mg/Ca] ratios. In a preliminary study different [K]/[Na] and [Mg/Ca] ratios were analysed in two royal jelly samples from different bee colonies in single measurement. A selective secretion of minerals depending on the age, physiology and activity of the honeybee was proposed (Nation and Robinson 1971; Serra-Bonvehi 1991). Nation and Robinson (1971) described highly varying [K]/[Na] ratios in two royal jelly samples with 3.2 respectively 12.9 and also varying [K]/[Na] ratios honeybees bees collected in spring (approx. 5) and lower in autumn (approx. 8.6). The [Mg/Ca] ratios in two royal jelly samples were 4.3 respectively 1.8 (Nation and Robinson 1971). Homeostatic effects of the cations K, Na and Ca, Mg across the midgut epithelium of the American cockroach (*Periplaneta americana*) depending on gut lumen composition and concentration of these cations are described by Sauer and Mills (1969 a,b)

Aluminium (Al) concentrations show highly differing values in royal jelly samples. Values from 54.7 to 27.64 mg/kg harvested on the same place are most likely an aerosol contamination whereas values from 8.49 to 0.87 mg/kg seem more reliable. Exogenous contaminations of Al and Si with aerosol dust are well known in trace element analysis. In Vendée-Maine open cast mining of uranium were used before some decades and closed mines can still play a role in Al and Si aerosol

contaminations. The honeybee is probably capable to adjust Al in lower concentrations. High Al amounts lead certainly to elevated Al concentrations in the royal jelly.

Homeostatic adjustments of trace elements Zn, Fe, Cu and Mn are as evident as observed for main elements. The trace elements' Zn concentrations are stronger adjusted as concentrations of Fe, Cu and Mn (fig 3.2). Fe, Cu and Mn concentrations are evidently adjusted but less strong as the main elements and Zn (fig. 4.1 and 4.2), very similar in the royal jelly samples from the crystalline rocks of the Vendée-Maine. The nurse bee seem to have a physiological regulation of the zinc concentrations due to the needs of the bee larvae. Due to only slighter homeostatic adjustments the slightly varying concentrations of Fe, Cu and Mn can be found in royal jelly samples depending on different source rocks (table 4.3, fig. 4.4) as a consequence of different bioavailability of the elements.

The concentrations of several elements in royal jelly analysed in the present investigation are accordant to previous analysis of royal jelly samples from mountain and hill flora (Bonomi et al. 1986). Ca, P, Mg, K, Na, Co, F, Mn, Cu and Fe values in royal jelly samples (drymatter) showed only little variations. Benfenati et al. (1986) investigated royal jelly samples from several Italian regions with different alimentations like additional pollen and corn sirup or without any additional semi-artificial feeding. The elemental concentrations of K, Ca, Na, Mg, Zn, Fe, Cu and Mn in royal jelly showed highest similarity of values for samples of the same region. With exception of this finding no significant difference between the samples could be found.

Chromium (Cr) values in royal jelly showed parallels to Cu and Fe. The royal jelly samples with low concentrations of Cr were also low in Cu and Fe (table 4.2, fig. 4.4). Ni and Cr in royal jelly samples from Vendée-Maine showed low concentrations in the beginning and higher concentrations in the middle and end of the season. This effect of lower concentrations of several trace element in the beginning of the season (sample 2) could be observed for several other trace elements, especially the heavy metals (table 4.2). This can be due to a late arrival of the trace elements in the pollen

and nectars in the mid of May. Sn values show that the plants have a fractionation capacity from the phloem to the analysed product the royal jelly.
For the heavy metals Pb, Ti, Sn, Hg, Cd, W, Sb, Tl and Bi lowest concentrations were found in the beginning of the season (sample 05/04/01 Vendée-Maine). Higher concentrations could be found in samples from the same regional origin but later harvesting date. Due to the bioavailability and physiologic effects of plants heavy metals appear in groups into royal jelly.

In honey samples from different botanical origin highly varying concentrations of main and trace elements could be found depending on the elemental fractionation of the plant and bioavailability of the elements in source rocks of different age and geochemistry. The correlation of several elements e.g. K, Sr and Ba could be shown (fig. 4.5 to 4.8).
Sr concentrations in royal jelly samples in this investigation serie were below the limit of determination. Sr stable isotope ratio analysis of royal jellies (chapter 5) using thermal ionisation mass spectrometry (TI-MS; Horn et al. 1993a,b, 1994 and 1998) showed highly varying total Sr concentrations in royal jellies from different geographical origin, depending on the prevailing rock types. Royal jellies and bee tissues had approximately 10-fold lower Sr total concentrations compared to associated honeys. Homeostatic adjustments of the uptake of strontium into royal jelly are therefore highly likely.

Elias et al. (1982) described the natural biopurification of Pb, Ba and Sr in relation to Ca along nutrient pathways. From rocks to sedge leafs Sr/Ca ratios showed that Sr was biopurified 3-fold. From sedge leaf to vole Sr decreased 4-fold. From meadow vole to pine marten Sr decreased 4-fold. Similar values were received for the food chains of rocks / soil and detritivorous insects. For herbivorous insects (*Hymenoptera* and *Coloeoptera*) and amphibians like frog and snake similar factors of biopurification of Sr along the foodchain were observed (Elias et al. 1982). Physiological regulation of Cd in human milk was described by Schramel et al. (1988). Nascarella et al. (2003) described hormesis and stage specific toxicity of Cd in the queen blowfly, *Phormia regina*. Homeostatic effects in the distribution of metals in organs of the beetle *Chrysolina pardalina* are described by Przybylowicz et al. (2003). Cesco et al. (1994) investigated Cd and Pb in honey bees and bee products as possible

indicators of environmental pollution. No correlation between Cd and Pb contents in evironmental indicators as *Trifolium pratense* and rainwater and that in bee products as pollen, propolis and royal jelly was found (Cesco et al. 1994).

Homeostatic effects of the main elements and metals in royal jelly secretion are highly likely. The element contents of the rocks are slightly fractionated from the plants and can be found in the honeys. The concentrations of several main and trace elements can be used to give indications to geographical origins of honeys. The variation of main and trace element content of honeys depend on the bioavailibility in the prevailing rock types and fractionations of the plant growing on the resulting soils. This is accordant to literature data from elemental concentrations in all flower honeys from 4 mountain valleys in Northern Italy (Bontempo 2002). Highly varying elementary concentrations were also described for honeys produced in Galicia (North-west Spain) by Rodriguez-Otero et al. (1994 and 1995). The significant influence of botanical origin on main and trace element concentrations in honeys was described by Bengsch (1992 and 1993). Trace element concentrations in royal jellies show slight seasonal and geographical differences but are considerably more homogeneous as the associated honeys. Royal jelly origin can not be assigned with main and trace element contents. Homeostatic effects of the honey bee on main and several trace element concentrations are evident.

5 Multielement stable isotope ratio analysis – authenticity and origin

Summary

Royal jelly is a honey bee secretion which is highly appreciated as health food product (pharmaceutical product) especially in Japan and China. Due to semi-artificial production in large scale methods for the reliable assignment of authentic production and geographic origin are requested. Multi-element stable isotope ratio determinations with the elements carbon, nitrogen, and strontium have been applied for testing authenticity and geographical origin of royal jelly samples.

Carbon and nitrogen isotope contents (given as delta values relative to a standard, $\delta^{13}C$, $\delta^{15}N$) of royal jelly samples from several regions and floral origins were analysed. Honeys, pollen, beeswax and bee tissue were investigated additionally to confirm regional origin and in order to recognize isotope effects in different trophic steps. Seasonal effects respectively the botanical origin and the climatic situation were confirmed to show up as variations in carbon and nitrogen stable isotope ratios. Furthermore, royal jelly samples from authentic and from adulterated products could unambiguously be differentiated.

Additional investigation of the trace elements' strontium stable isotope ratio was applied for royal jelly samples, associated honeys and bee tissue from areas with well defined geological substrata. Strontium stable isotope ratios proved to provide a method for the geological situation of royal jelly origins. Stable isotope ratios of the elements carbon and nitrogen are sufficient to determine authentic or adulterated production of royal jelly. For the assignment of regional origin of royal jelly samples further isotope ratios of elements like strontium, sulphur, oxygen and hydrogen have to be investigated.

5.1 Introduction

Royal jelly is a bee product, formed under partial digestion of honey and pollen (according to chapter 2). It consists of approximately 66 % water, 15% sugar, 5% lipids and 13 % of proteins (Lercker et al. 1993). Most of all commercially available royal jelly samples (more the 100 tons each year) are produced in China possessing

more than 70 million hives (Ianuzzi 1990) - and in other Asian countries. Semi-artificial production of commercial royal jelly samples is described by Chen (2002). High fructose corn sirup (HFCS) as a sugar source and biotechnologically produced yeast powder are in suspicion to be used for high-yielding royal jelly production under adulterated conditions.

The properties of the natural raw product royal jelly however is correlated significantly to the origin of its natural precursors nectar and pollen. Nectar is the main sugar source and pollen with his multitude of components (proteins, glycosides and their derivates), in particular the secondary metabolites, is the essential precursor product of the royal jelly.

Investigations of adulterations and geographical origin of royal jelly are therefore of high interest for the consumer. Until now the concentrations of the 10-hydroxy-Δ2-decenoic acid were analyzed to investigate the authenticity of royal jelly samples (Bloodworth et al. 1995). These analytical methods are adapted to determine percentage content of royal jelly in pharmaceutical mixtures. For the verification of adulterated feeding methods or geographical origin, however, these methods are not qualified.

Other methods like morphological pollen analysis are capable to indicate regional origin of royal jelly samples (Louveaux et al. 1978, Ricciardelli et al. 1978). Also adulterations like starch feeding can be recognized by light microscopical inspections together with the pollen forms. Adulterations with sugars like corn sirup, beet sugar and several yeast preparations cannot be uncovered by microscopical investigations.

The ^{13}C analysis was widely used in earlier works to differentiate the plant metabolism (C3 or C4 plants) and the origin of compounds as sugars thereof (Korakli et al. 2001). Also the climatic and agricultural conditions of milk production could roughly be estimated (Kornexl et al.1997, Rossmann et al. 1998).

The ^{15}N analysis is known to be useful in the characterization of production conditions like extensive agriculture (without fertilizers), application of organic fertilization (e.g. animal manure) or differentiation of plant sources like legume

(Rossmann 2001). For verification of authentic production of royal jelly without adulterating additional feeding the combination of C and N stable isotope ratios can be sufficient.

For honey samples a large data pool of isotopic ratios of the elements C,H,N already exists (Rossmann 2001). Measurements of the royal jelly samples and the associated honeys can also be useful to supplement these investigations

A study of honeys from several regional origins in Northern Italy (Bontempo 2000) investigated botanical defined honeys with multi-element stable isotope and trace element analysis. Two element isotope blots of the carbon, nitrogen and hydrogen stable isotope ratios of the honeys and honey proteins were used to differentiate regional origin.

Trace elements play an important role for the quality and biological properties of royal jelly. For example the boron supply which is warranted from the crystalline rocks. The aim of the Sr isotope investigation was the characterization of several geological different regions which were found to differ in the biological properties of the royal jelly samples, too.

In addition, stable isotope ratios of the elements hydrogen, oxygen and sulfur can be taken into account for the determination of regional origin of royal jelly samples. Multielement stable isotope ratio analyses of the elements H, C, N, O, S and Sr were successfully applied for geographical origin assignment of milk, butter and cheese (Kornexl et al. 1997, Rossmann et al. 2000, Rossmann 2001). A combination of carbon and sulphur stable isotope ratios was capable to differentiate feed and breed of Iberian swines (Gonzalez-Martin et al. 2001).

The aim of the following investigation was therefore to test and improve the application of multi-element stable isotope ratio analysis as a tool for the examination of authenticity and geographical origin of royal jelly samples.

For the differentiation of origins from similar climatic regions but different geological features the analysis of Sr isotope ratios ($^{87}Sr/^{86}Sr$) was found to be a useful method

(Rossmann et al. 2000). We examined royal jelly samples from clearly defined regional origins with given geological features. Royal jelly samples from the acidid primordial granite rocks of the Vendée-Maine, France, from carbonate rocks in Centre, France and the loess soils of the Picardie, France. Strontium isotope ratios ($^{87}Sr/^{86}Sr$) from the associated honeys and bee tissues were also measured with the aim to control the agreement of values.

5.2 Methods and Materials

Freshly harvested royal jelly samples were produced as described in chapter 2. We produced botanically defined royal jelly samples in cooperation with apiaries in St. André de la Marche, Vendée-Maine, France, from Aisne (Lugny, Voharies, Marle, Bois les Pargny, Cilly et Toulis), Picardie, Northest-France, from Chézelles, Centre, France and several other places in Europe as indicated. Samples were produced in areas with geologically well defined substrata. Samples were kept at refrigeration temperature (4-7°C) until analysis. Samples from Asian provenance like China, Northern Thailand and Indonesia were commercial samples. These samples constitute the major part of worldwide produced and sold royal jelly. Honeys, wax, pollen and bee tissues derived from same provenance as the associated royal jelly samples.

Honey, wax and pollen were prepared as indicated for royal jelly samples. Honey bees were additionally homogenised by vigorous comminution by ultraturrax in order to obtain homogeneous sample material. In cooperation with A. Roßmann, who is the leading scientist in this field, I have shown ^{13}C, ^{15}N and ^{87}Sr in bee products.

$\delta^{13}C$-values of a carbon sample or its compound signifies its $^{13}C/^{12}C$ ratio relative to the V-PDB standard ($[^{13}C]/[^{12}C]=0.0112372$). In accordance with the different photosynthetic pathways employed by C3- and C4-plants, the $\delta^{13}C$ values from these plants differ (Schmidt 1986). ^{13}C and ^{15}N analysis was performed on CO_2 and N_2, respectively, which was obtained in an elemental analyzer directly connected to an isotope ratio mass spectrometer (IRMS), where the gas to be analyzed is transported into the ion source by helium carrier gas, the socalled continuous flow isotope ratio mass spectrometry (CF-IRMS; Barrie et al. 1989, Kornexl et al. 1995, 1997, Rossmann 2001).

For the measurement of $\delta^{13}C$-ratios royal jelly bulk material were weighed into tin capsules for combustion. With an average content of 20 % carbon weighed samples of 4 mg royal jelly were measured. The working standard was wheat starch ($\delta^{13}C$ value -24.60 %o), which was calibrated with NIST-22 with a $\delta^{13}C$ value of –29.80 %o (Koziet et al. 1993). The determination of $\delta^{13}C$ values was reproducible with a analytical uncertainty ±0.1%o (abs.). For the carbon isotope measurement of defatted drymatter, sugars and proteins a sample preparation procedure widely used for milk investigations (Kornexl et al. 1997) was applied to royal jelly.

The $\delta^{15}N$ analysis of dry matter was performed on N_2 (Standard= $^{15}N/^{14}N_{AIR}$) as described above using a sample size of 4-5 mg fresh royal jelly, which were weighed into tin capsules for combustion (nitrogen content of the fresh material was approx. 2.2%). The working standards were wheat flour and casein with $\delta^{15}N$-value of +3.27 %o and +6.15 %o, respectively. The determinations of $\delta^{15}N$ values were reproducible with an analytical uncertainty of ±0.2 %o (abs.).

$\delta^{87}Sr$: For royal jelly samples methods were used as described for the analysis of milk products and other biological material (Horn et al. 1993a,b, 1994 and 1998; Rossmann et al. 2000). Fresh royal jelly samples (approx. 200-500 mg) were dry-ashed at 600°C for several hours in quartz vials (Sr losses were not observed). The light, white ashes were dissolved quantitatively in HNO_3. Sr was separated from these solutions by means of ion chromatography and analysed by thermal ionisation mass spectrometry (TI-MS; Horn et al. 1998). All chemicals used for sample preparation were of ultrapure grade in order to avoid contamination of the samples.

Filaments of a thermal-ionization solid state mass spectrometer (MAT 261®) were loaded with approximately 100 ng Sr for isotopic ratio determinations. Measured values were corrected for mass fractionation by using the factor $^{86}Sr/^{88}Sr=0.1194$. International $SrCO_3$ standards (Eimer and Amend and NBS 987) were analysed ($0.707990\pm8\times10^{-6}$,n=47, and $0.710220\pm6\times10^{-6}$; n=56, respectively) to check the accuracy of the instrument. The determinations of $\delta^{87}Sr$ values were reproducible with an analytical uncertainty (2σ mean) of ±0.02 %o (abs.) of the results given as deviation vs. the reference ratio $^{87}Sr/^{86}Sr = 0.7093$.

The complete strontium concentration of royal jelly samples varied strongly depending on the rock types of the regions. The total strontium concentration was approximately tenfold higher for bee tissues and honeys compared with corresponding royal jelly samples. A physiological regulation of the uptake of strontium into royal jelly is therefore highly likely. This is in accordance to the trace element data of royal jelly samples of chapter 4 and literature data (Elias et al. 1982).

5.3 Results

Stable isotope ratios of light bio-elements C and N were analyzed in botanical defined royal jelly samples from different regions (together with the associated biochemical precursors honey and pollen, and fractions thereof). Results are given in table 5.1 and figure 5.1.

Carbon stable isotope ratios depend on the botanical origin. C_3 and C_4 plants have different δ^{13}C-values due to kinetic isotope effects in the different pathways of carbon fixation reactions (Schmidt 1986). The organic material (in this case pollen and nectar) deriving from C_3-plants is isotopically lighter, having lower ^{13}C contents with δ^{13}C-values from –32 to –25‰. C_4-plants are “heavier”, having higher ^{13}C content with δ^{13}C-values from –15 to -9‰ (Schmidt 1986). Furthermore the climatic situation plays a role. Dryness and high summer temperature of continental climates influence the metabolic pathway of plants, because under water stress conditions the carbon isotope fractionation of fixation is reduced, resulting in higher ^{13}C content of plant products (Farquhar et al. 1982; Miller et al. 2001). The δ^{13}C-values measured (table 5.1 and 5.2) show significant differences between the individual geographical provenance regions as far as they are different concerning the climate.

The lowest ^{13}C-contents among our samples were found for samples from the humide, cool (maritime) climate area (Picardie and Vendée-Maine, France) whereby also the differences between Picardie and Vendée-Maine are still significant. Samples from Centre, France and the Bavarian Molasse (Weilheim, Alpine foreland) possess clearly higher contents in ^{13}C as a result of a more continental climate (dryness) and higher (summer)-temperatures.

Isotope ratios of samples from China and Thailand are even higher (subtropical or tropical climates). The ^{13}C value for sample “China B” is so high, that a feeding with sugar from C_4-plants is very likely (which is well-known to be applied in China for honey production, too).

Botanical compositions of pollen harvested by honeybees have little effects on the carbon isotopes but strongly on those of nitrogen. This can be seen with the 5 royal jelly samples from Vendée-Maine 1999 from 27 April to 29 June. The royal jelly samples had the very same (regional) but had different seasonal harvesting dates (i.e. botanical origin of the nectars). Each of the 5 samples derived from a different main pollen composition (*Brassica group, Pyrus group, Genista ssp. Heracleum sphondylium, Sambucus spec., Castanea sativa*).

The chemical composition of the biological material is also one reason for differences in $\delta^{13}C$-values. Proteins, lipids and sugars have different $\delta^{13}C$-values due to kinetic isotope effects in the biochemical pathways (Schmidt et al. 1995). Usually the lipids and phenolic substances are most depleted in ^{13}C, while carbohydrates contain the highest amount of ^{13}C, whereas proteins are intermediate. Differences in $\delta^{13}C$-values concerning the 3 bee tissues can be a result of the different geographical origin and different lipid contents in the bee tissue as a feature of seasonally differing nourishment situation.
The beewax, a lipid ($\delta^{13}C$ -29.90 %o), and the pollen sample, a substance containing lipids and phenolics ($\delta^{13}C$ -27.83 %o) from Picardie (Lugny) 2001 showed both lower $\delta^{13}C$-values as the royal jelly samples (containing proteins, sugars and lipids), honeys (mainly sugars) and bee tissues (proteins, chitin, and some lipids) from Picardie. The beewax from the Bavarian Molasse (Alpine foreland, $\delta^{13}C$= -27.89%o) was also depleted in ^{13}C as compared to the royal jelly, honey and bee tissues from the same region.

The ^{13}C content of the defatted drymatter and sugar of the royal jelly samples from Vendée-Maine 5/2001 and Centre, France 7/2001 were measured for the comparison with the complete material. For both 2 royal jelly samples the defatted dry matter was enriched in ^{13}C as compared to the royal jelly dry matter. The sugars

of royal jelly were again “heavier” in ^{13}C content as the defatted dry matter. Kinetic isotope effects with incomplete conversions in the biochemical pathways from carbohydrates to lipids, proteins and other secondary products are the reason for these differences in ^{13}C content (Schmidt et al. 1995).
Lower contents in ^{13}C of the lipid fractions in the above mentioned two royal jelly samples must therefore be assumed compared to the royal jelly total organic material.

$\delta^{13}C$-values from 3 honeys and the protein fraction thereof were measured. The honey samples were slightly different in $\delta^{13}C$-values compared to the associated royal jelly samples from the same regional origin. The differences in chemical composition of the bee products are given here in the different $\delta^{13}C$-values. The protein fraction of the honey sample from the Bavarian Alps is heavier in ^{13}C content compared to the associated honey. For the 2 other honeys the proteins are “lighter” in ^{13}C content as the honey complete material. In honey analysis, the comparison of carbon isotope ratio of honey and protein from a honey is applied to detect addition of C4 plant sugars to honey or for feeding bees (method of internal standard, White and Winters 1989, White et al. 1992 and 1998). For that a maximum acceptable difference between authentic honey and protein from honey of 1 ‰ has to be taken into account.

The $\delta^{13}C$-values of all commercial royal jelly samples from Asian provenance are very high. Even with a feeding with predominantly “heavy” C_4-plants $\delta^{13}C$-values from –15 to –9 ‰ (Schmidt 1986) lower $\delta^{13}C$-values of the resulting royal jelly samples can be assumed. Taking this into account the nitrogen isotope ratios however are not characteristic for a C_4-plant feeding, they are significantly too low. The ratios of carbon and nitrogen stable isotopes are not characteristic for authentic royal jelly samples. A feeding with corn sugars and yeast proteins at these commercial samples from China, Thailand, Indonesia or beet invert sugar or HFCS from potato starch and similar proteins in Europe is therefore most likely.

The nitrogen isotope data show also a considerable differences between different geographical sample origins; additionally within one regional provenance but different

seasonal harvesting dates the $^{15}N/^{14}N$ ratio varies (results from Picardie, Northern France and Vendée-Maine, France). The reason for this are different main pollen in different floral seasons (according to chapter 2). A correlation between the pollen composition and nitrogen stable isotope ratios was found. The main pollen data of royal jelly samples from Vendée-Maine are given in chapter 2: (*Brassica group, Pyrus group, Genista ssp. Heracleum sphondylium, Sambucus spec., Castanea sativa*).

The influence of the botanical origin (main pollen) is therefore higher for nitrogen stable isotope data as for carbon stable isotope data. According to our experience and literature data (Rossmann et al. 1998) for example legume show $\delta^{15}N$-values from 0 to +2 ‰ whereas for cruciferous plants $\delta^{15}N$-values up to +8 ‰ were measured. The $\delta^{13}C$-values of the different plant groups show likewise certain differences, but these are less pronounced as at the nitrogen isotopes. Samples from Vendee-Maine, France derived from different main pollen origins (according to chapter 2).

In hotter and dryer areas the same plant groups deliver higher ^{15}N-contents as in cooler, more humid zones. In addition, effects of fertilization and nitrogen turnover in the soils can affect the nitrogen isotope ratio of soil nitrate and as a consequence in plants growing on the soils (Amberger and Schmidt 1987).

Further on in Middle Europe lower ^{15}N-contents for grasses were observed at the beginning of the growth period compared to the end. For regions at higher altitudes, being extensively farmed (for example the Alps) lower ^{15}N-contents were found as for intensively utilised regions for example in the lowland regions (Kornexl et al. 1996).

Stable isotope ratios of the heavy element strontium were measured on geologically defined royal jelly samples, honey, wax, pollen and bees from different regional origins. Results are given in table 5.2 and figure 5.2. Geological and pedological informations were received from geological maps (BRGM, France). Geological data were accordant to observations of the source rocks on the production sites. The purpose of the strontium isotope measurement was to show the capability of this method to indicate the regional origin of the royal jelly samples depending on the rock types. We produced royal jelly samples on acidic magmatic (granite) rocks of

the Vendée (France), on carbonate bearing rocks (Centre, France and Bavarian Alpine foreland) and on loess (Picardie, France).

