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Chiral and Nonchiral GC×GC/TOFMS Analysis of Natural Compounds: The Case of Possible Aggregation Pheromones of Chinese Bark Beetles *Ips shangrila* and *Ips nitidus*

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1. Introduction

Semiochemicals (compounds used for chemical communication within and between species) are usually produced in tiny amounts that vary from picograms to nanograms. As a consequence, pheromones are rarely isolated in quantities sufficient to carry out the full range of spectroscopic tests (^{1}H and ^{13}C NMR, infrared, and mass spectrometry) routinely used in structural organic analysis. Pheromone identifications predominantly rely on gas chromatography coupled with mass spectrometry (GC-MS) and electroantennographic detection (GC-EAD) (Struble & Arn, 1984). The GC-EAD analysis, where insect antenna is used as specific biological detector, helps to focus the identification effort only on a particular part of the chromatogram. Quite often, however, the GC-EAD indicates an activity in chromatogram areas where FID or MS detects no compound (Svatoš et al., 1999, 2006; Kalinová et al., 2006) or the active compound is overlapped by higher amount of non-active one(s).

Enantiomeric composition of a pheromone is instrumental with respect to the behaviour mediating capacity of the signal. This especially stands for bark beetles (Coleoptera: Curculionidae, Scolytinae) where even different populations of the same species employ pheromone of different enantiomeric composition (Seybold, 1993; Miller et al., 1996). Enantioselective production of, and response to pheromones has been demonstrated in many species of Scolytinae subfamily (Birch, 1984; Borden, 1985; Byers, 1989). Electrophysiological studies have revealed that species such as *Ips pini* Say, *I. typographus* (L.), *I. paraconfusus* Lanier, *Scolytus multistriatus* (Marsham), *S. scolytus* (F.) and *Trypodendron lineatum* (Olivier) and many others have olfactory receptor cells specific to optical isomers of aggregation pheromones (Mustaparta et al., 1980, 1984; Wadhams et al., 1982; Tømmeras et al., 1984).

Conventional chiral analysis provides data of a high complexity with high extent of peak overlap even for moderately complex mixtures. This complexity makes the chiral analysis of

low quantities of insect pheromones quite difficult. The comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC-TOFMS) is the most advanced analytical technique of high sensitivity and selectivity. It is also a technique which provides a substantial enhancement of peak capacity and signal intensity over conventional GC analysis. Signal capacity and intensity enhancements are achieved by using a modulator which is capable to trap and concentrate effluent portions from the first column and deliver these "samples" into the second column (Lee et al., 2001). Several books and reviews focusing on the two dimensional gas chromatography with FID or MS detection have been published to familiarize the reader with the method's fundamentals (Adahchour et al., 2008; Dallüge et al., 2003; Mondello et al., 2008; Panić & Górecki, 2006; Ramos, 2009). The most advanced of these methods, comprehensive GC×GC-TOFMS, was successfully used for separation of many groups of natural compounds or xenobiotics (Dallüge et al., 2002; Gogus et al., 2006, Kalinová et al., 2006, 2009; Matamoros et al., 2010; Rocha et al., 2007 and many others). The using of chiral column in one of two dimensions of the comprehensive two-dimensional gas chromatography thus adds a new "dimension" into the separation process and offers another, more intimate view into 2D/3D-visualizations of chromatograms (Shellie et al., 2001).

China has large mountain regions of conifer habitat with unexpectedly rich and still largely unknown Scolytid fauna (Critchfield & Little, 1966; Cognato & Sperling, 2000; Cognato & Vogler, 2001; Cognato & Sun, 2007). In north-eastern margin of the Tibetan Plateau (NW China), where remnants of the former mountain forest occur, bark beetles cause severe damages. The main target of bark beetle infestation is the thick leaf spruce, *Picea crassifolia* Komarov (Pinaceae). The major *Ips* species in these areas are *Ips nitidus* and newly discovered *Ips shangrila* (Cognato & Sun, 2007), monophyletic sister species of *Ips typographus* (L.) and *Ips amitinus* (Eichhoff), respectively (Cognato & Sun, 2007). Both bark beetle species are serious pests of *Picea crassifolia* in NW China mountain areas.

In the present chapter we want to demonstrate in the form of chemo-ecological/analytical study the applicability of the GC×GC-TOFMS for achiral and chiral insect semiochemicals analysis together with another key instrumental technique in this research - the gas chromatography with electroantennographic detection (GC-EAD). The insects we described in the study were the two above mentioned Chinese bark beetle species - *Ips nitidus* and *Ips shangrila*.

2. Brief introduction into used instrumental techniques

2.1 TOFMS

Time-of-flight mass spectrometry (TOFMS) is probably the simplest method of mass spectrometric measurement by the physical principle. The key features of TOFMS are extreme sensitivity (all ions are detected), practically unlimited mass range and as well as high-speed analysis (recent TOFMS instruments are able to measure hundreds full spectra per second). This all makes TOFMS one of the most desirable methods of mass analysis (Schlag, 1994; Guilhaus, 1995). The general scheme of TOFMS is shown in Scheme 1.

The ions are introduced either directly from the ion source of the instrument as a very short pulse. This results in all the ions receiving the same initial kinetic energy. As they then pass
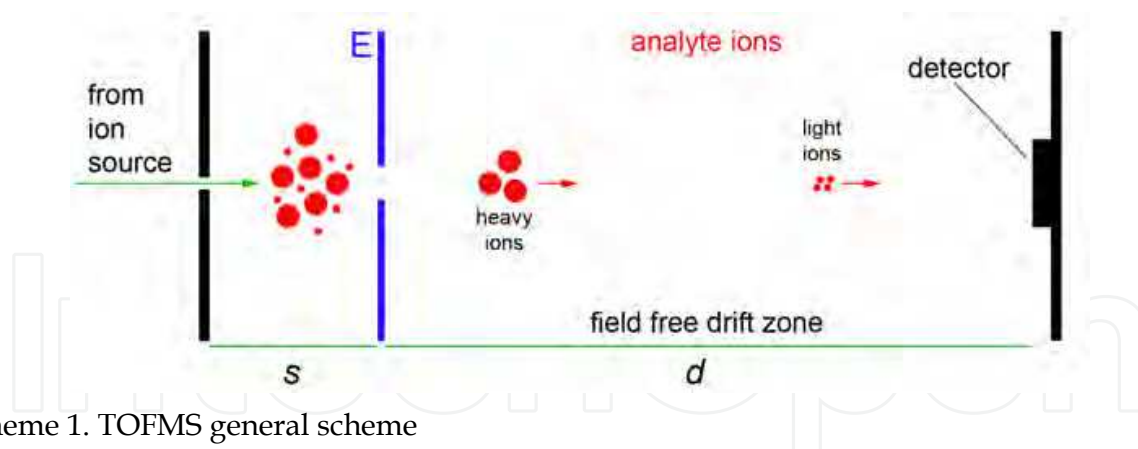
Scheme 1. TOFMS general scheme

along the field free drift zone, they are separated by their masses, lighter ions travel faster, reaching the detector earlier. This enables the instrument to record all ions as they arrive at the detector and so accounts for the techniques high sensitivity. The equation describing the time-of-flight separation is:

$$\frac{m}{z} = 2eEs\left(\frac{t}{d}\right)^2$$

Where m/z is mass-to-charge ratio of the separated ion, E is the extraction pulse potential, s is the length of the flight tube over which E is applied, d is the length of the field free drift zone and finally, t is the recorded time-of-flight of the ion.

2.2 GC×GC-TOFMS

The used comprehensive GC×GC-TOFMS is a recently developed analytical technique which offers a solution to the co-elution problem and provides high sensitivity and selectivity (Dallüge et al., 2003; Dimandja, 2003; Mondello et al., 2008). In principle, the instrumental system consists of two GC ovens equipped with columns of different manner of separation (by a boiling point, polarity, chirality…) connected by an interface equipped with a non-moving quad-jet cryomodulator. The modulator's cryogenic trap (using two cold jets) repeatedly condenses compounds eluting from the primary column and releases them (using two hot jets) periodically as short pulses to the secondary column. Parameters like duration and frequency of both condensation and injection pulses are variable and allow precise tuning of the instrument according to the requirements of the analysis. Since the GC×GC system produces very narrow peaks (mostly narrower than 50 ms, depending on the frequency of modulation) a TOFMS detector with a high acquisition rate (up to 500 full spectra per second) is required. The pulsed nature of the TOFMS source of ionisation further enhances the system accuracy by avoiding spectral skewing common in a continuous ionisation mode. GC×GC with TOFMS detection thus operates with a high precision independent of concentration range.

