Journal of Chromatography A, 1347 (2014) 17–29

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Preparative mass-spectrometry profiling of bioactive metabolites in Saudi-Arabian propolis fractionated by *high-speed countercurrent chromatography* and *off-line* atmospheric pressure chemical ionization mass-spectrometry injection

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ARTICLE INFO

Article history:
Received 20 January 2014
Received in revised form 17 April 2014
Accepted 18 April 2014
Available online 28 April 2014

Keywords:
Saudi Arabian propolis
Diterpene
Triterpene
Preparative *high-speed countercurrent chromatography*
Off-line APCI-MS/MS injection metabolite profiling
1D/2D-NMR

ABSTRACT

Propolis is a glue material collected by honeybees which is used to seal cracks in beehives and to protect the bee population from infections. Propolis resins have a long history in medicinal use as a natural remedy. The multiple biological properties are related to variations in their chemical compositions. Geographical settings and availability of plant sources are important factors for the occurrence of specific natural products in propolis. A propolis ethylacetate extract (800 mg) from Saudi Arabia (Al-Baha region) was separated by preparative scale *high-speed countercurrent chromatography* (HSCCC) using a non-aqueous solvent system *n*-hexane–ACN (1:1, v/v). For multiple metabolite detection, the resulting HSCCC-fractions were sequentially injected *off-line* into an atmospheric pressure chemical ionization mass-spectrometry (APCI-MS/MS) device, and a reconstituted mass spectrometry profile of the preparative run was visualized by selected ion traces. Best ion-intensities for detected compounds were obtained in the negative APCI mode and monitored occurring co-elution effects. HSCCC and successive purification steps resulted in the isolation and characterization of various bioactive natural products such as (12*E*)- and (12*Z*)-communic acid, sandaracopimaric acid, (+)-ferruginol, (+)-totarol, and 3β-acetoxy-19(29)-taraxasten-20*a*-ol using EI-, APCI-MS and 1D/2D-NMR. Cycloartenol-derivatives and triterpene acetates were isolated in mixtures and elucidated by EI-MS and 1D-NMR. Free fatty acids, and two labdane fatty acid esters were identified by APCI-MS/MS. In total 19 metabolites have been identified. The novel combination of HSCCC fractionation, and APCI-MS-*target-guided* molecular mass profiling improve efficiency of lead-structure identification.

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1. Introduction

Propolis is a regional strongly differing heterogeneous glue material generated by honeybees using plant resins and beeswax. It is used to seal cracks in hives and to protect the bee populations from bacterial and fungal infections [1,2]. Bees collect the material from leaves, leaf buds, bark resins, and exudates [3,4]. Since many different plant sources can be used by the honeybees the chemical profiles are as complex as mixing different medicinal plants to produce a decoction for a phytomedical formulation.

Propolis was already used in the antique by Egyptians, Romans and Greeks as a medicine to treat various diseases, e.g. disinfection and treatment of open wounds [5]. Geographical settings, plant sources, and the collecting season are important factors in the diversity of the propolis samples regarding their chemical profiles, and their multiple biological activities which are of interest to researchers [5].

Up to now, mass spectrometry with ESI- and APCI-MS has been used intensively for metabolite profiling- and fingerprinting

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http://dx.doi.org/10.1016/j.chroma.2014.04.068
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-studies of propolis. HPLC–DAD–ESI-MS/MS implemented to a study of Italian propolis [6], resulted in 40 metabolites such as phenolic acids, and flavonoids. The investigation of propolis samples from different locations in Brazil using LC–APCI-MS (negative ion mode) led to a large amount of metabolites such as diterpenes and phenolic compounds. For the respective aqueous extracts, dicaffeoyl-quinic acids were identified [7]. Gardana et al. compared the profiles of propolis from Europe, Latin American countries, Russia and China using HPLC–ESI-DAD–MS, and identified mainly flavonoids and phenolic acid derivatives [8]. Volpi and Bergonzini quantified flavonoids in propolis from different regions of the world by LC–ESI-MS [9]. The mass spectrometric fingerprinting could be seen as a reasonable approach to correlate specific geographical origins, regional variations and to prove authenticity of propolis samples [10]. Fingerprinting and chemical profiling has been applied to a set of 49 ethanolic extracts of propolis samples collected worldwide (North and South America, Europe, Asia and Oceania) using ambient sonic-spray ionization mass spectrometry (EASI-MS) in the negative ionization mode [11].

Investigations and analysis of very complex constituted crude extracts (plants, terrestrial microorganism, and marine organism) are strongly dependent on efficient preparative chromatographic separation methodologies. Techniques such as *countercurrent chromatography* (CCC) and *countercurrent partition chromatography* (CPC) using solely liquid chromatographic phases are versatile in its use and increasingly implemented to lab-scale isolation protocols for the recovery of bioactive natural products [12–14]. A specific spectrometric detection of substances is also required for easy and fast tracing of lead- or target-compounds in order to facilitate the isolation process.

In the past we have used a direct *on-line* coupling of *high-speed countercurrent chromatography* (HSCCC) to an ion-trap ESI-MS/MS device or APCI-MS/MS as a fast screening method for acquisition of structural information of compounds eluting from preparative chromatography [15,16]. This method collected essential data to perform accurate preparative fractionations. Furthermore, it facilitated the specific detection of potential target molecules. Co-elution effects of up to 10 compounds could be easily monitored by selective ion traces including the respective MS/MS fragmentation data. In the present study we applied a novel *off-line* APCI-MS monitoring approach to characterize apolar metabolites recovered from the HSCCC fractions [17–19]. Sequential injections of collected fractions to the APCI-MS/MS mass spectrometer resulted in a reconstituted molecular weight profile for the separated and recovered compounds. It is important to recognize the presence of so far unknown metabolites by the acquired molecular weight data. Knowing specific bioactivities for the extracts and the CCC or CPC fractions, bio-assay-guided fractionation could correlate interesting biological data to mass spectrometric profiles.

There are many studies focusing on the metabolite profiles of propolis for elucidation of lead structures responsible of bioactivities (anti-inflammatory, anti-tumor, anti-bacterial) [20–23]. Honeybees harvest from available plant resources. The influence on the chemical constitution of propolis, plant biodiversity in the respective geographical regions is a strong limitation to elucidate or compare compound profiles of materials coming from not intensively investigated geographical areas. Especially, classical honey production areas in Persia, Arabia, and East-Africa will require much more efforts to build up a chemical data base for these regional propolis products.

In the presented study we investigated propolis from Al-Baha region (Saudi Arabia), a well-known honey production area. One principal direction of our investigation was to correlate the elucidated metabolites with existing phytochemical knowledge of local plants in order to trace the origin of the propolis product in this specific area.

A large amount of studies and surveys focused on therapeutic activities of propolis was given by Marcucci [21]. Recent progress in pharmacological research on propolis was summarized by Banskota et al. [1], and recent trends to elucidate the biological lead structures using bio-assays were surveyed by Bankova [22].

One issue regarding propolis is also toxicity due to the possibility that it could contain toxic plant metabolites. This aspect was reviewed by Burdock [23].

Interesting biological activities against parasites of so-called 'neglected tropical diseases (NTDs'), such as *Leishmania tropica* [24] were found. Also strong inhibitory activity of ethanolic extracts of a Bulgarian propolis material against proliferative epimastigotes of *Trypanosoma cruzi* (Chagas disease) was observed [25].

The all-liquid HSCCC study – which has a lower risk of chemisorptive losses – was initiated to open an effective route for fractionation, isolation and the recovery of bioactive metabolites from propolis without the potential loss of the target compounds responsible for observed bioactivities.

2. Materials and methods

2.1. Reagents

For the solvent extraction of the propolis material ethylacetate was used (Chromasolv® for HPLC, Sigma Chemical Co., St. Louis, USA). The preparative HSCCC separation was done with HPLC gradient grade solvents *n*-hexane, acetonitrile (Sigma Chemical, Deisenhofen, Germany). For the make-up solvent mixture being used for the APCI-MS/MS continuous *off-line* injections *tert*.-butylmethylether (Chromasolv®, Sigma, Deisenhofen, Germany), methanol (LC–MS-grade, Fisher Scientific, Loughborough, UK), and water (Nanopure®, Barnstead, USA) were used.

2.2. Propolis material and extraction of metabolites

All propolis material has been collected by the project team from our hives located in Al-Baha region. The total area of the patio area is comprising 10,362 km^2 and is located south-west of Saudi Arabia (coordinates 41°27′ E/20°0′ N) with an altitude range from 1550 to 1900 m including mountains areas up to 2215 m. The local bee colonies were classified as *Apis mellifera jemenitica*.