The trace elements' strontium isotope ratios depend on the age and Rb/Sr chemistry of the rocks (Horn et al. 1993a,b, 1994 and 1998). In fact, δ^{87}Sr are dependent on the weathering behaviour of Sr bearing minerals. Old, acidic rocks like granites show high δ^{87}Sr-values. Loess can also have high amounts of ^{87}Sr, depending on the parent rocks of the loess and on the carbonate contents. Basic and/or young rocks like carbonates or basalts have low δ^{87}Sr-values due to their low Rb/Sr (Horn et al. 1993a,b, 1994 and 1998). In addition, the uses of phosphate fertilizers have to be considered in that these may alter the original δ^{87}Sr-values, if they stem e.g. from phosphate mines in (northern Finland)/Russia (Kola peninsula) with very low ^{87}Sr/^{86}Sr. In case of wine, this effect was found not to be very pronounced (Horn et al, 1991). Ultimately, the effect depends on the ratio of plant available Sr concentrations and related ^{87}Sr/^{86}Sr and these parameters in the fertilizers (Horn et al. 1993 a,b, 1994 and 1998).

The high δ^{87}Sr-value of royal jelly from Vendée-Maine 5/2001 (St. André de la Marche) with δ^{87}Sr +2.15 ‰ is characteristic for acidic crystalline rocks. The δ^{87}Sr-values of the associated honey and bee tissue showed also high δ^{87}Sr-values and confirm therefore tentatively the accordance of the strontium isotope results from Vendée-Maine. The royal jelly sample from Centre (Chézelles), France derives from an acidic rock with carbonates. A δ^{87}Sr-value of the sample δ^{87}Sr= +1.67 ‰ was measured.

The samples from the Bavarian Molasse (Alpine foreland, Weilheim) 2001 stem from regions underlayed by sediments of mixed compositions. The δ^{87}Sr-value of the royal jelly sample was –1.39 ‰ and therefore characteristic for rocks with much carbonate. The honey from the same apiary showed absolute concordance in their δ^{87}Sr-values. The bee tissues were significantly "heavier" (more radiogenic) with a δ^{87}Sr-value of +0.02 ‰. The latter has to be explained by the fact that the wider region is heterogeneous in ^{87}Sr/^{86}Sr and the growing animal's food was higher in ^{87}Sr/^{86}Sr.

The δ^{87}Sr-values of the honey (δ^{87}Sr= -1.30) and the bees (δ^{87}Sr= -0.83) from the Picardie, Aisne (Lugny, Voharies, Marle, Bois les Pargny, Cilly and Toulis) are characteristic for carbonates. The δ^{87}Sr-value of the corresponding royal jelly sample from July 2001 with δ^{87}Sr=+5.11 however is more typical for acidic magmatic rocks. Hereupon 4 additional samples from the royal jelly production of the preceding year were investigated. Highly varying ^{87}Sr/^{86}Sr-ratios were found (Table 5.2 and figure 5.2). Ultimately the strontium stable isotope situation of the samples from Picardie could be explained with a heterogeneous geological situation.

Table 5.1: Carbon and nitrogen stable isotope analysis of royal jelly samples and associated honey, (wax, pollen) and bee tissue from different geographical and seasonal origin:

sample	δ^{13}C [‰] $_{V\text{-}PDB}$ bulk material	δ^{15}N [‰]$_{AIR}$ bulk material
royal jelly Vendée-Maine 04/27/99	-25.19	+ 3.37
royal jelly Vendée-Maine 05/07/99	-25.74	+ 4.56
royal jelly Vendée-Maine 05/17/99	-26.41	+ 3.49
royal jelly Vendée-Maine 06/03/99	-26.74	+ 5.28
royal jelly Vendée-Maine 06/29/99	-26.45	+ 1.66
royal jelly Picardie 5/2001	-25.50	+ 4.32
royal jelly Picardie 6/2001	-26.22	+ 3.56
royal jelly Picardie7/2001	-26.83	+ 3.60
royal jelly Picardie 8/2001	-26.66	+ 6.44
royal jelly Bavaria 8/01	-25.35	+ 3.08
royal jelly Hannover, Germany 7/2001	-25.77	+ 4.67
royal jelly Syria 2001	-25.18	+ 2.61

royal jelly China A 2001	-22.48	+ 1.62
royal jelly China B 2001	-20.68	+ 0.24
royal jelly China C 2001	-21.50	+ 2.14
royal jelly Thailand 2001	-22.53	+ 1.51
royal jelly Indonesia 2001	-13.35	+ 0.30
royal jelly Commercial 2001	-24.20	+ 2.08
royal jelly Vendée-Maine 5/2001	-26.16	+ 4.74
Defatted drymatter	-25.96	
Sugar	-25.10	
Centre 7/01, France	-23.73	+ 1.08
Defatted drymatter	-23.37	
Sugar	-22.96	
bee tissue Vendée-M. 2001	-25.26	+7.57
bee tissue Picardie 2001	-26.82	+6.64
bee tissue Bavaria 2001	-26.89	+7.63
bee wax Picardie 2001	-29.90	
bee wax Bavaria 2001	-27.89	
pollen Picardie 2001	-27.83	

	$\delta^{13}C$ [‰] $_{V\text{-}PDB}$		$\delta^{15}N$ [‰]$_{AIR}$
	honey	protein	protein
honey Bavaria 2001	-26.59	-26.14	+2.81
honey Picardie 2001	-25.93	-26.34	+3.32
honey Vendée 2001	-25.52	-26.53	+4.92

All values are means of at least 3 individual measurements.

Table 5.2: Strontium stable isotope analysis of royal jelly samples and associated honey, (wax, pollen) and bee tissue from different geographical and seasonal origin:

Sample	$\delta^{87}Sr$ [‰]* bulk material
royal jelly Vendée-Maine 5/2001	+2.15
honey Vendée-M. 2001	+4.25
bee tissue Vendée-M. 2001	+2.63
royal jelly Centre 7/01, France	+1.67
royal jelly Picardie 7/2001	+5.11
royal jelly Picardie 5/2000	+2.47
royal jelly Picardie 6/2000	+1.33
royal jelly Picardie 7/2000	-0.73
royal jelly Picardie 8/2000	+2.04
honey Picardie 2001	-1.30
bee tissue Picardie 2001	-0.83
royal jelly Bavaria 8/2001	+0.02
honey Bavaria 2001	-1.39
bee tissue Bavaria 2001	-1.29
royal jelly China B 2001	+2.93
royal jelly China C 2001	+2.75

* relative to $^{87}Sr/^{86}Sr = 0.7093$

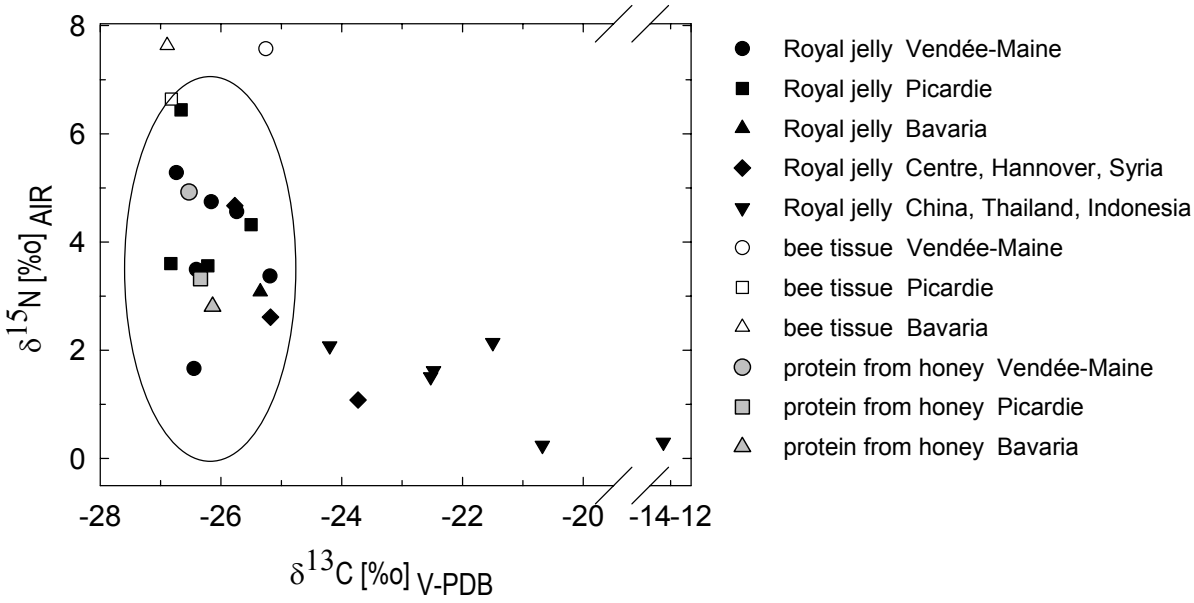

Figure 5.1: $\delta^{13}C$ / $\delta^{15}N$ –values of royal jelly, bee tissue and protein from honey; the ellipse indicates the confidence area for authentic bee products.

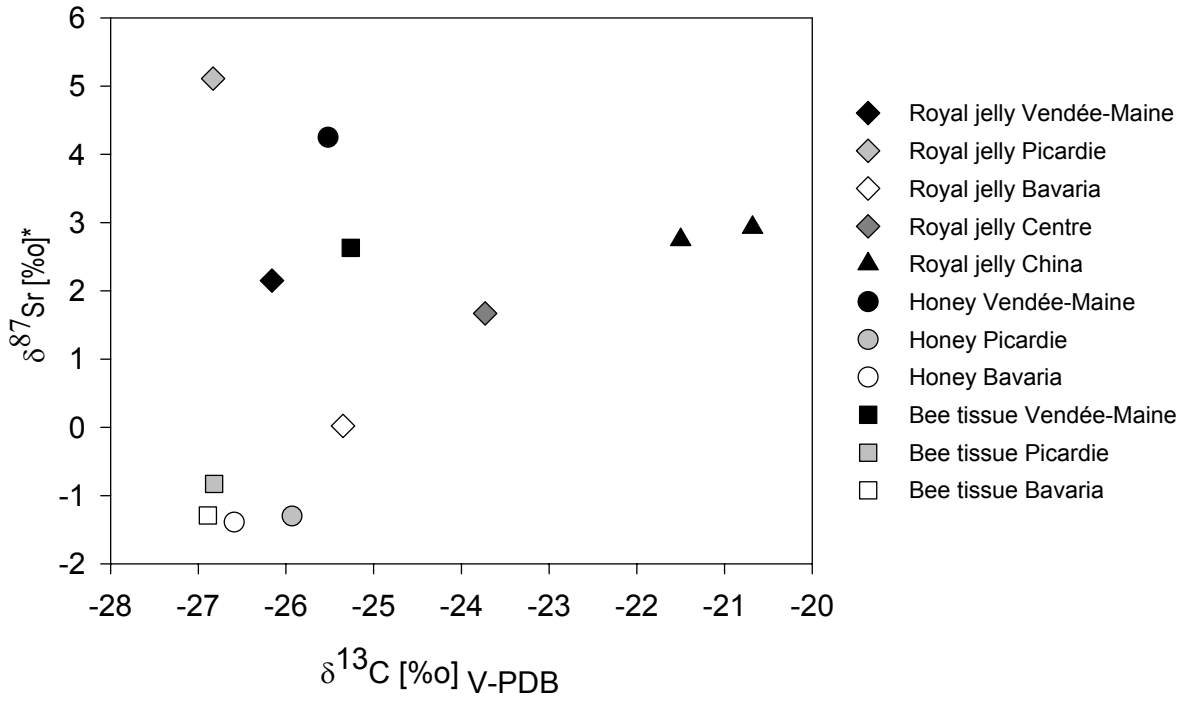

Figure 5.2: $\delta^{13}C$ / $\delta^{87}Sr$ –values of royal jelly, bee tissue and honey;

* relative to $^{87}Sr/^{86}Sr = 0.7093$

5.4 Discussion

Carbon und nitrogen stable isotope ratios of royal jelly samples from different botanical origins, climate zones and production situations were analysed in order to give indications to authentic production and regional origin (Table 5.1 and figure 5.1).

$\delta^{13}C$ values give informations to the botanical origin from C_3-plants, C_4-plants or CAM-plants (crassulacean acid metabolism). The organic materials (pollen and nectar) from C_3- or C_4-plants or CAM-plants have different contents in ^{13}C (Schmidt 1986) due to kinetic isotope effects in carbon fixation pathways. The climatic conditions influence the $\delta^{13}C$ values, because the carbon isotope fractionation in plants changes with the temperature and with water stress (Schmidt et al. 1995). A system for the classification (Bengsch-Grivet rules) of the non-statistical label distribution in biosynthetic ^{13}C enriched amino acids, fatty acids and further organic substances is described by Bengsch et al. (1981, 1982, 1983 and 1986).

$\delta^{13}C$-values of royal jelly samples with different seasonal (i.e. botanical) origin from Vendée-Maine 1999 and Picardie 2001) showed decreasing values at the beginning and increasing values at the end of the vegetation period (Table 5.1). This is accordant to trend lines for $\delta^{13}C$-values of milk from grassland during the vegetation period (Rossmann et al. 1998).

Royal jelly samples which we produced together with apiarist as described in chapter 2 and royal jelly samples from Asian provenance showed enormous differences in $\delta^{13}C$-contents (tab. 5.1 and fig. 5.1). The European royal jellies had $\delta^{13}C$ contents mostly from –25 to –27 (fig. 5.1, indicated with an ellipse), commercial royal jelly samples from Asian provenance ranged from –13 to –23. The $\delta^{13}C$-value of the Indonesian royal jelly is like the commercial royal jellies from China likely not authentic. One royal jelly sample from Centre had $\delta^{13}C$ contents of nearly –24 which is well known as the limit value for authenticly produced honeys. The $\delta^{13}C$ value would be authentic for the solely provenance of C_4-plants like maize or CAM-plants like agave, sukkulents, orchids as vanilla. In this respect the $\delta^{15}N$-value of the Indonesian sample of +0.30 is not accordant to a derivation of C4 plant. The morphological pollen analysis of the Indonesian royal jelly sample showed *Mimosa*

pudica as the most frequent pollen form of the Indonesian royal jelly which is to our knowledge a C_3-plant.

Feeding of sugars (hydrolysate of maize starch and cane sugar sirup) is most likely for the Indonesian sample as well as the other commercial sample of Asian provenance. Additional feeding in high yielding royal jelly production was described by Chen (2002). In honey samples 1‰ difference in the $\delta^{13}C$ content signifies the additional feeding of 7 % sugars from C_4-plants (White and Winters, 1989). Therefore analysis of carbon isotope ratios is sufficient to uncover samples with adulterated production with C4 or CAM plant sugars.

$\delta^{13}C$-values of the bee tissues from Vendée-Maine and Picardie are in accordance with the $\delta^{13}C$-values of the associated royal jelly samples (fig.5.1). Bee tissue from Bavaria (Weilheim, Alpine foreland) is isotopically about 1.5‰ lighter in $\delta^{13}C$ as the associated royal jelly sample. Bee tissue from Bavaria (Weilheim) was collected 3 months later in the season and differences in the chemical composition of the bee tissue (more lipids) can be the reason for the difference in $\delta^{13}C$-values.

Beewax from Picardie and Bavaria (table 5.1) are lighter in $\delta^{13}C$-values as the associated royal jelly samples and also bee tissues. Royal jelly sugars from Vendée-Maine and Centre, France were about 1‰ “heavier” in $\delta^{13}C$ as its bulk material. $\delta^{13}C$ stable isotope ratios in beewax (predominantly lipids) and sugars can be explained with kinetic isotope effects in the metabolic pathway of sugars and lipids. The sugars in royal jelly derive completely from the honey. Separate analysis of the sugars, bulk material and proteins of royal jelly can therefore give indications to the derivation of the sugar fraction. The sugar fractions of the royal jelly sample from Vendée-Maine, Picardie and Bavaria are accordant to the honey samples from the same regional origin (table 5.1).

$\delta^{15}N$-values are highly differing within plant species. Examples for $\delta^{15}N$ $[‰]_{AIR}$ values are red clover –0.27 (*Trifolium pratense*), beans +0.31 (*Vicia faba*), grass +3.21 (mixture), maize silage +4.24 (*Zea mays*) and rape +8.73 (*Brassica napus*). Climatic

conditions like temperature and dryness play a further role for nitrogen isotope ratios. Use of agricultural fertilizers with δ^{15}N=+0 ‰ change also the δ^{15}N-values.

Indonesian and Chinese royal jelly had extremely low δ^{15}N-values which are only authentic with legumes as N-source (fixation of N from air). The symbiontic bacteria in the roots of legumes have a preference for ^{14}N. Plants utilise nitrate produced by symbionts and the ^{15}N values are increasing. Morphological pollen analysis showed that the nectar source were not legumes. *Mimosa pudica* was the only frequent pollen. Ultimately the explantion for the low ^{15}N values is additional feeding of fermentative produced yeast (on the base of C_4 plant material, ammonium salts). Maize starch hydrolysate (HFCS) and glucose sirup can be supposed as C_4 plant material for the production of yeast.

Nitrogen stable isotope ratios of royal jelly samples which we produced under defined conditions in cooperation with apiarists are given in figure 5.1. Due to different seasonal (i.e. botanical) origins variations in δ^{15}N-values could be observed (tab. 5.1, fig. 5.1). In this respect “extremely low” nitrogen stable isotope ratios of commercial royal jellies e.g. samples “China B 2001, Indonesia 2001” with δ^{15}N= +0.24 respectively δ^{15}N= +0.30 give further proof of semi-artifical production. These low values are even for a hot climate not characteristic. Use of agricultural fertilizers with δ^{15}N=+0 ‰ has presumably no influence at the low ^{15}N values in this case.

Differences in δ^{15}N of honeys and royal jelly samples from the same origin can be explained with different botanical origin of honeys and royal jellies (tab. 5.1). Two element blot of honey protein and royal jelly samples showed that honeys are within the authentic area (fig 5.1).

The δ^{15}N-values of the bee tissues are roughly 3 ‰ higher as the associated royal jelly samples. The reason are higher trophic steps, because the δ^{15}N content increases at each step of metabolism about 3‰. The plant (pollen) has a nitrogen stable isotope ratio of δ^{15}N=+3 ‰. The royal jelly, a partly modified secretion made from pollen and honey has δ^{15}N=+4 ‰, which corresponds to a slight increase of trophic step. The bee tissue with δ^{15}N=+7 ‰ is a whole trophic step higher. The excrements of the honeybee can therefore be assumed to be accordingly lower in

^{15}N content (about $\delta^{15}N$=+0 ‰). These nitrogen stable isotope effects in increasing trophic steps are also well known from investigations of porc meat $\delta^{15}N$=+8 ‰ and the hairs of the human consumers $\delta^{15}N$=+11 ‰.

Analysis of carbon and nitrogen stable isotope ratios is sufficient to check authenticity of royal jelly samples. For the confident assignment of the geographical provenance stable isotope ratios of further elements are necessary. Royal jelly samples from different geographical and botanical origins proved to have different biological activities. Samples from regions with crystalline magmatic (granite) basements with high contents of trace elements showed high activities. High amounts of trace elements available for the plant have evidently influence on biological properties of the resulting royal jelly samples. One purpose of the present study was the regional assignment of royal jelly samples which we produced on granites, carbonates and loess.

The heavy trace element strontiums' isotope ratio $^{87}Sr/^{86}Sr$ was succesfully applied for the proof of the regional provenance in biologic materials (Horn et al. 1993a,b, 1994 and 1998; Roßmann 2000). The total strontium concentration of the source rocks and resulting soils depend on the geological age and chemistry of the rocks. The strontium isotopes ^{86}Sr and ^{88}Sr are primordial. The strontium isotope ^{87}Sr is radiogen and derives from the ß-radiator ^{87}Rb. Rubidium accompanies potassium which appears in higher concentration in acid, old crystalline rocks and less in the basic, younger carbonate rocks.

Additionally to the rock types anthropogenic sources of Sr in form of (phosphate-) fertilizers which enter in the soils have to be considered (Horn et al. 1993). Intensive agriculture lead to higher ^{87}Sr values. Plant nutrition via arerosol uptake of the leaves is a further source of Sr which can differ in isotopic ratio of Sr from the local soil (Horn et al. 1993). Thorough knowledge of the geological and pedological situation in the regions was therefore necessary.

Examples for $^{87}Sr/^{86}Sr$ stable isotope ratio in different rock types are: acidic magmatites (>+2.1), loess (-0.5 – +1.0), molasse sediment (-2.4 – +1.0) and limestones e.g. jurassic carbonates (-1.7) or Keuper, Trias (+2.1).

Acidic crystalline rocks of Vendée-Maine are high in ^{87}Sr deriving from ^{87}Rb. Royal jelly , honey and bee tissue from Vendée-Maine showed therfore high ^{87}Sr values (tab. 5.2 and fig. 5.2). Royal jelly and bee tissue are accordant. Higher $^{87}Sr/^{86}Sr$ ratio of the honey bee can be explained with extraneous influence factors as the aerosol uptake of Sr as described.
Bee tissue and honey from the Alpine foreland (Weilheim, Bavaria) had low ^{87}Sr values which are typical for the Bavarian Molasse Bassin. Associated royal jelly had a slightly higher $\delta^{87}Sr$-value of +0.02‰. Concerning the regional assignment the ^{87}Sr values are roughly accordant (fig. 5.2).

The $\delta^{87}Sr$-values of the Asian commercial samples “China B” ($\delta^{87}Sr$=+2.93) and “China C” ($\delta^{87}Sr$=+2.75‰) are accordant to ^{87}Sr values received from apple juices from Chinese loess ($\delta^{87}Sr$=+2.25‰, Roßmann, unpublished).

The ^{87}Sr value of the royal jelly from the Chézelles, Centre, France with $\delta^{87}Sr$=+1.67‰ deriving from acidic rocks with carbonates is lower in ^{87}Sr as royal jelly from crystalline rocks (fig. 5.2).

C and N isotope ratios proofed authentic production of the royal jelly samples from Picardie (Aisne). Carbon and nitrogen isotope ratios referred to location typical pollen and climatic conditions (fig 5.1).
The $\delta^{87}Sr$ strontium stable isotope ratio of the royal jelly samples, honeys and bee tissue from Picardie (Bois Les Pargny, Toulis et Attencourt) showed highly differing values (table 5.2 and fig. 5.2). Thorough query of the geological and pedological situation of the region (Picardie) showed a complex geological situation. Within only few kilometers the locations Lugny, Voharies, Cilly, Marle respectively Bois Les Pargny, Toulis et Attencourt are harvesting places with various geological differences. Besides the loess (loess-like silt) also other soils and rocks are present in terasses (sandy silt, colluvium of depression, old alluvium, modern alluvium, higher thanetien and higher ypresien): The loess has a deepness from 0.5 to 1 meter (Cartes géologiques BRGM, Orléans, France) and underlaying rocks appear in many places at the surface. The bee colonies of the royal jelly samples from Picardie (Aisne) 2000 are positioned within a distance of some kilometers. Ultimately the

reason for the varying ^{87}Sr values is the highly variable geological situation of the region.

Multi-element stable isotope analysis with carbon and nitrogen stable isotopes was shown as an applicable and useful method for the proof of authenticity of royal jelly samples. The trace elements' strontium stable isotope ratios could indicate the regional assignment of royal jelly samples from areas with geologically well-defined substrata. For a doubtless regional assignment of royal jelly samples stable isotope ratios of further elements like oxygen, sulphur and hydrogen have to be investigated. Actual developments in analyzing instruments allow unproblematic high-throughput of samples in deuterium ratio analysis as described for the regional assignment of butterflies (Wassenaar et al. 1998). A successful application of further elements' investigation can carefully be predicted.

6. Analysis, isolation and characterisation of proteins and peptides – detailed investigations of protein components

Summary

Royal jelly protein and peptide fractions were separated with analytical and semi-preparative HPLC, gelfiltration, ultrafiltration, anion and cation exchange chromatography, electrophoresis and subsequent microbiological tests of the proteins fractions. Numerous totally or partially purified proteins and peptides involved in microbiological activities were characterised and correlated with their N-terminal sequence analysis (Edman degradation), mass spectrometry (ESI-MS and MALDI-TOF-MS) and data base search. Royal jelly contains a high number of native and derivated proteins, dominated by 4 large major protein families. These Major Royal Jelly Proteins (MRJP's) contain more than 80% of the total protein mass. Electrophoretical investigation (2-DE) of active HPLC peaks and electrospray mass spectrometry (ESI-MS) showed the presence of families of small royal jelly peptides in active samples. MALDI-TOF-MS analysis of royal jelly showed that the preponderating part of royal jelly peptides and proteins are cleavage products of abundant protein families. Antibacterial activity tests shows that mainly short peptides are biological active. Such peptides are released from the major royal jelly proteins by proteolytic cleavage. Other small peptides are independent from major proteins and show therefore no sequence homology to the major royal jelly proteins. Some of such small peptides were synthesized and investigated by NMR spectroscopy. The synthetic product shows similar activity compared to those isolated from royal jelly.