Another important advantage of the comprehensive GC×GC, especially significant while analyzing the complex mixtures of natural origin with trace concentrations of target analytes, is the second dimension separation of the background from the compounds of our interest. The background components (solvent traces, cyclosiloxanes of septum and column bleed, etc.)

have significantly different retention and elute from the 2nd dimension column much earlier. The high "wall" of column bleed is usually perfectly separated in the second dimension allowing MS analysis without any interference (Kalinová et al., 2006; Rocha et al., 2007).

The standard GC×GC-TOFMS experiment involves MS analysis of hundreds "cut-outs" of the primary column chromatogram. Any analysis thus can be seen as a series of 2D-chromatograms eluting one after another. Depending on modulation parameters and peak widths, each compound eluting from the primary column shows up as several consecutive narrow peaks. These complex and voluminous data are further converted into a contour plot with the primary column retention plotted along the X-axis, and the secondary column retention plotted along the Y-axis (2D-visualization of GC×GC-TOFMS analysis), and eventually detector response on the Z-axis (3D-visualization of GC×GC-TOFMS analysis). On such chromatograms, compounds characterised by the same mass spectrum appear as one spot. As the quantity of compounds can be colour coded, the colour of individual spots continuously changes from peak base to its top. Such coloured contour plots make searching for possible active compounds much easier, especially when a display based on characteristic ion current is selected instead of a total ion current (TIC). The pattern analysis of 2D-chromatogram allows fast and effective comparison of many sets of obtained analytical data as well as a quick search of any differences. Thanks to set of two retention parameters, GC×GC-MS also makes identification more reliable in comparison with a standard one dimensional GC. The technique provides identification capabilities similar to performing two separate 1DGC separations on different columns, as required by some analytical methods. The identification by two dimensional retention times and mass spectral match between an analyte and a spectrum, offered either by a library or obtained by an injection of available standard, is very robust and the possibility of misidentification is low.

2.3 GC-EAD

GC-EAD is gas chromatography method in which a unique biological detector, based on living insect antenna, is used as one of two detection systems. GC-EAD is an analytical method which offers very fast and reliable identification of compounds in complex natural mixtures that stimulate the olfactory sensilla of insect antennae (Struble & Arn, 1984). In other words, the GC-EAD helps to discover what specific chemicals in tested mixture an insect can smell, respective, which compound elicits the electric antennal response.

It has been known for more than 50 years that the electric potential difference (voltage) measured by two tiny microelectrodes between the tip and the base of an insect's antenna changes measurably when the antenna is exposed to compounds of biological significance for the insect. This voltage represents the sum of potentials of all responding olfactory neurons around the recording electrode, and the voltage's amplitude approximately corresponds to an insect's sensitivity to a particular compound. The voltage produced by an antenna can be easily amplified, measured and recorded together with corresponding data delivered by a classical FID detector. The GC-EAD system is in principle very simple apparatus based on a standard chromatograph with FID detector. The only important change is a post-column splitter followed by doubled detection system. After the separation is the column effluent split in two parts, with the first half transported to a FID detector and the other one transmitted to the EAD detector. The effluent is cooled by mixing with purified air and blown over a live insect antenna attached to a high impendence amplifier.

The amplified electrical signals of both detectors are subsequently digitized, processed and recorded simultaneously against the time (Scheme 2).
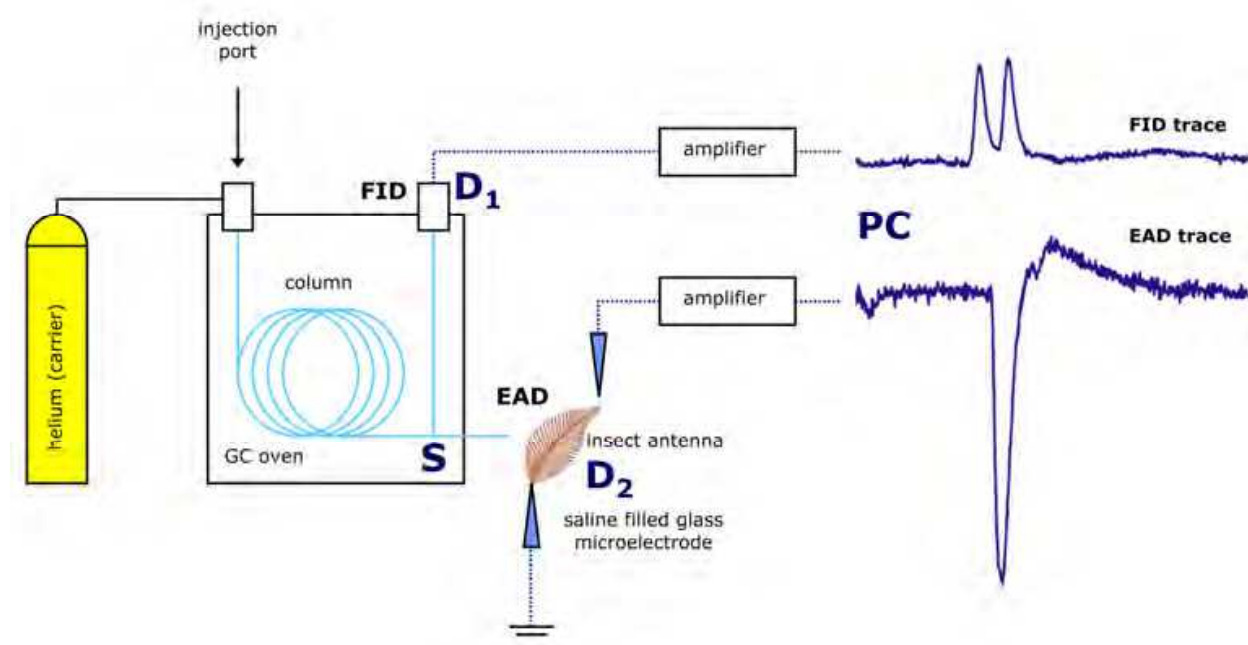
(S) Splitter - column effluent is split in two halves; (D_1) FID produces a signal proportional to the amount of volatile in the column effluent; (D_2) EAD detection – with air mixed column effluent is blown over an insect's antenna; (PC) combined FID/EAD chromatogram.

Scheme 2. A standard GC-EAD system

3. Experimental part

3.1 Insects

3.1.1 Determination of target bark beetle species

Bark beetles colonizing *Picea crassifolia* were collected randomly on several locations within the territory of two villages of Maixiu and Sibosha in Tongren and Beishan counties of Qinghai province (NW China). The specimens were collected in May 2007 and May 2008 directly from freshly infested spruce logs. The morphological characters of individual beetles were inspected and determined based on available taxonomic bark beetles literature and when possible also by comparison with the holotypes of particular species located in different natural history museum's collections (Naturhistorisches Museum Wien - Austria, National Museum Prague - Czech Republic, etc.). Parental and larval galleries of each respective species were investigated on both freshly infested and dead trees and their characteristics were associated with the identified species.

3.1.2 Sex determination of target bark beetle species

For physiological experiments sex determination of living specimens was necessary since males and females often produce different sets of semiochemicals. In general, sex determination of living individuals of some bark beetles species is morphologically quite

straightforward (*Polygraphus*, *Pityogenes* etc.). In other species however, it can be rather difficult or impossible without the destructive method of dissection of male aedeagus.

In *Ips nitidus*, sex of beetles was determined under stereomicroscope by means of sex specific differences of hair density on head and pronotum as described for *Ips typographus* by Schlyter & Cederholm (1981). *Ips nitidus* females had more densely haired frontal part of pronotum and the frons from forehead view then males. In *I. nitidus*, other morphological sexual differences exist in the shape and size of the frontal and lateral tubercles on lateral margins of the elytral declivity, but these characters are more difficult for practical use.

In *Ips shangrila*, sex can be determined only under stereomicroscope based on sex-specific differences in the size of the frontal tubercle from lateral view. In males, this tubercle is remarkably large and its base is wider. Cognato & Sun (2007) reported other sex-specific differences, but the morphological features described by these authors were quite difficult to follow.

After sex determination, beetles were removed from infested treed during initial infestation phase, when males construct nuptial gallery and emit aggregation pheromone that serves as sex pheromone for females and synchronizes mass attack of bark beetles aiming to kill the tree. The beetles were then dissected and gut extracted the same day.

3.2 Guts and frass extraction

Guts of males and females of both species were dissected under stereomicroscopic control. Excised guts were placed in hexane (10 µL of hexane per one gut) and left in ambient temperature 24 hrs. Then, the extracts were filtered, transferred into glass capillaries, flame sealed and stored in a freezer until analysis. Except guts, we extracted also frass (a mixture of beetle-produced sawdust and excreta that accumulated at the entrance hole of the beetle gallery), collected from freshly colonized logs of *Picea crassifolia* using glass exhaustor. Obtained material was extracted by trace analysis grade hexane under ambient temperature for 1 hr. Extracts were filtered, transferred into glass break-seal ampoule and sealed until analyses.