2.3. High speed countercurrent chromatography apparatus (HSCCC)

The separation of the propolis extract was performed on a preparative triple multilayer coil planet J-type *HSCCC* instrument (model CCC-1000, Pharma-Tech Research Corp., Baltimore, MD, USA). The three preparative separation coil columns were connected in series and were equipped with polytetrafluorethylene (PTFE) tubings: 165 m × 2.6 mm i.d. with 865 mL theoretical total volume (given by manufacturer). The measured total volume was 850 mL. The distance (revolution radius = *R*) of the holder axis of the coils to the central (solar) axis of the instrument was 7.5 cm. The inner β_r-value was measured to be 0.53 at the internal end of the coil and the outer β_r-value was 0.8 (equation: $\beta_r = r/R$; *r* is defined as the distance from the coil (planetary) axis to the nearest and farthest layer of the PTFE tubes wound on the coil system). The *HSCCC* system's direction of rotation determined the *head* locations at the periphery of the three coils.

2.4. HSCCC separation of the ethylacetate extract of propolis – biphasic solvent system

A suitable liquid–liquid separation system for the medium polar to apolar propolis metabolites in the ethylacetate extract was the

biphasic non-aqueous solvent system *n*-hexane/acetonitrile (1:1, v/v).

The distribution of compounds between the two liquid phased was evaluated by thin-layer chromatography (silica gel 60 TLC plates F254, Merck KGaA, Darmstadt, Germany) and visualized by anisaldehyde-universal reagent [26].

After equilibration of the two solvents in a separatory funnel, the two resulting phase layers were separated shortly before use in the *HSCCC* separation. For the separation, the upper *n*-hexane phase was used as stationary phase and the lower acetonitrile phase as mobile phase in the '*head-to-tail*'-mode.

The multilayer coil columns of the *HSCCC* were filled with upper phase using a Biotronik BT 3020 HPLC pump (Jasco, Grossumstadt, Germany). The *HSCCC* separation was performed at ambient temperature (outside the machine: approx. 28 °C; inside: approx. 40 °C) with no active temperature control during the separation.

The dried resinous ethylacetate extract of propolis (800 mg) was dissolved in 10 mL each of upper and lower phase in accordance to Ito [12,13], and filtrated. The sample was introduced via a plastic syringe to a 25 mL sample loop and then directly injected into the separation column through a manual low-pressure sample injection valve (Rheodyne, Cotati, CA, USA) without prior column mobile phase equilibration [12,13]. The lower phase was used as mobile phase and was pumped at a flow rate of 3.0 mL/min in the '*head-to-tail*' mode. A *HSCCC* rotation velocity of 1000 rpm was used for the separation in the *elution* mode of compounds.

For recovery of the whole polarity window of existing propolis metabolites, the two-column volume method of Berthod et al. with *elution* and *extrusion* mode for HSCCC was applied [27]. Running the HSCCC experiment, the 'break-through' of mobile phase occurred after 40 min (loss of 14% stationary phase from the HSCCC system). Beginning with this event, the *elution* of compounds started. 85 fractions were collected (numbered F1–F85) (HSCCC fractions cf. Tab. supplement 1, Fig. Supplement 1). Then the system was changed to the *extrusion* mode with pumping of stationary phase (upper phase), the use of a lower spinning velocity (400 rpm), and a flow rate of 6.0 mL/min. Every 2 min the *extrusion*-fractions were collected (F86 until F134).

Supplementary Fig. 1 and Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.04.068.

Aliquots of every second HSCCC fraction were filled to HPLC vials and diluted (cf. 2.5). For the APCI-MS profiling all recovered HSCCC fractions from the *elution* and *extrusion* mode were injected in sequence to generate a full mass spectrometry profile of the compounds existing in the propolis ethylactetate extract.

2.5. Off-line APCI-MS/MS injection analysis for HSCCC molecular weight profiling of propolis metabolites

The molecular weight profiling of the metabolites from recovered propolis *HSCCC*-fractions (*elution* and *extrusion mode*) were determined by injection in *off-line* mode to an ion-trap atmospheric pressure chemical ionization (APCI) mass spectrometer (HCT-Ultra ETD II, Bruker Daltonics, Germany) in the sequence of the collection of chromatographic fractions [17–19]. The acquired data were recorded in one data file. For sample preparation, an aliquot volume of 300 μL from every second *HSCCC*-fraction (*elution* and *extrusion* mode) was directly filled to HPLC vials and then diluted with 900 μL ACN. These *profiling* samples containing the potential target metabolites were stored frozen (-30 °C) until analysis by APCI-MS.

Some fast pre-injection experiments using some selected *HSCCC* fractions were done to evaluate the correct injection volumes later being used in the main APCI-MS/MS profiling experiment. This approach avoided the potential risk of highly concentrated MS-overloading injections. This protocol had been shown to be a practicable and safe operation procedure for preventing any risk of contamination in the APCI-MS-interface or the high vacuum ion compartment during HSCCC profiling experiments. This aspect could be seen as a major advantage of profiling of HSCCC fractions by sequential injections in *off-line* mode compared to *on-line* hyphenation experiments of HSCCC with a direct hyphenation to an APCI- or ESI-MS spectrometer [15,16].

The injections of fractions of the preparative *HSCCC* samples resulted in APCI-MS-ion-abundancies in the base peak chromatogram in the range between 1.0×10^6 and $\times 10^8$ (Fig. 1) and were similar to observed ion abundances for routine HPLC-APCI-MS/MS experiments.

The evaluation of ideal injection volumes resulted in excellent ion intensities, therefore every second fraction from the *HSCCC* run (*elution*- and *extrusion* mode) was sequentially injected using an injection volume of 100 μL. The interval time setting between the re-occurring injections was set to 2 min. Every observed injection peak was corresponding to one injected vial from the *HSCCC* run carrying the complete molecular weight information of the ionizable compounds in a single fraction. The selected APCI-MS traces (Fig. 1) displayed the mass spectrometry profile information of the compounds located in a respective *HSCCC* fraction.

The *off-line* injections to the APCI-MS/MS were conducted by an independently working, and programmable autosampler system (AS-2000A, Merck-Hitachi, Tokyo, Japan). The injected fractions were then delivered to the APCI-MS by a normal HPLC-pump (binary pump, G1312 A, 1100 Series, Agilent, Waldbronn, Germany) using a make-up solvent system with a flow rate of 0.7 mL/min composed of a mixture of solvent system A and B (50:50, v/v). System A was constituted of *tert.*-butylmethylether–methanol–water (4:92:4, v/v/v), and system B in the ratios of 90:6:4 (v/v/v). The medium apolar make-up solvent system enabled the transfer of all compounds separated by HSCCC.

APCI-MS/MS parameter settings: negative ionization mode for the detection of propolis compounds was used due to best ion responses and in general $[M-H]^-$ and $[M-H_2O]^-$-ion signals were assumed. Interestingly, positive APCI ionization was not very effective to the fractionated metabolites (pre-evaluation data not shown). The scan-range was set between *m/z* 150 and 2200. The rapid '*Ultra*'-mode with a mass scanning rate of 26.000 *m/z* per second was chosen. Drying gas was nitrogen (flow rate 5.0 L min^{-1}, 350 °C), vaporizer heated to 400 °C, and nebulizer pressure was set to 40 psi. Ionization voltage at HV capillary +800 V, HV end plate off set −500 V, trap drive 79.1, octopole RF amplitude 187.1 Vpp, lens 2 + 60.0 V, Cap Ex −115.0 V, max. accumulation time 200 ms, averages 5 spectra, trap drive level 120%, target mass range: *m/z* 500, compound stability 80%, Smart ICC target 70000, ICC charge control on, smart parameter setting active. To monitor co-elution of metabolites, 5 precursor ions were selected to obtain specific APCI-MS/MS fragmentation data. The MS/MS fragmentation amplitude value was set to 3 V.

2.6. Nuclear magnetic resonance spectroscopy (1D/2D-NMR)

The 1D- (including ^1H-, ^{13}C-, DEPT135), and 2D-NMR experiments (COSY, HSQC, HMBC) were measured on a 500 MHz spectrometer (Bruker Avance III HD, magnet Ultrashield Plus 500, probehead PABBO BB/19F-1H/D Z-GR, Rheinstetten, Germany). All samples were measured in $CDCl_3$ (99.95%) and were referenced against the solvent signals (^1H: δ 7.26 ppm, ^{13}C: δ 77.2 ppm).

