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9 Detection of Physiologically Active Flower Volatiles Using Gas Chromatography Coupled with Electroantennography

F.P. Schiestl[1] and F. Marion-Poll[2]

9.1 Introduction

Most insect-pollinated plants produce floral odour to attract their pollinators. Fragrances and floral morphology typically advertise the presence of food rewards, i.e. nectar, pollen, or oil (Vogel 1983). Floral volatiles, however, can also be the reward themselves: some euglossine bees and fruit flies actually collect the fragrance from certain flowers and thereby pollinate them (Dodson et al. 1969; Sazima et al. 1993; Tan and Nishida 2000). Some orchids emit floral odour, typically not detectable by humans, which mimics the sex pheromone of insects, the males of which pollinate the flowers in an attempted copulation or precopulatory routine (Kullenberg 1961; Peakall and Beattie 1996; Schiestl et al. 1999). These orchids exploit their pollinators as they do not provide any reward for them. On the other hand, herbivorous insects can utilise floral volatiles to find their host plants, particularly if they feed or oviposit on the flowers (Blight et al. 1995). Floral odour is thought to have originally evolved to deter insects that fed on reproductive parts of the plant (Pellmyr and Thien 1986). Accidental pollination by these insects may have outweighed the disadvantages of their feeding activity, thus leading to a selection for a means of increasing insect attraction to the flowers.

The chemical composition of floral fragrances has been investigated in great detail in numerous plant species (e.g. Knudsen et al. 1993; Kaiser 1995). However, to understand how plants communicate with their pollinators, or how insects exploit floral signals, we need to know more about the insects' olfactory detection of floral odour compounds and how volatiles mediate their behaviour. This knowledge increases our comprehension of the evolution of certain pollination syndromes and helps us to interpret and manipulate insect behaviour (Agelopoulos et al. 1999).

The investigation of insect chemical communication has been greatly facilitated by the use of electrophysiological methods. In insect olfaction, these techniques were pioneered by Schneider (1957) who, for the first time, recorded an "electroantennogram" (EAG), i.e. a difference in potential between the tip and the base of an excised antenna of a *Bombyx mori* male stimulated with the female sex

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pheromone. Moorhouse et al. (1969) invented the coupling of the EAG with a gas chromatograph (GC). The GC column was split, with one branch leading to the detector of the GC and the other to a micro-collector in the GC oven. The effluent was collected for 15 s and then flushed over an antenna prepared for EAG recordings. The signals of the GC detector and the EAG responses were recorded simultaneously. Arn et al. (1975) improved the method by using a capillary GC column and continuously monitoring the GC effluent for EAG active compounds using an insect antenna mounted between two silver wires. This allowed components of an odour blend to be separated and tested individually for their potential to elicit EAG responses. This setup was termed the electroantennographic detector (EAD) since it could be used selectively to detect minute amounts of pheromone components. Some authors prefer the term GC-EAG, which refers to the same technique. The GC-EAD method provided an easy and very efficient tool for identifying physiologically active components (those compounds that release receptor potentials in olfactory neurons) within a blend. It has been used widely for the identification of semiochemicals in insects (Struble and Arn 1984). A further development was achieved by coupling single sensillum recordings (SSR) with gas chromatographic analyses (GC-SSR), which allowed a more sensitive and specific detection of physiologically active odour components (Wadhams 1984).

A thorough analysis of the behaviour-mediating functions of semiochemicals, including floral odour compounds, will optimally involve the following sequential steps: scent collection, evaluation of physiologically active compounds, identification and synthesis of compounds, and behavioural tests. In this chapter, we describe the methods used for these steps, with the exception of compound identification and synthesis, providing examples particularly related to floral scents. Further, we list physiologically active compounds emitted from flowers that have been identified using GC-EAD, GC-SSR, EAG, and SSR.

9.2 Collection of Floral Scent

Floral odours can be sampled by extraction of a flower or flower parts using solvent or a polymer-coated fibre (solid-phase microextraction, SPME; see Sect. 9.2.3). Typically, flower extracts predominantly comprise compounds of low volatility. Alternatively, fragrances can be collected from living flowers over longer periods of time, providing more realistic quantitative data on volatile emissions and the ratios of airborne compounds, and avoiding artefacts attributable to extraction of plant tissue. These methods also yield greater amounts of more highly volatile compounds, since floral odour is usually not stored but emitted as it is produced (Vogel 1983). In so-called headspace collections, the compounds in the headspace above a flower can be trapped on polymer (headspace sorption), in a cold trap (headspace cold trapping), or on a SPME fibre. Methods for floral scent collection have been reviewed in great detail (e.g. Dobson 1991; Kaiser 1991; Agelopoulos and

Pickett 1998; Millar and Sims 1998; Raguso and Pellmyr 1998). In the following sections we point out some important considerations to ensure that ecologically relevant odour samples are obtained, and describe the relatively new method of SPME.