Introduction

The microbiological properties of royal jelly are the most evident biological properties of royal jelly. The antibacterial activity of royal jelly was formerly assigned to a fatty acid, the 10-hydroxy-Δ2-decenoic acid. Other highly active molecules as native and derivated proteins became now more and more evident (Fujiwara 1990; Sauerwald 1997). Recent investigations (Stocker 1999, Sauerwald 1997; Sauerwald et al. 1998) showed the essential contribution of protein fractions and peptides to the antibacterial activity. Growth-inhibition assays with protein fractions separated with gelfiltration and polyacrylamide gels were successfully applied to indicate the antibacterial

activity of royal jelly peptides (Stocker 1999). Gram-positive bacteria are inhibited strongly and most of the gram-negative bacteria significantly less (Stocker 1999, Sauerwald 1997; Yatsunami and Echigo 1985). Fungicidal activity of royal jelly fractions was described for several fungi, in particular *Fusarium subspecies*. (Sauerwald 1997; Sauerwald et al. 1998). The microbiological properties of royal jelly are of particular interest for pharmaceutic applications. Further characterization of active proteins and peptides with bioanalytical methods was therefore one of the aims of the present study. The significance of particular proteins in respect of their contribution to the antibacterial effect should to be specified and is subject of this work.

Methods and Materials

Separation of water-soluble and water-insoluble royal jelly components: To separate the water-soluble supernatant from the insoluble pellet, the royal jelly sample was diluted threefold with distilled water (4°C) and centrifugated at 27 000×g, 15 minutes at 4°C. The supernatant was concentrated in a vacuum dryer to obtain roughly the original concentration before dilution of royal jelly (Stocker 1999; Sauerwald et al. 1998, Sauerwald 1997). The water-insoluble pellet was threefold washed with cold (4°C), distilled water and centrifugated. For agar diffusion tests one drop of pure royal jelly, approx. 100 mg of the water insoluble wet pellet and 40 µl the water-soluble supernatant were separately applied on agar plates.

Gel filtrations were carried out using Sephadex G-50, Sephadex G-10 and Sepadex-G25 and Sephacryl S-300 materials from Pharmacia, Freiburg, Germany as described by Stocker (1999) on a FPLC from BioRad, München, Germany. Column sizes were 100x2 cm. 4mM Na-Citrate buffer pH 4 at a flowrate of 4 ml/min was applied. Fractions were collected and vacuum concentrated for antibacterial tests. For subsequent agar diffusion tests *Micrococcus luteus* DSM 348 was used as indicator strain.

Ultra filtrations with centricon 3, centricon 10 und centricon 30 UF-tubes from Amicon-Millipore, were carried out with aqueous royal jelly suspensions. For this 500 mg of royal jelly were two-fold diluted with deionized water and the suspensions were

filtrated subsequently with the 30 kD, 10 kD and 3 kD-tube. Therewith 3 fractions were produced: one fraction (<3kDa), a second with 3-30 kDa and a third fraction (>30 kDa). Royal jelly (RJ) dilutions (500 mg RJ + 1 ml deionized H_2O) were filtrated according to the specifictions of the manufactur. Fraction volumes were approximately 120 µl for the fraction 3-30 kDa.

Ion exchange chromatography HQ and HS: Aqueous royal jelly suspensions were separated on a BioCAD™Sprint™, Perfusion Chromatography® System from PerSeptive Biosystems Inc., USA. Column material was a strong anion exchange column POROS 20 HQ (20 µm; 4.6x100 mm) (Quaternized polyethyleneimine) from Perseptive Biosystems Inc. using a 50 mM Tris-buffer with pH 7.5 at a flow rate of 5 ml/min and a NaCl-gradient from 0 –1 M in 17 minutes (50 column volumes). Protein fractions of 1.7 ml were collected and vacuum concentrated. Agar diffusions tests with *Micrococcus luteus* DSM 348 were applied according to chapter 2.2.

A strong cation exchange column POROS 20 HS (20 µm; 4.6x100 mm; Sulphopropyl) from Perseptive Biosystems Inc was used with a 50 mM MES-HEPES-acetate buffer pH 7 at a flow rate of 5ml/min and a NaCl-gradient from 0-1 M in 17 minutes (50 column volumes). UV-absorbance was measured at 220 nm. 50 µl sample volume of royal jelly supernatant were applied for ion exchange chromatography with HQ and HS. The separations were also combined with preceding UF-filtration and subsequent analytical HPLC analysis and antibacterial tests.

Purification of Royal jelly proteins and their sequence analysis:

Analytical C8 RP-HPLC - Royal jelly was diluted 3 fold with water, vortexed to homogenity and solids removed by centrifugation at 18 000 x g, 4°C, 15 minutes. Injections of 20 µl supernatant were purified by HPLC on a aquapore RP-300 column, C8, 7 microns, 2.1 x 220 mm, with the Applied Biosystems µHPLC system 140 B. Absorbance was monitored at 220 nm, and the elution solvents consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). Samples were eluted at a flow rate of 120 µl/min using the following solvent gradient: 0':0%B; 60':10%B; 155':30%B; 185':45%B. Fractions

were collected and dried under vacuum for further analysis by Edman degradation and Agar diffusion test.
Up to 40 fractions were collected. Volumes were collected between 100 µl and 1400 µl. About 30 significant peaks were detected. There was no antibacterial activity without peak.

Semi-Preparative C8 RP-HPLC – Water-soluble royal jelly supernatant was prepared as described for analytical C8 RP-HPLC and 1 ml of supernatant was purified by semi-preparative RP-HPLC on a C8 column (Vydac RP-C8, 5 microns, 250x10 mm) with the Merck-Hitachi HPLC system. Absorbance was monitored at 214 nm. The elution solvents were 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). Samples were eluted at a flow rate of 1 ml/min using the following solvent gradient: 0':0%B; 60':10%B; 155':30%B; 185':45%B.

N-terminal Sequence Analysis - The sequence of HPLC purified peptides was determined by automatic Edman degradation using a Procise 492 microsequencer from Applied Biosystems, Foster City, USA, running in a pulsed-liquid mode. N-terminal sequences of royal jelly proteins are given in tables 6.2.1.
Extraction of coomassie stained royal jelly proteins from two-dimensional electrophoresis gels for subsequent N-terminal sequencing (Edman degradation): 2-DE-gels were rinsed 1-2 hours in bi-distilled water and protein spots were excised. Several spots of one protein from different gels were shaken overnight in a Eppendorf tube with 200 µl extraction buffer (sodium acetate 0.1N; pH 8.5; SDS 0.1%) at 37°C.

Sequence alignements of the N-terminal protein sequences (obtained from Edman degradation) with Swiss Prot and TrEMBL databases were performed according to the computer program BLASTP. All the comparisons were made using the computer facilities of infobiogen (Evry, France).

Protein concentrations were determined using the Bio-Rad Protein Assay (Bradford test) and the BC Assay (Bichionic acid test), Uptima, Montluçon, France.

Synthesis of a tetrapeptide: A tetrapeptide with the sequence NEVN was syntesized by solid phase peptide synthesis according to Merrifield method (Merrifield 1963, Merrifield 1965; Merrifield and Stewart 1965). The Fmoc / wang strategy was used. The Fmoc group (9-fluorenyl–methyl–oxy–carbonyl) is the temporary protection of N alpha amino function of the peptide in elongation (Atherton 1981) and the wang resin (Wang et al.1973) is the solid support . The peptide was synthetized in 0.1 mmole scale (=100 µmol scale) with a peptide synthesizer ABI 431A Version 2.0 (Applied Biosystems).

For Fmoc deprotection, 20% piperidine/NMP was used as basic solvent . The amino acid was activated with a 1:1mixture of two solutions : DCC 1 M in NMP and HOBt 1M in NMP (1 mol.l-1 in NMP solvent) (DCC : dicyclohexylcarbodiimide, HOBt : 1-Hydroxybenzotriazol, NMP : N-methylpyrrolidon).

After synthesis, the mixture of TFA (trifluoroacetic acid) / water / TIS (tri-isopropylsilane) (90/5/5) was used for the peptide cleavage and for the peptide deprotection (4 hours). A second deprotection was necessary for the trityl group deprotection. The peptide was cristallized in ether. All amino acid derivates used were provided from Novabiochem: Fmoc-Asn(Trt)-OH, Fmoc-Glu(tBu)-OH and Fmoc-Val-OH, Fmoc-Asn(Trt)-OH.
For NMR spectrometry the peptides NEVN, NEVNF and NEVNNEVN were synthesised in the Genzentrum of the Ludwig Maximilian Universität, München (Arbeitsgruppe Arnold) using Fmoc / wang strategy as described above with following modifications: For activation 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexyfluorophosphat (HBTU) in combination with 1-Hydroxybenzotriazol (HOBt) as activator and Diisopropylethylamin as base were used.

Purification of the tetrapeptide: The synthesized tetrapeptide was purified by RP-HPLC on a C18 column (Lichrospher 100, RP-C18, 5 microns, 250x4 mm) with the Merck-Hitachi HPLC system. Absorbance was monitored at 214 nm. The elution solvents were 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in a solution of 50% water and 50% acetonitrile (solvent B). Samples were eluted at a flow rate of 1 ml/min using the following solvent gradient: 0':0%B; 30':100%B; 35':100%A. The pureness of the synthesized tetrapeptide was verified

and the molecular mass measured by ESI-MS mass spectrometry on a triple quadrupole mass spectrometer (QuattroII, Micromass, Manchester, UK).

Electrospray ionisation-mass spectrometry (ESI-MS) of HPLC-samples: Analyses were performed on a triple quadrupole mass spectrometer (Quattro II, Micromass, Manchester, UK) equipped with a nebulizer assisted electrospray source. The high-flow nebulizer was operated in standard mode with N_2 as both nebulizing (20 L/h) and drying (250 L/h) gas. A voltage difference of 3 kV was applied between the capillary and the counter electrode. The cone voltage was of 25 V. The ion source was kept at 80 °C. Instrument control and data analysis were accomplished using Masslynx application software, version 3.4, from Micromass (Manchester, UK). Calibration of the mass spectrometer was performed using myoglobin.

MS experiments were achieved on samples collected during the analytical HPLC separation and 3 µL of each peptide were injected into a Rheodyne 7125 injection valve with a 10 µL sample loop. The compound was eluted into the mass spectrometer using 80/20 acetonitrile/water as mobile phase delivered by an isocratic LC-10AD pump (Shimadzu, Les Ulis, France) at a 20 µL /min flow rate. ESI mass spectra were obtained in positive ion mode.

Bacterial growth-inhibition assays on polyacrylamide gel and IPG gel strips: Antibacterial activity of royal jelly proteins and peptides against *Micrococcus luteus* DSM 348 was tested using growth-inhibition assays on 1-DE polyacrylamide gels (SDS-PAGE) according to Stocker (1999). Under identic conditions active peptides were isoelectric focused using immobiline dry strips (IPG gel strips) as described for two-dimensional (2-D) electrophoresis without the denaturing agent dithiothreitol (DTT) in the sample buffer. 1-DE gels and IPG gel strips were rinsed in deionized water to remove the most part of the buffer substances before antibacterial tests.

Antibacterial tests of RP-HPLC purified proteins and peptides were realized using agar diffusion tests as described in chapter 2 with *Micrococcus luteus* and *Escherichia coli* as two indicator strains. Aqueous peptidic solutions (10 µl) were additionally tested in microtiter plates with 90 µl of a bacteria culture (midexponential-phase) diluted with nutrient broth to a starting absorbance of 0.001 at 600 nm.

Microbial growth was measured by the increase of absorbance after 16h incubation at 37°C.

Two-dimensional (2-D) polyacrylamide gelelectrophoresis of royal jelly proteins with Immobilized pH-gradients was carried out according to Görg et al. (1995, 1999 and 2000, mod.). Protein compounds are separated in 3 steps: isoelectric focussing, equilibration and the separation by molecular mass using sodium dodecyl sulphate polyacrylamide gelelectrophoresis (SDS-PAGE).

Sample preparation: For micro-preparative 2DE-gels royal jelly samples were solved 1.5 fold respectively 3-fold in lysis buffer (6 M urea, 2 M thiourea, 1% CHAPS, 0.4% DTT, 0.5 % Pharmalyte 3-10) and sample volumes from 40 to 100 µl were loaded into the sample cups, corresponding to approximately 1 to 5 mg of protein were focused with IEF as described.
Active protein peaks separated with analytical HPLC were visualized with 2D-electrophoresis and stained with silver nitrate. The HPLC-peaks were vacuum dried and solved in 60 µl lysis buffer (DryStrip Swelling Buffer) and 20 µl SDS-buffer (1% SDS, 100 mM Tris-HCl, pH 9.5) before focusing with the analytical IEF-protocol.

Isoelectric Focusing (IEF): IPG (immobilized pH gradient) gel strips from Amersham Biosciences,Freiburg with pH gradient 3-10, 18 cm were used. At each time respectively 10 identic IPG gel strips were focused synchronously. Immobiline DryStrips pH 3-10 were rehydrated overnight in a Immobiline DryStrip Reswelling Tray in 350 µl Dry Strip swelling solution. Firstly the swelling solution was applied. Gel strips were applied with the gel side below. Gel surface was protected by a layer of silicone oil (1.5 ml). Reswelled strips were rinsed with deionized water and applied on the temperated (20^0C) plate cooler. Sample cups were applied close to the anode with electrode strips impregnated with 1 ml water. Electrodes were carefully mounted as described by the manufacturer. IPG gel strips were protected with a layer of silicone oil. The electric field was applied as described for analytical and preparative gels. Electrode strips were changed every two hours. After isoelectric focalisation IPG strips were frozen at -80^0C or immediately used for second dimension of separation.

Dry Strip Swelling solution (lysis buffer):

6 M urea
2 M thiourea
1% CHAPS
0.4% DTT
0.5 % carrier ampholytes (Pharmalyte 3-10)

Materials:

Immobiline DryStrip pH 3-10, 18 cm (Amersham Biosciences,Freiburg)
Immobiline DryStrip Reswelling Tray
Immobiline DryStrip Kit
Inert oil: PlusOne Immobiline DryStrip Cover Fluid

Equipment:

Multiphor II
Electrophoresis Power Supply-EPS 3501 XL
MultiTempIII

Parameters of IEF:

	voltage	amperage	power	time
Analytical IEF				
Immobiline DryStrip pH 3-10, 18 cm	50V 150 V 300 V 600 V 1.500 V 3.500 V	0.5 A 0.5 A 0.5 A 0.5 A 0.5 A 0.5 A	50 W 50 W 50 W 50 W 50 W 50 W	16 1 h 1 h 1h } 42.000 Vh
Micropreparative IEF				
Immobiline DryStrip pH 3-10, 18 cm	50 V 150 V 300 V 600 V 1.500 V 3.500 V	0.5 A 0.5 A 0.5 A 0.5 A 0.5 A 0.5 A	50 W 50 W 50 W 50 W 50 W 50 W	16 h 3 h 3 h 3h } 42.000 Vh

Times in initial stages of IEF were prolongated in order to improve sample entry.

Equilibration: Before the separation of royal jelly proteins according to their molecular weight (Laemmli 1970) IPG gel strips were equilibrated using SDS containing buffers under denaturing conditions. Dithiothreitol (DTT) was used as denaturing agent. Further treatment with iodacetamide has the function to avoid new linkage of cystein-bonds. After removal of the inert oil the IPG strips were equilibrated in the Immobiline DryStrip Kit. For fixation of the IPG gel strips an electrode was

used. IPG gel strips were equilibrated for 15 minutes in equilibration solution 1 and subsequently 15 minutes in equilibration solution 2.

Solutions (1. Dimension):

Equilibration buffer:	6 M urea 30% glycerol 2% SDS 50 mM Tris-HCl, pH 8.8	
equilibration solution 1 :	equilibration buffer DTT	100 ml 1g
equilibration solution 2 :	equilibration buffer iodacetamide	100 ml 4 g

After removing the oil (dry strip cover fluid) dry strips were equilibrated 15 minutes in both equilibration solutions 1 and 2. The dry strips were equilibrated in the Immobiline DryStrip Kit using an electrode for fixation of the IPG gel strips.

SDS-PAGE as second dimension of separation: The equilibrated dry strips were separated in the second dimension (SDS-PAGE) as described by Görg et al. (1999 and 2000). Sample entry occured for one hour at 80 mA, 200 V, the electrophoretical separation until the discharge of the bromphenolblue front after approximately 16 h at 165 mA and 200 V in a vertical system Hoefer DALT multiple Electrophoresis Tank (Amersham Biosciences, Freiburg) as described by Anderson et al. (1978), Berkelman et al. (1998). 10 gels of the size 20x23 cm run simultaneously overnight at a temperature of 20°C. Molecular weights were determined with Biorad Precision Protein Standards, from Bio Rad, Germany. 2-DE gels were documented with a transmitted light scanner 1600 Pro from Epson, Düsseldorf, Germany. Wet gels were stored in plastic films for subsequent analysis or conservation.

Solutions (2. Dimension):

Glycerol solution:	glycerol 87% bromphenol blue $aqua_{deion.}$	87 ml 1 ml 62 ml
Buffer 1:	Tris SDS	90.83 g 2.00 g

Dissolved in 300 ml deionized water, adjusted with HCl to a pH of 8.6 , filtrated and filled to 500 ml.

Buffer 1-saturated butanol: 20 ml Buffer 1 shaken vigourously with 30 ml 2-Butanol and decanted 2-Butanol.

Gel solution (12%):	Acrylamide solution (29/1)	389.5 ml
	Buffer 1	250 ml
	$Aqua_{deion.}$	315 ml
	Glycerol	50.0 g
	TEMED	55 µl
	Ammonium persulphate (10%)	7.0 ml
Gel solution (18%):	Acrylamid solution (29/1)	584 ml
	Buffer 1	250 ml
	$Aqua_{deion.}$	120.5 ml
	Glycerol	50.0 g
	TEMED	55 µl
	Ammonium persulphate (10%)	7.0 ml
Tank buffer:	SDS	19.9 g
	Glycine	299.6 g
	Tris	58 g
	$Aqua_{deion.}$	19.9 l
Agarose solution:	Agarose	250 mg
	Tank buffer	49 ml
	Bromphenol blue	1 ml
Equipment:	Hoefer Dalt Gel Caster	
	10 x Hoefer Dalt Gel Cassettes, 1.5 mm thickness	
	Hoefer Dalt multiple Electrophoresis Tank with circulation pump	
	Water bath Multi Temp III Thermostatic Circulator	
	Power Supply EPS 3501 XL	

All mentioned equipment used for two-dimensional electrophoresis derived from Amersham Biosciences, Freiburg.

Realisation of two-dimensional electrophoresis gels using the above mentioned equipment is described by Görg et al. (1999 and 2000) and appropriate laboratory manuals. Vertical SDS electrophoresis with Hoefer Dalt, Amersham Biosciences,

Freiburg was realised as described by Anderson (1978) and Berkelman (1998). Further descriptions are given in several recent PhD-theses (Steudel 2001, Scheyhing 2003; Wildgruber 2003).

Coomassie-staining: PhastGel® Blue R (Coomassie R350 stain) from Amersham Biosciences, Freiburg and Roti-Blue Coomassie stain, (CBBG-250 stain) Roth, Karlsruhe were used for visualization of the proteins in the gel matrix with colloidal coomassie stainings. Coomassie stained micro-preparative gels were used for subsequent sequencing of the excised proteins using automated Edman degradation and MALDI-MS.

- Roti-Blue Coomassie stain was diluted with methanol as described in the enclosed protocol of Roth, Karlsruhe. The gels were stained for 24 hours without previous fixation. Then the gels were destained in deionized water for 24 hours.
- PhastGel® Blue R (Coomassie R350 stain) was used as 0.1% solution in ethanol/acetic acid/water (3/1/6) for 3 hours. Gels were stained in approximately 200 ml coomassie solution to cover the gels completely and shaken slowly at 60 RPM. Gels were destained with ethanol/acetic acid/water (4/1/5). The background was hereby not completely destained. Then gels were immersed overnight in deionized water containing few destaining solution to intensify the spot colour.

Silver nitrate staining of two-dimensional gels according to Blum et al. (1987, mod.) was applied for the determination of isoelectric point and molecular mass of royal jelly protein peaks from analytical HPLC:

Used Solutions:

Fixing solution:	40% ethanol (v/v), 10% acetic acid (v/v)
Thiosulphate reagent:	0.02% (w/v) sodium thiosulfate
Silver nitrate reagent:	0.2% (w/v) silver nitrate (Merck) 0.02% (v/v) formaldehyde (37%)
Developer:	3% (w/v) sodium carbonate 0.05% (v/v) formaldehyde (37%) 0.0005% (w/v) sodium thiosulfate
Stop reagent:	0.5% (w/v) glycine
Shrinking solution:	30% ethanol 12% (w/v) glycerol

All solutions were prepared with deionized water. Reagents should have analytical grade pureness. The silver nitrate reagent and the developer solution should be kept cool. Formaldehyde solution should be added straight before use of the reagents.

Staining:

Fixing:	3 hours
Washing:	3x20 minutes
Sensibilisation:	1 minute (thiosulphate solution)
Washing:	3x20 seconds
Silver stain:	20 minutes
Washing:	2x20 seconds
Developing:	approx. 5 minutes
Washing:	1x20 seconds
Stop reagent:	5 minutes
Washing:	3x10 minutes
Shrinking:	5 minutes

Wet gels were stored in plastic films for conservation.

MALDI-MS and microsequencing of proteins (in gel cleavage of royal jelly proteins separated with two-dimensional electrophoresis): MALDI-MS analysis and internal sequencing of royal jelly proteins were applied after in-gel cleavage of the proteins with the endoproteinase Lys-C (sequencing grade) as described by Decker et al. (2000). The received peptides were separated with a 300 µm x 150 mm capillary-HPLC-column (Vydac RP18, LC Packings) and the collected fractions were tested on their applicability for sequencing. One peptide was electrophoretically transferred on a Polyvinylidene Fluoride (PVDF) membrane (Applied Biosystems) and sequenced N-terminally with Edman degradation according to Hunkapiller (1983). An amino acid sequencer Procise™ 492A from Applied Biosystems (Weiterstadt, Germany) was used with an Online PTH Analyser 140 C and UV Detektor 785 A (Applied Biosystems, Weiterstadt). For sequence analysis the chemicals and reagents from the manufacturer (Applied Biosystems, Weiterstadt) were applied using the programme Pulsed Liquid (reaction cycle, converting cycle and gradient programme for the PTH-separation).
MALDI-MS was performed on a Bruker Reflex III TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). Dihydroxybenzoic acid was used as matrix and peptides were desorbed with a 337 nm nitrogen laser. Peptides were measured in

the positive reflector mode. An acceleration voltage of 20 kV and a reflector voltage of 22.8 kV were applied as described by Decker et al. (2000).

6.1 Gel filtration, ultrafiltration and ion exchange chromatography

Gel filtration of royal jelly supernatant was combined with antibacterial agar diffusion tests and growth inhibtion assays on polyacryamide gels as described by Stocker (1999). Gel filtrations showed poor seperativity (control with SDS-PAGE) but remained to be a separation methods which maintains integral activity of active royal jelly samples. IPG gel strips were used for further separation of active fractions of gel filtration as described before without the denaturing agent Dithiothreitol (DTT). Growth inhibition assays with the IPG gel strips were applied using agar diffusion tests with *Micrococcus luteus* DSM 348 as an indicator strain (Stocker 1999). Independent activity tests of active protein fractions with IEF and SDS-PAGE indicated molecular weight and isoelectric point of the active peptides. A molecular weight of the activity below 20 kDa and an isoelectric point of approx. pH 9 in the growth inhibiton tests allow the conclusion that only the chemically very stable insect defensin Royalisin can be found with this seperation methods. Therfore further column chromatography was applied together with antibacterial tests.

Ultrafiltrations (UF) of aqueous royal jelly suspensions were carried out using centricon 3, 10 and 30 UF-tubes (Amicon-Millipore) in order to get a priliminary separation of royal jelly for further chromatography. Ultrafiltrations were combined with antibacterial tests and analytical C8 RP-HPLC (according to chapter 2.2). Fractions were microbiologically tested after UF-separation and further after analytical HPLC. The fraction below 3 kDa showed the least antibacterial activity against *Micrococcus luteus* the fractions 10-30 kDa and >30 kDa higher activity. Analytical HPLC runs of the received UF-fractions showed differences in shape of several peaks, but altogether only poor separation was received with ultrafiltrations. Antibacterial tests of protein peaks received from analytical HPLC separations of the above mentioned UF-frations showed activities in all UF-fractions. Comparison of active peaks in HPLC chromatograms of the UF-fractions indicated that activities derived also from low-molecular peptide fractions (<3 kDa).