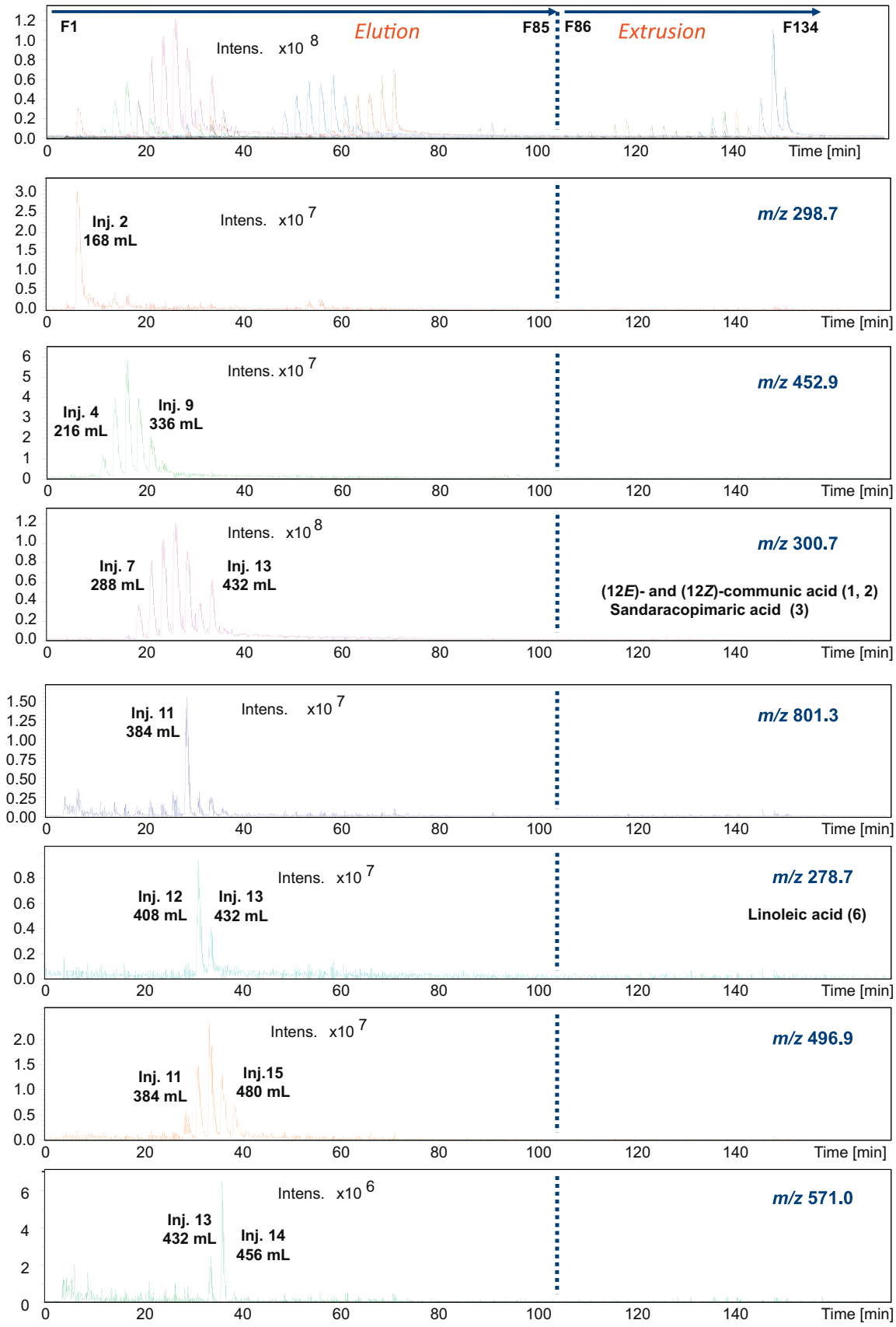

Fig. 1. HSCCC off-line APCI-MS (neg. mode) with selected ion traces $[M-H]^-$ (scan range *m/z* 150–2200) with experimental time for profiling in minutes. Real HSCCC experimental *elution*- and *extrusion*-volumes are displayed for the chromatographic retentions and elution intervals of detected metabolites. The flow rates for elution were 3.0 mL/min, and extrusion 6.0 mL/min, respectively (Inj. = injected tube from fraction collector).