3.3 SPME

SPME analyses of infested bark strips (males and females kept separately) were performed using CAR-PDMS fibre (Supelco, Sigma-Aldrich Group, St. Louis, MO, USA). For SPME experiments, sexed beetles were allowed to bore into 15×20 cm fresh strips of *Picea crassifolia* bark (separated sexes), placed in 20×25 cm containers. SPME fibres were exposed 60 minutes and then immediately analysed using for GC-EAD and/or GC×GC-TOFMS analysis, respectively.

3.4 GC-EAD

In GC-EAD experiments, isolated head-antennal preparations of bark beetles were used. Glass Ag/AgCl microelectrodes filled with insect saline solution were used (Roelofs, 1984). The reference electrode was inserted into the head, the recording one was brought into contact with antennal sensory epithelium on antennal club. Analysed samples (5-10 beetle equivalents) were injected splitless into a 5890A Hewlet-Packard gas chromatograph

equipped with an Agilent J&W DB-5 column (Agilent, Santa Clara, CA, USA; 30 m × 250 μm i.d. × 0.25 μm film) column. The column was splitted at the end by Graphpack 3D/2 four-arm splitter allowing divide the eluate to FID and EAD detectors. The GC was operated at an initial temperature 50 °C for 2 min then ramped at a rate 10 °C/min to 270 °C (with 10 min hold). The temperature of GC inlet and detector was set to 200 °C and 260 °C, respectively. A series of C_8-C_{22} alkane standards was co-injected with analyzed samples to allow calculate Kovats' indices (I_K) of EAD active peaks and to check the correspondence of retention behaviour and antennal activity of authentic compounds with synthetic standards.

3.5 Achiral GC×GC-TOFMS analysis

The analyses were performed using a LECO Pegasus 4D Instrument (LECO Corp., St. Joseph, MI, USA). A slightly polar Agilent J&W DB-5 column (Agilent, Santa Clara, CA, USA; 30 m × 250 μm i.d. × 0.25 μm film) was used for GC in the first dimension. It was the same type of column used for GC-EAD. The second dimension analysis was performed on a polar BPX-50 column (SGE Inc., Austin, TX, USA; 2 m × 100 μm i.d. × 0.1 μm film). Helium was used as a carrier gas at a constant flow of 1 mL/min. The temperature program for the primary GC oven was as follows: 50 °C for 2 min, then 50–280 °C at 7 °C/min, and finally 10 min hold at 280 °C. The program in the secondary oven was 7 °C higher than in the primary one and was operated in an iso-ramping mode. The modulation period, the hot-pulse duration and the cool time between stages were set at 4.0, 0.8 and 1.2 s, respectively. The transfer line to TOFMS detector source was operated at 260 °C. The source temperature was 200 °C with a filament bias voltage of −70 eV. The data acquisition rate was 100 Hz (scans/s) for the mass range of 29–400 amu. The detector voltage was 1450 V. One microlitre samples were manually injected in the splitless mode. The inlet temperature was 220 °C. The purge time was 30 s at a flow of 80 mL/min. Data were processed and consecutively visualised on the 2D and 3D chromatograms (as TIC intensity) using the advanced LECO ChromaTOF™ software. As in GC-EAD experiments, C_8-C_{22} alkane standards were analyzed under the same parameters to allow the comparison of Kovats' indices (I_K) with GC-EAD experiments. Two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST)V. 2.0- Mainlib and Replib) and our own-made database of terpenoids comprising 100 compounds were used. Mass spectral match factor, similarity >850, was used to decide whether a peak was correctly identified or not.

3.6 Chiral GC×GC-TOFMS analysis

The absolute configuration of antennaly active compounds was assigned using LECO Pegasus 4D Instrument (LECO Corp., St. Joseph, MI, USA). The chiral analysis was performed on a β-cyclodextrin HP-Chiral β column (Agilent, Santa Clara, CA, USA; 30 m × 250 μm × 0.25 μm film). The chiral column was used as the first dimension column. The second dimension column was used the same as described in non-chiral analysis section above. Helium was used as a carrier gas at a constant flow of 1 mL/min. The temperature program for the primary GC oven was as follows: 50 °C for 2 min, then 50–180 °C at 2 °C/min, then 180–210 °C at 15 °C/min and finally 35 min hold at 210 °C. The program in the secondary oven was 7 °C higher than in the primary one and was operated in an iso-ramping mode. The cryomodulator settings and all other GC×GC-TOFMS parameters were the same as described in non-chiral analysis section above.

3.7 Chemicals

Synthetic enantiomerically pure terpene standards (Contech Inc., Delta, British Columbia) were dissolved in hexane and analyzed or co-injected with extracts under the same conditions as authentic samples.

4. Results

4.1 GC-EAD and achiral GC×GC-TOFMS analysis

4.1.1 *Ips nitidus*

The SPME GC-EAD analyses of volatiles emanating from male infested bark consistently showed two areas of EAD activities (EAD1 and EAD2) characterized by $I_{K(EAD1)}$ = 1099 and $I_{K(EAD2)}$ = 1150. These two active EAD areas were observed also in GC-EAD analyses of male and female guts and frass extracts. Except EAD1 and EAD2, GC-EAD analysis of guts extracts showed the 3rd area of EAD activity, EAD3, characterized by $I_{K(EAD3)}$ = 1175. Finally, GC-EAD analyses of male frass extract provided another two EAD peaks EAD4 and EAD5 with $I_{K(EAD4)}$ and $I_{K(EAD5)}$ calculated as 948.9 and 991.6, respectively. A typical section of the GC-EAD recording in area of EAD1-EAD3 is demonstrated in Figure 1.
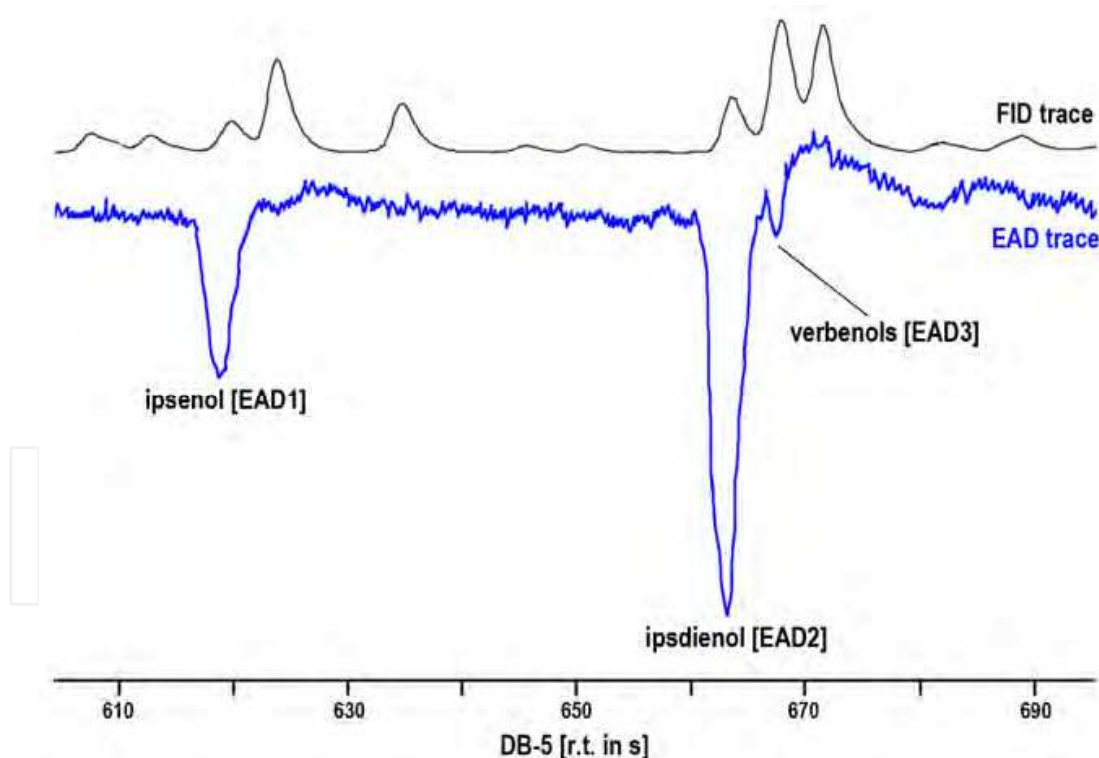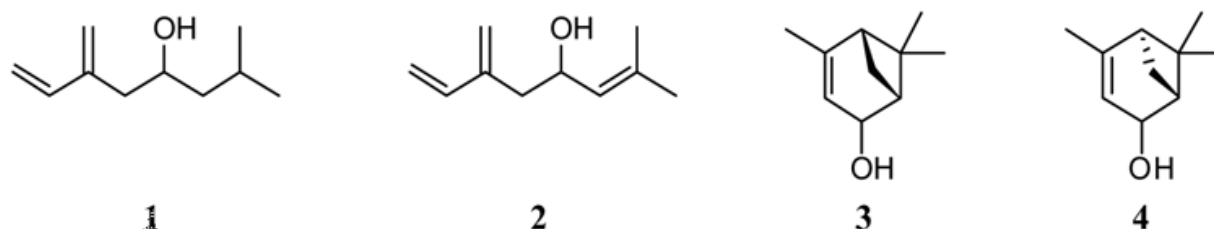

Fig. 1.