Ion exchange chromatography with a strong anion exchange column POROS HQ (quaternized polyethyleneimine) and a strong cation exchange column POROS HS (sulphopropyl) were applied for separation of royal jelly proteins. The separations

were combined with above described Ultra-Filtration and subsequent analytical C8 RP-HPLC analysis and antibacterial tests.

Separations with the cation exchange column HS showed poor separativity with only two broad peaks. Separations with the anion exchange column HQ were more separative showing several distinct peaks. A HQ ion exchange-chromatogram of a protein fraction (120 µl sample volume) received with Ultrafiltration (3-30 kDa) of aqueous royal jelly suspension is shown in fig. 6.1A. Agar-Diffusion tests with *Micrococcus luteus* DSM 348 as indicator strain showed that the preponderant part of the activity is concentrated in one peak in the void volume (fig. 6.1A; signed with an arrow). The other peaks of the chromatogram showed only few activity.

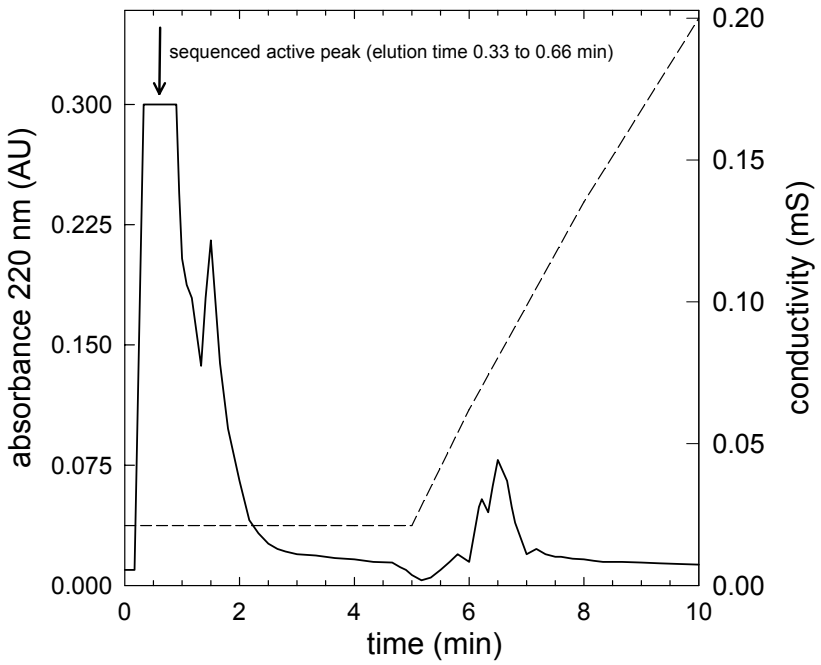

Figure 6.1A: HQ anion exchange-chromatogram of royal jelly proteins received from ultrafiltration (3-30 kDa) of aqueous royal jelly suspension

N-terminal sequencing of the most active HQ-peak (Edman degradation using Prosorb-filters) up to 17 amino acids showed following mixed sequence: 1X_2X_3Gln_4Asn,Asp_5Leu_6Gln_7Phe_8Lys_9Ala_10X_11Gly_12Val_13Ile,Asn _14Asp,Ile_15Gln_16Ala_17Ala

Amino acid positions with no UV-signal were signed with X. Several amino acids after one number indicate that the sequenced peak contained different proteins. No

homology of the sequence to known royal jelly protein families (MRJP's) was found in sequence alignements. Repeations of the separation showed high reproducibility of the described separation. To specify the activity sites the described most active peak of the HQ chromatography was also aliquoted for further µHPLC-separations as described in chapter 6.2.

6.2 Analytical C8 RP-HPLC and N-terminal sequencing (Edman degradation) of royal jelly peptides

Gel filtration and ultrafiltration proved applicability as preliminary purification of royal jelly peptides for subsequent microbiological tests. For characterisation of the activity further analysis of the protein fractions was necessary. The most active peak of the ion exchange separation HQ (fig. 6.1, peak signed with arrows) was therefore aliquoted and further separated with analytical C8 RP-HPLC runs as described in chapter 2.2 with gradients from 3 to 60% acetonitrile in 60 minutes runs. Eluted HPLC peaks (fig. 6.2) were collected seperately and analysed with antibacterial tests and N-terminal sequencing (Edman-degradation).

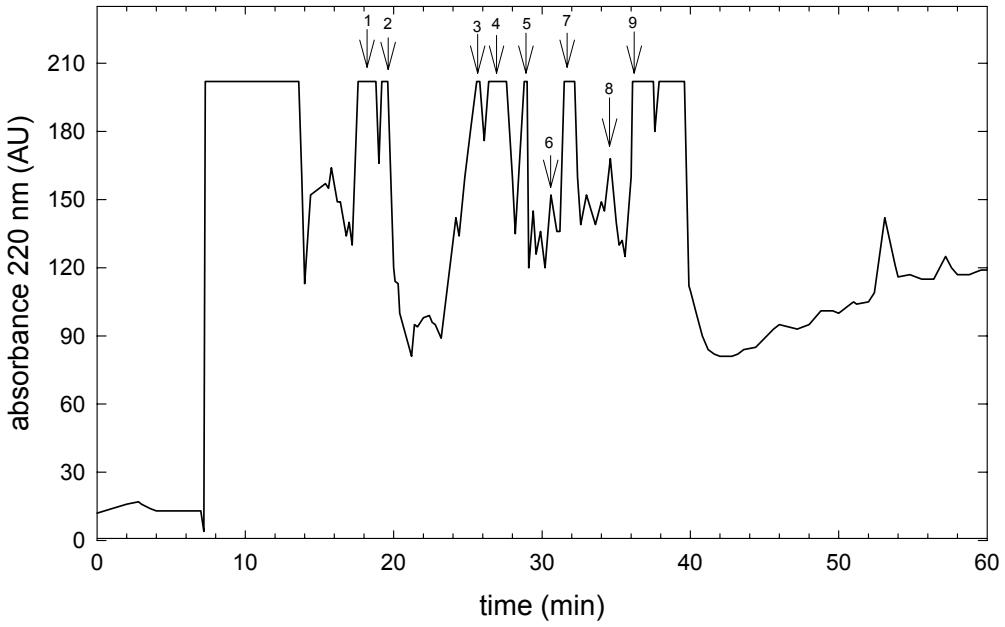

Figure 6.2: Analytical C8 RP-HPLC of a royal jelly protein peak from ion exchange chromatography HQ (according to 6.1). Proteins were separated as described in chapter 2.2 using a gradient from 3-60% acetonitrile within 60 minutes runs. Arrows indicate partially (N-terminal) sequenced proteins.

The analytical RP-HPLC chromatogram (fig. 6.2) shows several distinct separated broad protein peaks. Antibacterial activity against *Micrococcus luteus* DSM 348 was found in the double-peak 9 (fig. 6.2) and at the beginning of the chromatogram (9

minutes; fig. 6.2). Several peaks were sequenced N-terminally (Edman degradation) in order to characterise protein and peptide compounds of royal jelly. Homology of the N-terminal sequences was searched with several data bases. The knowledge of the N-terminal sequences of pure and derivated proteins in the HPLC-fractions is of substantial interest for the complete knowledge of royal jelly proteins.

Table 6.2A: N-terminal sequences of royal jelly proteins, separated with analytical C8 RP-HPLC; results of homology search are indicated; peak 1-9 derived from the chromatogram shown in fig. 6.2; peaks a,b,c derived from similar HPLC runs.

Peak-number	Gradient [%] acetonitrile	N-terminal sequences (Edman degradation) of royal jelly proteins (multi-sequences) and data base entries
1	20.7%	1 X- 2 Lys,Gln- 3 Gln,Arg,Val- 4 Leu- 5 Asp- 6 Asp- 7 Ala,Arg- 8 Lys,Gln- 9 X- 10 Asp- 11 Lys- 12 Ser- 13 Arg- 14 X- 15 Gly- 16 X- 17 Arg,Lys- 18 Gln,Lys → hypothetical protein of *Arabidopsis thaliana*
2	21.9%	1 Ala,Lys- 2 Ile,Met,Gln- 3 Val- 4 Arg- 5 Leu,Glu,Asp- 6 Asp- 7 Gln- 8 Gln,Lys- Leu- 9 Asp,Asn- 10 X- 11 Ala,Lys
3	27.9%	1 Lys- 2 Ile- 3 Leu- 4 Arg,Lys- 5 Ala,Asp,Arg- 6 Ile,Trp- 7 Ala,Lys,Met- 8 Met,Thr- 9 X- 10 Lys- 11 X- 12 X → transcription regulator of chloroplastes
4	29.2%	1 Lys- 2 Val- 3 Leu- 4 Pro,Leu,Asn- 5 Ala- 6 Ile,Lys,Asn- 7 Phe,Ala,Glu- 8 Ile- 9 Met- 10 Lys,Asp- 11 X- 12 X
5	30.6%	1 Ile- 2 His- 3 Leu,Phe- 4 Lys- 5 Ile- 6 Ala- 7 Asn- 8 Ile- 9 Asn- 10 Lys- 11 Gly- 12 Gly
6	32.6%	1 X- 2 Ile- 3 Gln- 4 Asn,Pro- 5 Glu,Gly,Ala- 6 Gln- 7 Ala,Phe- 8 Lys- 9 Ala,Glu,Asp,Met- 10 Lys,Gly- 11 Gly- 12 Gly,Phe
7	33.8%	1 X- 2 Ile- 3 Gln,Glu- 4 Asn,Pro- 5 Glu,Ala- 6 Gln,Asn- 7 Phe,Ala- 8 Lys- 9 Ala,Ile,Glu- 10 Lys- 11 Gly- 12 Gly, Phe
8	36.2%	1 Ile,Val- 2 Thr- 3Ile,Gln- 4 His,Asn,Asp- 5 Leu,Glu- 6 Leu,Gln- 7 Phe,Ser,Leu- 8 Lys,Phe- 9 Lys,Ala,Glu- 10 Gly,Lys- 11 Gln,Gly- 12 Val → mixture of peak 7 and royalisin
9	38.2%	royalisin – 35 amino acide positions sequence

a	26-30%	1 Lys,Asp- 2 Asn,Val- 3 Gln,Ala- 4 Ile,Pro- 5 X- 6 Lys- 7 Asn,Lys- 8 Gln- 9 Gly,Asp,Asn- 10 Asp → *Plasmodium falciparum* fragment
b	30-40%	1 Ile,Lys,Val- 2 Ser- 3 Ile,Gln- 4 His,Asn- 5 Leu,Glu- 6 Gln- 7 Phe,Ser- 8 Lys- 9 Ala- 10 Gly- 11 Gln,Gly- 12 Val
c	45%	1 Ala- 2 Ile- 3 Leu- 4 Arg- 5 Gly- 6 Glu- 7 Ser → Major Royal Jelly Protein 1 (MRJP1)

The Edman-sequencing of protein peaks which contain several proteins shows several amino acids at one step of degradation respectively. The amino acids are ordered according to decreasing signal intensity. The first amino acid position respectively can therefore be assumed as the sequence of the most abundant protein of the HPLC peak. Amino acid positions with no UV-signal were signed with X. Several amino acids after one number indicate different peptides in the HPLC peak. The sequences are different in some amino acid positions in the N-terminal ends and other positions are equal. Several amino acids at one position indicate that the HPLC peak contain several peptides which are potentially very similar and only varying in some amino acid positions. Due to the uncertainty of several amino acids at one step of degradation and the comparison of only one sequence segment results of homology search in data bases have to be regarded as vague homologies.

N-terminal sequences (multi-sequences) of HPLC-peaks (peak 1-9; fig. 6.2) are shown in table 6.2.A. Peak 2 shows homology to the N-terminal end of MRJP 2 (from Major Royal Jelly Protein). For several other sequences (1; 3-8; table 6.2A) no homology to N-terminal ends of MRJP's were found. The sequenced proteins can be derivated (proteolytic cleavage products) from the abundant high molecular MRJP's.
One active peptide peak (Nr. 9, fig. 6.2) was sequenced until the 35th amino acid position and the sequence was found identic to royalisin, an antibacterial peptide belonging to the insect defensin family (Fujiwara et al. 1990). Both peaks of the double peak (Nr. 9) were antibacterially active and contained royalisin. A further active peak was found at the beginning of the chromatogram. Sequencing of the active peak at the beginning of the chromatogram showed no clear sequence due to poor separation. The N-terminal sequence of royalisin was not found in the first unseparated broad protein peak. In order to improve separation of several peaks

HPLC conditions were modified towards longer separation times (more flat gradients).

The last 3 sequences (peaks a,b,c) derived from identic analytical C8 RP-HPLC separation chromatograms as fig. 6.2 which were found to be highly reproducible. Sequence of peak a derives from pooled peaks (within 26-30% acetonitrile), repurified with µHPLC using a gradient of 15 to 40% within 60 min - the sequence of a predominant peak at the gradient of 21% is given. The peak b derived also from pooled peaks (within 30-40% acetonitrile; repurified as peak a) - the sequence of a peak at the gradient of 25-27% is given. Peak a and b showed no homology to known royal jelly proteins. The peak c at 45% was pre-purified with gelfiltration sephadex G50 before analytical HPLC. Peak c showed homology to a Major Royal Jelly Protein 1 (MRJP1).

For following analytical RP-HPLC separation royal jelly supernatant (RJ sample collected 05/16/1998) was prepared and separated directly on HPLC without previous ion exchange chromatography (HQ). For preparations of royal jelly supernatant 500 mg royal jelly and 1.5 ml deionized water were vortexed vigorously and centrifugated (18 000xg; 15 min, 4°C). 20 µl of supernatant were directly injected in the µHPLC system 140 B from Applied Biosystems at a flow rate of 120 µl/min (table 6.2B) as described using following gradient of water/acetonitrile: 0':0%B; 90':10%B; 240':30%B; 270':45%B.

Table 6.2B: N-terminal sequences of royal jelly proteins, separated with analytical C8 RP-HPLC; results of homology search are indicated

Gradient [%] acetonitrile	N-terminal sequences (Edman degradation) of royal jelly proteins (multi-sequences) and data base entries
5.5%	1 X- 2 X,Met- 3 Arg,Glu- 4 X,Ala- 5 Gly,Met- 6 Lys,Ala- 7 Gln,Arg, Lys- 8 Gln,Lys- 9 Asp,Gly- 10 X-11 X,Gly- 12 Gln,Lys- 13 Arg- 14 X,Asp- 15 Gly
9.7%	1 X- 2 Met,Glu,Pro,- 3 Asp,Leu- 4 Met,Arg- 5 Met- 6 Asp,Asn- 7 Arg,Lys- 8 Met- 9 X-10 Asn- 11 Met → yeast plasma membran iron permease
10.4%	1 Lys,Thr- 2 Lys,Met,Asn,Asp,Glu- 3 Gln,Ser,Leu- 4 Gly,Ile,Gln- 5

	Asp,Leu,Met- 6 Val- 7 Lys- 8 Thr,Asn,Met- 9 Ala,Glu- 10 X- 11 Asn- 12 Lys- 13 X- 14 X-15 X → virus capsid protein VP2, current in virus capsid proteins
10.6%	1 Asn- 2 Val- 3 Glu,Phe- 4 Ala,Arg- 5 Glu,Met- 6 Asn,Asp- 7 Lys,Ser- 8 Pro,Gln- 9 Arg- 10 Asn- 11 Leu- 12 Glu
17.1%	1 Val- 2 Gly- 3 Gln- 4 Asn- 5 Glu- 6 Gln- 7 Phe- 8 Lys- 9 Gly,Ile- 10 Lys- 11 Gly- 12 Gly- 13 Ile- 14 Arg- 15 Asn → guanine nucleotide binding proteine
18.4%	1 X- 2 Val- 3 Ile- 4 Pro- 5 Gly- 6 Val- 7 Ala- 8 Ser- 9 X- 10 Gly- 11 X- 12 X- 13 X- 14 Asp- 15 Gln → several data base entries
19.5%	1 X- 2 Ile,Gly- 3 Gln,Leu- 4 Asn,Asp,Gly,Pro- 5 Glu,Ala- 6 Gln,Asn, Ile,Tyr- 7 Phe,Lys,Val- 8 Lys,Ala,Asn- 9 Glu,Ala,Pro- 10 X- 11 Gly → similar to peak 7 of tab. 6.2.A and peak 17,1% of table 6.2.B
29%	1 Val- 2 Thr- 3 X- 4 Asp- 5 Leu- 6 Leu- 7 Ser- 8 Phe- 9 Lys- 10 Gly- 11 Gln- 12 Val → royalisin

Analytical C8 RP-HPLC with royal jelly supernatant (RJ sample collected 05/16/1998) as described for table 6.2B were carried out with further prolongated running times in order to obtain favorable separation of selected peaks (gradient: 0':0%B; 105':7%B; 235':14%B; 375':22%B; 525':30%B). N-terminal sequencings showed following results (table 6.3.C):

Table 6.2.C: N-terminal sequences of royal jelly proteins, separated with analytical C8 RP-HPLC; results of homology search are indicated

Gradient [%] acetonitrile	N-terminal sequences (Edman degradation) of royal jelly proteins (multi-sequences) and data base entries
3.9%	1 Asn,Lys- 2 Gln,Lys- 3 Gln- 4 Asn- 5 Gly,Asp- 6 Asn- 7 Gln,Arg- 8 Gln- 9 Asn- 10 Asp- 11 Gly- 12 Lys,His
4.6%	1 Ala- 2 Ile- 3 Val- 4 Arg- 5 Asp- 6 Asn- 7 Gln- 8 Val- 9 His- 10 His- 11 Ser- 12 X- 13 Lys- 14 Leu → several homologies
5.3%	1 X,Gly- 2 Lys- 3 Gln,Arg- 4 Gln,Glu- 5 Gly- 6 Asp- 7 Arg- 8 Lys,Gln- 9 Arg- 10 Asp- 11 Gly- 12 Lys- 13 Arg-14 Gln- 15 Gly motif: Lys, Arg, Gly, Gly

	→ *Heliobaoter pylori*
7.5%	1 Lys- 2 Met- 3 Asn,Asp,Gln- 4 X,Arg- 5 Met,Leu- 6 Asn,Asp,Ala- 7 Gln,Arg- 8 Gln,Met- 9 Leu- 10 Gln- 11 X- 12 Ala → several homologies → Lys,Met,Asn,Asp,Arg,Met,Asn,Asp,Gln,Met
8.4%	1 X-2 Gln,Met,Glu,Asp- 3 Asn,Asp- 4 Leu,Pro,Arg- 5 Lys,Met- 6 Asp,Asn- 7 Val,Gln- 8 Leu,Met- 9 Pro,Gln
8.5%	1 X- 2 Glu- 3 Gln,Asn,Asp- 4 Asn- 5 Met,Gly,Lys,Leu- 6 Asn- 7 Ile- 8 Gln- 9 Leu- 10 Gln- 11 X- 12 Ala
9.4%	1 Ile- 2 His- 3 Leu
10%	1 Ile- 2 His- 3 Leu,Arg- 4 Pro,Met- 5 Met,Asp- 6 Asn,Lys- 7 Arg,Ala- 8 Met
13.1%	1 X- 2 Val- 3 Gln- 4 Arg,Ala,Phe- 5 Glu- 6 Lys,Ala- 7 Ser,Gln- 8 Ile- 9 Asn
14.3%	1 X- 2 X- 3 Gln- 4 Asn,Pro- 5 Met,Asp- 6 Lys,Gln- 7 Phe,Ala- 8 Lys- 9 Val,Ile- 10 Lys- 11 Gly- 12 Phe → peak 17.1% of table 6.2.B: Val- Gly- Gln- Asn- Glu- Gln- Phe- Lys- Glu- Lys- Gly- Ile- Arg- Asn
15.4%	1 Lys- 2 Met,Val- 3 Gln,Asn,Ala- 4 Asn,Pro- 5 Glu,Gly,Lys- 6 Gln,Val- 7 Ala- 8 Lys- 9 Asn,Glu,Asp- 10 Lys,Asn- 11 Gly → peak 17.1% of table 6.2.B
15.9%	1 X- 2 Pro- 3 Asn,Pro- 4 Glu- 5 Ile,Lys,Gln- 6 Gly,Pro- 7 Lys,Ala- 8 Asn- 9 Gln,Pro- 10 Arg- 11 Asp- 12 Val

Prolongation of analytical C8 RP-HPLC gradients showed further improving of separations. The multitude of proteins and peptides in royal jelly taken into account analytical HPLC (under described conditions) showed favorable separation of peptides and proteins for subsequent sequence and microbiological analysis. N-terminal sequences of HPLC purified royal jelly proteins (table 6.2A-C) showed some homologies to several data base entries. Homologies to proteins of several species are shown (fig. 6.3A-C). Due to uncertainties of several amino acids at one step of degradation and the comparison of only one sequence segment results of data base searches have to be regarded as vague homologies. N-terminal sequences of already described royal jelly proteins as royalisin (Fujiwara et al. 1990) or the most abundant royal jelly proteins (MRJP1, 3 and 5; Schmitzova et al. 1998) were also found in the HPLC separations.

Optimised analytical HPLC conditions for N-terminal sequencing were succesfully applied. Resulting protein sequences can be rationed in two groups according to their degree of purification. N-terminal sequence was characterised from approximately one dozen of predominant proteins:

Table 6.2.D: N-terminal sequences of selected royal jelly proteins from tables 6.2.A-C, separated with analytical C8 RP-HPLC; only amino acids with the most intensive signals in each step of Edman degradation were taken into account in order to receive the predominant sequences; results of homology search are indicated.

Number	N-terminal sequences (Edman degradation) of royal jelly proteins (multi-sequences) from tables 6.2.A-C and data base entries
1	Lys-Asn-Gln-Ile-X-Lys-Asn-Gln-Gly-Asp (6.2A-a) → Asparagine-rich blood stage proteins of *Plasmodium falciparum* (position 107)
2	Val-Ile-Pro-Gly-Val-Ala-Ser-X-Gly-X-X-X-Asp-Asn (6.2B-18.4%)
3	Val-Gly-Gln-Asn-Glu-Gln-Phe-Lys-Glu-Lys-Gly-Gly-Ile-Arg-Asn (6.2B-17.1%)
4	Ala-Ile-Val-Arg-Asp-Asn-Gln-Val-His-His-Ser-X-Lys-Leu (6.2A-a) → N-terminal end of Major Royal Jelly Protein 3
5	Gly-Lys-[Glu, Arg]-Gln-Gly-Asp-Arg-[Lys,Gln]-Arg-Asp-Gly-Lys-Arg-Gln-Gly (from 6.2C-5.3%)
6	royalisin
7	[Ile,Lys]-Ser-[Ile,Gln]-[His,Asn]-[Leu,Glu]-Gln-[Phe,Ser]-Lys-Ala-Gly-[Gln, Gly]-Val (from 6.2A-b)
8	Met-[Arg,Glu]-X-[Gly,Met]-[Lys,Ala]-[Gln,Arg]-[Gln,Lys]-[Asp,Gly]-X-[X,Gly]-[Gln, Lys]-Arg (from 6.2B-5.5%)
9	Asn-Val-[Glu,Phe]-[Ala,Arg]-[Glu,Met]-[Asn,Asp]-[Lys,Ser]-[Pro,Gln]-Arg-Asn-Leu-Glu (from 6.2B-10.6%)
10	Ile-His-[Leu,Arg]-[Pro,Met]-[Met,Asp]-[Asn,Lys]-[Arg,Ala]-Met → internal part from Major Royal Jelly Protein 5 (from 6.2C-10%)

From our sequence results of HPLC fractions the described abundant royal jelly proteins (MRJP1, 3 and 5; Schmitzova et al. 1998) and royalisin (Fujiwara et al.

1990) could be determined. Furthermore our sequence studies (tables 6.2.A-D) showed that several HPLC peaks constitute derivated proteins, i.e. proteolytic fragments of major royal jelly proteins (MRJP's); others are independent proteins without sequence homology to MRJP's. The knowledge of the proteolytic processes and thereby produced derivated proteins and peptides is of substantial interest with regard to the biological activities of the royal jelly protein compounds.

6.3 Analytical 2-D electrophoresis of selected C8 RP-HPLC peaks: from HPLC to 2-DE gel

Antibacterial activity against *Micrococcus luteus* and *Escherichia coli* was determined in several peaks of analytical C8 RP-HPLC (as described in chapter 2). Active proteins peaks were electrophorized with 2-D electrophoresis and stained with silvernitrate (acc. to Blum et al.1987, mod.) in order to examine molecular mass and isoeletric point.
10 selected analytical RP-HPLC peaks were vacuum-concentrated, solved in sample buffer and 2-DE gels were prepared as described (acc. to Görg et al. 2000, mod.).

