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RESEARCH PAPER

Identification of 1-methyloctyl butanoate as the major sex pheromone component from females of the saddle gall midge, *Haplodiplosis marginata* (Diptera: Cecidomyiidae)

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Abstract The saddle gall midge, *Haplodiplosis marginata* (von Roser) (Diptera: Cecidomyiidae), has undergone a resurgence recently as a pest of cereals in Belgium and other European countries. An effective monitoring tool of saddle gall midge flights is needed to understand the enigmatic population dynamics of this pest, and to design an integrated management strategy. Therefore, volatile compounds emitted by females (alkan-2-ols and alk-2-yl butanoates) were identified, and the chirality of the emitted esters was determined to be the *R* absolute configuration. In field-trapping experiments, racemic non-2-yl butanoate attracted substantial numbers of *H. marginata* males. Thus, this compound will be useful in baited traps for monitoring seasonal flight patterns, and improving integrated management of the saddle gall midge in agricultural systems.

Keywords *Haplodiplosis marginata* · Sex pheromone · Alk-2-yl butanoates · Chiral gas chromatography · Pheromone synthesis · Field-trapping experiment

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Introduction

Since 2010, a resurgence of the saddle gall midge, *Haplodiplosis marginata* (von Roser, 1840) (Diptera: Cecidomyiidae), has been observed in several European countries, particularly in Belgium, the Netherlands, the United Kingdom and France (Censier et al. 2012; Dewar 2012). This small univoltine pest can cause severe damage in cereal crops, mainly in wheat and barley, but also in spelt or rye (De Clercq and D'Herde 1972; Skuhravý et al. 1983).

Haplodiplosis marginata had not been reported for several decades in some countries. In Belgium, for example, the last outbreak dates back to the 1960s (De Clercq and D'Herde 1972; Latteur 1972; Skuhravý et al. 1983). While this species is usually a minor pest in Western Europe, the saddle gall midge is considered to be a major pest in Central Europe (Skuhravá 2000).

Adults of this midge, which generally emerge from May to the beginning of June, have a very short lifespan, ranging from 3 to 5 days. Mated females lay eggs on cereal leaves and once the eggs have hatched, young larvae crawl to the stem and creep under the leaf sheath where they feed

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causing the plant to develop saddle-shaped galls. This larval damage causes yield losses and, moreover, galls facilitate mould development and broken stalks, which can lead to the complete crop loss in extreme cases. Fully-grown larvae leave the stems after rain, usually from mid-June to mid-July, and enter the soil where they hibernate. While a portion of the population can remain in diapause up to 6 years, most larvae move up to the surface of the soil the following spring to pupate and emerge as adults 14–25 days later (Nijveldt and Hulshoff 1968; De Clercq and D'Herde 1972; Golightly and Woodville 1974; Skuhravý et al. 1983, 1993).

Thus far, *H. marginata* was very difficult to detect and, therefore, to study. Only if saddle gall midge adults are abundant can they be detected at the bottom of the vegetation by well-trained observers. At other stages, it is even more difficult to detect because eggs are very small, feeding larvae are hidden under leaf sheaths, and they eventually bury themselves in the soil.

More precise monitoring tools are needed to better define threshold at infestation levels requiring implementation of integrated pest management control measures and, in addition, to understand long-term population dynamics of this pest.

Flight monitoring using sex pheromone-baited traps has proven useful for other midges. Indeed, in the Cecidomyiidae, sex pheromones have been identified for several species, and it is well established that sexual chemical communication in many cecidomyiid species is based, at least in part, on the presence of saturated and/or unsaturated volatile esters or keto-esters (Hall et al. 2012).

Pheromone-baited traps have successfully been developed and commercialised for the orange wheat blossom midge, *Sitodiplosis mosellana* (Géhin), a gall midge closely related to *H. marginata* (Gries et al. 2000). Promising results have also been reported for other cecidomyiids, such as the Hessian fly, *Mayetiola destructor* (Say) (Andersson et al. 2009), the pea midge, *Contarinia pisi* (Winnertz) (Hillbur et al. 2000) or the raspberry cane midge, *Resseliella theobaldi* (Barnes) (Hall et al. 2009).

In this paper, we report on gas chromatography–mass spectrometry (GC–MS) analyses of the volatiles emitted by *H. marginata* females and on their identification, chemical synthesis and biological activity, to develop efficient and selective flight monitoring protocols for this pest.

Methods and materials

Insect rearing

Soil infested with *H. marginata* larvae was collected in Blankenberge (Belgium; geographical coordinates 51°17.5′N, 3°8.9′E) in January 2013. This soil was kept in a cold storage at 6 °C until diapause was completed. The larvae were then extracted from the soil and individually transferred to small transparent plastic tubes (3.5 cm × 5 cm diameter; Visalux SA, Kortrijk, Belgium), on a moistened soil layer 1 cm deep. These tubes were held on plastic trays in a chamber at 18 ± 1.6 °C and 16:8 h L/D. Newly emerged (0–24 h), virgin adults were separated by sex daily for experiments.