Reconstructed GC-EAD recording from isolated antennae of *Ips nitidus* in response to male guts extract. *I. nitidus* antennae responded to 3 compounds from the extract (EAD1-EAD3) corresponding to ipsenol (**1**), ipsdienol (**2**) and *cis/trans*-verbenol (**3**, **4**), respectively.

Compound EAD1 subjected to GC×GC-TOFMS analysis gave the following diagnostic ion peaks; m/e 41, 53, 79, 85, 93, 121, 136, 154 (M+), 68 (base ion peak). The I_K was calculated 1111. This value, with counting the longer retention cause by the presence of the secondary column, matched well with $I_{K(EAD1)}$ = 1108 confirming the same identity in both GC-EAD and GC×GC-TOFMS analysis. Spectrum, retention and GC-EAD behaviour of compound EAD1 was identical to that obtained for synthetic ipsenol (2-methyl-6-methylene-7-octen-4-ol, **1**).

Compound EAD2 subjected to GC×GC-TOFMS analysis gave the following diagnostic ion peaks; m/e 41, 57, 67, 91, 109, 119, 134, 152 (M+), 85 (base ion peak). The I_K was calculated 1151. This value matched well with $I_{K(EAD2)}$ = 1140 confirming the same identity in both GC-EAD and GC×GC-TOFMS analysis. Spectrum, retention parameters and GC-EAD behaviour of compound EAD1 was identical to that obtained for synthetic ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol, **2**).

Compound EAD3 subjected to GC×GC-TOFMS analysis showed after spectra deconvolution process presence two close eluting geometrical isomers of verbenol (*cis* and *trans*), which were not separated well during the GC-EAD experiments. Were obtained the following diagnostic ion peaks; m/e 41, 55, 59, 67, 79, 91, 109, 119, 134, 137, 152 (M+), 94 (base ion peak; for *cis*-isomer) and m/e 41, 55, 67, 81, 91, 109, 119, 137, 152 (M+), 109 (base ion peak; for *trans*-isomer). The I_K were calculated 1158 for *cis*-isomer, and 1164 for *trans*-isomer, respectively. These value matched well with $I_{K(EAD3)}$ = 1155 (unseparated *cis/trans*-isomers) confirming the same identity in both GC-EAD and GC×GC-TOFMS analysis. Spectra and retention behaviour of compounds hidden in peak EAD3 were identical to that obtained for synthetic *cis*-, respectively *trans*-verbenol (*cis*-4,6,6-trimethyl- bicyclo[3.1.1]hept-3-en-2-ol, **3** / *trans*-4,6,6-trimethylbicyclo[3.1.1]- hept-3-en-2-ol, **4**)

Compounds EAD4 and EAD5 subjected to GC×GC-TOFMS analysis gave the following diagnostic ion peaks; m/e 41, 53, 67, 77, 79, 105, 121, 136 (M+), 93 (base ion peak; EAD4), and m/e 41, 53, 69, 77, 91, 107, 121, 136 (M+), 93 (base ion peak; EAD5), respectively. The I_K were calculated 947 and 989, respectively This value matched well with $I_{K(EAD4)}$ = 949 and $I_{K(EAD5)}$ = 992 confirming the same identities in both GC-EAD and GC×GC-TOFMS analysis. Spectrum, retention and GC-EAD behaviour of compound EAD4 and EAD5 were identical to that obtained for standards of α- and β-pinene.

4.1.2 *Ips shangrila*

The SPME GC-EAD analyses of male and female infested bark and male and female guts extracts consistently showed one area of EAD activities with retention time corresponding to EAD2 from analysis of *I. nitidus* extracts. $K_{I(EAD2)}$ in SPME GC-EAD experiments using *I.*

shangrila antennae was calculated as 1155 and subsequent GC×GC-TOFMS analyses gave identical results as in *I. nitidus* case – the active compound is ipsdienol (**2**). Activity in area of EAD1, also caused by identical compound as in *I. nitidus* – ipsenol (**1**), was observed only in few analyses.

The GC-EAD analyses of male frass extract showed in addition to EAD1/EAD2 one more EAD active area (EAD3) eluting later and characterized by $I_{K(EAD3)}$ = 1178. Compound EAD3 subjected to GC×GC-TOFMS analysis gave the same results as in the case of *I. nitidus* – under one GC-EAD peak were hidden both geometrical isomers of verbenol (**3**, **4**). Spectra and retention behaviour of compounds hidden in peak EAD3 were identical to that obtained for synthetic *cis-* (**3**), respectively *trans*-verbenol (**4**).

4.1.3 Frass extracts GC×GC-TOFMS analysis in both *Ips* species

GC×GC-TOFMS showed that frass extracts in both *Ips* species are more complex compared to guts ones, especially in the area of monoterpene hydrocarbons with $I_K \sim 900\text{-}1100$, where analyses in both species gave practically identical results (Table 1). This finding is not surprising. While guts extracts contain putative pheromone together with the food, frass represents sawdust produced while beetles excavate their galleries (containing mainly host volatiles released from damaged bark tissue) plus beetles excreta enriched with pheromone. A typical section of GC×GC-TOFMS chromatogram in area $I_K \sim 900\text{-}1100$ is showed in Figure 2.

Name of Compound	Peak No.	Ret. Time [s] 1st/2nd dimension	$I_{K\text{-EXP}}$	$I_{K\text{-LIT}}$ *	Identification **
Tricyclene	**5**	684 / 1.970	935	926	L, A, O
α-Pinene	**6**	700 / 2.190	647	939	S, L, A
Camphene	**7**	724 / 2.070	963	954	S, L, A
Sabinene	**8**	749 / 2.060	981	975	L, A, O
β-Pinene	**9**	763 / 2.070	989	979	S, L, A
Δ3-Carene	**10**	800 / 2.050	1018	1011	S, L, A
α-Terpinene	**11**	808 / 2.060	1024	1029	L, A, O
p-Cymene	**12**	820 / 2.170	1033	1024	S, L, A
Limonene	**13**	828 / 2.130	1039	1029	S, L, A
γ-Terpinene	**14**	864 / 2.130	1066	1059	L, A, O
Sabinene hydrate	**15**	884 / 2.230	1080	1070	L, A
Terpinolene	**16**	904 / 2.190	1095	1088	L, A, O
Nonanal	**17**	920 / 2.190	1108	1100	S, L, A
Ipsenol	**1**	924 / 2.170	1111	1100	S, L, A
Pinene oxide	**18**	928 / 2.330	1114	1099	L, A
Thujol	**19**	940 / 2.360	1123	1109	L, A

* Adams (2007);

** L – ChromaTOF™ software with NIST / Wiley MS spectra libraries installed on LECO Pegasus 4D data station, S – standard, A - Adams (2007), O – own MS spectra library measured on LECO Pegasus 4D