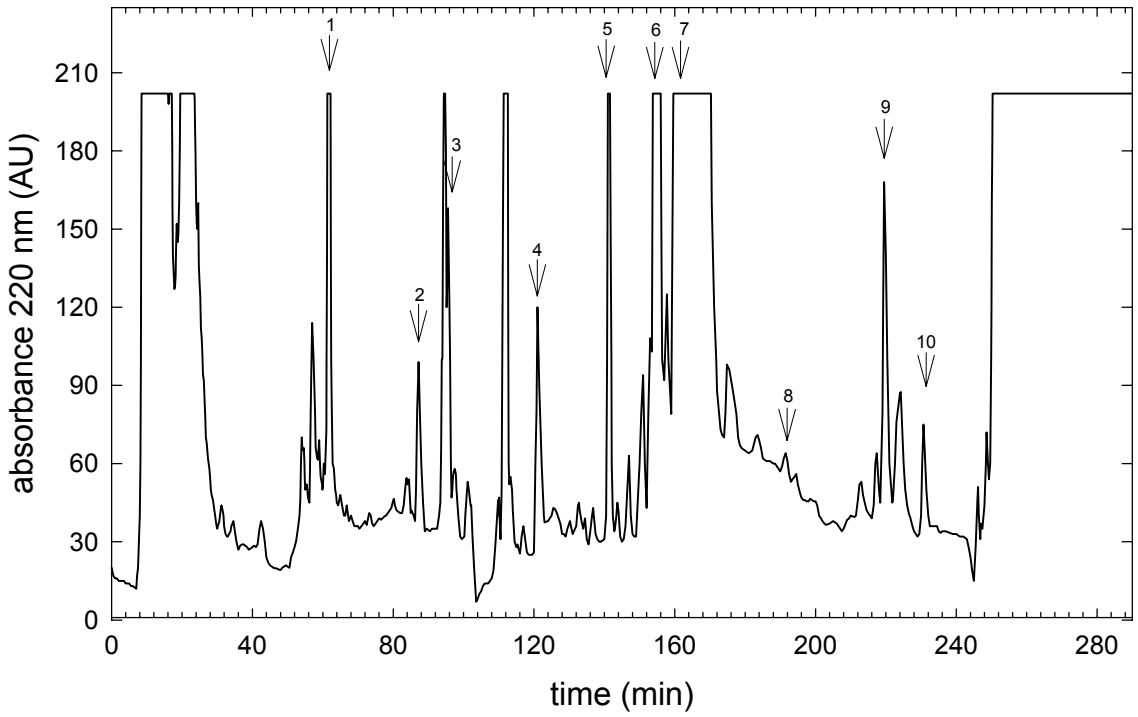

Figure 6.3.1: Analytical C8 RP-HPLC of a highly active royal jelly sample (RJ sample collected 05/18/2000) used for 2-D electrophoresis; the chromatogram was pepared with an analytical µHPLC system from Applied Biosystems with following gradient: 0':0%B; 90':10%B; 240':30%B; 290':45%B.

The above mentioned HPLC peaks number 1-10 from figure 6.3.1 were separated with 2-D electrophoresis. Some of the received 2-DE gels are displayed in the following:

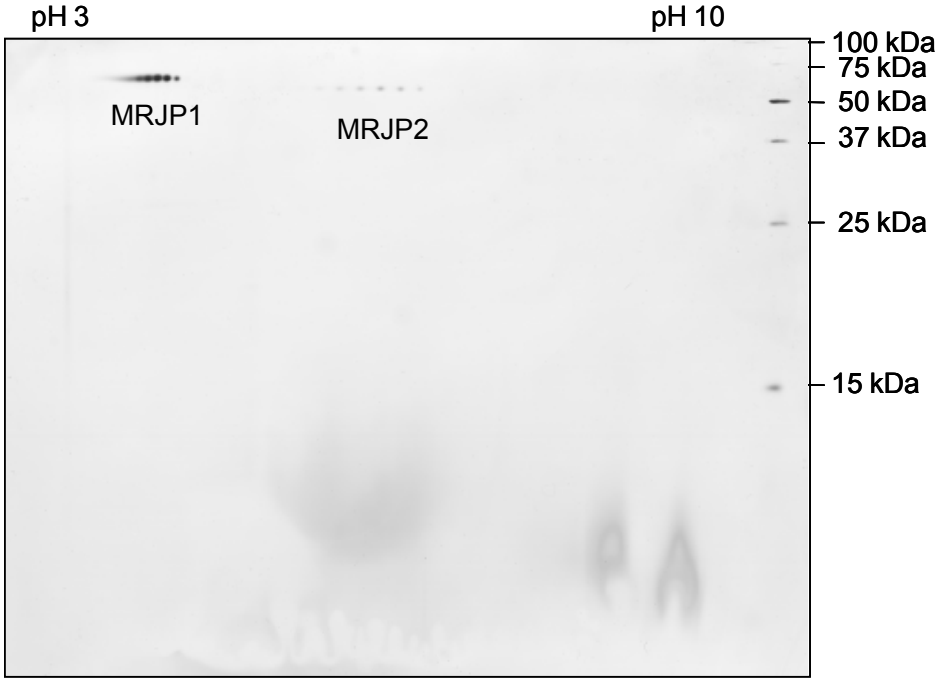

Figure. 6.3.2A: 2-D electrophoresis gel (silver nitrate stain) of a protein fraction purified with analytical C8 RP-HPLC (peak 1; fig. 6.3.1); 1. Dimension IPG 3-10; 2. Dimension T=18%; 2 protein families composed of respectively at least 6 proteins with same molecular weight but different isoelectric points.

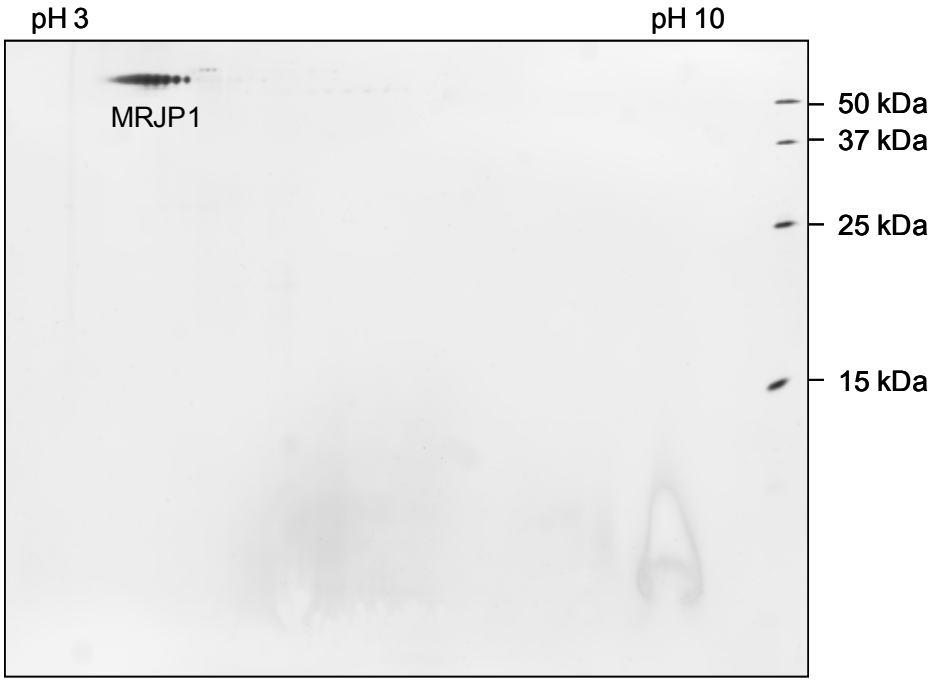

Figure 6.3.2B: 2-D electrophoresis gel (silver nitrate stain) of peak 3 (fig. 6.3.1)

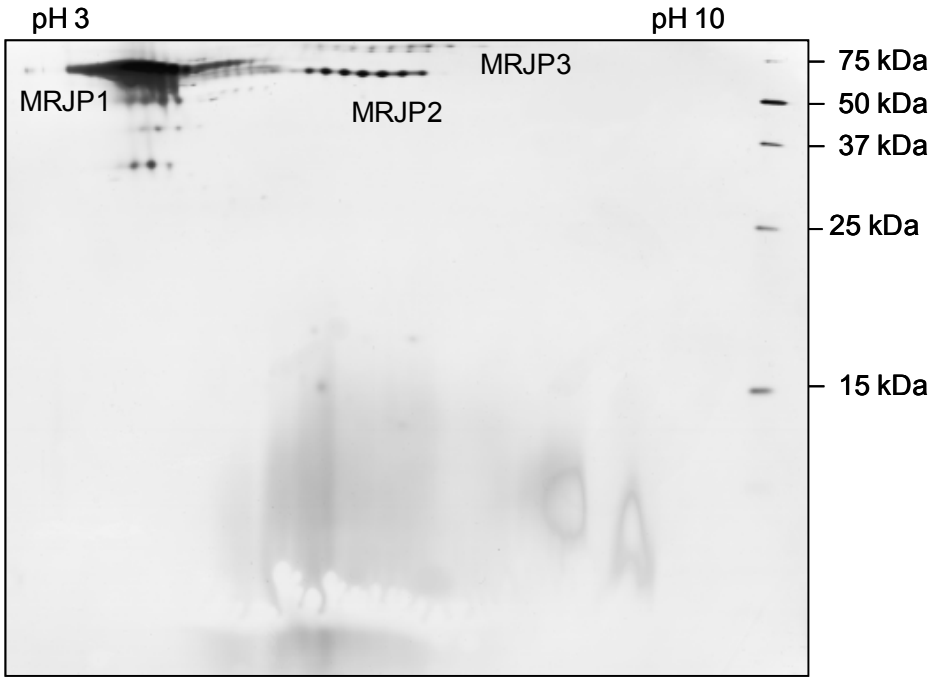

Figure 6.3.2C: 2-D electrophoresis gel (silver nitrate stain) of peak 6 (fig. 6.3.1)

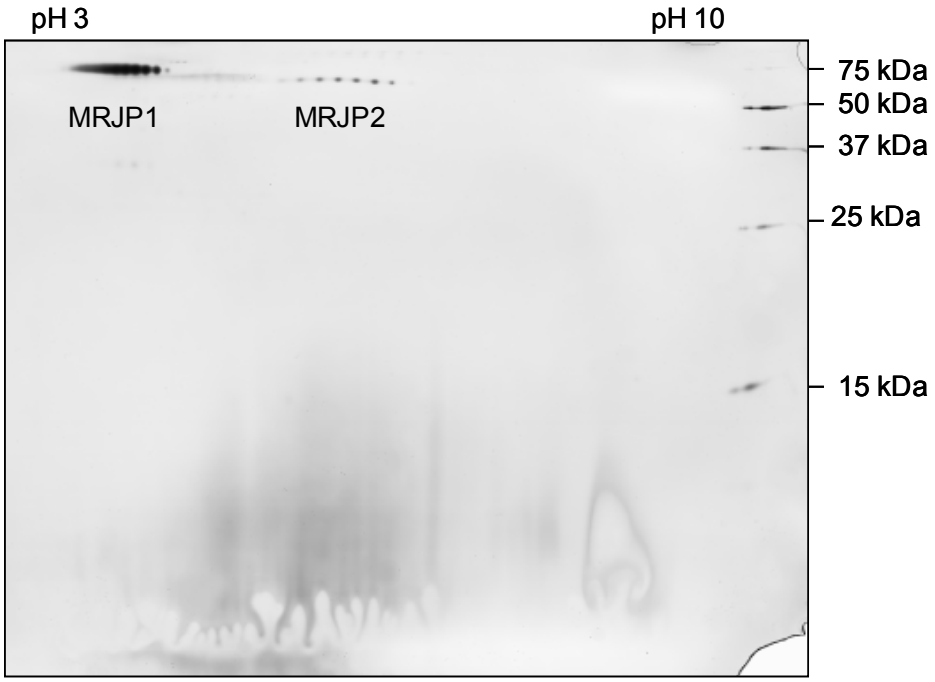

Figure 6.3.2D: 2-D electrophoresis gel (silver nitrate stain) of peak 7 (fig. 6.3.1)

2-D electrophoresis with subsequent silver nitrate staining showed the presence of protein peaks with middle and higher molecular weight in antimicrobial active RP-HPLC eluates. Proteins with a molecular weight above 50 kDa were found in HPLC peaks in the first part and in the last part of the chromatogram. According to well-

known characteristics of reversed phase HPLC hydrophilic peptides can be expected in the first part of the chromatogram and middle and high molecular proteins with hydrophobic characteristic are eluted at the last part. In this regard the visualisation of HPLC eluates with 2-D electrophoresis was succesfully applied to reveal unexpected characteristics of analytical HPLC separations.

The obtained HPLC-basic profile (fig. 6.3.1) therefore differ from the standard schema of common HPLC separations. In our investigations of HPLC peaks most of the proteins develop in 2-DE gels a serie of closely related spots (fig. 6.3.2A-D). These abundant proteins with higher molecular weights (50-80 kDa) are represented as protein families with several isoelectric points (fig. 6.3.2A-D).

HPLC separations of royal jelly from one botanical source (i.e. seasonal origin) were highly reproducible. Royal jelly sorts from various seasonal (i.e. botanical) origins showed a constant HPLC-basic profile with different shape of certain peaks (as described in chapter 2).

Royal jelly is a quasi-cellular mixture of materials with numerous interactions between the individual compounds. Several „pure“ HPLC peaks (fig. 6.3.2A; peak1) show only one abundant protein with several isoelectric points (fig. 6.3.2A). Unseparated „expanded“ HPLC peaks showed a mixture (peak 6) of several abundant protein families (>50 kDa) and their cleavage products, single positioned proteins with a molecular mass from 30 to 50 kDa (peak 6 and 7). Royal jelly proteins form a matrix in the sense of protein groups with variations in isoelectric point as well as molecular weight (two-dimensional polymorphism). As we have shown (fig. 6.3.2 D) this two-dimensional polymorphism is clearly visualised also for MRJP1.

In peaks 4, 5 and 10 respectively one protein family was found. In peak 9, which was identified as royalisin-peak no protein spot could be visualised. Proteolytic effects towards smaller molecular weight of the peptide royalisin can be the reason. Combination of analytical C8 RP-HPLC and 2-D electrophoresis provided valuable predications for the comprehension of chemical composition of antimicrobial active HPLC-peaks.

The most abundant royal jelly proteins, the major royal jelly proteins MRJP 1,2,3 and 5 (Schmitzova et al. 1998) were found in the 2-DE gels as a matrix of protein families with various isoelectric points and molecular weights (peak 6; fig. 6.3.2C). Proteolysis and post-translational modifications create chemically similar protein groups which are eluted together in HPLC and visualised with analytical 2-D electrophoresis.

6.4 Analysis of C8 RP-HPLC protein peaks with electrospray ionisation-mass spectrometry (ESI-MS)

Electrospray ionisation-mass spectrometry is a widely applied method in peptide characterisation (Mann and Wilm 1995; Buré et al. 2001). Antibacterial active protein peaks from analytical C8 RP-HPLC were analysed using electrospray ionisation-mass spectrometry (ESI-MS) in the positive ion mode to determine molecular weights of proteins and peptides involved in antibacterial activity. Altogether approximately 30 protein peaks were analysed. Molecular masses of peptides from 400 to 740 Da were found in the majority of the peaks. Due to a unfavorable signal / noise ratio the masses of several peaks were hardly determinable. Therefore the investigation was repeated with a semi-preparative HPLC system. Particularly peaks in the first part of the chromatogram were analysed. Protein peaks with unknown sequences in the first part of HPLC chromatograms were involved in antibacterial activity (according to chapter 2).

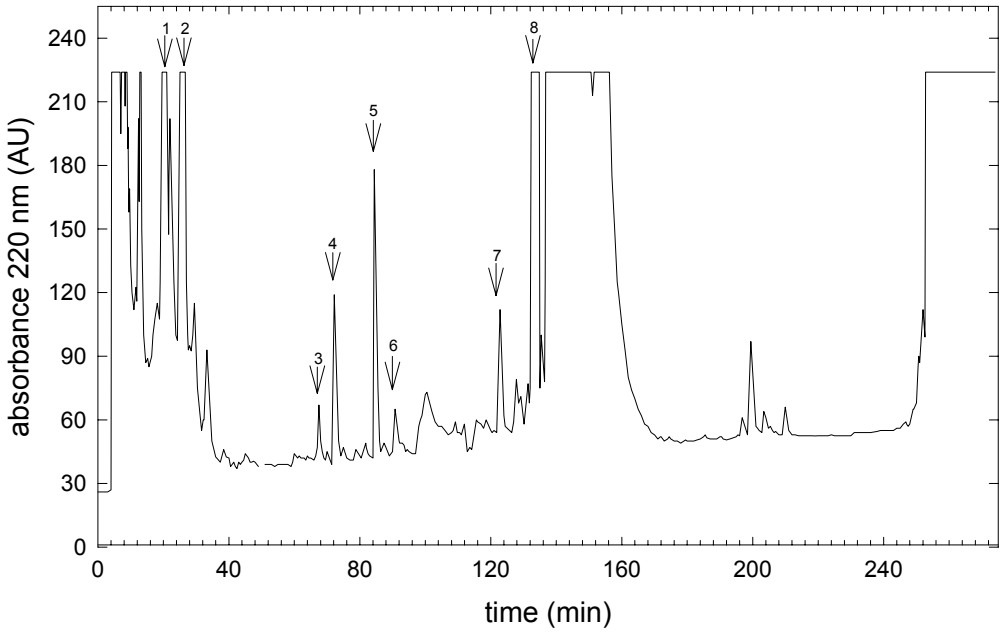

Figure 6.4.1: Semi-preparative C8 RP-HPLC of royal jelly supernatant; peaks analysed with ESI-MS are signed with arrows.

Protein peaks from semi-preparative C8 RP-HPLC (fig. 6.4.1) analysed with electrospray ionisation-mass spectrometry showed the presence of small peptides in

the samples (table 6.4.1; fig. 6.4.2A-D). Due to higher amounts of peptides and proteins in the samples better resolved mass spectra were received.

Table 6.4.1: ESI-MS positive ion (m/z) results from semi-preparative C18 RP-HPLC derived peptide fractions (fig. 6.4.1):

peak	m/z [Dalton]
1	303, 348; 367, 459, 695
2	309; 393, 461, 487; 535, 754, 802
3	322, 381; 450; 635,822, 1392
4	314, 408; 464, 635; 822, 1243
5	328, 461; 475, 663; 645, 661, 865
6	305, 346, 410, 463, 486, 667, 859, 1353
7	349, 366, 433, 550, 542, 697, 717
8	323, 373; 486, 613; 784, 800, 969

Electrospray mass spectra of royal jelly peptides are shown in fig. 6.4.2 A-D:

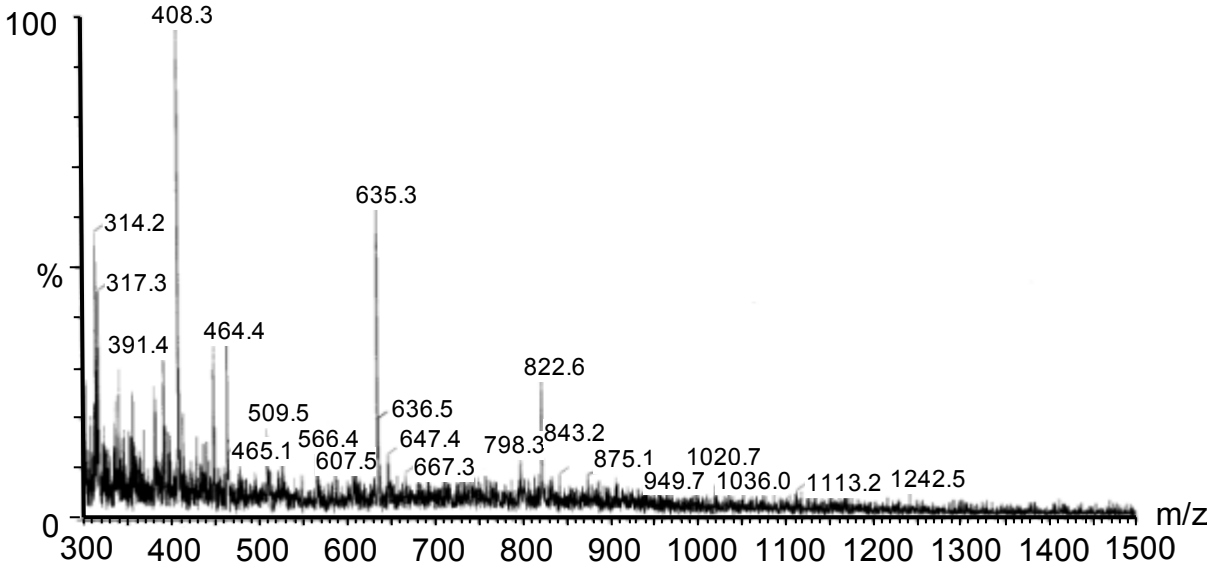

Figure 6.4.2A: ESI-MS positive ion spectrum of peptides derived from semi-preparative C8 RP-HPLC (peak 4; chromatogram fig. 6.4.1). Relative abundance of the ions is given in [%].

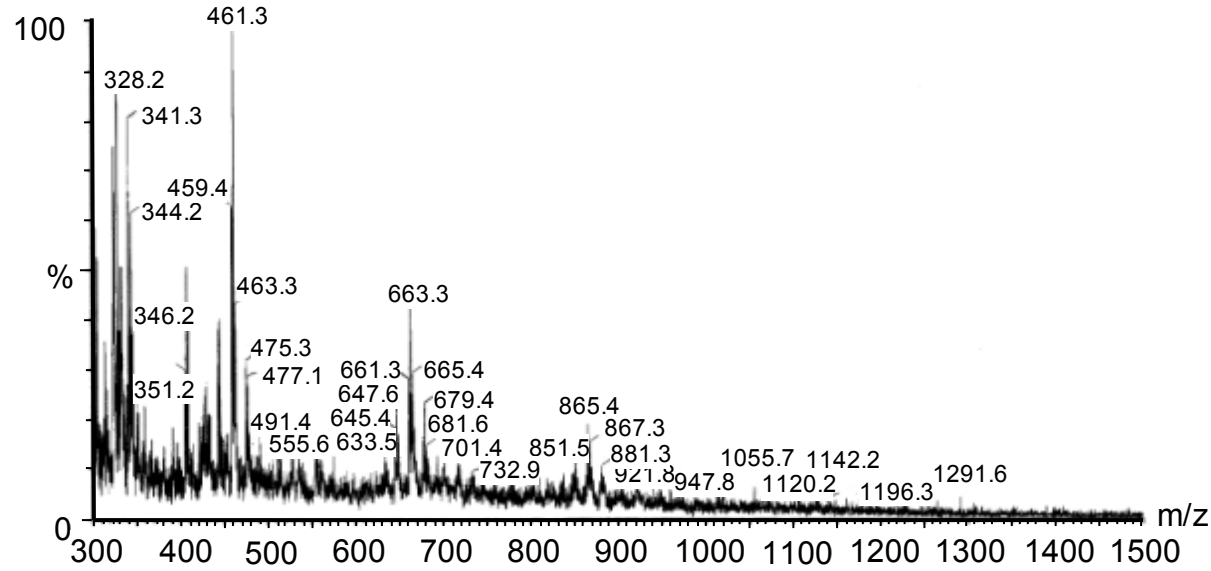

Figure 6.4.2B: ESI-MS positive ion spectrum of peak 5 (chromatogram fig. 6.4.1)

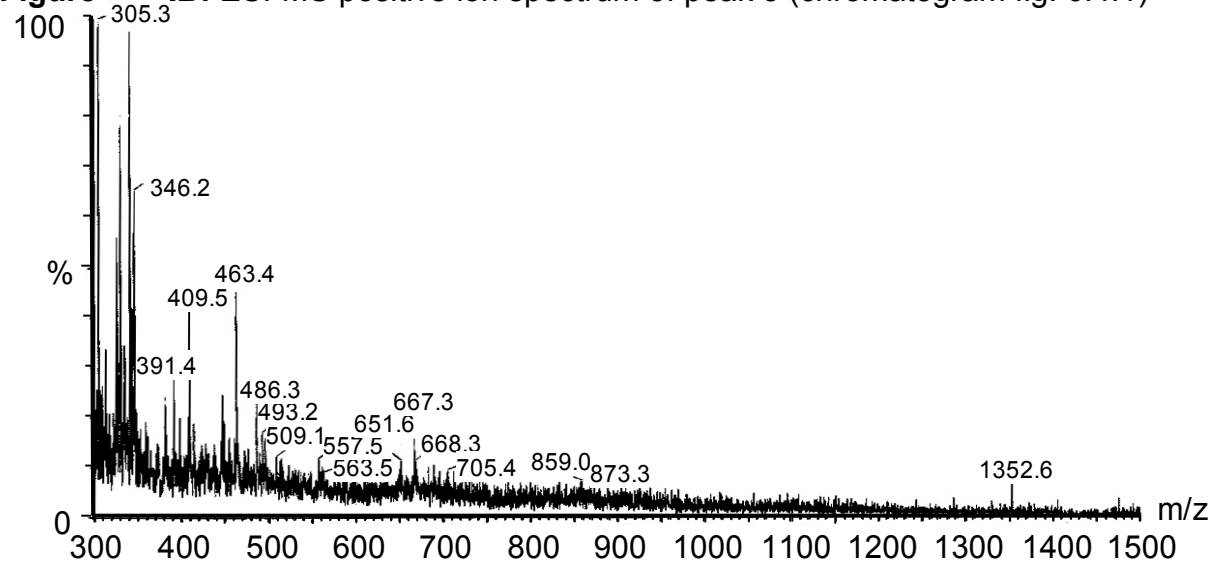

Figure 6.4.2C: ESI-MS positive ion spectrum of peak 6 (chromatogram fig. 6.4.1)