Chemicals

All chemicals (reactants, reagents and solvents) were purchased from Sigma-Aldrich BVBA (Diegem, Belgium), VWR International Europe BVBA (Leuven, Belgium) or Merck SA/NV (Overijse, Belgium). Their purities were >99.9 % and, therefore, they were used without further purification.

Collection of volatiles from virgin midges

Volatiles were collected from newly emerged, virgin females and males of *H. marginata*, by placing each individual into a sealed glass vial (internal volume 20 mL). The volatile organic compounds (VOC) emitted by midges were sampled at 24 °C during a 24-h period, using an SPME fibre (divinylbenzene/carboxen/polydimethylsiloxane, 50/30 µm coating, 1 cm length; Supelco, Bellefonte, Pennsylvania, USA).

Under the same conditions, VOC samples were also prepared from newly emerged, virgin females and males crushed in sealed sampling vial using a magnetic pestle device, according to the method developed by Fischer and Lognay (2012).

Analytical methods

GC–MS chiral analyses were performed with a Finnigan Trace MS apparatus coupled to a GC (Interscience, Louvain-la-Neuve, Belgium). GC–MS analyses were conducted via splitless injection for SPME samples, and split injection for liquid samples (split ratio 20:1, split flow at 20 mL/min) at 225 °C on a CP-cyclodextrin-β-2,3,6-M-19, 25 m × 0.25 mm I.D., 0.25-µm film thickness (Chrompack, Middelburg, The Netherlands), using He at 1 mL/min as the carrier gas, with a temperature programme from 40 °C for 2 min to 220 °C at 5 °C/min, and a final hold of 12 min. MS conditions were as follows: EI mode at 70 eV; source temperature: 220 °C; and scanned mass range $m/z = 35$–400. GC-FID analyses were performed with a Hewlett Packard HP 6890 apparatus (Agilent Technologies SA/NV, Diegem, Belgium), using the following conditions: splitless injection for SPME/split injection for liquid

samples (split ratio 20:1, split flow at 20 mL/min) at 220 °C; column: HP5-MS, 30 m × 0.25 mm I.D., 0.25 μm film thickness; carrier gas: He at 1 mL/min; temperature programme 40 °C during 2 min then a raise to 320 °C at 4 °C/min held 1 min at 320 °C; detector at 320 °C. For chromatographic analyses of synthetic standards, 1 μL of a 0.2 mg/mL ether solution was injected.

NMR spectra (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC) of synthesised compounds were recorded at 25 °C in $CDCl_3$ at 600 MHz (^{1}H) and 150 MHz (^{13}C) with a Varian instrument (Oxford, UK), and are reported in ppm from internal TMS on the δ scale. Data are reported as follows: chemical shift [multiplicity (d, doublet; t, triplet; sext, sextet; m, multiplet), coupling constants in Hertz, number of protons, attribution].

IR spectra were recorded with a Bruker IFS 25 instrument. Optical rotations were recorded at 589 nm (sodium D line) in a 2 dm cell at 25 °C on a ADP220 polarimeter (Bellingham and Stanley, UK).

Chemical syntheses

*Hept-2-yl butanoate (**1**).* A solution of commercial racemic heptan-2-ol (0.1195 g, 1.03 mmol) in dichloromethane (5 mL) was added dropwise to a stirred solution of butyryl chloride (0.2161 g, 2.03 mmol) in dichloromethane (5 mL). After stirring overnight at room temperature, the mixture was quenched with a saturated aqueous sodium hydrogencarbonate solution (20 mL). The organic layer was then separated and washed with saturated aqueous $NaHCO_3$ solutions (3 × 20 mL). The organic layer was finally filtered through a Whatman® filter paper (hydrophobic phase separation filter paper, impermeable to water, Sigma-Aldrich, Diegem, Belgium) and the solvent evaporated under vacuum to afford **1** (0.1705 g, 0.916 mmol, 89 %) as a colourless liquid.