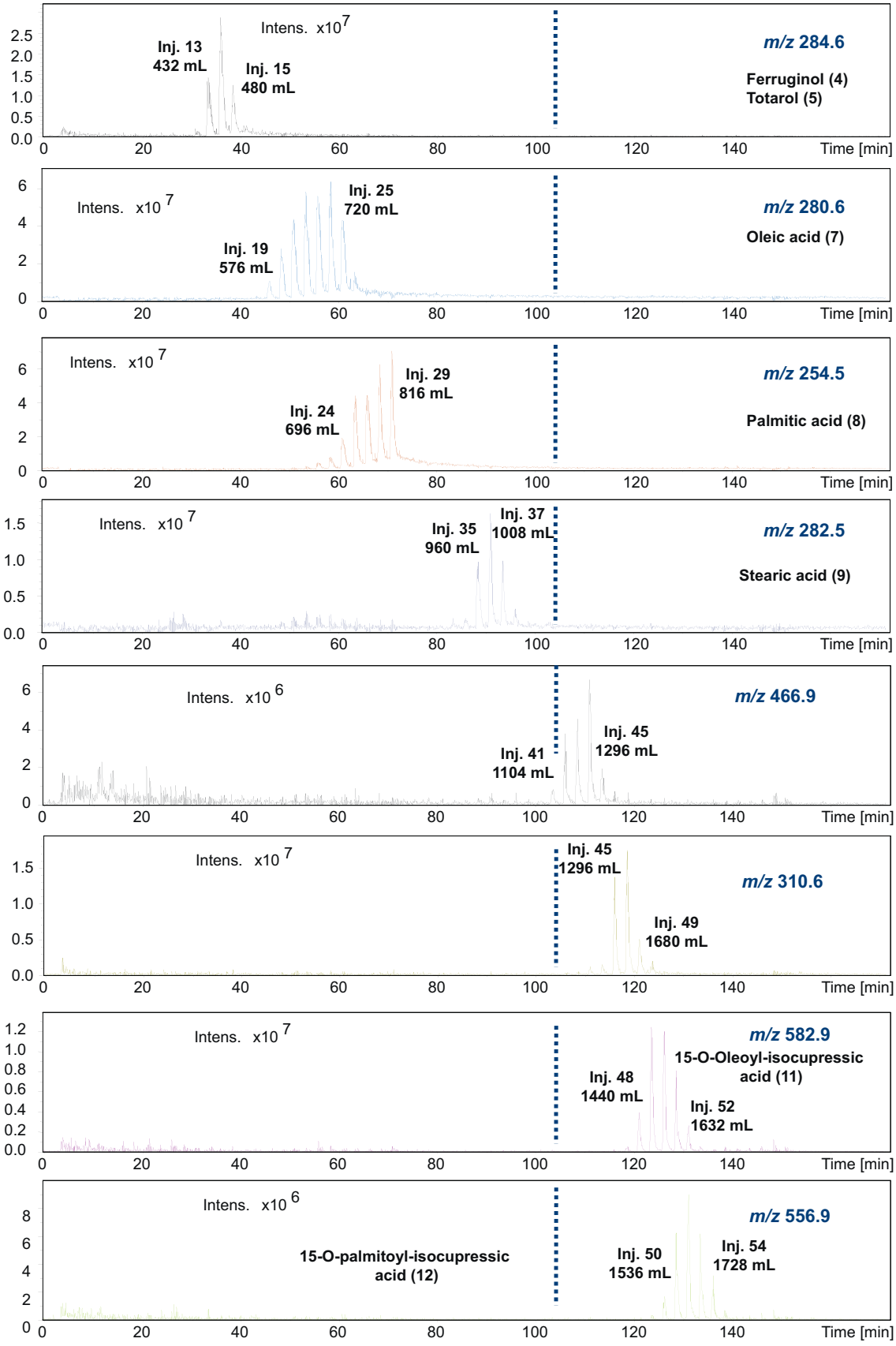

Fig. 1. *Continued*

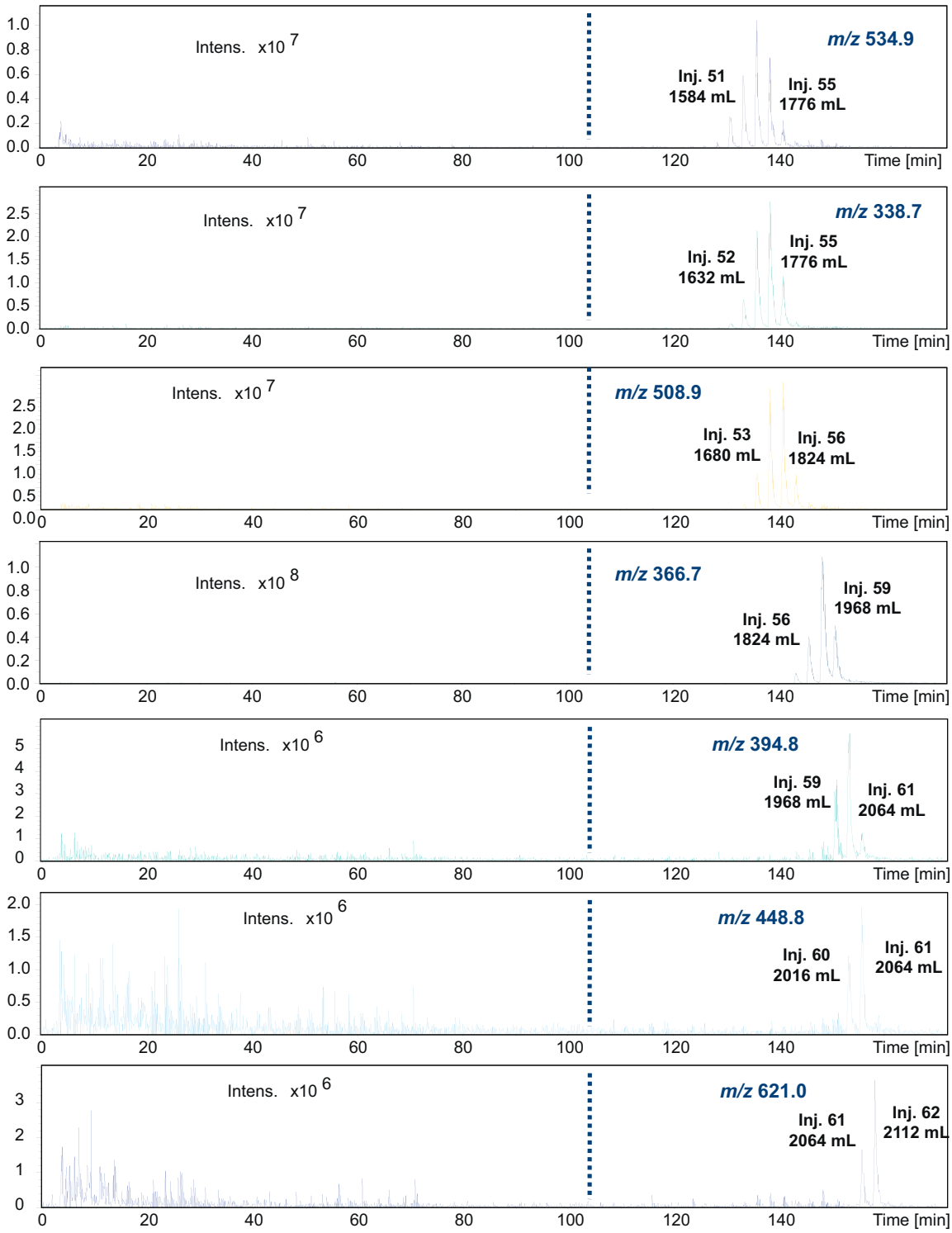

Fig. 1. *Continued*

2.7. Lyophilization of HSCCC fractions containing propolis metabolites

The HSCCC fractions containing *n*-hexane and acetonitrile were gently evaporated with a SpeedVac Plus concentrator equipped with a rotor for fraction collector tubes (SC210A, and refrigerated vapor trap RVT 400, Thermo Savant, Holbrook, NY, USA).

Recovered fractions from the *elution* and the *extrusion* mode of the HSCCC separation are displayed in Tab. supplement 1. 800 mg ethylacetate soluble propolis extract resulted in a recovered yield of 620 mg for all HSCCC fractions (recovery ~77.5%).

3. Results and discussion

3.1. HSCCC separation and off-line APCI-MS/MS detection of metabolites from propolis

The focus of this investigation on propolis from Saudi Arabia was the efficient chromatographic fractionation and isolation

of potentially bioactive metabolites combined with a fast mass-spectrometry detection method. In general, the use of all-liquid chromatography phases in preparative HSCCC minimize the potential loss of target compounds by omitting chemisorptive effects. The sensitive detection and profiling for metabolites in the obtained HSCCC fractions was achieved by *off-line* injections to APCI-MS/MS mass spectrometry (cf. 2.5). The resulting information was used for further silica gel chromatographic purification steps on fractions containing metabolites of interest. Nevertheless, as shown in the past, solely ESI-MS or APCI-MS methodology used for molecular weight determination especially for isobaric compounds had quite often been insufficient for unambiguous identification of the constitution of already known natural products. Therefore our principal approach was to perform very accurate fractionation steps – in this study by use of TLC and APCI-MS profiling of the HSCCC fractions (Fig. 1, Fig. Supplement 1) – and then the complete characterization of pure compounds by 1D/2D-NMR which in general requires sample amounts in the range of 1–2 mg for a single structure.

The described metabolite profiling procedure for preparative HSCCC fractions using sequential *off-line* injections to APCI-MS is not time consuming (cf. 2.5). Decisions how to cut fractions can be easily retrieved and time consuming multiple LC–MS runs are not required at this isolation stage. Getting a quick overlook on preparative HCCCC-fraction combined to mass-spectrometry data is so far not being used by routine in the field of countercurrent chromatography. Every generated injection peak contains the profile information of ionized compounds located in the respective fraction. The high detection sensitivity known for LC–MS with a chromatographic peak focusing of specific compounds will for sure not be reached.

The large advantage of this *off-line* approach is the enormous reduction of required MS-routine experimental time. Such in our case, the APCI-MS experiment only required 180 min for the full profile of eluted and extruded HSCCC fractions using every second tube fraction for *off-line* injection (65 tubes). Comparing this to fast UHPLC–MS with an approximate chromatographic running time of 15–20 min per injection, this amount of fractions would require up to 16–22 h MS-experimental time.

3.2. APCI-MS and NMR structure elucidation of fractionated propolis metabolites

Each propolis sample harvested in a different geographical environment will present its unique chemical compound profile. These profiles could differ over the seasons and years, and the availability of plant sources visited by the honeybees determined the composition of the final product. Therefore every propolis product is characterized by a *fingerprint* metabolite profile. Collected chemical data will not necessarily prove or clarify authenticity or a certain geographical origin. Nevertheless, a large data set from different propolis production areas in the world would help to understand the diversity of biological properties known for this natural remedy.

Actually the already known huge amount of metabolites identified in propolis samples from Latin America (mainly Brazil), Europe, and other locations were not of great help to indicate metabolites occurring in propolis from Saudi Arabia (Al-Baha region) [6–11,28]. Especially, chemical data for propolis samples with origin Persia, Arabia, and East-Africa are barely available [29]. Thorough investigations with modern methods such as mass-spectrometry, 1D/2D-NMR need to be conducted to see correlations in the chemical profiles. The flora of these arid and semi-arid areas might be somehow similar and Cupressaceae plant species might be of high importance for the honeybees.

3.2.1. (12*E*)- and (12*Z*)-communic acid (**1**) (**2**)

A mixture of (12*E*)- and (12*Z*) -communic acid (**1**) (**2**) was isolated and detected by the APCI-MS profiling in the HSCCC fractions F14–F23 (Fig. 1, with APCI-MS injection signal No. 8–12) in higher amounts with the selective ion trace $[M-H]^-$ at *m/z* 300.7 and MS/MS fragment ions at *m/z* 219.4, 204.2. As seen in Fig. 1 for the following injection of HSCCC fraction No. 13, ion intensity for *m/z* 300.7 increased significantly. The TLC chromatography in combination concluded that after F23 potentially an isobaric metabolite eluted closely after communic acid (Fig. supplement 1).

Depending on the differing TLC and APCI-MS results only F14 until F23 were combined, and cleaned by SiO_2-short column chromatography to yield 5 mg. The ^1H and ^{13}C NMR identified (*12E*)- and (12*Z*)-communic acid (**1**) (**2**). The mixture of isomers was recognized by signal doublings for the double bond protons H-14, H-15_a and H-15_b (Tab. Supplement 2). Corroborating the proposal for the constitution of communic acid isomers, the HMBC showed the relevant $^{2,3}J$-CH long-range correlation signals (1D/2D-NMR data cf. Fig. 2, Tab. Supplement 2). The ratio of the isomers in the mixture was determined by the integration of intensities of the H-14 protons of both compounds and led to a 12*E*/12*Z*-value of 2.1:3. The published NMR reference data were in good accordance to our findings [30–32].

Tab. Supplement 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.04.068.

The mixture (12*E*)- and (12*Z*)-communic acid (**1**) (**2**) had shown the same molecular weight as sandaracopimaric acid in the EI-MS with $[M]^+$, solely the strong fragment ions at *m/z* 256 and 241 were missing.

Direct inlet EI-MS (70 eV): *m/z* 302 $[M]^+$ (69), 287 (50), 273 (8), 257 (18), 246 (22), 241 (25), 201 (18), 175 (83), 161 (33), 147 (72), 135 (74), 134 (74), 133 (54), 121 (82), 119 (100), 107 (57), 105 (68), 93 (84), 91 (65), 81(90), 79 (85), 67 (28), 55 (42).

Communic acid in the (12*Z*)- and (12*E*)-double bond isomeric configurations had been isolated from about nine *Juniperus* species [33] including *Juniperus procera* which might be one of the sources where the honeybees of Al-Baha region collected the resins for the propolis. Antibacterial and cytotoxic activities had been documented for these isomeric labdane derivatives [31]. (12*E*)- and (12*Z*)-communic acid isolated from *Juniperus communis* was described as well as an inseparable 2:3 mixture (12*E*/12*Z*) [32], and it was concluded that these compounds were one of the responsible metabolites in the activity observed against *Mycobacterium tuberculosis* H37Ra, and (12*Z*)-communic against *Mycobacterium aurum* [34].