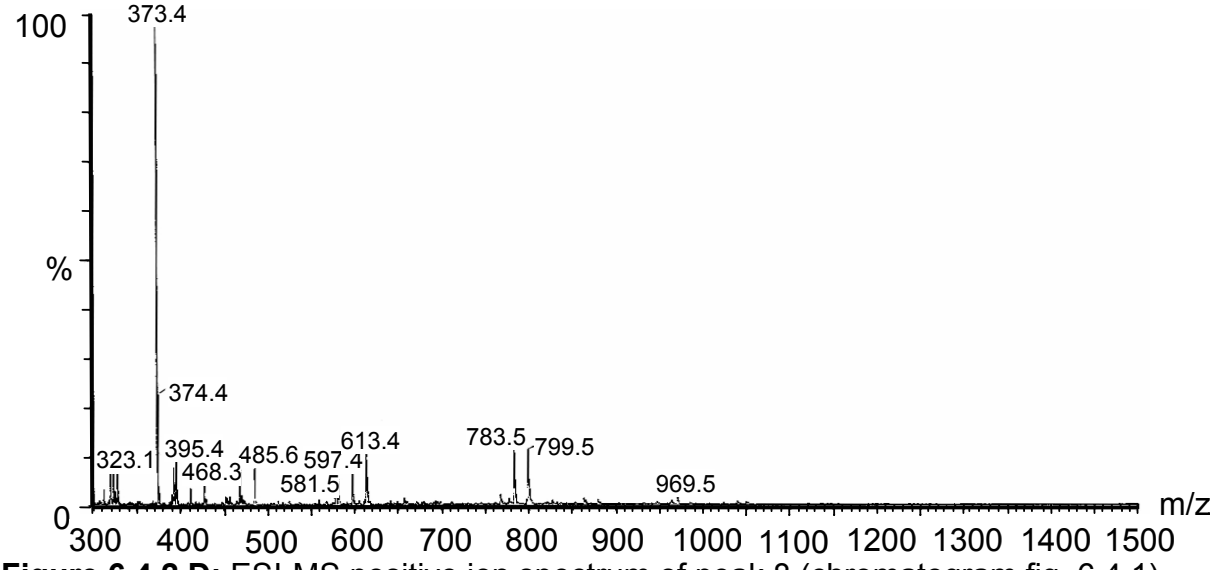

Figure 6.4.2 D: ESI-MS positive ion spectrum of peak 8 (chromatogram fig. 6.4.1)

A particular microbiological activity outside of the already investigated royalisin region can be assigned to the proteins isolated in peak 5 (semi-preparative C8 RP-HPLC column; fig. 6.4.1). The corresponding separation fraction was isolated in an analytical C8 RP-HPLC run as peak 3 (fig. 6.3.1). Edman sequencing of this peak showed a protein mixture. The signal intensity, however characterizes NEVN (molecular weight = 474 Da) as the dominating sequence. The ESI-MS spectrum (fig. 6.4.2B) indicates the same result. For the identification of the active component however, the possibility of a pentapeptide remains, because the sequencing mechanism (Edman degration) discriminates the last remaining amino acid. The suppressed terminal amino acid woud be preferentially F, so that the pentapeptide NEVNF (molecular weight = 621 Da) can be considered.

N-terminal sequencing (Edman degradation) of peak 5 (fig. 6.4.1) showed following sequence (weak signals due to smaller becoming amounts of amino acids are shown in brackets):
1 Asn- 2 Glu [Val,Lys]- 3 Val [Gln]- 4 Asn- 5 [Phe, Met, Asp]- 6 [Lys,Asn]- 7 [Gln,Val]- 8 [Met-Gln]- 9 [Leu]
The first amino acid positions showed intense measurement signal, the following amino acid positions in brackets showed much weaker signals. The principal sequence is Asn-Glu-Val-Asn (NEVN). The sequence was evidently a sequence mixture of a short peptide together with larger proteins.

The received sequence NEVN is part of the larger royal jelly protein MRJP5 at amino acid position 563 (including signal peptide). The calculated molecular weight of NEVN is 474 Da which is accordant with the measured m/z results (fig. 6.4.2B; table 6.4.2, peak 5). In figure 6.4.2B the signal at m/z 475 corresponds to the $[M+H]^+$ ion of NEVN. This is also in accordance to the m/z of the synthetic peptide NEVN of 475.3 measured with ESI-MS. For the abundant ion at m/z 461 (fig. 6.4.2B) no correspondency was found. Due to characteristics of the Edman degradation the last amino acid of a peptide can not be determined with N-terminal sequencing. The next amino acid after NEVN in MRJP5 is F. Therefore the peptide in peak 5 could also be NEVNF (molecular weight = 621 Da) which is also accordant to sequence data and m/z results (fig. 6.4.2B; table 6.4.2). The m/z 645 corresponds to $[M+Na]^+$ and m/z 661 to $[M+K]^+$ of the peptide NEVNF. Homology to following sequence of MRJP5

(position 550 to 580) was found in protein data bases (NCBI; AAD01205; Albert et al. 1999):

H_2N…….MKLQKFINNDYNF↓**NEVN**F↓RILGANVNDLIMN……COOH

NEVNF (fig. 6.4.3 A) could result from a nonspecific chemical or a proteolytic cleavage. The present cleavage is specific for chymotrypsine (after two phenylalanines).

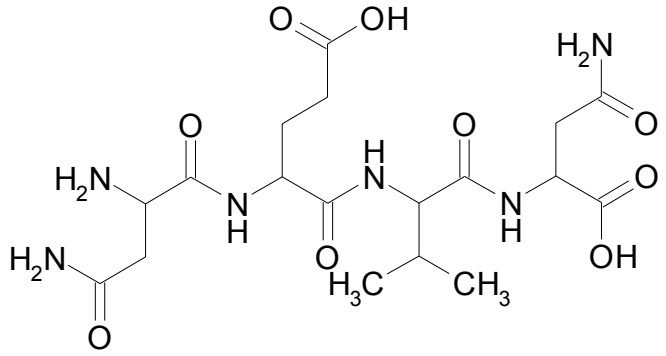

Figure 6.4.3 A: Chemical structure of the peptide NEVN

In fact, preliminary agar diffusion tests with the synthesized tetrapeptide NEVN (fig. 6.4.3A) showed antibacterial activity against *Micrococcus luteus* DSM 348, *Micrococcus varians* TMW 2.121 and *Escherichia coli* K12JM83. Antibacterial investigations with the related peptide NEVNF (fig. 6.4.3 B) are still under progress, so that Minimal Inhibitory Concentrations (MIC's) are reported subsequently.

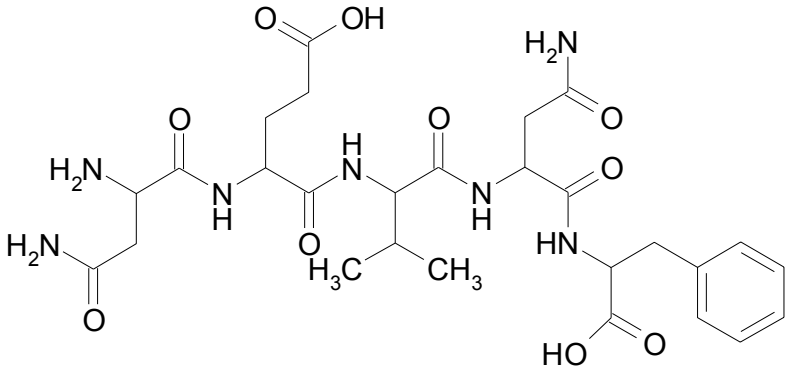

Figure 6.4.3 B: Chemical structure of the peptide NEVNF

Storage function of the large proteins and biological activity of small proteins and peptides which are cleavaged therefrom are a general principle. The microbiological results of chapter 2 taken into account, abundant large royal jelly proteins (49-87 kDa) provide peptides and small proteins with antibacterial properties. The cleavaged peptides appear in active HPLC fractions together with the intact proteins. These

findings establish an antibacterial role of peptides derived from the most abundant royal jelly proteins additionally to the nutrition of larvae.

Other highly active biological mechanisms of action are based on the same principle of gradually proteolysis of active small molecules which derive from high moleculare protein storage molecules (e.g. encephaline, endorphine).

Our results of small royal jelly proteins and peptides are accordant to literature data. Jingwei (1996) described a peptide composed of 8 amino acids with activity against grampositive bacteria. Bilikova et al. (2001) described antibacterial and antifungal activity of a small fragment from the peptide royalisin. Insulin-like hypoglycemic and immunological activities of peptides in royal jelly are described by Kramer et al. (1977). Takenaka (1987) described 5 peptides isolated from royal jelly with a molecular weight of about 1.6 kDa and different amino acid composition.

6.5 Micro-preparative 2-D electrophoresis and N-terminal sequencing (Edman degradation)

Protein fractions of royal jelly play an essential role in antibacterial activity (Bengsch et al. 2000; Fujiwara et. al. 1990; Sauerwald 1998; Stocker 1999). The predominant part of the proteins in royal jelly are abundant protein families within a molecular mass range from 40 to 90 kDa (fig. 6.5.1). We have shown that these large proteins are present in antibacterial active RP-HPLC fractions together with small peptides which are cleavaged thereof (chapter 6.1-6.4). Therefore we characterised the protein families with micropreparative 2-D electrophoresis and subsequent sequencing (Edman degradation) followed by data base search. We sequenced at least one protein of every identified protein familiy. For several proteins with modifications in molecular weight and isoelectric point several spots (two-dimensional polymorphism) were N-terminally sequenced. N-terminal sequences and results of data base search are given in table 6.5.1.

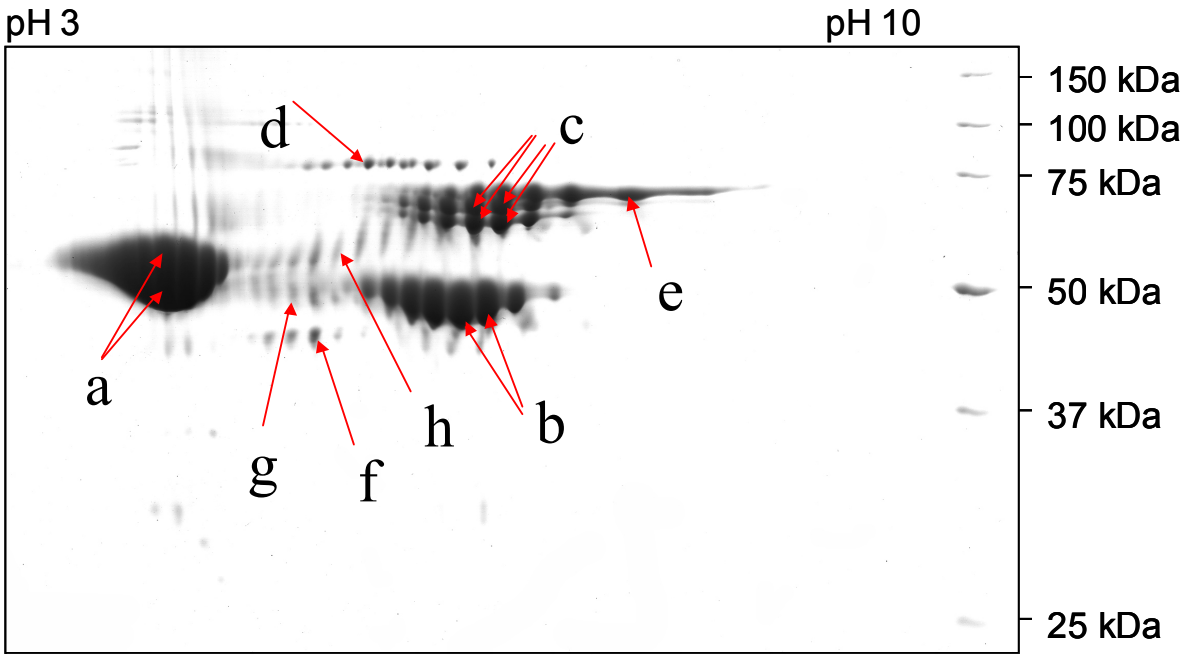

Figure 6.5.1: micropreparative 2-D electrophoresis (Coomassie R250 stain) of royal jelly (sample was collected 05/18/2000) and N-terminal characterisation (Edman degradation) of proteins: Polymorphic proteins grouped in at least 6 series are visualised.

The gel shows a matrix of protein families which differ in isoelectric point and molecular weight. N-terminally sequenced proteins (Edman degradation) are signed with arrows (fig. 6.5.1). Sequences of the proteins (table 6.5.1) showed no variation in the N-terminal ends within spots of one protein with differences in molecular weight (spot a) or isoelectric point (spot b,c).

Table 6.5.1: N-terminal sequences (Edman degradation) of royal jelly proteins

spot	N-terminal sequences (data base entries)
a	1 Asn- 2 Ile- 3 Leu- 4 Arg- 5 Gly- 6 Glu- 7 Ser- 8 Leu- 9 X- 10 Lys- 11 Ser- 12 Leu- 13 Pro- 14 Ile- 15 Leu- 16 His- 17 Glu- 18 X- 19 Lys- 20 X- 21 Phe- → Major Royal Jelly Protein 1 (MRJP 1); glycosylation in position 9
b	1 Ala- 2 Ile- 3 Val- 4 X- 5 Glu- 6 Asn- 7 Ser- 8 Pro- 9 X- 10 Asn- 11 Leu (Asn)- 12 Glu- 13 Lys- 14 Asp- 15 Leu- 16 Asn- 17 Val- 18 Ile → Major Royal Jelly Protein 2 (MRJP 2)
c	1 Ala- 2 Ala- 3 Val- 4 Asn- 5 X- 6 Gln- 7 Arg- 8 Lys- 9 Ser- 10 Ala- 11 Asn- 12 Asn → Major Royal Jelly Protein 3 (MRJP 3)
d	1Val- 2 Thr- 3 Val- 4 X- 5 Glu- 6 Asn- 7 Ser- 8 Pro- 9Arg- 10 Lys- 11 Leu- 12 Leu → Major Royal Jelly Protein 5 (MRJP 5)
e	1 Ala- 2 Ala- 3 Val- 4 Asn – 5 X- 6 Gln – 7 Arg- 8 Lys - 9 Ser 10 Ala – 11 Asn → Major Royal Jelly Protein 3 (MRJP 3)
f	1 Ala- 2 Ala- 3 Val- 4 Asn – 5 X- 6 Gln – 7 Arg- 8 Lys - 9 Ser- 10 Ala – 11 Asn- 12 Asn- 13 Leu- 14 Ala- 15 His → Major Royal Jelly Protein 3 (MRJP 3)
g	1 Ala- 2 Ile- 3 Val- 4- Asn- 5 Glu – 6 Asn → Major Royal Jelly Protein 2 (MRJP 2)
h	1 His- 2 Ile- 3 Val – 4 Asn- 5 Glu- 6 Gln – 7 X- 8 Lys- 9 Ser- 10 Ala- 11 Asn- 12 Asn → similarities to Major Royal Jelly Protein 3 (MRJP 3)

In our investigations we characterised the most abundant royal jelly protein (spot a) within 21 amino acid positions at the N-terminal part and sequence identity was found to the royal jelly protein MRJP1 (Simuth 2002; Klaudiny 1993). In our investigations N-terminal sequences of spot a with variation in molecular weight but same isoelectric point showed the same N-terminal ends. Spot a (MRJP1) occurs in several modifications of molecular weight and isoelectric point (two-dimensional

polymorphism). The modifications in molecular weight of MRJP1 are preferentially glycosylations.
Yonekura (1998) described a royal jelly glycoprotein (55 kDa) and its N-linked mannose type sugar chain with proliferation stimulating activity for human monocytes. This glycosylations can cause the slight variations of molecular weight of MRJP1 within a coherent protein spot (fig. 6.5.1). Several changes in isoelectric point can result from various posttranslational modifications which are ubiquitary in proteoms of most organisms. The content of essential amino acids of MRJP1, calculated from cDNA is 48% (Schmitzova et al. 1998). An important role of MRJP1, 2, 4 and 5 in the nutritive supply of the bee larvae is therefore indicated. It is of high interest, that royal jelly proteins play a role in the insect nervous system: The expression of MRJP1 cDNA in a subset of neuronal cells in the honeybee brain in addition to the hypopharyngeal glands (Kucharski et al. 1998) reveal further polyvalent functions of this protein.

N-terminal sequence of spot b is identic to major royal jelly protein 2. N-terminal sequences of 2 spots with different masses showed identic N-terminal sequences. Spot b (MRJP2) occurs in 7 main variants of isoelectric points (fig. 6.5.1). For MRJP2 and MRJP1 no sequence polymorphism of cDNA clones is described (Schmitzova et al. 1988). Variations in isoelectric point result therefore preferentially from posttranslational modifications. Post-translational modifications as e.g. phosphorylations, glycosylations, deaminations and cleavages result in the highly polymorphic protein pattern of royal jelly shown in fig. 6.5.1.

From our results a protein family with high polymorphism in isoelectric point and molecular weight (spot c) 4 protein spots were characterised by Edman sequencing. N-terminal sequences of the protein family were identic and homologous to MRJP3. This protein shows the highest variability of all protein families (fig. 6.5.1). Three protein families with at least 7 spots with different isoelectric points form a matrix of closely related proteins with modifications in molecular weight and isoelectric point. Molecular weights calculated from cDNA clones are in the range from 60 to 70 kDA. This is in good accordance with protein families shown in fig. 6.5.1. Albert et al. (1999) described at least 10 alleles of MRJP3 DNA with distinct sizes. Polymorphism was described as a result of tandem repeats of the sequence (VNTR) located at the

C-terminal part of MRJP3. Highly polymorphic variable tandem repeat (VNTR) markers were previously described in human DNA (Wolff et al. 1991). In opposite to RNA and DNA tandem repeats are not frequent in proteins. The nitrogen-rich amino acids in the repetitive regions were proposed as storage of nutritive components for the fast developing bee larvae.

From the protein family with the highest molecular weight (spot d) of approximately 80 kDa the most abundant protein spot was sequenced at the N-terminal part. The protein sequence was identic to the N-terminal part of MRJP5. Two protein sequences with 77 kDa and 87 kDa are suggested from cDNA sequences (Schmitzova et al. 1998). In our investigations one protein family is visualised in coomassie-stained 2-D electrophoresis gels (fig. 6.5.1) corresponding preferentially to the 77 kDa protein. In our investigation we found cleavaged peptides from MRJP5 with the sequences NEVN and/or NEVNF in RP-HPLC fractions. Antibacterial activity of these peptides was shown in preliminary tests.

Spot e is one of two proteins on the basic side of the MRJP3 group. This protein belongs to the most basic proteins among the abundant large royal jelly proteins (fig. 6.5.1) and shows no visible vertical variation in molecular weight as other proteins belonging to this group. N-terminal sequence of spot e was identic to MRJP3. Evidently the closely related spots e and c are belong to the same protein family.

A group of proteins with at least 5 spots distinctly lower in molecular weight (approx. 40 kDa) as the protein families mentioned above was assumed to contain unknown proteins. The most abundant spot of the protein group (spot f) was characterised by Edman degradation (fig. 6.5.1). N-terminal sequence of spot f was identic to MRJP3. The protein group of spot f is considerably smaller in molecular weight as the main group of MRJP3 (spot c and e). Several 2-D electrophoresis runs with royal jelly samples from different botanical origin (and sampling dates) showed high reproductivity of the given protein pattern (fig. 6.5.1). This protein group (of spot f) derives evidently from the larger protein group MRJP3, presumably created by proteolytic or chemical cleavages. Chemical cleavages are e.g. Asp-Pro cleavages which are well-known in the acid environment. This finding indicates exemplarily that proteins groups lower in molecular weight as the abundant MRJP's derive from larger

proteins. Beside the nutritive function large royal jelly proteins are the storage material for smaller proteins and peptides with potential biological activities which are released after proteolytic or chemical cleavage. The cleavage products (group of spot f) form also closely related groups of protein families.

N-terminal part of spot g was homologous to the described protein MRJP2. The horziontal chain of proteins (between spot a and b; fig. 6.5.1) consitutes evidently a group modified MRJP proteins with small abundance between the very abundant proteins MRJP1 and MRJP2 (spot a and b). Evidently a certain MRJP's receive various different post-translational modifications, changing both isoelectric point and molecular weight. As 2-D electrophoretical runs of different royal jelly samples protein patterns were very reproducible these highly modified patterns can be assumed to occur universally in royal jelly samples. Slight modifictions in the amounts of certain proteins may depend on the botanical origin or physiological conditions of the nurse bee.

A further serie of highly modified proteins appear between the abundant proteins MRJP1 and MRJP3 (spots a and c). Spot h (fig. 6.5.1) belongs to this serie of proteins with increasing molecular weight. At least 8 proteins belong to the protein serie with several spots of similar molecular weight and then increasing molecular weight. Under our 2-D electrophoretical conditions there is a seamless transition from this protein serie into the abundant group of MRJP3 proteins (spot c and e). Therefore it can be assumed that several proteins are overlaid by more abundant spots with similar molecular weight and isoelectric point. N-terminal sequence of this protein (spot h) showed slight homology to MRJP3 but several amino acids are different. Comparison of the sequence (table 6.5.1) show that the sequence can be regarded as a mixture of MRJP3 and MRJP2. Exchange of several amino acids in the N-terminal part of the proteins can be the reason.

No protein sequence corresponding to the cDNA of MRJP4 was found in our investigations of royal jelly proteins. In accordance with investigations of Schmitzova et al. (1998) only MRJP's 1,2,3 and 5 were N-terminally sequenced as a protein. MRJP4 in small quantities can be overlaid in the 2-D electrophoresis gel by another protein.

Royal jelly proteins play an essential role in many biological processes. Royal jelly protein appear as a pattern of highly modified protein families. Several modifications

may play an important role in biological functions of royal jelly. From our results 2-D electrophoresis of royal jelly proved to be the most successful method for the characterisation of the proteins fraction.

6.6 Maldi-MS characterisation of royal jelly proteins

Micropreparative 2-D electrophoresis was used to separate royal jelly proteins for subsequent sequence analysis (chapter 6.5). High purification of the sequenced spots could be observed. All described protein families could be detected among the analysed spots. 2-D electrophoresis and sequence analysis was described for the investigation of an antiviral royal jelly oligopeptide (Bengsch et al. 2000; Sauerwald 1997). One purpose of the analysis of small royal jelly peptides, which appear in 2-DE gels as single spots, was the investigation of potentially unknown royal jelly peptides with biological activities.

For proteins which constitute evidently highly modified protein series MALDI-MS analysis was used for characterisation of these protein families which appear in small amounts between the most abundant protein families (fig. 6.6.1). N-terminal sequence analysis (Edman degradation) of these intermediate protein spots (fig. 6.6.1; Nr. 1 and 2) showed also similarity to major royal jelly proteins. For further analysis 2 spots were additionally characterised with MALDI-TOF-MS.