1: EIMS: *m/z* (%) 186 [$M^{+\cdot}$] (<1), 115 (13), 98 (11), 89 (10), 71 (100), 70 (13), 69 (10), 57 (30), 56 (22), 55 (12), 43 (34), 41 (15); IR (film) ν = 2,926, 2,855, 1,736, 1,460, 1,379, 1,254, 1,184, 961, 725 cm^{-1}; ^{1}H NMR: δ = 4.87 (sext, J = 6.6, 1H, H-2′), 2.21 (t, J = 7.8, 2H, H-2), 1.61 (sext, J = 7.2, 2H, H-3), 1.53 (m, 1H, H-3′), 1.43 (m, 1H, H-3′), 1.25 (m, 6H, H-4′–H-6′), 1.15 (d, J = 6.6, 3H, H-1′), 0.91 (t, J = 7.8, 3H, H-4), 0.84 (t, J = 6.6, 3H, H-7′); ^{13}C NMR: δ = 175.94 (CO), 73.29 (C-2′), 39.23 (C-2), 38.52 (C-3′), 34.23 (C-5′), 27.68 (C-4′), 25.14 (C-6′), 22.61 (C-1′), 21.17 (C-3), 16.56 (C-7′), 16.23 (C-4). Racemic sample: R_t (*S*)-**1** = 16.60 min; R_t (*R*)-**1** = 16.82 min. Calculated RI = 1,216.

*Non-2-yl butanoate (**2**).* The same protocol was used to synthesise non-2-yl butanoate starting from butyryl chloride (0.2352 g, 2.21 mmol) and commercial racemic nonan-2-ol (0.1509 g, 1.05 mmol). This afforded **2** (0.2067 g, 0.966 mmol, 92 %) as a colourless liquid.

When using commercial optically pure (*S*)-(+)-nonan-2-ol (0.1518 g, 1.05 mmol, 99 % optical purity), the optically pure ester (*S*)-(+)-**2** was obtained in a 92 % yield (>99 % ee).

2: $[\alpha]_D^{25}$ = +21° (c = 0.62; $CHCl_3$); EIMS: *m/z* (%) 214 [$M^{+\cdot}$] (<1), 126 (11), 115 (19), 97 (11), 88 (12), 84 (10), 71 (100), 70 (13), 69 (10), 57 (15), 56 (18), 55 (19), 43 (63), 42 (11), 41 (28); IR (film) ν = 2,929, 2,857, 1,735, 1,460, 1,379, 1,256, 1,186, 1,123, 1,088, 1,043, 952, 723 cm^{-1}; ^{1}H NMR: δ = 4.85 (sext, J = 6.0, 1H, H-2′), 2.19 (t, J = 7.8, 2H, H-2), 1.59 (sext, J = 7.2, 2H, H-3), 1.51 (m, 1H, H-3′), 1.42 (m, 1H, H-3′), 1.22 (m, 10H, H-4′–H-8′), 1.14 (d, J = 6.6, 3H, H-1′), 0.89 (t, J = 7.2, 3H, H-4), 0.82 (t, J = 6.0, 3H, H-9′); ^{13}C NMR: δ = 175.86 (CO), 73.24 (C-2′), 39.20 (C-2), 38.56 (C-3′), 34.37 (C-7′), 31.99 (C-5′), 31.79 (C-6′), 28.01 (C-4′), 25.22 (C-8′), 22.58 (C-1′), 21.15 (C-3), 16.62 (C-9′), 16.20 (C-4). R_t (*S*)-**2** = 21.58 min; [racemic sample: R_t (*S*)-**2** = 21.59 min; R_t (*R*)-**2** = 21.73 min]. Calculated RI = 1,409.

*Undec-2-yl butanoate (**3**).* The same protocol was used to synthesise undec-2-yl butanoate starting from butyryl chloride (0.2438 g, 2.29 mmol) and commercial racemic undecan-2-ol (0.1725 g, 1.00 mmol). This afforded **3** (0.2257 g, 0.933 mmol, 93 %) as a colourless liquid.

3: EIMS: *m/z* (%) 242 [$M^{+\cdot}$] (<1), 115 (15), 89 (11), 83 (10), 71 (100), 70 (18), 69 (14), 57 (24), 56 (15), 55 (18), 43 (36), 41 (15); IR (film) ν = 2,928, 2,855, 1,731, 1,462, 1,380, 1,255, 1,185, 1,124, 1,087, 1,044, 955, 726 cm^{-1}; ^{1}H NMR: δ = 4.88 (sext, J = 6.6, 1H, H-2′), 2.23 (t, J = 7.8, 2H, H-2), 1.63 (sext, J = 7.8, 2H, H-3), 1.53 (m, 1H, H-3′), 1.43 (m, 1H, H-3′), 1.24 (m, 14H, H-4′–H-10′), 1.17 (d, J = 6.6, 3H, H-1′), 0.92 (t, J = 6.6, 3H, H-4), 0.86 (t, J = 7.2, 3H, H-11′); ^{13}C NMR: δ = 176.00 (CO), 73.35 (C-2′), 39.27 (C-2), 38.60 (C-3′), 34.51 (C-9′), 32.17, 32.15, 32.08, 31.92 (C-5′–C-8′), 28.04 (C-4′), 25.29 (C-10′), 22.64 (C-1′), 21.20 (C-3), 16.72 (C-9′), 16.27 (C-4). Racemic sample: R_t (*S*)-**3** = 26.10 min; R_t (*R*)-**3** = 26.20 min. Calculated RI = 1,604.