3.2.2. Sandaracopimaric acid (**3**)

The profiling of the HSCCC fractions F13–F25 detected $[M-H]^-$ at *m/z* 300.7 (APCI injection signals No. 7–13, cf. Fig. 1). The earlier eluting highly concentrated compounds with lila coloring spots on TLC were already identified as (12*E*)- and (12*Z*)-communic acids (**1**) (**2**). The blue coloring spots in the following fractions (F24–F27) were combined (Fig. supplement 1), purified by a SiO_2-short column chromatography and were identified by 1D/2D-NMR as sandaracopimaric acid (**3**) (Fig. 2). ^1H, and ^{13}C NMR and 2D-NMR correlation data are given in Tab. Supplement 3. The observed $^{2,3}J$-long-range correlations (HMBC), and the published ^{13}C NMR data clearly elucidated this compound and distinguished it from the C-13 epimer pimaric acid [35]. The yield of pure substance was 2 mg (0.25% of injected propolis extract). Sandaracopimaric acid had been isolated from more than twelve *Juniperus* species [33,36]. In case of *J. procera* and *Juniperus excelsa* we suspected that these plants could have been potential honeybee collecting sources in Al-Baha region for this propolis [37].

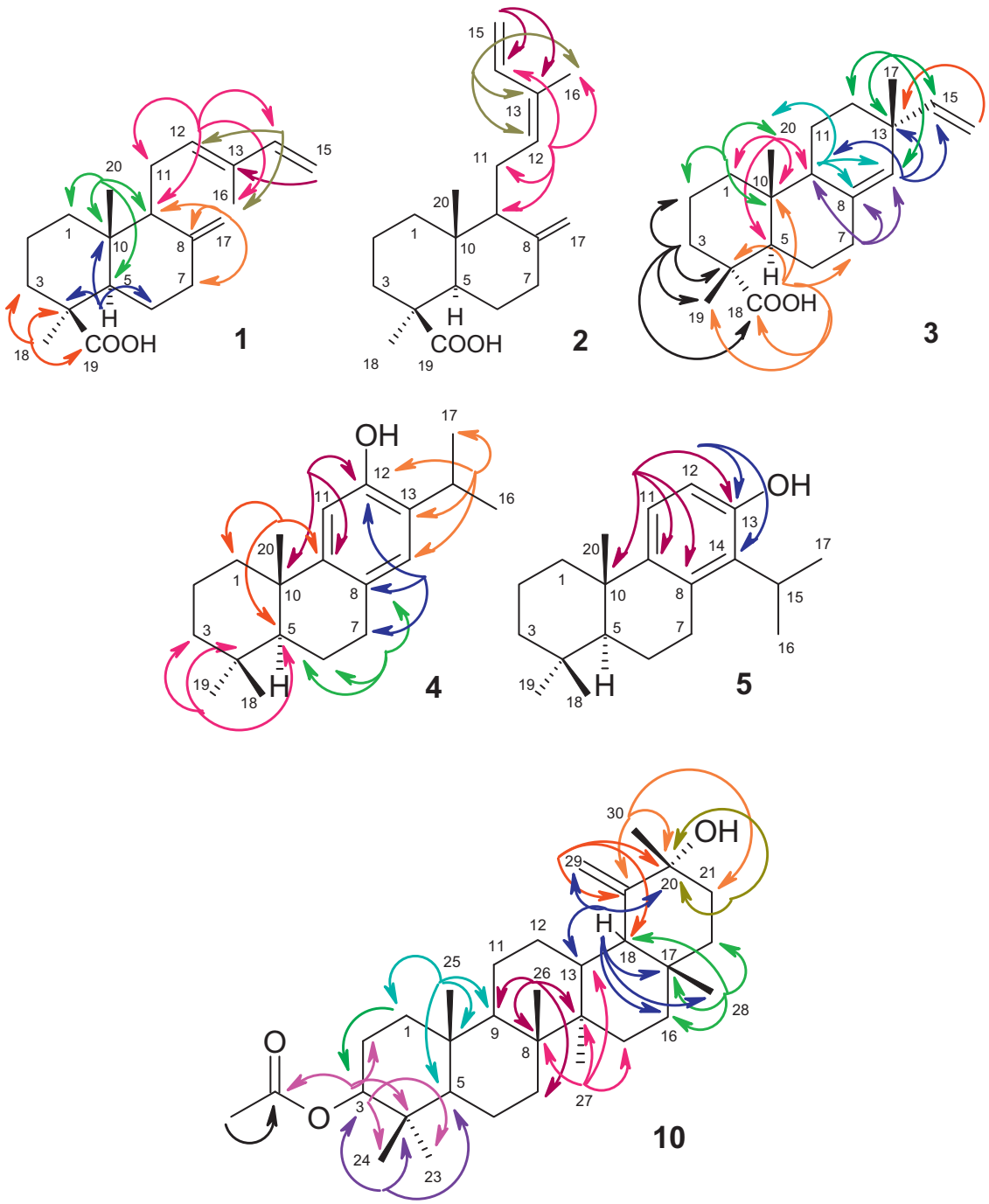

Fig. 2. Important $^{2,3}J$-CH correlations (HMBC) in (12*E*)-communic acid (**1**) and (12*Z*)-communic acid (**2**), sandaracopimaric acid (**3**), (+)-ferruginol (**4**) and (+)-totarol (**5**), 3β-acetoxy-19(29)-taraxasten-20*a*-ol (**10**) (^{1}H: 500 MHz, $CDCl_3$).

Tab. Supplement 3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.04.068.

*3.2.3. (+)-Ferruginol (**4**) and (+)-totarol (**5**)*

The APCI-MS profiling (Fig. 1) detected a selected ion trace with $[M-H]^-$ at *m/z* 284.6 in F25–F30 (injection signals No. 13–15, cf. Fig. Supplement 1). The combined fractions were cleaned by a SiO_2-short column chromatography and led to the mixture of substitutional isomers (+)-ferruginol (**4**), and (+)-totarol (**5**) (8 mg) which were not separated by HSCCC nor by silica gel.

The ratio with ~3.5:1 of the positional isomers **4** and **5** was determined by the ^{1}H NMR spectrum and integration of the aromatic proton signals. The signals of both compounds were distinguished by the coupling pattern where ferruginol showed solely singlet signals (H-11: δ 6.61, H-14: 6.83 ppm), and totarol had two doublet signals for H-11 and H-12 (δ 6.99 and 6.50 ppm). 1D/2D-NMR data revealed the $^{2,3}J$-CH long range correlation data and corroborated the mixture (+)-ferruginol and (+)-totarol (Fig. 2). Also the ^{1}H, and ^{13}C NMR (cf. Tab. Supplement 4) confirmed the two compounds and were in good accordance to reference data [38–42].

Tab. Supplement 4 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.04.068.

Ferruginol and sandaracopimaric acid from *Juniperus* spp. had shown anti-bacterial activity against *Staphylococcus aureus* and

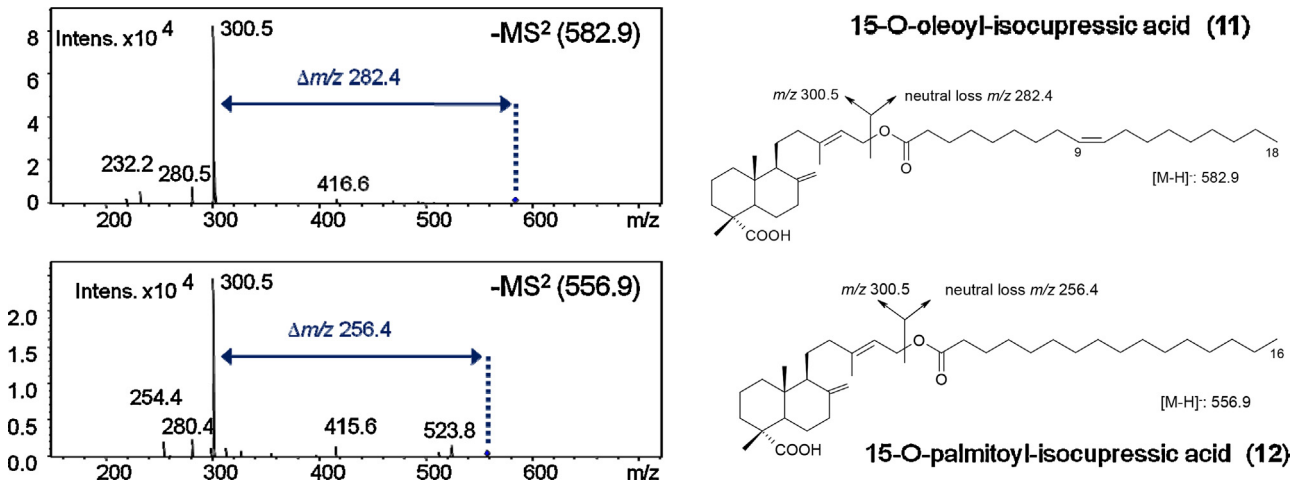

Fig. 3. APCI-MS/MS fragmentation (negative mode) of labdane-fatty acid esters: 15-O-oleoyl-isocupressic acid (**11**), and 15-O-palmitoyl-isocupressic acid (**12**).