Table 1. Identified compounds ($I_K \sim 900\text{-}1100$) typical for frass of *I. nitidus* and *I. shangrila*.
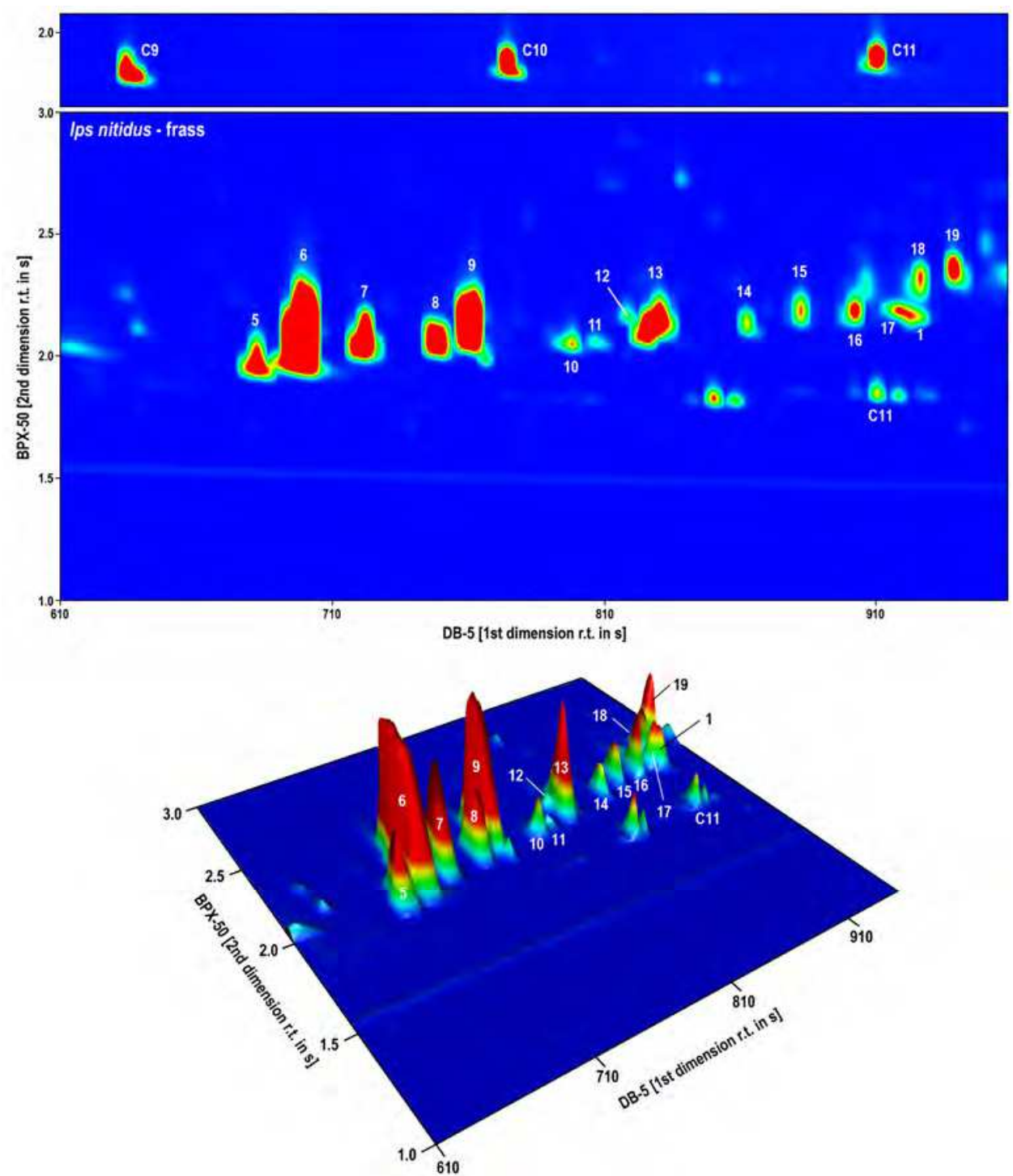
Fig. 2. Section of GC×GC-TOFMS TIC-chromatogram of *Ips nitidus* frass extract I_K range ~ 900-1100 visualized as contour plot (with *n*-alkane standards in the upper part) and as 3D-view, respectively. Each spot in the upper 2D-chromatographic plane represents a single compound, the intensity of which is colour coded. White, yellow and red represent low, middle and high intensity, respectively. On the plane, the individual compounds are distributed based on their volatilities (X-axis) and polarities (Y-axis). The more polar and less volatile compounds elute at later retention times. The numbers indicate the identity of the compounds as listed in Table 1.

4.1.4 Species and sex specific differences

GC×GC-TOFMS analyses show that ipsenol (**1**), ipsdienol (**2**) and verbenols (**3**, **4**) were present in both *I. nitidus* and *I. shangrila*. GC-EAD tests aimed to test the cross reactivity of *I. nitidus* extracts on *I. shangrila* antennae (example in Fig. 3) and vice versa supported the assumption.
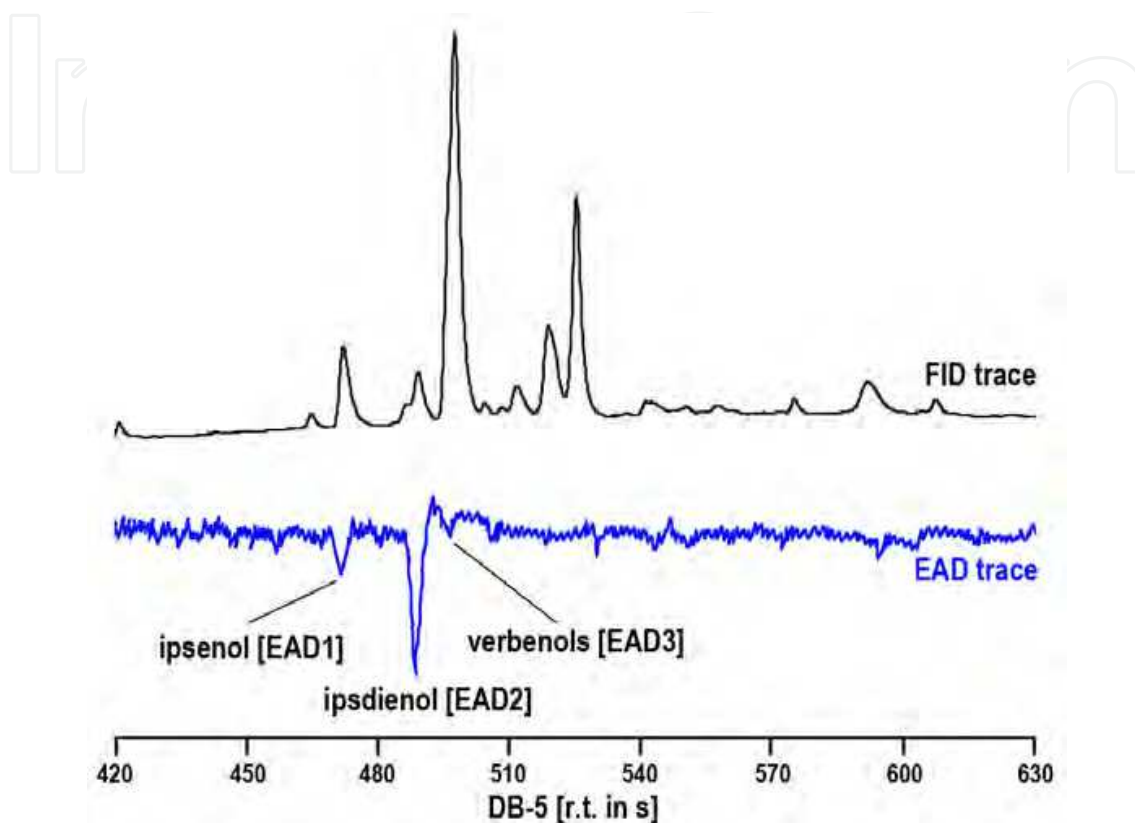

Fig. 3. Reconstructed GC-EAD recording from isolated antennae of *Ips shangrila* in response to *Ips nitidus* male guts extract. *I. shangrila* antennae responded to 3 compounds from the extract (EAD1-EAD3) corresponding to ipsenol (**1**), ipsdienol (**2**) and *cis/trans*-verbenol (**3**, **4**), respectively.

Thus, both GC×GC-TOFMS and GC-EAD analysis show that pheromones of the two species share common compounds. GC×GC-TOFMS and GC-EAD analysis also showed that identified EAD active compounds were present in guts extracts of both sexes. Specifically, ipsenol, ipsenol and *cis/trans*-verbenol were found both in *I. nitidus* male and female guts extracts. Available GC×GC-TOFMS analyses for *I. nitidus* guts extracts (N = 5) showed that male extracts contained more ipsenol and ipsdienol then female ones. On the other hand in *I. shangrila* guts extracts, ipsenol was equally present in traces in both sexes, while the content of ipsdienol was higher in males than in females. Due to limited number of analysis available, more data is needed to confirm the observed trends in pheromone sex-specific differences.