All chromatographic analyses were conducted using the conditions described above.

Chemical identification

Analyses of the mass spectra were indicative of alcohols or butyroxy esters. Components were identified by comparing recorded mass spectra with the NIST08 and Wiley275 spectral databases. Identifications were confirmed by injection of the available commercial alcohols and synthetic ester standards for all candidate pheromone compounds.

As the same chromatographic conditions with the same column were used for the analyses of both standards and volatile compounds emitted by *H. marginata* females, identification of the natural products were confirmed by comparing their retention times and mass spectra with those of the reference compounds. Further identification was carried out by calculating non-isothermal retention indices by injecting saturated *n*-alkane standard solution (C_7–C_{30}, 1 µg/mL in hexane, Supelco, Belgium) under the chromatographic conditions described above, using the definition of Van den Dool and Kratz (1963).

Field-trapping experiments

Observing normal behaviour in laboratory conditions has been reported to be difficult for certain cecidomyiid species (Hall et al. 2012, according to Hillbur, unpublished). After failing to gain a Y-tube olfactometer response for *H. marginata* virgin males and our compounds using a Y-tube olfactometer [arms and stem were 14 and 12 cm long, respectively, with an inner diameter of 3 cm (Euroscientific, Antwerpen, Belgium)] based on Andersson et al. (2009) (Censier, unpublished), we moved directly to field testing. Field experiments using traps baited with commercial (±)-nonan-2-ol (**5**) and synthetic (±)-non-2-yl butanoate (**2**) were thus carried out in cereal fields in Belgium, to determine whether *H. marginata* males responded to at least one of these compounds.

Preparation of prototype dispenser

Prototype dispensers were made with blotting paper sheets (3 cm × 1 cm) in sealed plastic bags (4 cm × 1.5 cm). The blotting paper was impregnated with the pheromone [20 mg synthetic (±)-non-2-yl butanoate (**2**)] or with its precursor [20 mg commercial (±)-nonan-2-ol (**5**)] applied in solution in pure diethyl ether (0.1 mL). Blank dispensers, loaded only with diethyl ether (0.1 mL), were also prepared as unbaited controls. After the evaporation of the solvent (1 min), the dispensers were placed in field traps. This type of dispenser was chosen because of its high release rate (Bruce et al. 2007) to increase the probability of saddle gall midge catches, as the majority of adults had already emerged at the beginning of the field trials. That is also the reason why dispensers were loaded with 20 mg of (±)-non-2-yl butanoate (**2**), an unusually high loading for pheromone dispensers.

Field experiment 1

The first trial was conducted in a winter wheat (*Triticum aestivum*) field lightly infested with *H. marginata* in Gembloux (geographical coordinates 50°33.8′N, 4°42.5′E), in May and June 2013. Traps were white delta traps with sticky inserts (Suterra Europe Biocontrol Espana SL, Gavà, Barcelona, Spain) suspended at 20 cm above ground level and at least 6 m apart. Baited traps with (±)-non-2-yl butanoate (**2**) dispensers and unbaited controls were arranged in a randomised block design with five replicates. Trap catches were checked daily, sticky inserts replaced and *H. marginata* adults counted by sex. The dispensers were renewed weekly, and the experiment lasted 5 weeks.

The release rate of (±)-non-2-yl butanoate (**2**) was roughly assessed by weighing the dispensers just before their use and after their removal from traps, using a Sartorius research R200D balance. In addition, four delta traps with (±)-nonan-2-ol (**5**) dispensers were placed in the same field during 3 weeks to assess the potential attractiveness of the precursor pheromone.

Field experiment 2

The second trial was carried out in a winter wheat field infested with *H. marginata* in Blankenberge (geographical coordinates 51°17.5′N, 3°8.9′E). White delta traps suspended at 20 cm above ground level with sticky inserts were disposed in the field at least 10 m apart. Baited traps with (±)-non-2-yl butanoate (**2**) dispensers and unbaited controls were arranged in a randomised block design with three replicates. The dispensers were renewed three times during the experiments (after 1, 3 and 5 weeks). Trap catches were checked daily, sticky inserts replaced, and *H. marginata* adults counted by sex. This experiment took place between May 29 and July 10 2013.

Results

Identification of volatiles emitted by *H. marginata* females and synthesis of alk-2-yl butanoates

The volatile compounds emitted by live *H. marginata* females were investigated by SPME–GC–MS. This showed the occurrence of 1-methyloctyl butanoate (non-2-yl butanoate) (**2**) and nonan-2-ol (**5**) as prominent constituents (Fig. 2, R_t = 14.32 min; R_t = 9.92 min, respectively).