Bacillus subtilis comparable to the standard drugs such as cephotaxime and chloramphenicol [43,44].

3.2.4. Free fatty acids

Linoleic acid (**6**) was detected by APCI-MS at *m/z* 278.7 in the HSCCC fractions F23–F25 (with APCI injection signals No.12–13, cf. Fig. 1, and Fig. Supplement 1). The elution time is quite short in comparison to oleic (**7**) and palmitic acid (**8**) on the HSCCC device due to two isolated *cis*-configured double bonds and therefore a lower van-der Waals force field to the Teflon surface of the CCC-tubing material [16].

Oleic acid (**7**) was detected in the HSCCC-fractions F38–F49 with the APCI-MS profiling experiment (Fig. 1) with $[M\text{-}H]^-$ at *m/z* 280.6 (and APCI-MS injection signals No. 19–25). The observed retention time window for **7** on HSCCC (*elution* section) was in good accordance to the elution time of identified oleic acid in a previous HSCCC-APCI-MS profiling experiment of apolar constituents in cashew nuts (*Anacardium occidentale*) [16]. Palmitic acid (**8**) (hexadecanoic acid) eluted shortly after oleic acid in F48–F57 (Fig. Supplement 1) with the selected ion trace $[M-H]^-$ at *m/z* 254.5 (and APCI-MS injection signals No. 24–29). Stearic acid (**9**) with *m/z* 282.5 eluted latest of all free fatty acids in F72–F75 (APCI-MS injection signals No. 35–37) (Fig. 1). Free fatty acids **6**–**8** had been detected before in various propolis samples by GC–MS and LC–MS. Oleic- and palmitic acid had also been identified in various *Juniperus* species [33]. This was corroborating the proposal that honeybees harvested for the propolis on *Juniperus* spp.

3.2.5. Triterpene acetate

*3.2.5.1. 3β-Acetoxy-19(29)-taraxasten-20a-ol (**10**).* The compound 3β-acetoxy-19(29)-taraxasten-20*a*-ol (**10**) (2 mg) was isolated from the HSCCC-fractions F64–F74 and was solely detected by TLC visualization as blue spots with anisaldehyde–sulfuric acid–glacial acid universal reagent [26] (Fig. Supplement 1). The detection by APCI-MS profiling (neg. mode) of the respective injected fractions (82–92 min) (Fig. Supplement 1) failed similar to the other triterpene acetates (**15**–**19**) later identified by ^{13}C NMR.

Only the direct-inlet measurement by EI-MS (70 eV) of the purified compound led to the molecular weight of $[M]^+$ at *m/z* 484. The search for a respective $[M-H]^-$ selective ion trace at *m/z* 483, and the ion $[M-H-H_2O]^-$ in the APCI-MS profiling (neg. mode) failed due to insufficient ionization under the chosen conditions or low concentration in the HSCCC fractions.

Direct-inlet EI-MS (70 eV), *m/z* (rel. int.): 484 $[M]^+$, 466, 451, 426, 406, 391, 363, 337, 295, 255, 227, 217, 203, 189, 175, 147, 135, 133, 121, 107, 95, 81.

For 1D/2D-NMR analysis, the compound was purified on a short silica-gel column (eluent: CH_2Cl_2 100%). The ^{13}C NMR detected 32 carbon resonances, combined with the results of DEPT135 and gHSQC-NMR eight quaternary, five CH-, ten CH_2-, and eight CH_3-groups were recognized. In the tritepene moiety one exo-methylene- (δ 151.6, 107.1 ppm), two carbinol- (δ 81.0, 72.7 ppm) and one acetate function (δ 171.0, 21.3 ppm) were seen.

The gHMBC enabled to elucidate the constitution of the oxygenated triterpene. Indicative long-range correlations such as observed from H-18 (δ 2.42 ppm), H_a-29 and H_b-29 (δ 4.49, 5.06 ppm), CH_3-30 (δ 1.35 ppm), CH_3-25 (δ 0.87 ppm) and H-3 (δ 4.45 ppm) confirmed the constitution (Tab. Supplement 5, Fig. 2). The absolute coherence with the published ^{13}C NMR and NOESY suggested that the absolute stereochemistry of 10 from propolis was identical to the compound isolated from *Ficus microcarpa* [45].

Tab. Supplement 5 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.04.068.

To the best of our knowledge 3β-acetoxy-19(29)-taraxasten-20*a*-ol (**10**) had been isolated only once from the ornamental plant *F. microcarpa* (Moraceae) collected in Taiwan with a series of five more oxygenated cytotoxic triterpenes [45]. In the herbarium collection of the Al-Baha region *Ficus salicifolia*, *Ficus ingens*, *Ficus carica*, *Ficus palmate*, *Ficus vasta*, *Ficus glumosa* and *Ficus sycamorus* were listed and could be a potential source for this compound in the investigated propolis from this region [46].

3.2.6. Labdane-fatty acid esters

The isocupressic acid – oleic acid ester (**11**) (15-O-oleoyl isocupressic acid) was detected in the negative APCI-MS profiling (*extrusion* mode) in the HSCCC fractions F97–F105 (Fig. 1, APCI-MS injection signals No. 48–52, and Fig. Supplement 1) with the $[M-H]^-$ signal at *m/z* 582.9. The MS/MS showed the fragment ion of *m/z* 300.5 for isocupressic acid and the neutral loss of $\Delta m/z$ 282.4 for oleic acid (Fig. 3).

Isocupressic acid – palmitic acid ester (**12**) (15-O-palmitoyl isocupressic acid) eluted with slightly longer retention time from the HSCCC and was detected in F99–F107 (APCI-MS injection signals No. 50–54 cf. Fig. 1, and Fig. Supplement 1) with $[M-H]^-$ at *m/z* 556.9. The most prominent MS/MS fragment ion was *m/z* 300.5 and $\Delta m/z$ 256.4 indicated the cleavage of palmitic acid (Fig. 3).

Previously, these two components were fully characterized by 2D-NMR in propolis from Crete [47]. There are only few reports about diterpenic structures linked to fatty acids [47]. 15-O-palmitoyl-isocupressic acid (**12**) was identified in leaves of *J. communis* subsp. *Hemispaerica* [48]. Various dehydroabietane and sandaracopimaric acid esters of hexadecanoic acid had been

characterized by Seca et al. [49] from leaves of *Juniperus brevifolia*. This is corroborating our postulate that the bees in the Al-Baha region could have visited *a Juniperus* spp., e.g. *J. procera* tree for collection of the bark resins for the propolis production. From viewpoint of botanical diversity there might be similarities between the Mediterranean area, such as Crete and the west-coast of Saudi Arabia.

As seen in Fig. 1 free oleic (**7**) and palmitic acid (**8**) had been detected in the propolis sample. So in case the bees had collected plant resins from the Cupressaceae family it might have been possible that the diterpene structures were enzymatically esterified by existing phospholipases in the bee organism. Free iso-cupressic acid was isolated from Greek propolis and this material had been shown a 90% concentration of Pinaceae pollen [42], as well as in Brazilian propolis [28]. Some constituents seem to be reoccurring in propolis independent of the origin.

The Cretean propolis delivered various antimicrobial active terpenes [47]. However, these labdane terpene acid esters **11** and **12** had shown a weak antibacterial activity.

3.2.7. Cycloartenol derivatives

Due to similar appearance on TLC the HSCCC fractions F114–F121 (Fig. Supplement 1) (*extrusion* section) were combined and directly measured by 1D-NMR, and identified cyclartenol (**13**) and 24-methylene-cycloartenol (**14**) (Fig. 4).