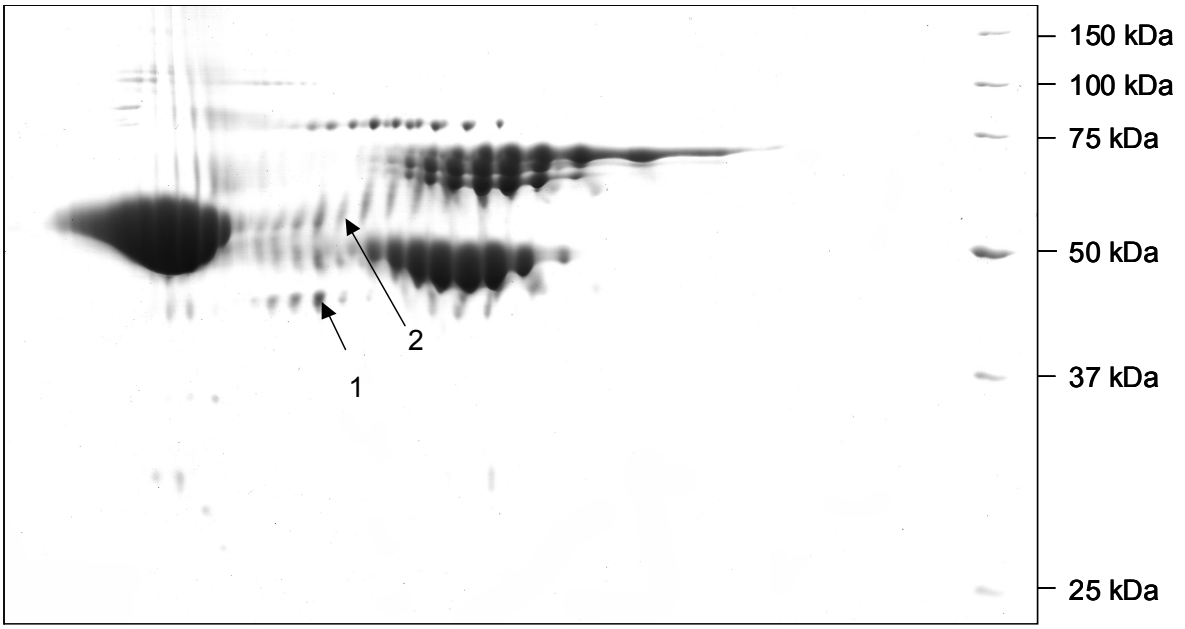

Figure 6.6.1: Maldi-MS analysis of two protein spots 1 and 2 from modified protein groups intermediated between the most abundant protein families; 1. Dimension IEF 3-10; 2. Dimension T=12%; Royal jelly was collected (05/18/2000).

Spots (fig. 6.6.1) were excised, digested with endoproteinase Lys-C and resulting peptides were analysed with MALDI-TOF-MS. Both two spots were identified as homologous to MRJP3 (RJP57-1). MALDI-MS spectra of peptides from spot 2 are shown in fig. 6.6.2 A-C.

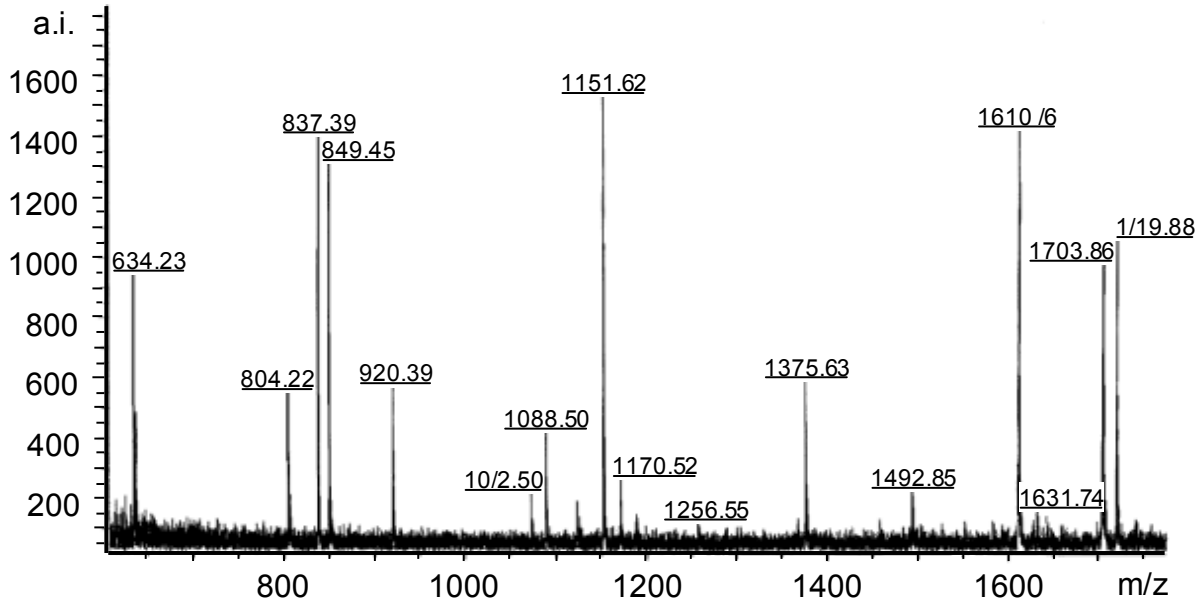

Figure 6.6.2 A: Maldi-MS spectrum of royal jelly protein-spot 2 (fig. 6.6.1); 1st mass range

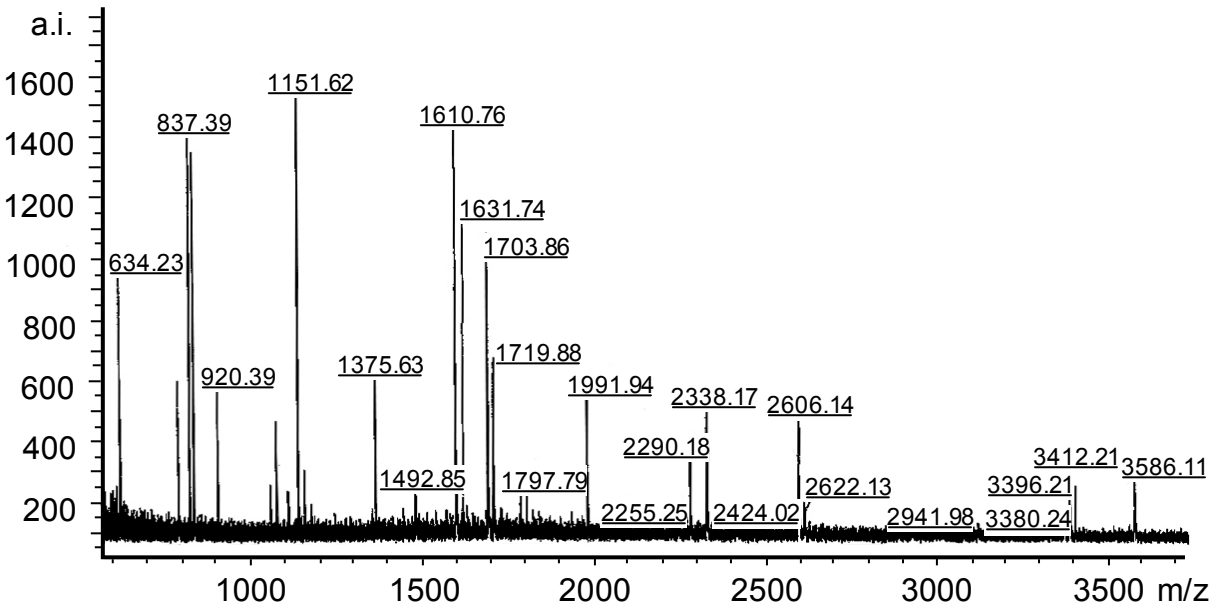

Figure 6.6.2 B: Maldi-MS spectrum of royal jelly protein-spot 2 (fig. 6.6.1); 2nd mass range

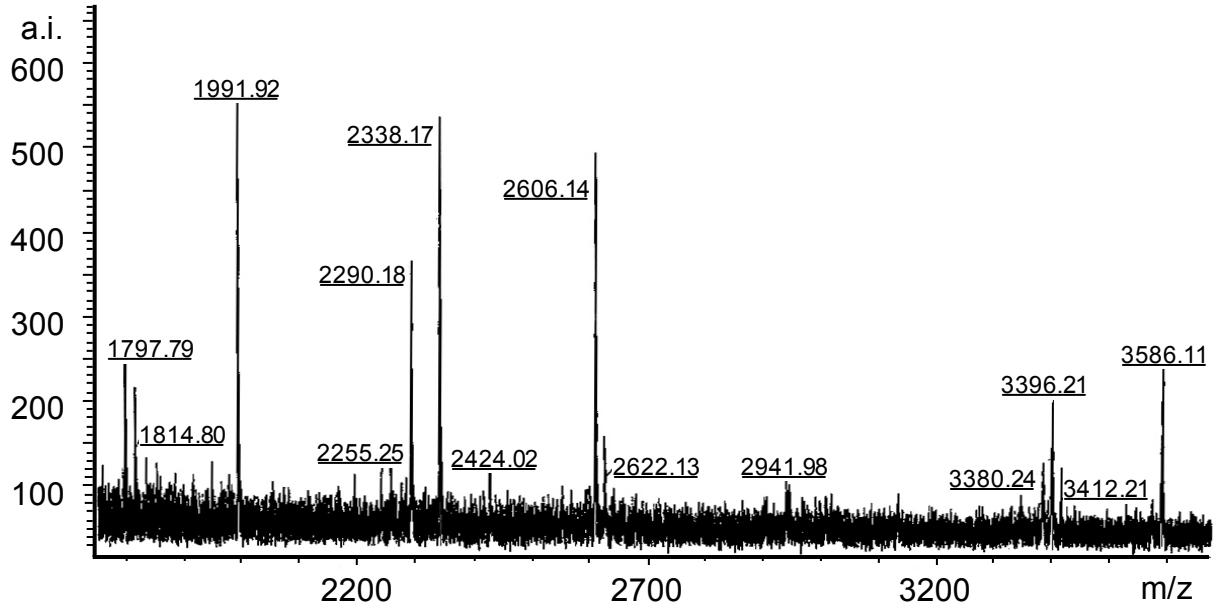

Figure 6.6.2 C: Maldi-MS spectrum of royal jelly protein-spot 2 (fig. 6.6.1); 3rd mass range

The homology to MRJP3 of the two proteins (fig. 6.6.1 and 6.6.2) is in accordance with our results from N-terminal sequencing (Edman degradation). Taken the results of Edman sequencing into account N-terminal sequence and peptide mass fingerprint of spot 1 (fig. 6.6.1) has homology to MRJP3 with differences in several N-terminal amino acid positions. A lower molecular weight of this protein serie (approx. 40 kDa) compared to the MRJP3 protein group (60-70 kDa) can be explained with proteolytic cleavages at the C-terminal end.

Proteolytic cleavage of small, active sequence motifs appears again as a general systematical mechanism. Other highly active biological mechanism of action are based on the same principle of gradual proteolysis of active small molecules from high molecular protein storage material (e.g. encephaline, endorphine).

The protein spot 2 belongs to a serie with proteins in low abundance between the adjoining abundant protein families (fig. 6.6.1). As we have shown the highly modified spots are evidently modifications of the protein group MRJP3. It can be predicted that these spots in the intermediate range between high-molecular weight and abundant protein families contain no unknown protein sequences. The two-dimensional pattern of protein series with modifications in isoelectric point and increasing molecular weight can be formed by several modifications. Variable numbers of tandem repeats

at the C-terminal end change molecular weight and isoelectric points. Phosphorylations and decarboxylations change the isoelectric point of the proteins. Groups of glycosides can change the molecular weight of protein series. Modifications with glycosides in the range of several kiloDalton can result in the observed steps of molecular weight (fig. 6.6.1). Kimura et al. (1995 and 1996) described N-linked sugar chains of the oligomannose-type in 55-kDa and 350-kDa royal jelly glycoproteins.

Protein patterns of 2-D electrophoretically separated royal jelly proteins showed several spots with molecular weights below 40 kDa (fig. 6.6.3). Micropreparative 2-D electrophoresis gels were prepared with high amounts of total protein to receive adequate protein amounts of spots in the low molecular range.

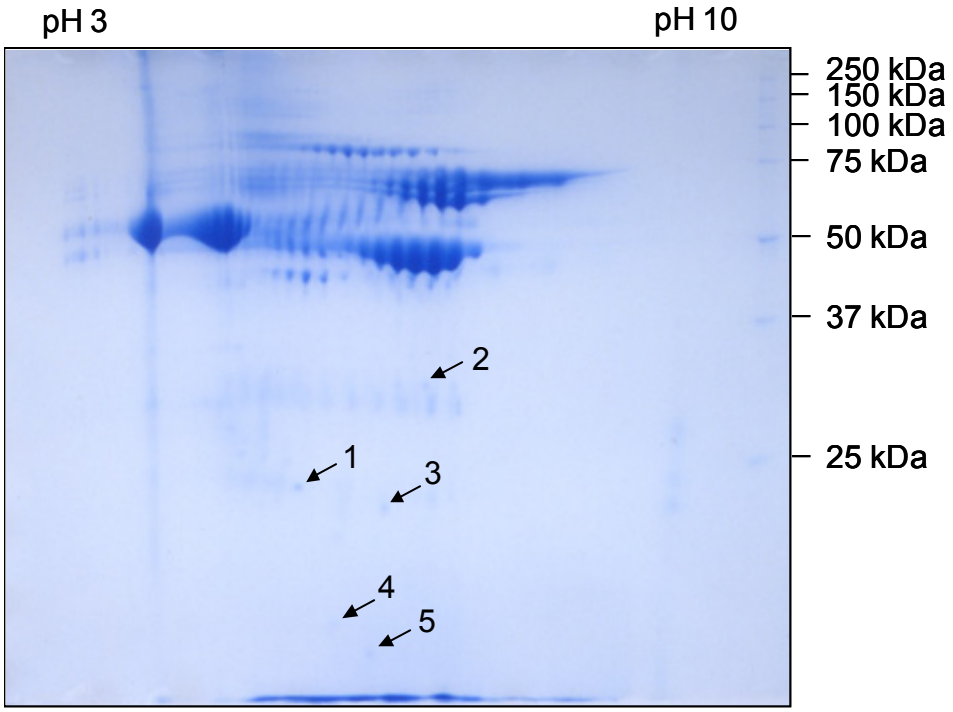

Figure 6.6.3: MALDI-TOF-MS characterisation of royal jelly protein spots (Nr.1-5) which appear single standing in the low molecular weight region; 1. Dimension IEF 3-10; 2. Dimension T=12%. Royal jelly was collected (05/16/2001)

As known generally small proteins and peptides are predominantly responsible for biological activities of royal jelly (Bengsch et al. 2000; Fujiwara 1990; Sauerwald 1997; Stocker 1999). Further protein spots with a small molecular weight below 37

kDa were therefore analyses with MALDI-TOF-MS with the purpose to find out proteins with sequences related to antiviral and antibacterial activities. Five protein spots were analysed (fig. 6.6.3; Nr.1-5) with molecular weights below 37 kDa.

Peptide mass fingerprints of spots 1 to 5 showed sequence homology to the cDNA of MRJPs. MALDI-MS analysis of spot 1, the most abundant of the low moleculare royal jelly proteins was homologous to MRJP1. Spot 1 was additionally blotted onto a PVDF membrane and sequenced N-terminally (Edman degradation). The resulting protein sequence PKFTKMTIDGESY was accordingly found homologue to the cDNA sequence of MRJP1. MRJP1 has a molecular weight of approximately 47 kDa (Schmitzova et al. 1998) and is N-glycosylated. The peptide is cleavaged between the amino acids asparagine acid and proline at amino acid position 216 (without signal peptide). These D-P cleavages are well-known to occur in acid milieu. Spot 2 is homologous to MRJP2 and derives from any cleavage. Spot 3 and 4 derive from MRJP1 from D-P cleavage as described for spot 1. Spot 5 could not be identified (background noise). Taken these results into account it can be predicted that most of all royal jelly peptides are cleavage products of larger, highly abundant proteins. Both enzymatical proteolysis and chemical cleavages in the acid milieu can be involved in the digestion of the large proteins which can be regarded as the storage material for the production of biologically active peptides.

7 General discussion, final remarks and perspectives

Identification of active substances from the large diversity of compounds in royal jelly and biochemical investigation of their potential for the development of pharmaceutic use was the purpose of the present work. Therefore royal jelly raw material with reproducible effects is required. Parameters for the activity of royal jelly were predefined to optimize royal jelly samples and their biochemical activity.

Methods of royal jelly production were perfected under attention, investigation and development of following considerations:

Habitat:

- vegetation choice, wild plants, variable small-scale agriculture hedgerow landscapes in Vendée and Bretagne without industrial agriculture (exhausted basements, pesticides)
- unspent acidic magmatic rocks
- permanent and abundant disposability of trace elements

Climatic Factors:

- high luminosity
- balanced alternating athmosphere over the whole season
- lack of stress factors for the plant like heat, coldness, dryness, permanent wetness
- proximity of the ocean and prevailing sea winds provide regular ventilation and lack of air pollutants
- no airborne transport of pesticides

Under abidance of these conditions for the habitat selection royal jelly samples optimised in this manner are significantly more active than royal jelly samples which are produced under non-observance of these conditions. Royal jelly samples were harvested in systematical variation of seasonal (i.e. botanical) origin within several years and tested onto their microbiological activity. The royal jelly production season in Central Europe lasts 3-4 months and 40 to 50 botanically different samples were produced annually.

For open questions answers could be found. The activity of royal jelly varies in the course of the season also at a constant location. There are optimal production dates in the middle of the season. These variations are essentially determined from the changing pollen disponibility, as pollen morphological analyses have shown.

Examination of authenticity:
The botanical origin of the seasonally produced royal jelly series was examined with pollen morphological (palynological) analyses of the pollen exines. The pollen exines remain after the transformations in the intestine and postcerebral glands as empty cages in the royal jelly emulsion. The pollen exines indicate the botanical origin of the royal jelly sorts. Their enumeration enables a direct proof of origin.

Pollen constitutes the exclusive source of proteins which are contained in the resulting royal jellies. As we pointed out protein profiles of the royal jelly samples vary according to the seasonal harvesting date. Therefore this investigation of the seasonal variation of microbiological activities prove to be the first important step to the successful solution of this conceptual formulation. Thus royal jellies from the most active harvesting phase from 15 to 22 May of every year respectively were selected for the biochemical analyses. Based on this results (chapter 2) the works of the following chapters could be carried out under optimal conditions. It is the first time that such correlations could be shown in a systematical manner. The annual rhythm of royal jelly activity from our results is synchronous to the antibiotic effect in the honey bee (homogenized bee tissue) (Chauvin 1968).

The above described biologically optimised royal jelly raw materials with high and reproducible activities were tested against certain pathogen bacteria, several fungi and viruses. Approximately 150 bacteria and several fungi strains, thereof 70 human-pathogen strains were tested with soluble and insoluble protein fractions from royal jelly and the natural raw material itself.
As we pointed out gram-positive bacteria are more inhibited as some gram-negative (differentiated). For gram-positive bacteria a nearly general susceptibility was observed. Several nosocomial, multiresistant bacterial strains could be inhibited. Among the multitude of observed effects we have pointed out to results with

particular medical relevancy. Especially spectacular effects we observed for *Corynebacterium diphteriae*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Yersinia enterocholitica* which are of interest for the solution of future problems. Some of the bacteria cause particular problems in view of resistance against classic antibiotics. Especial attention in these investigations was concentrated to the resistance development of commensal and pathogen bacteria, the potential nosocomial and here especially septicaemic bacteria which are responsible for the most part of cases of death at the intensive-care units of the hospitals. These bacteria are therefore under special monitoring in the public health system. It has to be remembered, that the present 25 families of clinical active antibiotic agents lose increasingly their efficacy (Levy 1998; Jungkind 1996). This is caused by the fulgurating development of multi-resistance in bacteria and fungi (hospital, animal mast and human therapy effects).
As we pointed out protein fractions take effect especially against gram-positive bacteria, significant infective agents as well as opportunistic bacterial strains. Inhibitory activities of royal jellies against these nosocomial pathogenic bacteria from our results are contributions to the development of new antibiotics.

Soil bacteria regulate their respective population density with the aid of antibiotics. For the producer strain they are not toxic. Developments of resistant strains are preprogrammed.
New antibiotics:
A number of active substances of royal jelly derive indirectly from soil bacteria and fungi. The active agents were developed to a concentrate with highly protective properties via the serie bacteria-roots-juvenescent leafs-blossoms-insect glands. Royal jelly was optimized to this function during a co-evolution plants / insects of 140 million years (Ruttner 1992). The close symbiosis plant / bee is exemplified by the diversification of the herbal species. During this co-evolution the bee promoted the dominance of the flowering plants. In return for this the plants provided the bee with more and more elaborated substances. This made royal jelly to a particular qualified source of biological active molecules.

Among all investigated and disposal natural material royal jelly offers probably the greatest potential to isolate new antibiotic substances:

- enormous long evolution time of the system *Apis mellifera* and evolutionary perfection
- potency of the active compounds
- quantities of the disposal natural raw material.

Flavonoids are cofactors of antibacterial and antiviral activities. Catechins as model compounds showed distinct inhibition of pathogen and commensal bacteria: Histochemical staining reactions visualised that the active compound catechin can be effectively accumulated in the bacteria.
The anti-viral properties of some compounds of royal jelly are of particular future interest. There are no antiviral substances for virus treatment post infection without cytotoxic adverse reactions. Royal jelly as raw material is effective against viruses. The antiviral activity appears to correlate with the antibacterial activity. We have pointed out some particular interesting examples which deserve further intensive attention. Results and developed methods of our investigations should provide new perspectives for the study of antiviral substances, particularly antiviral proteins.

As already discussed active royal jelly samples depend on the composition of soils which are rich in trace elements. These trace elements are recovered in the royal jellies via the path soil-plant-insect gland. It was therefore obvious to investigate the correlation between the activity of royal jelly and disponibility of trace elements in the soils with the determination of trace elements in royal jelly. Particularly noticeable is the constance of the content of trace elements in royal jelly in comparison to the contents in honeys deriving from the same plant sources. These are homeostatic effects which were already observed in mammalian and human breast milk (Schramel et al. 1988). This is comprehensible under the aspect that royal jelly excretion is a form of lactation on the insect level.

From our results royal jellies exist from small to high activities in view of a multitude of biological properties. The less active royal jelly samples are received with semi-artificial feeding methods of the honey bee but they are of minor interest with regard to various biomedical activities. With exception of the microbiological tests and the palynological observations there are no appropriate methods for the differentiation of royal jellies received under natural entomological conditions and semi-artificial

samples. Both test methods are not fraud resistant. The multielement stable isotope analyses allows to differentiate authentic royal jellies from semi-artificial samples. The guarantee of authentic samples is an important precondition for microbiological and biochemical studies we have shown. They are of trading interest (adulteration of food) and certainly also precondition for the works which are realized in these studies.

The anti-bacterial activity of royal jelly was formerly assigned to a fatty acid, the 10-hydroxy-Δ2-decenoic acid. Highly active molecules as native and derivated proteins became now more and more evident (Fujiwara 1990; Sauerwald 1997). Protein fractions and peptides contribute essentially to the antibacterial activity (Stocker 1999; Sauerwald 1997; Sauerwald et al. 1998). The contribution of of particular proteins and peptides to the antibacterial effect was investigated with a multitude of phyico-chemical and microbiological methods.

Royal jelly contains a high number of native and derivated proteins, dominated by 4 large major proteins. These abundant proteins with a molecular weight from 40 to 80 kDa contain more than 80% of the total protein mass. As we pointed out major proteins appear in 2-D electrophoresis gels as protein families with modifications in isoelectric point and molecular weight. For major royal jelly protein 3, the most polymorphic protein in royal jelly, a variable number of tandem repeats (VNTR) located at the C-terminal part of MRJP3 is described by Albert et al. (1999).
In our 2-D electrophoretical investigations we visualized the polymorphic pattern of MRJP's. MALDI-MS investigations of several low molecular weight royal jelly proteins which appear solitary in the 2-DE gels showed that these low molecular RJ proteins are cleavage products of higher molecular MRJP's.
Combinations of C8 RP-HPLC, Edman sequencing, ESI-MS and microbiological tests showed that small royal jelly peptides are involved in antibacterial activity. Enzymatical proteolysis and chemical cleavages in the acid milieu digest a part of the abundant high molecular proteins which are the storage material for the production of biologically active peptides. The peptides and small proteins stay inactive when part of a high molecular protein, and contribute to the antimicrobial activities after cleavage in the form of active peptides and small proteins. This principle is known for

the antibiotic substance group of Encephaline: petides are cleavaged gradually and temporarily.

Active short sequence motifs were synthesized, and firstly examined with NMR spectroscopy: Two-dimensional NMR-techniques (COSY, TOCSY) were performed. Preliminary results reveal the identity of the synthesized molecules. In fact the molecules were synthesized with great purity. These small peptides develop no distinct steric structure. Enlarged and detailed investigations are still under progress, so that for the moment concluding results are not possible to report.

Application potential with corporative / economic value of benefit:
An actual subject which corresponds to a urgent social / economic requirement:
10 000 viruses, transposable genetic elements and subviral particles are actually known and more or less identified. In humans they are responsible for a complete spectrum of classic acute diseases, a part of them with calculable consequences (children's diseases, influenza infections and rhinitis).

Persisting in the host or integrated in his genome, viruses arouse heavy disfunctions after partly long times of latency like the formation of tumors (adeno virus, papilloma virus SV 40 etc.), leukaemia (HTLV 1,2); cardiovascular diseases (coxsackie-viruses B1-6, cardio-viruses 1-40, juvenile diabetes (retroviruses), certain forms of nervous depression (borna-virus), excessive obesity (adeno-virus 36) and a range of neural-degenerative pathologies e.g. multiple sclerosis (theilers virus + herpes virus 6 + unknown retrovirus), which are incurable after appearance of the symptoms.