Other esters, namely 1-methylhexyl butanoate (hept-2-yl butanoate) (**1**) and 1-methyldecyl butanoate (undecen-2-yl butanoate) (**3**), as well as the nonesterified alcohols heptan-2-ol (**4**) and undecan-2-ol (**6**) were also detected as minor constituents (Figs. 1, 2).

Volatile alcohols were also abundantly observed from crushed females (up to 10 times more alcohols than for live females), while esters were only observed in trace amounts. None of the above mentioned compounds were observed in VOC released by living or crushed males.

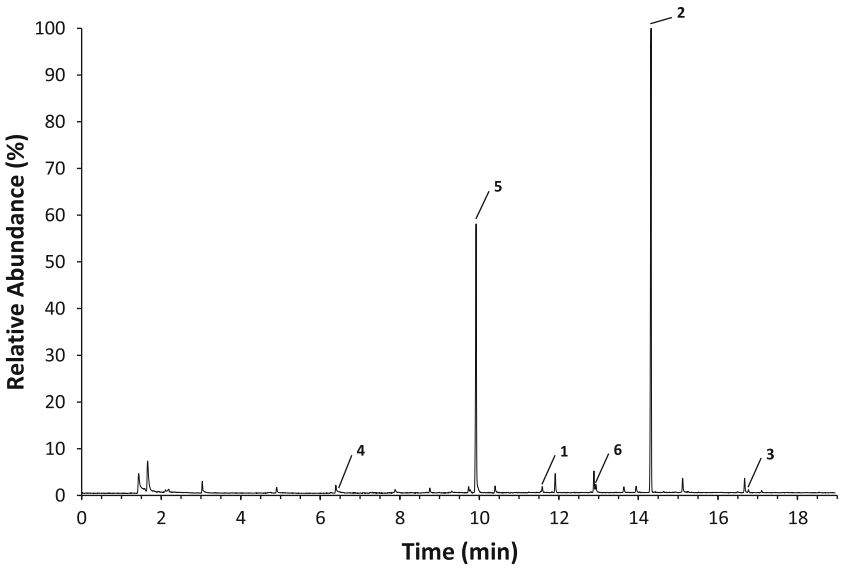
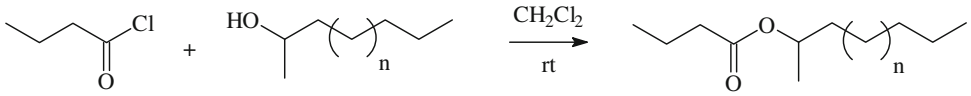


Fig. 1 Total ion chromatogram of volatile compounds emitted by *Haplodiplosis marginata* females (HP5 stationary phase)

1 n = 1
2 n = 3
3 n = 5

4 n = 1
5 n = 3
6 n = 5

Fig. 2 Chemical structures of esters and alcohols emitted by *Haplodiplosis marginata* females



1 n = 1 89 %
2 n = 3 92 %
3 n = 5 93 %

Fig. 3 Syntheses of esters **1–3**

Concerning the alcohols, their structures were assigned on the basis of their mass spectra and confirmed by injection of pure commercial samples. As butanoate esters are not commercially available, they were synthesised from butyryl chloride and the corresponding alcohol (Fig. 3).

This afforded the expected esters **1–3** as racemates in very good yields (89–93 %) or as optically pure (*S*)-**2** when using (*S*)-nonan-2-ol (**5**).

The spectroscopic properties (^{1}H and ^{13}C NMR, EI-MS and IR) of synthetic compounds **1–3** unambiguously proved their structure, and were in accordance with those described in the literature (Molinari et al. 1996; Hirata et al. 2002; Strohalm et al. 2007, 2010).

Table 1 provides the list of the volatile compounds emitted by *H. marginata* females.

Absolute configuration of the natural sex pheromone

Injection of the pure non-2-yl ester sample confirmed the proposed structural assignment for the sex pheromone of *H. marginata*.

Separation of the racemic esters **1–3** was achieved by GC using a column with a heptakis chiral phase: (2,3,6-tri-*O*-methyl)-β-cyclodextrin phase has been repeatedly successfully used for many similar separations (Betts 1996; Cserhati and Forgacs 2001; Cousin et al. 2009).