The ^{1}H NMR revealed the typical appearance of a phytosterol with a cyclopropane-ring system with two doublet signals with each J 4.0 Hz at δ 0.33 ppm and δ 0.56 ppm. The ^{13}C NMR spectra detected a mixture of cycloartenol (**13**) [50], and 24-methylene-cycloartenol (**14**) which is a C_{31}-phytosterol with an exo-methylene double bond. The ratio of **13**–**14** was determined of approximately 4:1 [51]. Significant chemical shift differences for metabolites **13** and **14** were observed in the chain C-21 to C-27. The ^{13}C-signals at δ 125.3 (*CH*) and 130.9 (*q*) ppm were related to the double bond C-24/C-25 in cycloartenol (**13**), and the exomethylene double bond C-24/C-31 in **14** gave signals at δ 157.0 and 106.0 ppm. All other ^{13}C-resonances of the two identified compounds were in absolute accordance to published reference data [50,51]. Structurally closely related is cycloeucalenol with only one methyl group at C-4. This compound was excluded from the mixture because of missing ^{1}H-NMR signals of the cyclopropane-ring at δ 0.15 and 0.39 ppm. The low polarity difference of the compounds was not sufficient to separate these two double bond isomers with HSCCC, nor by SiO_2-column chromatography. The separation would have required a C_{18}-preparative HPLC clean-up which was not further conducted in this study. The molecular weights for the compounds are M_r 426 for **13** and M_r 440 for **14**, respectively. Direct EI-MS analysis of this fraction pool detected only $[M]^+$ at *m/z* 426, and the much lower concentrated compound was not seen. EI-MS fragment ions at *m/z* 411, 393, and 286 corroborated the identified structure. The APCI-MS (neg. mode) profiling approach for the HSCCC fractions completely failed to detect the expected molecular ions of these compounds with $[M-H]^-$ at *m/z* 425 and *m/z* 439. Ions *m/z* 366.7 and *m/z* 393 (low signal abundance) were detected as potential in-source fragments and could not be securely correlated to the NMR identified compounds **13** and **14**. In this case the APCI-MS approach could be more effective in the positive ion mode.

Two cycloartan-type compounds, the 7-hydroxy-cycloartenol derivative with additional carboxyl-function at C-28, and the opened A-ring metabolite 3,4-seco-cycloart-12-hydroxy-4(28),24-diene-3-oic acid had been elucidated in propolis from Crete [47]. In that case the botanical origin of these compounds remained unconfirmed but it was suggested that the bees visit plants of Cupressaceae or Pinaceae families. So far cycloartenol derivatives had been barely identified in propolis. One study of Brazilian propolis described six oxygenated cycloartenol derivatives with carboxy-functional groups [52] with the origin of *Mangifera indica* (Anacardiaceae). The respective fractions for the recovery of cycloartenols F114–F121 had a total yield of approximately 47 mg (yield ~6% of injection).

3.2.7.1. Mixture of triterpene–acetates. The final HSCCC fractions (*extrusion* section) F122–F130 (APCI injection signals No. 61–63, cf. Fig. 1, Fig. Supplement 1) contained the most apolar compounds of the separation, and appeared as a single blue-lila spot on the TLC after visualization with anisaldehyde [26] (Fig. supplement 1).

The APCI-MS (neg. mode) profiling of these fractions yielded molecular ions $[M-H]^-$ at *m/z* 621.0, 448.8, and 394.8 which did not correlate with the NMR data.

The ^{1}H, and ^{13}C NMR spectra revealed a complex mixture of five similar triterpene actetates. Small changes in the constitutions and stereochemistry of the triterpenes induced characteristic ^{13}C NMR chemical shift variations for the specific regions. The constituents of the mixture were characterized by reference data and identified stereoisomers and substitutional isomers (Fig. 4). The ^{13}C NMR signals for the acetate function (δ 171.0, δ 21.4 ppm) existing in the five compound mixture for all esterified triterpene alcohols were very abundant.

β-Amyrin-3β-*O*-acetate (**15**) [50,53], and α-amyrin-3β-*O*-actetate (**16**) [53] were distinguished by different chemical shift data due to isomeric pattern of methyl-functionality in the ring E and were in good accordance to the published ^{13}C NMR reference data. In the same manner taraxasterol-3β-*O*-acetate (**17**) [50,54,55], and pseudotaraxasterol-3β-*O*-acetate (**18**) [53] were identified differing in an exomethylene and a ring enclosed double bond, respectively (Fig. 4). Lupeol-3β-O-actetate (**19**) was identified with the ^{13}C NMR signals and referenced to lupeol [56,57].

Triterpene compounds such as α-and β-amyrin, four oxygenated triterpenes (ursane- and oleanane-type with $[M]^+$: *m/z* 468), lupeol had been detected in propolis from Egypt by GC–MS, and also a cycloartenol derivative was considered [58].

The total amount of these triterpene actetates was around 100 mg (~12% of the injected ethylacetate extract) and comprised the major constituents in the propolis sample.

From alecrim-propolis (Brazil) non-esterified triterpene alcohols such as β-amyrin, α-amyrin, lupeol, cycloartenol were isolated. Additionally procrim a and procrim b, lupeol-3β-O-esters with β-hydroxy-hexadecanoic and β-hydroxy-octadecanoic acid were isolated [59]. In our study these esters were not detected in the propolis. Lupeol is a widely distributed pentacyclic triterpene, and it was isolated from the medicinal plant *Cadaba farinosa* Forssk. from Al-Baha region [60].

Interesting fact seems that until the year 2004 no triterpene compounds had been found and documented in *Juniperus* species. So this would imply that the detected triterpenic compounds most likely were collected by the honeybees from other plant sources [33].

3.2.7.2. Postulates for unknown components. The selected APCI-MS ion traces (negative ion mode) with the MS/MS fragmentation data showed for most of the components good ion abundances. Not necessarily, good ion yields had been correlated to high compound concentrations of target metabolites. Ion abundances are strongly depending on the ionization capacities of the respective compounds of interest.

Especially for the triterpene and sterol compounds the correct molecular weight data, and structural identifications were solely achieved by EI-MS measurements, such as in case of 3β-acetoxy-19(29)-taraxasten-20*a*-ol (**10**).

Various selected ion traces seen in the APCI-MS profiling (Fig. 1) of HSCCC fractionation could not be correlated with already known compounds. The quite large amount of metabolite mass

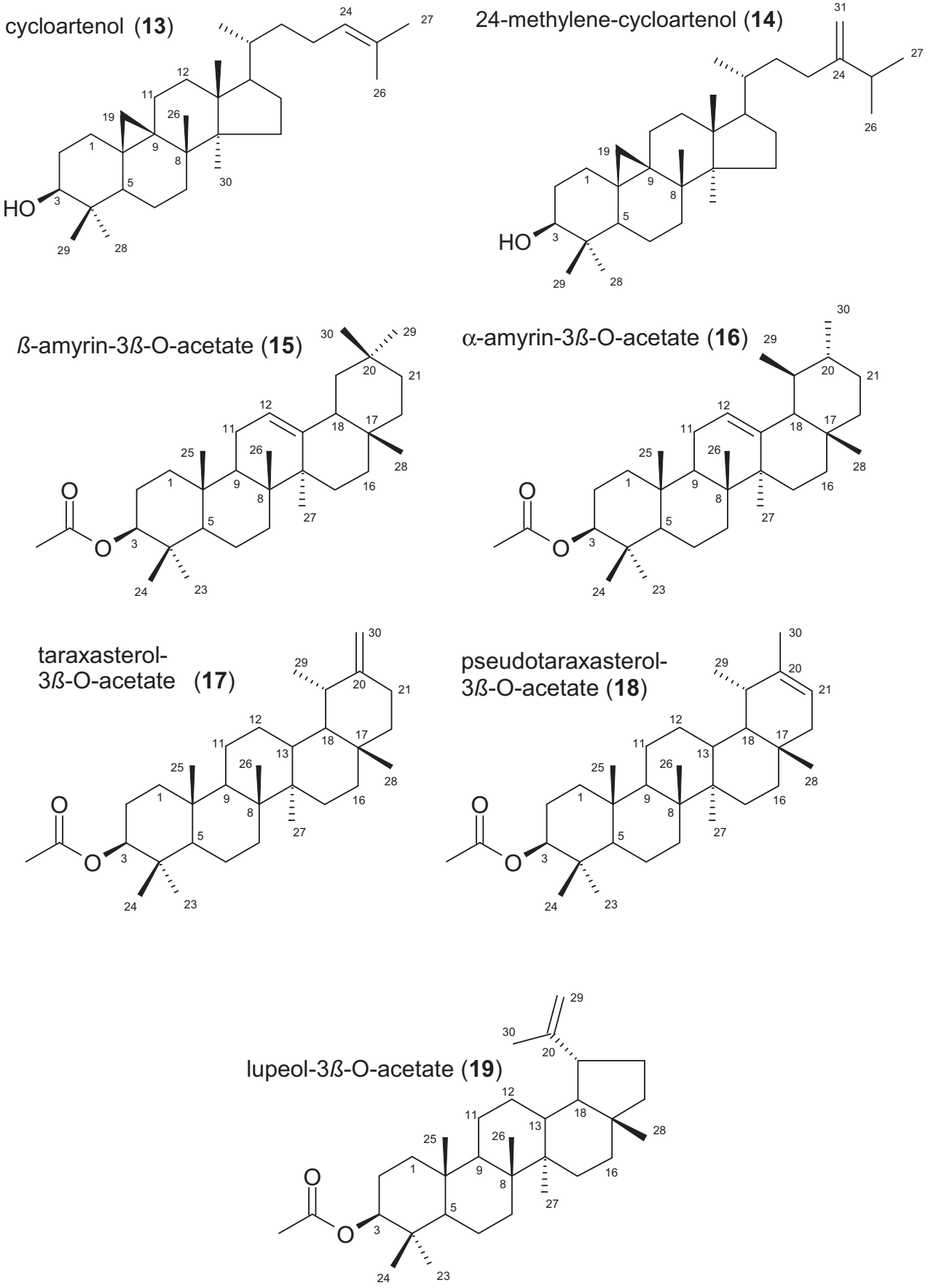

Fig. 4. Structures of cycloartenol (**13**), and 24-methylene-cycloartenol (**14**), and the mixture of triterpene acetates **15**–**19** recovered from the extruded HSCCC fractions.

spectrometry data of propolis metabolites from Europe and Brazil did not match with the unknown compounds from the Saudi Arabian propolis.