Following two figures (Fig. 4 and Fig. 5) are depicting complete achiral GC×GC-TOFMS separations in the area of EAD activity of both studied *Ips* species displayed as total ion chromatograms (TIC). The series of *n*-alkane standards (A), male frass (B), male guts extract

(C) and female guts extract (D) were analysed. The figures show differences in composition of frass and guts extracts of both sexes. EAD active compounds ipsenol (**1**), ipsdienol (**2**) and *cis/trans*-verbenols (**3**, **4**) are present in all analyzed samples of both *Ips* species.
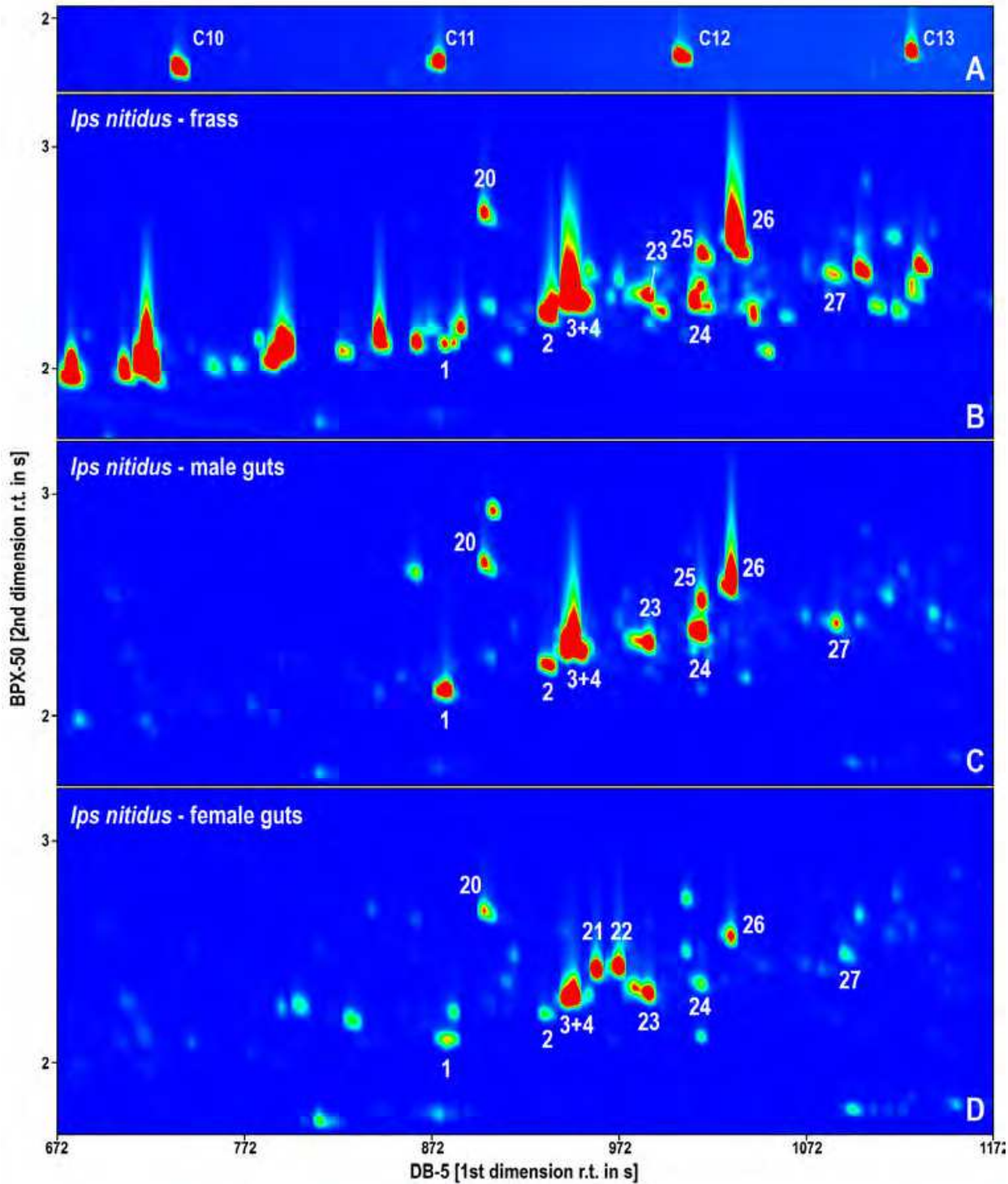

Fig. 4. Section of GC×GC-TOFMS TIC-chromatograms of *n*-alkane standards (A), *I. nitidus* male frass (B), *I. nitidus* male guts extract (C) and *I. nitidus* female guts extract (D) in I_K range ~ 1000-1300.
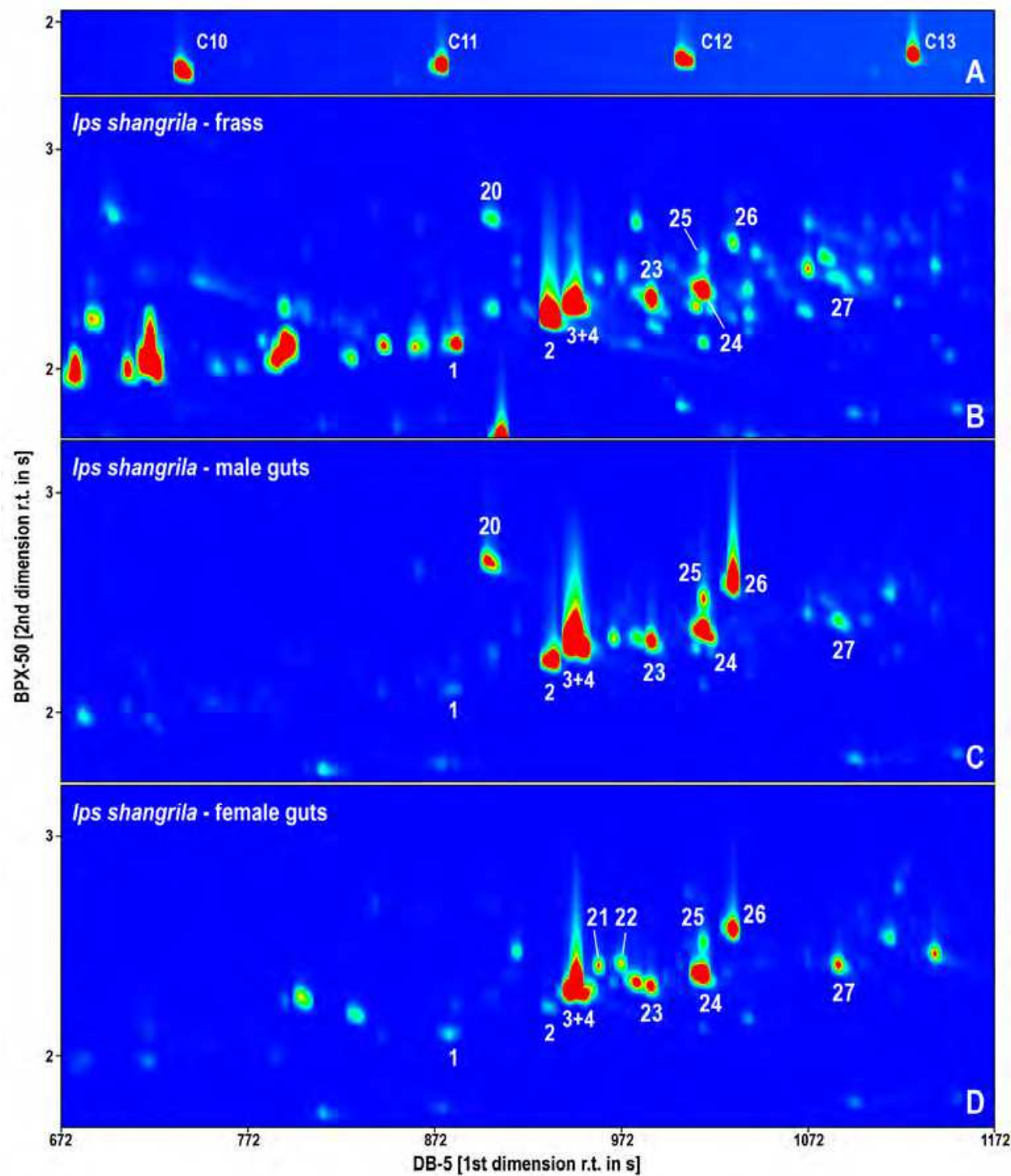
Fig. 5. Section of GC×GC-TOFMS TIC-chromatograms of *n*-alkane standards (A), *I. shangrila* male frass (B), *I. shangrila* male guts extract (C) and *I. shangrila* female guts extract (D) I_{K} range ~ 1000-1300.

Except discussed possible pheromonal components **1** - **4** we identified in this I_K range of all samples also several, mostly oxygenated terpenoic compounds, originated from host tree: phenylethanol (**20**), borneol (**23**), myrtenol (**24**), myrtenal (**25**), verbenon (**26**) and myrtanol (**27**).

In *I. nitidus* samples, chromatograms of examined extracts partially overlap, EAD active compounds ipsenol, ipsdienol and *cis/trans*-verbenols are present in all three sample groups with relatively lowest concentration in female guts extracts. However, two still unidentified female specific compounds (**21** and **22**; M^+ 154) from group of monooxygnated monoterpenes were found. In *I. shangrila* samples was the situation practically the same, except generally low (almost trace) amounts of ipsenol (**1**) in all three sample groups.