Using this permethylated β-cyclodextrin as stationary phase, baseline separations could be achieved for the enantiomers of the complete series of alk-2-yl esters. Co-injection with a synthetic sample of optically pure (*S*)-**2** indicated that the (*S*) enantiomer is the fastest-eluting

Table 1 Volatile compounds emitted by *Haplodiplosis marginata* females

CAS number [a]	IUPAC name	Identification[b]	Sample RI[c]	Reference RI[d]	Observed mass spectrum[e]
543-49-7	Heptan-2-ol (**4**)	MS, STD, RI	904	905[f]	116 (<1), 83 (10), 55 (24), 45 (100), 43 (12), 41 (11)
628-99-9	Nonan-2-ol (**5**)	MS, STD, RI	1,130	1,120[g]	144(<1), 69 (28), 57 (12), 56 (13), 55 (18), 45 (100), 43 (17), 41 (15)
39026-94-3	Hept-2-yl butanoate (**1**)	MS, STD	1,216	1,199[h]	186 (<1), 115 (13), 98 (11), 89 (10), 71 (100), 70 (13), 69 (10), 57 (30), 56 (22), 55 (12), 43 (34), 41 (15)
1653-30-1	Undecan-2-ol (**6**)	MS, STD	1,346	1,311[i]	172 (<1), 97 (11), 83 (18), 70 (12), 69 (18), 57 (21), 56 (14), 55 (24), 45 (100), 43 (21), 41 (17)
69727-42-0	Non-2-yl butanoate (**2**)	MS, STD	1,409	–	214 (<1), 126 (11), 115 (19), 97 (11), 88 (12), 84 (10), 71 (100), 70 (13), 69 (10), 57 (15), 56 (18), 55 (19), 43 (63), 42 (11), 41 (28)
55193-05-0	Undec-2-yl butanoate (**3**)	MS, STD	1,604	–	242 (<1), 115 (15), 89 (11), 83 (10), 71 (100), 70 (18), 69 (14), 57 (24), 56 (15), 55 (18), 43 (36), 41 (15)

[a] CAS number of compounds listed in order of elution from an HP-5 column. Source CAS: Scifinder® (Chemical Abstract Service, Columbus, USA)

[b] Identification methods: MS, comparison of mass spectra with those of Nist08 and Wiley275 libraries; RI, comparison of retention indices with those reported in the literature; STD, comparison of retention time and mass spectra of available standards

[c] Retention indices on an HP-5 column, experimentally determined using a saturated *n*-alkane standard solution C_7–C_{30}

[d] Retention indices taken from

[e] EI-MS, 70 eV, source at 220 °C

[f] Wang et al. (2005) (measured on a HP-5 column)

[g] van Ruth et al. (2001) (measured on a BPX-5 column)

[h] Brat et al. (2000) (measured on a DB-1 column)

[i] Congiu et al. (2002) (measured on a DB-5 MS column)

compound for the ester homologues. Figure 4 shows the enantiomeric separation of alk-2-yl butanoates **1**–**3**.

Moreover, as natural butanoates emitted by *H. marginata* females proved to be chromatographically indistinguishable from the slower eluting compounds (Fig. 4), this unequivocally established the absolute configuration of the major sex pheromone blend compound to be the (*R*)-enantiomer.

Identification of *H. marginata* sex pheromone by field experiments

In the literature, Bruce et al. (2007) did not find significant differences in the capture levels of the orange wheat blossom midge, *S. mosellana*, between lures with racemate and enantiomerically pure major component sex pheromone. The racemate was thus favoured for common use in field, as it is less costly to synthesise than the enantiomerically pure material. The attractiveness of (±)-non-2-yl butanoate (**2**) to *H. marginata* males has, therefore, been tested in field experiments in Gembloux. These clearly demonstrated the attractiveness of **2**. Indeed, a total of 1,655 males were caught in traps baited with (±)-non-2-yl butanoate (**2**) over the whole trial period, with between 282 and 426 males caught in each baited trap, while no males and only one female were caught in unbaited controls (Fig. 5). The non-variability of catches in unbaited traps did not allow statistical analyses for this trial.

The release rate of (±)-non-2-yl butanoate (**2**) was evaluated at 85.7 ± 2.9 % on average over 1 week. However, 83 ± 16.6 % of catches occurred on average within 3 days of the installation or renewal of the pheromone dispensers. So, it seems that this type of dispenser allows a very effective diffusion of the pheromone, but the diffusion rate rapidly decreases over time.

Furthermore, traps baited with (±)-nonan-2-ol (**5**) provided no evidence of specific attractiveness to *H. marginata*, catching only two males and two females during the 3-week trial.

In the second field trial in Blankenberge, comparisons were made only between catches of *H. marginata* adults in traps baited with (±)-non-2-yl butanoate (**2**) and in unbaited controls.