The most polar compound was detected by APCI-MS profiling in the fraction F3 (injection signal 2, cf. Fig. 1 and Fig. Supplement 1) with the $[M-H]^-$ signal at *m/z* 298.7. We suggested sugiol (7-oxo-ferruginol) as a possible metabolite as this substance was already identified for *J. procera* [33]. Unfortunately, the large compound diversity existing in fraction F3 was not displayed by the APCI-MS profiling experiment (Fig. Supplement 1).

In case of the $[M-H]^-$ signal from the profiling experiment at *m/z* 466.7 (Fig. 1) and the quite apolar character (*extrusion*) during the HSCCC-run we suggested the oxygenated triterpene acid 3,11-dioxo-oleanan-12-ene-30-oic acid which was isolated before from the Saudi Arabian plant *Maytenus undata* (Abha region) with antibacterial properties [61].

The compound with the selected ion trace $[M-H]^-$ at *m/z* 801.3 was detected in a low abundance in F21 (injection peak No. 11), and co-eluted from HSCCC with (12*E*)- and (12*Z*)-communic acid (**1**, **2**). The MS/MS data did not lead to a structural proposal.

Further unknown metabolites were detected by selected ion traces (Fig. 1) such as *m/z* 310.6, *m/z* 338.7, *m/z* 452.9, *m/z* 496.9, *m/z* 508.9, *m/z* 534.9, and *m/z* 571.

3.2.8. Performance of APCI-MS detection for compounds with different structural features

Mass spectrometry related profiling experiments on complex mixtures as seen here during HSCCC fractionation are strongly limited in performance due to the fact that every ionization method (ESI-, APCI-, EI-, or MALDI-MS) is designed for a specific polarity or molecular mass range. In certain cases the resulting ion yields are quite low, and the quantitative situation of detected metabolites in a mixture will not be correctly displayed. For the detection of the mixture (+)-totarol/(+)-ferruginol with 8 mg yield, the $[M-H]^-$ observed signal intensity at *m/z* 284.6 during the APCI-MS profiling was rather low. Otherwise the ion signal for the mixture (12*E*)- and (12*Z*)-communic acid with $[M-H]^-$ at *m/z* 300.7 was rather intense compared to a recovered sample amount of 5 mg of pure substance. Therefore MS-guided profiling experiments are highly dependent on structural features existing in the target molecules (e.g. oxygenation or nitrogen content).

Furthermore, as seen in the APCI-MS profile combined to TLC-analysis (cf. Fig. Supplement 1), many compound classes remained undetected in this MS experiment. Especially the early eluting fractions of the so-called mobile phase 'break-through' of the HSCCC chromatography (elution of most polar compounds) were invisible to APCI-MS detection. Classical TLC-analysis with the universal spray reagent of Stahl and Kaltenbach [26] detected very complex compound profiles (cf. Fig. Supplement 1). In case of the second injection signal, the APCI-MS detected solely one compound at *m/z* 298.7. But these complex fractions at the starting point of *elution* mode turned out to be the principal fractions from viewpoint of mass recovery (F1–F6: approx. 250 mg = 30% of injected extract). The complexity was high and oligomeric and polymeric plant constituents, such as waxes, proanthocyanidins, cutins might be present and invisible for APCI-detection. Ionization of low molecular weight compounds might be also significantly hampered by signal-quenching effects initiated by the presence of large matrix molecules.

We concluded that diterpene compounds in propolis had displayed a good ion response for APCI-MS detection in the negative ionization mode. In case of the phytosterols, and all triterpene actetates, the positive ionization mode is supposed to be more effective. Alternating measurements for APCI are difficult to manage due to different ionization voltages (approx. 800 V for negative APCI, and approx. 3500 V for positive APCI).

In the case of evaluation of very apolar mixtures coming from the *extrusion* part of the HSCCC run, analysis by NMR spectroscopy was definitely the most accurate methodology. Detection is not based on structural features and ionization results.

4. Conclusions

Mass spectrometry profiling and fingerprinting experiments by ESI- or APCI-MS methods are providing enormous metabolomic data sets. In case of propolis samples these approaches could document characteristic and unique changes in the chemical pattern.

In the presented metabolite study on Saudi Arabian propolis, the molecular mass profiles of recovered HSCCC fractions were monitored by *off-line* injections to an APCI-MS spectrometer. The selection of single ion traces $[M-H]^-$ combined with the results of compound visualization by thin-layer-chromatography (TLC) enabled the most accurate fractionation of potentially bioactive *target*-compounds from this propolis sample. Co-elution effects occurring for HSCCC were clearly detected by APCI-MS. The complex matrix most likely consisted of oligomers and polymers (waxes, suberins, resins) and the HSCCC chromatography step resulted in a good fractionation, and partly pure substances. The fortified HSCCC fractions with *target* compounds were further purified by silica gel column chromatography and 19 metabolites could be identified by means of EI-MS, APCI-MS and 1D/2D-NMR spectroscopy in the propolis ethylacetate extract.

Standardization and evaluation of propolis samples is frequently done on the basis of known and ESI-/APCI-MS-detectable compounds, such as flavonoids and phenolic acids. But it should be taken into account that larger amounts of non-detectable natural products might be present in the propolis, and that these compounds might significantly contribute to an observed specific bioactivity. Additional for propolis evaluation, we surely can recommend classical TLC-analysis with visualization using a universal spray-reagent.

Without the availability of molecular reference data which need to be connected to the geographical origin of propolis samples, laborious isolation procedures for the unknown compounds will be needed to recover pure material for any unambiguous identification by 1D/2D-NMR.

The chemical metabolite profile of the Saudi Arabian propolis from Al-Baha showed similarities to the results published for propolis from Crete [47], and Greece [42]. The occurrence of (+)-ferruginol (**4**), (+)-totarol (**5**), (12*E*)- and (12*Z*)-communic acid (**1**) (**2**), and sandaracopimaric acid (**3**) suggested that one of the potential plant sources visited by the honeybees in Al-Baha region could be *Juniperus* species, such as *J. procera*, *J. excelsa*, and *J. phoeniceae*.

Interesting aspect was that the known anti-bacterial (anti-tuberculosis) activities for the identified diterpene metabolites such as **1**–**5** suggested that the investigated propolis could also present potent action against bacterial growth [44].

So far it remained unknown which selection of plant families honeybees in the Al-Baha region visit for propolis generation. Depending on the identified diterpene chemical profile, the resin sources for propolis might be *J. procera* or any other plant from the Cupressaceae family [31,36,37,44]. The confirmation of these findings will require more field studies to trace the plants which are generally visited and this is also depending on the vegetation period.

Propolis is a very rare and expensive natural remedy and the chemical profile will alternate depending on the geographical area and availability of specific plants. A standardization procedure for a propolis product including its chemical bioactive metabolite profile is almost impossible.

For future studies and biological evaluation of the antimicrobial and also anti-parasite active metabolites (totarol and ferruginol

as active compounds against Leishmaniosis [62]) it might be required to provide much larger amounts of propolis. A larger scale extraction and isolation of active compounds could be also accomplished directly on plant basis. Then the chromatography could be conducted on larger lab-scale or industrial sized *countercurrent chromatography* techniques to use directly the active plant extracts for the recovery of bioactives [63,64].

In general, the presented novel method combination of HSCCC fractionation with *off-line* APCI-MS injection for *target-guided* molecular mass profiling could be easily combined with specific bio-assays and guide the fractionation to the active lead-structures. The large advantage of this *off-line* approach is the enormous reduction of required MS-routine experimental time for getting a significant metabolite overview including a fast molecular identification. Hence, it could be easily implemented for routine analysis in labs using preparative countercurrent chromatography techniques.

Acknowledgements

This work was supported by the National Plan for Sciences and Technology at King Saud University, Riyadh, Saudi Arabia (Grant No. 10-BIO1173-02). We are also grateful to Mrs. Christel Kakoschke (Helmholtz Centre for Infection Research – Braunschweig) for performing 1D/2D-NMR measurements, Mrs. Conny Mlynek and Dr. Uli Papke (Organic Chemistry Department – TU Braunschweig) for EI-MS measurements. We also are thankful to Mrs. Carola Balcke (TU-BS, Institute of Food Chemistry) for careful proofreading.

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