Overpopulation, modern means of transport (number and velocity of viral vectors), depletion of industrial foodstuffs in antiviral substances (e.g. the nearly complete absence of the 5000 natural flavonoids, the quasi-total elimination of boron compounds) and the existence of toxic residues in nutrition and environment implicate that viruses in increasing number and virulence confront a increasingly immune-enfeebled population.

Pathogen micro-organisms, which were so far controlled with classical antibiotics (26 commercialised active agent groups), develop multi-resistances (tuberculosis,

bacterial and fungal infections of all kind and bacteria which act lethal after phage-modification). They return with a force which necessitates new forms of therapy from the research.

Herbal monocultures and intensive mass animal farming relate to the appearance and proliferation of plant viroids as well as infection pathogen which destroy the nervous system of vertebrates. These pathogens escape the immune-mechanisms they resist all treatments and medications except for boron compounds wich can neutralise the virulence of viroids.

The colony of *Apis mellifera* constitutes the miniatur-model of a naturally overpopulated environment: 50 000 individuals are densely packed a volume of 60 l without the slightest metabolic disfunction, without infections, in a situation which poses no sanitary and epidemiological problems.

Pharmaceutical vocation:
Our investigations showed the potential of the highly efficient substances, which assure the function and the anti-parasitic defense of this system as well as the influence of the vegetation as raw material of the bee products onto their effects against viruses, micro-organisms and macro-parasites.

The research of the presented work is focussed at two primary important biological interfaces:

- Interface plant (pollen) / insect (postcerebral glands)
- Interface royal jelly / bee larvae

Highest metabolic activity occurs at these interfaces that is particularly numerous, rapid and intensive biochemical procedures take place. Therefore at this sites a particular large number of active molecule sorts can be expected which open series of new pharmaceutical and medical perspectives. The substances which are effective here, their transformation, processing and associated genetics form the central topic of further investigations.

8 Summary

Royal jelly is produced in the hypopharyngeal, mandibular and postcerebral glands of nurse bees (Apis mellifera) under partial digestion of pollen and honey. Pollen is the main source of proteins, peptides and secondary metabolites in royal jelly. Thus pollen is the essential precursor product of the resulting royal jellies and influences substantially the biological properties. The most important biological properties are the antibacterial properties. In the present study royal jellies were produced within several harvesting years in systematic seasonal variation for microbiological and pollenmorphological analysis.

Antibacterial screening tests showed highly sensible indicator strains for the microbiological analysis of seasonal (i.e. botanical) varyed royal jellies. *Micrococcus luteus* DSM 348 was the most sensitive of 12 tested commensal bacteria. Minimal Inhibitory Concentrations (MIC) against *Micrococcus luteus* of royal jellies from 3 consecutive harvesting years showed maxima of activity in the middle of the season and lower activities in the beginning and the end of the season. Activities (MIC) ranged from 40 µg/ml for highly active samples to 300 µg/ml for lower activities.

Pollenmorphological analysis of royal jellies from 3 consecutive harvesting years showed a yearly identic pollen pattern of seasonal changings. Royal jellies of every seasonal range have a characteristic botanical origin of the predominant pollen. Pollen patterns and microbiological activities correlate in yearly consistent trend lines. The most active royal jelly sorts in the middle of the season show respectively several pollen species. Activities can therefore not be assigned to only one pollen sort.

Analytical C8 RP-HPLC-analysis of highly active royal jellies from various botanical origin showed a constant HPLC basis profile and the variation of several protein peaks. Agar-diffusion tests of protein peaks with *Micrococcus luteus* show the involvement of proteins and peptides in antibacterial activity. The activity of several protein peaks was seasonally relatively constant, others varied seasonally. The result suggests, that different peptides respectively low molecular proteins are involved in activity. In accordance with the activity of royal jellies also the activity of the protein fractions varies with the seasonal (i.e. botanical) origin. Seasonal varying HPLC-peaks were sequenced N-terminally (Edman degradation) and showed slightly

varying peptide patterns. Certain homogeneities in the antimicrobial activities and HPLC profiles suggest, that de novo syntheses of the honeybee play a great role in royal jelly. Substantial correlation between botanical origin of the pollen species and biological activity of the resulting royal jellies could be shown.

Antibiotic screening tests with royal jelly and protein fractions thereof showed strong inhibitory antimicrobial activities against nosocomial pathogenic bacteria, several fungi and viruses. Altogether more than 150 bacteria , several fungi and viruses were tested. Activities against antibiotic resistant bacteria offer a great potential of applications. Flavonoids are co-factors of antimicrobial activities in royal jelly. Antibacterial screening tests with catechins as model compounds were used in the search of bacteria strains with high sensitivity for subsequent histochemical detection reactions and microscopic investigation. Accumulation of catechins in bacteria correlated unambiguously with inhibitory activity. Plaque assays showed the antiviral potential of royal jelly against Coxsackie B3 virus.

Concentrations of inorganic compounds in royal jelly are of considerable interest from various perspectives. Trace elements have a multitude of known and unknown biological functions. Trace element concentrations of 28 elements in botanically and geographically defined royal jelly samples were analysed. The elements K, Na, Mg, Ca, P, S, Cu, Fe, Zn, Al, Ba and Sr in royal jelly were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES). The elements Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Mn, Ni, Ti, V, Co and Mo in royal jelly were determined by sectorfield ICP-MS. Concentrations of 14 trace elements were measured in the associated honey samples. In honey samples trace element concentrations of several elements were strongly depending on botanical and geographical origin. The concentrations of several main and trace elements were highly constant in the associated royal jelly samples. Homeostatic adjustments of several main and trace element concentrations in royal jelly of nurse bees for needs of bee larvae are evident.

Multi-element stable isotope ratio determinations with the elements carbon, nitrogen, and strontium have been applied for testing authenticity and geographical origin of royal jelly. Carbon and nitrogen isotope contents (given as delta values relative to a

standard, $\delta^{13}C$, $\delta^{15}N$) of royal jelly samples from several regions and floral origins were analysed. Honeys, pollen, beeswax and bee tissue were investigated additionally to confirm regional origin and in order to recognize isotope effects in different trophic steps. Seasonal effects respectively the botanical origin and the climatic situation were confirmed to show up as variations in carbon and nitrogen stable isotope ratios. Furthermore, royal jelly samples from authentic and from adulterated production could unambiguously be differentiated. Additional investigation of the trace elements' strontium stable isotope ratio was applied for royal jelly samples, associated honeys and bee tissue from areas with well defined geological substrata. Strontium stable isotope ratios $^{87}Sr/^{86}Sr$ proved to provide a method for the geological situation of royal jelly origins.

Royal jelly protein and peptide fractions were separated with analytical and semi-preparative HPLC, gelfiltration, ultrafiltration, anion and cation exchange chromatography, electrophoresis and subsequent microbiological tests of the proteins fractions. Numerous totally or partially purified proteins and peptides involved in microbiological activities were characterised and correlated with their N-terminal sequence analysis (Edman degradation), mass spectrometry (ESI-MS and MALDI-TOF-MS) and data base search. Royal jelly contains a high number of native and derivated proteins, dominated by 4 high molecular major protein families. These Major Royal Jelly Proteins (MRJP's) contain more than 80% of the total protein mass. Two-dimensional electrophoretical investigation (2-DE) of active HPLC peaks and electrospray ionisation-mass spectrometry (ESI-MS) showed the presence of families of small royal jelly peptides in active samples. MALDI-TOF-MS analysis of royal jelly showed that the preponderating part of royal jelly peptides and proteins are cleavage products of abundant high molecular protein families. Antibacterial activity tests shows that mainly short peptides are biological active. Such peptides are released from the major royal jelly proteins by proteolytic cleavage. Other small peptides are independent from major proteins and show therefore no sequence homology to the major royal jelly proteins. Some of such small peptides were synthesized for antimicrobial tests and NMR spectrometry. The synthetic product shows similar activity compared to these isolated from royal jelly.

8 Zusammenfassung

Gelée royale wird in den Hypopharyngeal-, Mandibular- und Postcerebraldrüsen von Ammenbienen (*Apis mellifera*) unter teilweiser Verdauung von Pollen und Honig hergestellt. Pollen ist die Hauptquelle von Proteinen, Peptiden und Sekundärmetaboliten in Gelée royale. Damit ist Pollen das essentielle Vorprodukt der resultierenden Gelée royales und beeinflusst die biologischen Eigenschaften wesentlich. Die wichtigsten biologischen Eigenschaften sind die antibakteriellen Eigenschaften. In der vorliegenden Arbeit wurden Gelée royales während mehrerer Erntejahre in systematischer saisonaler Variation hergestellt und mikrobiologisch sowie pollenmorphologisch analysiert.

Antibakterielle Screeningversuche zeigten hochempfindliche Indikatorstämme für die mikrobiologische Analyse der saisonal (d.h. botanisch) variierenden Gelée royales. *Micrococcus luteus* DSM 348 war der empfindlichste von 12 getesteten kommensalen Bakterien. Die Minimal Inhibitorischen Konzentrationen (MIC) von Gelée royales von 3 aufeinander folgenden Erntejahren gegen *Micrococcus luteus* zeigten Aktivitätsmaxima in der Saisonmitte und schwächere Aktivität zu Beginn und Ende der Saison. Die Aktivitäten (MIC) bewegten sich zwischen 40 µg/ml für hochaktive Proben bis zu 300 µg/ml für schwächere Aktivitäten.

Pollenmorphologische Analysen der Gelée royales von 3 aufeinander folgenden Erntejahren zeigten ein jährlich identisches Pollenmuster der saisonalen Veränderungen. Gelée royales aus jedem Saisonbereich besitzen eine charakteristische botanische Herkunft an vorherrschenden Pollen. Pollenmuster und mikrobiologische Aktivitäten korrelieren durch jährlich gleichbleibende Trendlinien. Die aktivsten Gelée royale-Sorten der Saisonmitte weisen jeweils mehrere Pollen species auf. So können die Aktivitäten nicht nur einer einzigen Pollensorte zugeordnet werden.

Analytische C8 RP-HPLC-Analysen von hochaktiven Gelée royales verschiedener botanischer Herkunft zeigten ein konstantes HPLC-Basisprofil und die Variation einzelner Proteinpeaks. Agar-Diffusions-Tests der Proteinpeaks mit *Micrococcus luteus* zeigten die Beteiligung von Proteinen und Peptiden an der antibakteriellen Aktivität. Die Aktivität einiger Proteinpeaks war saisonal relativ konstant, andere variierten saisonal. Das Ergebnis legt nahe, dass verschiedene Peptide

beziehungsweise niedermolekulare Proteine an der Aktivität beteiligt sind. Übereinstimmend mit der Aktivität der Gelée royales variiert auch die Aktivität der Proteinfraktionen mit der saisonalen (d.h. botanischen) Herkunft. Saisonal variierende HPLC-peaks wurden N-terminal (Edman Abbau) ansequenziert und zeigten leicht variierende Peptidmuster. Gewisse Homogenitäten in den antimikrobiellen Aktivitäten und HPLC-Profilen legen nahe, dass de novo Synthesen der Honigbiene in Gelée royale eine große Rolle spielen. Ein wesentlicher Zusammenhang zwischen botanischer Herkunft der Pollen species und biologischer Aktivität der resultierenden Gelée royales konnte nachgewiesen werden.
Antibiotische Screeningversuche mit Gelée royale und Proteinfraktionen davon zeigten starke inhibitorische Aktivitäten gegen nosokomiale pathogene Bakterien, einige Pilze und Viren. Insgesamt mehr als 150 Bakterien-, einige Pilz- und Virusstämme wurden getestet. Aktivitäten gegen antibiotisch resistente Bakterien bieten ein großes Potential an Anwendungsmöglichkeiten. Flavonoide sind Co-Faktoren von antimikrobiellen Aktivitäten in Gelée royale. Antibakterielle Screening-Versuche mit Catechinen als Modellsubstanzen wurden verwendet auf der Suche nach Bakterienstämmen mit hoher Sensitivität für anschließende histochemische Nachweisreaktionen und mikroskopische Untersuchungen. Die Akkumulation von Catechinen in Bakterien korrelierte eindeutig mit der inhibitorischen Aktivität. Plaque-tests zeigten das antivirale Potential von Gelée royale gegen Coxsackie B3 Virus.

Die Konzentrationen an anorganischen Verbindungen in Gelée royale sind von erheblichen Interesse aus verschiedener Perspektive. Spurenelemente haben eine Vielzahl von bekannten und unbekannten biologischen Funktionen. Spurenelementkonzentrationen wurden von 28 Elementen in botanisch und geographisch definierten Gelée royale-Proben analysiert. Die Elemente K, Na, Mg, Ca, P, S, Cu, Fe, Zn, Al, Ba und Sr in Gelée royale wurden mit induktiv gekoppelter Plasma optische Emission Spektroskopie (ICP-OES) bestimmt. Die Elemente Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Mn, Ni, Ti, V, Co und Mo in Gelée royale wurden mit Sektorfeld ICP-MS gemessen. Die Konzentrationen von 14 Spurenelementen wurden in den zugehörigen Honigproben gemessen. In den Honigproben waren die Spurenelementkonzentrationen mehrerer Elemente stark vom botanischen und geographischen Ursprung abhängig. Die Konzentrationen von mehreren Haupt- und Spurenelementen in den zugehörigen Gelée royale Proben waren sehr konstant.

Homöostatische Einstellungen von mehreren Haupt- und Spurenelementen in Gelée royale der Ammenbienen für den Bedarf der Bienenlarven sind evident.

Multi-element Stabilisotopen Verhältnisse der Elemente Kohlenstoff, Stickstoff und Strontium wurden für den Test von Authentizität und die geographische Herkunft von Gelée royale angewendet. Die Gehalte an Kohlenstoff- und Stickstoffisotopen (als delta-Werte relativ zu einem Standard angegeben, $\delta^{13}C$, $\delta^{15}N$) von Gelée royales verschiedener geographischer und botanischer Herkunft wurden analysiert. Honige, Pollen, Bienenwachs und Bienengewebe wurden zusätzlich untersucht um die regionale Herkunft zu bestätigen und um Isotopeneffekte in verschiedenen Trophiestufen zu erkennen. Saisonale Effekte hinsichtlich der botanischen Herkunft und der klimatischen Situation, die als Variation in den Stabilisotopenverhältnissen von Kohlenstoff und Stickstoff erscheinen, wurden bestätigt. Darüberhinaus konnten Gelée royales aus authentischer und verfälschter Produktion eindeutig unterschieden werden. Zusätzlich wurden die Stabilisotopenverhältnisse des Spurenelements Strontium in Gelée royales, zugehörigen Honigen und Bienengewebe von Gegenden mit gut definierten geologischen Substrata untersucht. Die Strontium Stabilisotopen-verhältnisse $^{87}Sr/^{86}Sr$ zeigten, dass sie eine Methode für die geologische Situation der Gelée royale Herkunft darstellen.

Protein- und Peptidfraktionen wurden mit analytischer und semipräparativer C8 RP-HPLC, Gelfiltration, Ultrafiltration, Anionen- und Kationenaustaucher-Chromato-graphie, Elektrophorese und anschließende mikrobiologische Tests der Proteinfraktionen. Zahlreiche ganz oder teilweise aufgereinigte, an mikrobiologischen Aktivitäten beteiligte Proteine und Peptide wurden charakterisiert und korreliert mit ihrer N-terminaler Sequenzanalyse (Edman degradation), Massenspektrometrie (ESI-MS und MALDI-TOF-MS) und Datenbanksuche. Gelée royale beinhaltet eine große Zahl nativer und derivatisierter Proteine, dominiert von 4 hochmolekularen Haupt-Proteinfamilien. Diese Haupt-Proteine beinhalten über 80 % der gesamten Proteinmasse. Zweidimensionale elektrophoretische Untersuchungen (2-DE) von aktiven HPLC-Fraktionen und Electrospray-Ionisation Massenspektrometrie (ESI-MS) zeigen die Anwesenheit von Familien kleiner Gelée royale Peptide in aktiven Proben. MALDI-TOF-MS Analysen von Gelée royale zeigten dass der überwiegende Teil von Gelée royale Peptiden und Proteinen Spaltprodukte von überwiegend vorhandenen, hochmolekularen Proteinfamilien sind. Antibakterielle Aktivitätstests zeigten, dass hauptsächlich kurze Peptide biologisch aktiv sind. Solche Peptide

werden von den Haupt-Proteinen durch proteolytische Spaltung freigesetzt. Andere kleine Peptide sind unabhängig von den Haupt-Proteinen und zeigen deshalb keine Sequenzhomologie zu den Hauptproteinen. Einige solcher kleinen Peptide wurden für antimikrobielle Tests und NMR-Spektrometrie synthetisiert. Das synthetische Produkt zeigt ähnliche Aktivität verglichen zu jenen isoliert aus Gelée royale.

8 Sommaire

La gelée royale est produite dans les glandes hypopharyngiennes, mandibulaires et post-cérébrales des abeilles nourricières par digestion partielle de pollen et de miel. Le pollen est la source principale des protéines, des peptides et des constituants secondaires de la gelée royale. En tant que précurseur essentiel des gelées royales, le pollen conditionne fortement leurs propriétés biologiques. Parmi ces dernières, les propriétés antibactériennes sont de loin les plus importantes. La présente étude porte sur des récoltes pluriannuelles de gelées royales effectuées à différentes dates de la saison de prélèvement, ce qui a permis d'analyser l'activité anti-microbienne et la morphologie des pollens en fonction de la date de prélèvement.

Les tests de criblage antibactérien ont permis d'identifier des souches bactériennes de référence, hautement sensibles aux variations saisonnières des gelées récoltées. *Micrococcus luteus* DSM 348 est apparu comme la plus sensible parmi les 12 bactéries commensales testées. Les Concentrations Minimales d'Inhibition (MIC, pour *Minimal Inhibitory Concentration*) contre *Micrococcus luteus* de gelées royales récoltés sur trois années consécutives, montrent des maxima d'activité au milieu de la saison, comparés aux activités de début et de fin de saison. Les activités (MIC) s'échelonnent de 40 µg/ml pour les échantillons les plus actifs jusqu'à 300 mg/ml pour les activités les plus faibles. Les analyses morphologiques des pollens de gelées royales récoltés sur trois années consécutives font apparaître des profils identiques de variations saisonnières. Pour chacune des tranches saisonnières, la gelée royale peut être associée à un pollen dominant caractéristique. La même corrélation entre le type de pollen et l'activité microbiologique se retrouve d'une année sur l'autre. Les gelées royales collectées en milieu de saison sont associées à plusieurs type de pollens. De ce fait, les activités microbiologiques ne peuvent pas être assignées à une seule espèce de pollen.

Les spectres d'élution HPLC analytique en phase inverse sur C8 des gelées royales hautement actives de différentes origines botaniques présentent un profil de base qui

leur est commun mais des pics protéiniques différents. Les tests de diffusion dans l'agar effectués sur les pics protéiniques indiquent que l'activité antibactérienne contre *Micrococcus luteus* implique des protéines et des peptides. L'activité de plusieurs pics protéiniques est indépendante de la saison, tandis que celle d'autres pics présente des variations saisonnières. Ces résultats suggérent que différents peptides et/ou protéines de faibles poids moléculaires participent à l'activité biologique. L'activité biologique des fractions protéiniques varie selon la saison, et dépend, par conséquent, de l'origine botanique des échantillons de gelées royales, comme observé pour l'activité des gelées elles-mêmes. Les peptides des pics HPLC variables ont été séquencés par dégradation d'Edman. Leurs séquences N-terminales présentent des petites variations. Certaines homogénéités observées dans les activités antimicrobiennes et les profils HPLC suggèrent que les synthèses de novo réalisées par l'abeille jouent un rôle déterminant dans les propriétés de la gelée royale produite. Une corrélation significative entre l'origine botanique des espèces de pollen et l'activité biologique des gelées royales résultantes a pu être établie.

Les tests de criblage antibiotique réalisés sur la gelée royale et ses fractions protéiniques ont montré de fortes activités inhibitrices contre des bactéries pathogènes nosocomiales, plusieurs champignons et virus. Au total, plus que 150 bactéries, plusieurs champignons et virus ont été testés. L'activité inhibitrice contre les bactéries résistantes aux antibiotioques offre, de ce fait, un grand potentiel d'applications. Les flavonoïdes sont des cofacteurs de l'activité antimicrobienne dans la gelée royale. Des tests de criblage antibactérien ont été réalisés avec des catéchines (flavonoïdes) comme biomarqueurs afin d'identifier des souches de bactéries hautement sensibles permettant la détection histochimique et les investigations microscopiques. L'accumulation des catéchines dans les bactéries suit fidèlement l'activité inhibitrice. Des essais sur plaque ont montré le potentiel antiviral de la gelée royale contre le virus Coxsackie B3.

La présence de composés inorganiques dans la gelée royale est très intéressante à plusieurs titres. Les élements traces jouent de nombreuses fonctions biologiques dont toutes ne sont pas encore connues. Les concentrations de 28 éléments traces présents dans les échantillons de gelées royales, bien définies au plan botanique et géographique, ont été analysés. Les éléments K, Na, Mg, Ca, P, S, Cu, Fe, Zn, Al, Ba et Sr ont été dosés par spectroscopie d'émission optique par plasma couplé par

induction (ICP-OES). Les éléments Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Mn, Ni, Ti, V, Co et Mo ont été déterminés par spectrométrie de masse par plasma couplé par induction (ICP-MS). Les concentrations de 14 éléments traces ont été mesurés dans les échantillons correspondant de miel. Dans les miels, les concentrations des éléments traces dépendent fortement de l'origine botanique et géographique. Dans les gelées royales correspondantes, les concentrations de plusieurs éléments majeurs et traces sont remarquablement constantes. A l'évidence, des ajustements homéostatiques de plusieurs éléments majeurs et traces sont opérés dans la gelée royale par les abeilles nourricières pour les besoins nutritionnels des larves.

Les rapports des isotopes stables du carbone, de l'azote et du strontium ont été mesurés pour suivre l'authenticité et l'origine géographique des échantillons de gelée royale. Les proportions des isotopes stables du carbone et de l'azote (exprimés en valeurs relatives standard, $\delta^{13}C$, $\delta^{15}N$) de gelées royales de plusieurs origines géographique et botanique ont été determinées. Des miels, pollen, cire d'abeille et tissus d'abeille ont été analysés pour confirmer l'origine géographique mais également pour suivre d'éventuels fractionnements isotopiques au cours des différentes phases trophiques. Des effets saisonniers, liés à l'origine botanique et à la situation climatique, ont été confirmés au travers des variations des rapports isotopiques du carbone et de l'azote. Signalons que cette méthode isotopique permet de différencier nettement les gelée royales authentiques des productions falsifiées. Les rapports des isotopes stables de l'élément trace strontium ont été mesurés dans les gelées royales, miels et tissus d'abeille issus d'horizons géologiques bien définis. L'analyse des isotopes stables du strontium a permis de définir l'origine géologique des gelées royales.

Les fractions de protéines et de peptides de la gelée royale séparées par HPLC analytique et semi-préparative sur C8, gelfiltration, ultrafiltration, chromatographie échangeuse d'anions et de cations et électrophorèse, ont été soumises au test microbiologique. La plupart des protéines et peptides présentant une activité microbiologique, partiellement ou totalement purifiés, ont été caractérisés et comparés après analyse de leurs séquences N-terminales (par dégradation d'Edman), spectrométrie de masse (ESI-MS et MALDI-TOF-MS) et examen des bases de données. La gelée royale contient un grand nombre des protéines natives et dérivés protéiniques, dominé par 4 familles de protéines majeures de haut poids moléculaire. Ces protéines majeures de la gelée royale (MRJP, pour Major Royal

Jelly Protein) représentent plus de 80 % de la masse totale des protéines. L'électrophorèse bidimensionnelle (2-DE) et la spectrométrie de masse par ionisation électrospray (ESI-MS) appliquées aux pics actifs isolés par HPLC ont permis de mettre en évidence la présence de familles de petits peptides dans les fractions actives. L'analyse par MALDI-TOF-MS de la gelée royale a montré que la partie prépondérante des peptides et protéines de faible poids moléculaire de la gelée royale provient de la coupure protéolytique des protéines majeures de haut poids moléculaire. Les tests d'activité antibactérienne montrent que ce sont principalement les peptides courts qui sont biologiquement actifs. Ces peptides proviennent bien de la coupure protéolytique des protéines majeures de la gelée royale. D'autres petits peptides ne sont pas liés aux protéines majeures de la gelée royale car ils ne présentent aucune homologie de séquence avec ces dernières. Certains de ces petits peptides ont été synthétisés et soumis aux tests antimicrobiens et à l'analyse par spectrométrie RMN. Le produit synthétique présente une activité comparable à celle manifestée par les peptides isolés à partir de la gelée royale.

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