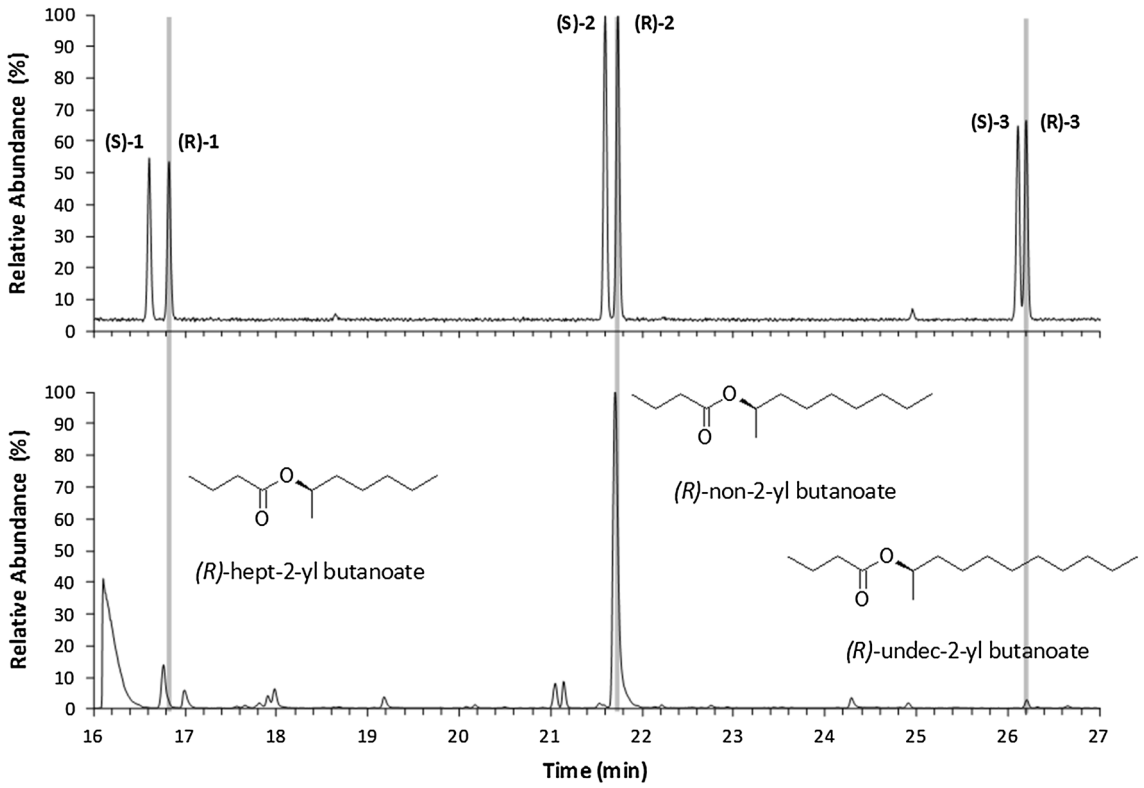


Fig. 4 Determination of the absolute configuration of *Haplodiplosis marginata* sex pheromone blend (upper trace: synthetic racemic compound; lower trace: insect pheromone) on a chiral heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin phase

A total of 2,418 adults of *H. marginata* were caught during this field experiment (Table 2). Although no statistical analyses were performed due to the excessive difference between variances of baited and unbaited trap catches, this trial confirmed the attractiveness of the (±)-non-2-yl butanoate (**2**). Indeed, the capture level of males was on average 112 times greater when traps were baited with (±)-non-2-yl butanoate (**2**), with 789 males on average per trap and at least 425 males in one trap, against a mean of only 7 males in unbaited controls. The sex ratio was also clearly different between treatments, as males represent 98 % of *H. marginata* adults caught, while this proportion falls to 54 % for traps without (±)-non-2-yl butanoate (**2**) dispensers.

Discussion

The major component of the saddle gall midge sex pheromone was identified as (*R*)-non-2-yl butanoate, and field testing of the racemic ester was proved highly attractive to *H. marginata* males.

As *H. marginata* is a pest of cereals that can cyclically represent a serious threat for crops, it was necessary to develop an effective tool for detecting the presence and abundance of this insect in fields, and for facilitating the development of a pheromone-based monitoring of the saddle gall midge.

Alk-2-yl butanoates were emitted only by *H. marginata* females. The alkan-2-ols detected among the VOC emitted by the saddle gall midge were probably precursors of the esters of interest, but this biosynthetic pathway still needs to be confirmed. Another explanation of the fact that the live insects produced both alcohols and butyrate esters, while the crushed insects only produced the corresponding alcohols would be that enzymatic hydrolysis is amplified with crushing (Ho and Millar 2002).

When VOC emitted by crushed females were analysed, no esters were detected although a substantial amount of nonan-2-ol (**5**) was observed. This suggests that the esters are produced in real time, and that there is little or no ester storage. This hypothesis is supported by several previous studies in which pheromones were extracted from whole insects or from excised glands, but sometimes required a huge number of females to obtain a very small amount of pheromone (Gries et al. 2000; Andersson et al. 2009; Hall et al. 2012). This may also explain the comparative efficacy of HS-SPME collection, as already emphasised by Hall et al. (2009).