4.2 Chiral GC×GC-TOFMS analysis

Chiral GC×GC-TOFMS analysis focused to the antennally active compounds **1** - **4** in *I. nitidus* frass and guts extracts revealed the presence of (*S*)-ipsenol (**1**), racemic ipsdienol (**2**), (*S*)-*cis*-verbenol (**3**) and racemic *trans*-verbenol (**4**) in all analyzed samples (Figure 6). The same analysis of *I. shangrila* frass and guts extracts gave different results, especially in the case of ipsdienol (**2**), where we found high excess of its (*R*)-enantiomer (Figure 7B-D). (*S*)-Ipsdienol (**2**) was present only in low amounts in guts extracts and in *R*:*S* ratio circa 10:1 in frass (Figure 7B). Also the amount of components **1** and **2** was substantially lower in female guts extracts in comparison with frass and male gut extracts (Figure 7D). The obtained results of all semiquantitative chiral analyses of compounds **1** - **4** in both *Ips* species samples are given in Table 2.

	ipsenol [1]		**ipsdienol [2]**		*cis*-**verbenol [3]**		*trans*-**verbenol [4]**	
	(*R*)-	(*S*)-	(*R*)-	(*S*)-	(1*R*)-	(1*S*)-	(1*R*)-	(1*S*)-
Ips nitidus								
frass	0	+++	++++ (racemic)		+	+++++	+++++ (racemic)	
male guts	traces	+++	++++ (racemic)		traces	+++++	+++++ (racemic)	
female guts	0	++	++++ (racemic)		0	+++++	+++++ (racemic)	
Ips shangrila								
frass	0	++	+++ *	+	+	++++	+++++ (racemic)	
male guts	0	traces	++	traces	+	++++	+++++ (racemic)	
female guts	0	+	+	traces	traces	++++	++	++++

+: 1 – 3%, ++: 3 – 10%, +++: 10 – 15%, ++++: 15 – 30%, +++++: >30% (percentage counts from sum of **1-4** TIC peak area); *R*:*S* ca 10:1; SD ~ 5-24%; N = 5

Table 2. Results of chiral GC×GC-TOFMS analyses of compounds **1 – 4** in frass and male / female guts extracts of *Ips nitidus* and *Ips shangrila*.
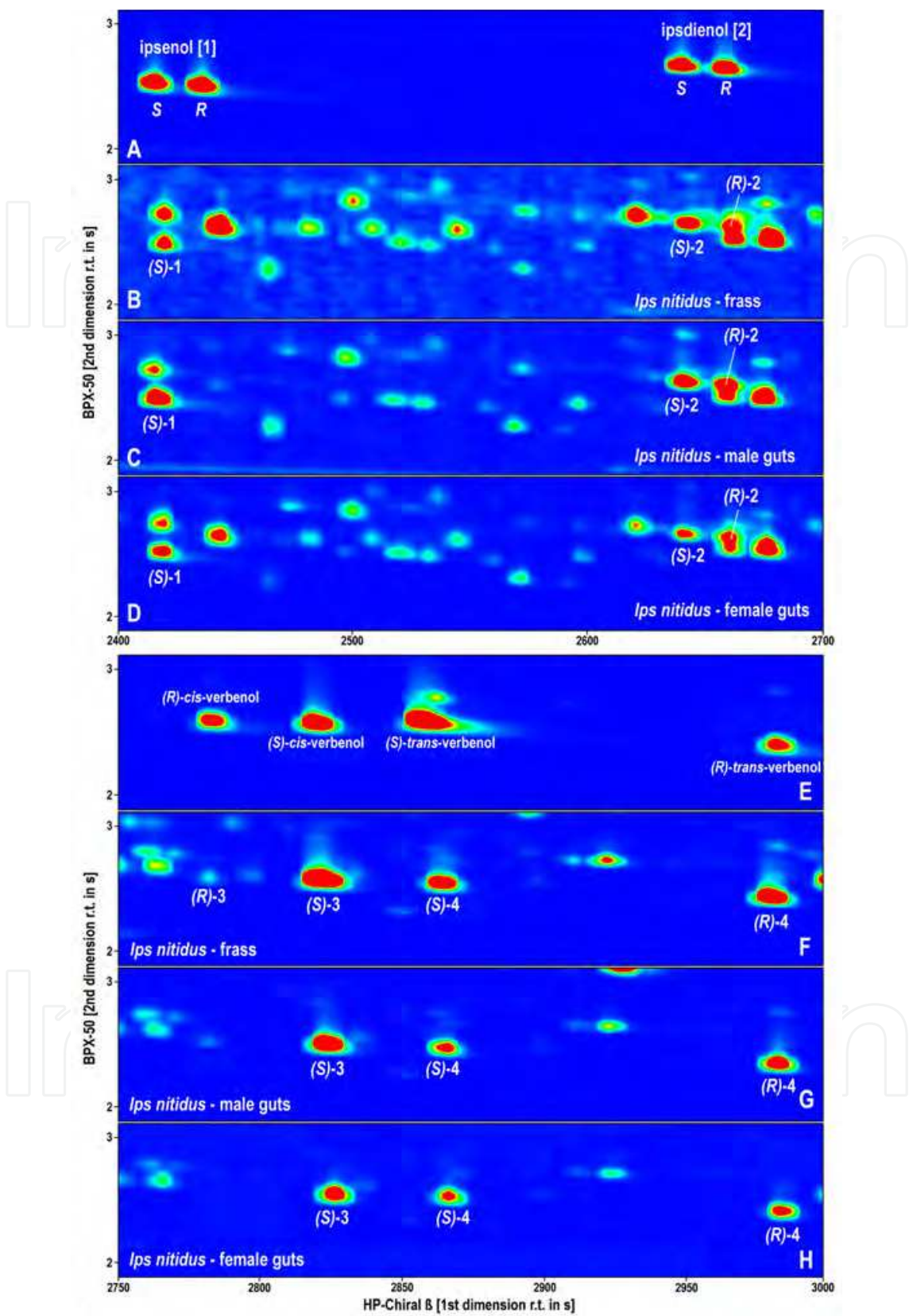
Fig. 6. Sections of GC×GC-TOFMS TIC-chromatograms (contour plots) depicting the chiral separation of compounds 1 - 4 in Ips nitidus samples (frass, males a females guts). Chromatograms with synthetic standards are assigned by letters A and E, respectively.
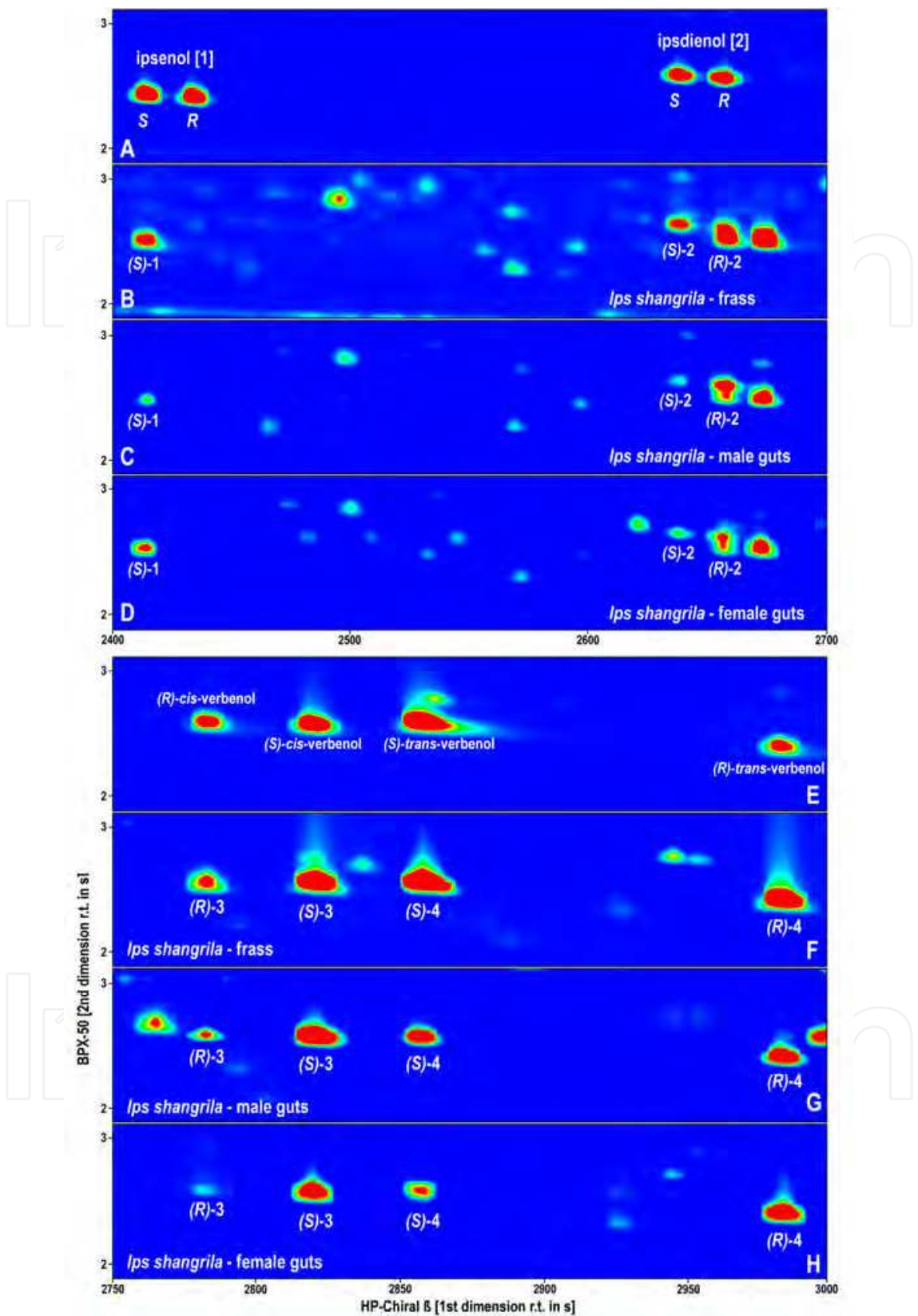
Fig. 7. Sections of GC×GC-TOFMS TIC-chromatograms (contour plots) depicting the chiral separation of compounds **1 - 4** in *Ips shangrila* samples (frass, males a females guts). Chromatograms with synthetic standards are assigned by letters A and E, respectively.