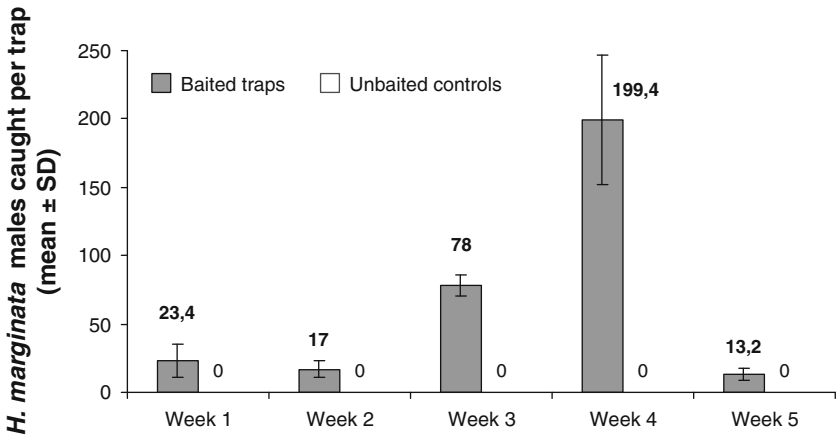


Fig. 5 Weekly mean numbers of *Haplodiplosis marginata* males caught per trap in a winter wheat field (Gembloux, Belgium) in May and June 2013. (±)-non-2-yl butanoate (**2**) dispensers were renewed weekly in baited traps. $N = 5$ for each treatment

Table 2 Mean total numbers (±standard deviation) of *Haplodiplosis marginata* adults caught per trap in a winter wheat field (Blankenberge, Belgium) between 29 May and 10 July 2013

	Baited traps (%)[a]	Unbaited controls (%)
Males	789 ± 316.2 (98)	7 ± 3.5 (54)
Females	4 ± 0.6 (2)	6 ± 3.0 (46)
Total	803 (100)	13 (100)

$N = 3$ for each treatment

[a] Traps with (±)-non-2-yl butanoate (**2**) dispensers

The main VOC emitted by *H. marginata*, namely (±)-non-2-yl butanoate (**2**) and its precursor, the (±)-nonan-2-ol (**5**), were subjected to biological tests in the field. These field experiments revealed that significant captures of *H. marginata* males required the presence of the ester, while the alcohol did not affect trap captures. This showed that non-2-yl butanoate (**2**) is the major component of the *H. marginata* sex pheromone.

The other esters, emitted in smaller proportions, are probably part of the sex pheromone blend of *H. marginata* and will be tested in further experiments. However, from a practical point of view, (±)-non-2-yl butanoate (**2**) showed sufficient efficacy to be used alone as a lure for monitoring the saddle gall midge in field traps. Indeed, use of a single racemate is much more convenient than using a pheromone blend, and much cheaper to produce.

While the attraction of males to sex pheromone is sometimes inhibited by the presence of a geometric isomer, as this is the case for the pea moth, *Cydia nigricana* (F.) (Witzgall et al. 1993) and for males and females to the aggregation pheromone of the western flower thrips, *Frankliniella occidentalis* (Hamilton et al. 2005; Dublon 2009), there seems to be no such limitation here.

Concerning the dispenser type, the prototypes used for the field tests were only intended to qualitatively demonstrate the attractiveness of saddle gall midge males to (±)-non-2-yl butanoate (**2**). As it clearly appeared that the diffusion of the sex pheromone with these prototype dispensers was too fast to allow the monitoring of *H. marginata* during the entire season, it is, therefore, necessary to develop a slow release dispenser that enables to quantitatively analyse the flight patterns of *H. marginata* over time.

In conclusion, despite the problems occurring when working with these small, delicate and short-lived insects that generally produce pheromone in very small amounts, we were able to identify the major sex pheromone component of *H. marginata*, (*R*)-non-2-yl butanoate. As in the majority of the Cecidomyiidae whose sex pheromones have been described (Hall et al. 2012), the major pheromone component of *H. marginata* is an ester produced only by females, which selectively attracts conspecific males. While most of these identified esters are acetoxy or diacetoxy compounds, *H marginata* produces butyroxy compounds. However, some other midges, such as the Chinese chrysanthemum midge, *Rhopalomyia longicauda* (Liu et al. 2009), and the orange wheat blossom midge, *Sitodiplosis mosellana* (Gries et al. 2000), are also known to produce butyroxy or dibutyroxy pheromone compounds.

Further experiments are now needed to optimise the dose of pheromone loading, dispenser type and trap design, and to investigate the effects of trap positioning, to allow an accurate monitoring of *H. marginata* in cereal crops.

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