5. Conclusion

The aims and conclusions of the presented study can be divided into two parts. The first part, in a majority methodological, verified the usability of modern gas-chromatographic techniques, achiral and especially chiral GC×GC-TOFMS in combination with GC-EAD, in the area of insect semiochemical research. The second part, more chemico-ecological, was directly focused on finding the possible aggregation pheromone components of two important spruce pests in NW China – bark beetles *Ips nitidus* and *Ips shangrila*.

In last 15 years, multidimensional GC technique has been used for analysis of a broad spectrum of organic compounds in many applications and has shown to be superior to standard one dimensional GC. The three principal advantages of comprehensive GC×GC are: a) separation of coeluting compounds; b) improvement of detection sensitivity (Górecky et al., 2004) and c) the second dimension separation of the background chemical noise from the biologically active compound, already mentioned in chapter introduction (Kalinová et al., 2006). These three advantages have a real key importance in the area of insect semiochemicals research, since volatile physiologically active compounds are often in extremely low quantities occurring components of complex mixtures. The small amounts of semiochemicals produced by pheromone glands make the analysis of insect semiochemicals challenging. While using standard GC/MS, we quite often faced a situation at which a biologically active compound was present, however the MS spectrum remained hidden in the chromatographic background noise (Svatoš et al., 1999). Here, in similar cases, where we are limited by an amount of experimental material or the concentration of the target analytes is extremely low, the use of the GC×GC-TOFMS technique is extremely helpful in the semiochemical identification.

During analyzing the biological samples obtained from bark beetles *Ips nitidus* and *Ips shangrila,* we faced another problem - whether the quantity of electrophysiologically active target components was relative high and thus sufficient for standard 1D-GC experiments, the analytes, moreover chiral, were hidden in complex mixtures of often coeluting terpenes and other natural compounds. In this case, the valuable GC×GC's advantage of offering an elegant and robust solution of the coelution, baseline separation in the second dimension, was really critical. Coelution problems, hardly avoided in 1D-GC, here were solved by 2nd dimension separation in all analyses of bark beetle samples. Nice examples of resolved coelution can be demonstrated on baseline separation of (*S*)-ipsenol (*S*-**1**) and 2,3-epoxypinane, or on separation of (*R*)-ipsdienol (*R*-**2**) and *trans*-2-caren-4-ol, respectively – both in chiral analyses of *I. nitidus* samples (Figure 6B-D). These two cases in a standard 1D-GC analysis will cause great troubles not only in compound identification, but also in the their semiquantitative (or quantitative) analysis. We found numerous similar troublemaking coelutions in other achiral and chiral analyses of *Ips* samples (see Figures 4-7).

Both achiral and chiral separations of all studied samples can also serve as typical examples of another great feature of GC×GC-TOFMS analyses, the possibility of fast and useful pattern analysis of obtained data visualized in the form of contour plot 2D-chromatograms (see Figures 4-7). These coloured contour plots make searching for target components much easier, with possibility of another improvement when display based on characteristic ion current is selected instead of total ion current (TIC) (Kalinová et al., 2009). This feature is closely connected with time-of-flight MS detector key attribute, the fast recording (up to 500

spectra per second) of full mass spectra in relative broad range (1 – 1000 amu), which is not reachable by standard quadrupole detectors. The helpfulness of 2D-chromatograms pattern analysis itself is clearly visible in Figures 6 and 7, where you are able to interpret the enantiomeric composition of analyzed samples "in-one-sight", by their simple and fast visual comparison. The same interpretation process in case of standard 1D-chromatograms ("a forest of hundreds peaks...") is more time demanding and often complicated by coelutions.

If the great usability of achiral and chiral GC×GC-TOFMS technique in the semiochemical research was clearly verified, the problem of the aggregation pheromone composition in both *Ips* species still would not be completely resolved. Our GC-EAD and subsequent achiral GC×GC-TOFMS analyses of bark beetle samples revealed the same four EAD active compounds in both species – ipsenol (**1**), ipsdienol (**2**), *cis*-verbenol (**3**) and *trans*-verbenol (**4**). The presence of these possible pheromone components in studied samples is not surprising – all of them were reported as pheromone components of the taxonomically closest Eurasian species – *Ips typographus* and *Ips amitinus* (Bakke et al., 1977; Francke et al., 1980; Schlyter, 1987). The observed strong *Ips nitidus* antennal response to pinenes also is not unusual. The similar reaction was reported in sister species *Ips typographus* (Dickens, 1981; Erbilgin et al., 2007), although the real biological significance of this phenomenon (possible synergistic effect to the aggregation pheromone?) is still questionable and unclear. The observed differences in the amounts of target compounds **1-4** in both species and also in their sexes (we found only traces of ipsenol in *I. shangrila* guts extracts) needs further detail study. Surprisingly we did not found any trace of 2-methyl-3-buten-2-ol in SPME-GC×GC-TOFMS analyses of both species frass. This compound, important pheromone component in many bark beetles species (Schlyter, 1987), was reported in two recently published studies dealing with pheromones of both studied *Ips* species (Zhang et al., 2009a,b). Early eluting highly volatile 2-methyl-3-buten-2-ol was not detected during our GC×GC-TOFMS analyses of the extracts thanks to longer solvent delay time (400 s) protecting sensitive and expensive CCD-array of Pegasus IVD TOFMS detector. However, it is also interesting that 2-methyl-3-buten-2-ol did not elicit any electrophysiological responses on both species antennae. From our point of view the occurrence and real biological function of 2-methyl-3-buten-2-ol in both studied *Ips* species deserves further experiments, both analytical and behavioural.

The chiral GC×GC-TOFMS analyses of bark beetle samples showed clearly the advantage of the comprehensive two dimensional gas chromatography - all obtained data were easily interpreted and results showed the strong enantioselectivity in the pheromone component composition, especially in ipsenol (**1**) and *cis*-verbenol (**3**). We found practically only (*S*)-**1** and high excess (>1 : 10) of (1*S*)-**3** in both species, in contrast to *trans*-verbenol (**4**) which was in examined samples present mostly only in racemic form. Racemic ipsdienol (**2**) we found in *I. nitidus* and predominantly as (*R*)-isomer in *I. shangrila.*

The obtained results of achiral and chiral analyses of both species samples differ from data published in works of Chinese colleagues (Zhang et al., 2009a,b). Except above mentioned absence of 2-methyl-3-buten-2-ol in our SPME samples we found other notable differences in amount and enantiomeric composition of compounds **1 - 4**. In *I. nitidus* we found in all samples only racemic ipsdienol (**2**) in contrast to data published by Zhang et al. (2009a), reporting very high excess of (S)-**2** enantiomer. Also the concentration of racemic *trans*-verbenol (**4**) in our samples was substantially higher in comparison with Zhang's results

(small amounts of (1*S*)-**4** enantiomer only). In *I. shangrila* we found a small, but well measurable amount of (*S*)-ipsenol (*S*-**1**), especially in frass samples. Also the enantiomeric composition of ipsdienol (**2**) and occurrence of the racemic *trans*-verbenol (**4**) were different from published results (Zhang et al., 2009b). All these observed differences in obtained results need further experimental confirmation. In our opinion the field tests results published by Zhang et al. (2009a,b) clearly inform us about yet undiscovered pheromone components and/or their possible different ratios in both *Ips* species aggregation pheromone systems.

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The aim of this book is to describe the fundamental aspects and details of certain gas chromatography applications in Plant Science, Wine technology, Toxicology and the other specific disciplines that are currently being researched. The very best gas chromatography experts have been chosen as authors in each area. The individual chapter has been written to be self-contained so that readers may peruse particular topics but can pursue the other chapters in the each section to gain more insight about different gas chromatography applications in the same research field. This book will surely be useful to gas chromatography users who are desirous of perfecting themselves in one of the important branch of analytical chemistry.

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