9.2.1 Location of Floral Scent Emission

Floral scents are produced in a variety of flower parts such as petals, sepals, stamens, pistil, and pollen (Vogel 1983; Dobson 1994). Additionally, some flowers bear morphologically distinct structures for scent production, the osmophores (Vogel 1962). The different flower parts can produce distinctive odours, which can trigger specific behavioural reactions in pollinator insects (Bergström et al. 1995; Dobson et al. 1996; Raguso and Pichersky 1999). Pollen odour, for example, can act as close-range attractant for pollen-eating bees or beetles (von Aufsess 1960; Dobson et al. 1999). While flowers are ordinarily the source of pollinator-attracting odours, in some instances volatiles emitted from vegetative parts have been suspected to play a role in pollinator attraction (Vogel 1983). Therefore, odour collection from different parts of a flower might be necessary to sample physiologically active compounds that trigger specific behavioural reactions. It is generally advisable to check samples/fractions by EAG and/or behavioural tests prior to GC-EAD analyses to detect which samples bear biological activity. In order to collect fragrances from specific floral organs, flowers can be dissected and the scent of specific parts sampled. This, however, disconnects the tissue from its water supply and may disrupt metabolic processes. SPME may be useful to extract from different flower parts of intact flowers, when the fibre is brought in contact with the desired floral parts.

9.2.2 Variation of Scent Emission

The production of floral volatiles can vary in both space and time, which is an important consideration. Variation can be found between individuals of one plant species and even between flowers of one plant (Dobson 1991; Ayasse et al. 2000). Scent emission can be dependent upon the age of flowers and has been shown to change after cutting and also after pollination, which can influence the pollinators' behaviour (Tollsten and Bergström 1989; Schiestl et al. 1997). Some flowers show rhythmic volatile emission, often dependent on environmental factors such as the photoperiod (Vogel 1983; Altenburger and Matile 1988, 1990). The emission often correlates with pollinator activity. In some species, rhythmic emission is only expressed when the flowers are still attached to the plant (Matile and Altenburger 1988). Choice of sampling time and the use of intact plants should therefore be considered. Particularly when dealing with quantitative analyses, repeated sampling, possibly from more than one individual plant, is recommended (Raguso and Pellmyr 1998).

9.2.3 Choice of Type and Amount of Adsorbent Material

The most frequently used method for collecting floral odours is headspace sorption with Porapak Q (Super Q being a refined version), Chromosorb, Tenax TA, or activated charcoal (e.g. Supelco, Alltech) as the adsorbent material. These adsorbents can also be used in combination (Williams and Whitten 1983). Fragrances can be desorbed using solvent or heat. Porapak Q is most commonly used and has been recommended for its superior adsorbing capacity and recovery features (Dobson 1991; Raguso and Pellmyr 1998).

Since the quantity of floral odours released differs widely within plant species, the correct amount of adsorbent material is important. In the case of large amounts of volatiles being emitted by the flower, too little adsorbent material might saturate, quickly leading to bleed-through and loss of compounds. With low scent emission, however, small amounts of adsorbent are advantageous, since the sampled compounds might be masked by contaminants emanating from the adsorbent material. Schiestl et al. (1997) collected volatiles from *Ophrys sphegodes* flowers, which emit only a total amount of only $70\,ng^{-1}\,h^{-1}\,flower^{-1}$, by using cartridges containing as little as 1.5 mg of activated charcoal (Brechbühler AG, Urdorf, Switzerland; Grob et al. 1984; Kaiser 1991). Adsorbent tubes with greater amounts of charcoal proved to be completely useless due to internal contamination. Similar effects were observed with Porapak Q and glass tubes filled with 5 mg adsorbent performed well (Schiestl, unpubl. data).

A relatively new method is SPME (Supelco; Zhang and Pawliszyn 1993). This consists of a polymer-coated fused silica fibre onto which volatiles are adsorbed from a headspace or the surface of the biological material. The compounds are desorbed from the fibre through direct exposure to a heated GC-injection port operating in the splitless mode. Different fibre coatings can be chosen according to the compounds to be sampled. Special field samplers are available where adsorbed compounds are sealed behind a replaceable septum. Technical bulletins that describe different fibre coatings, cleaning procedures, etc. can be obtained from Supelco. SPME is a solvent-free method, which has the advantage of excluding interactions of solvent with odour compounds and avoiding a solvent peak in the GC analysis, which can easily mask other peaks. However, it does not provide a liquid sample, which is necessary for adding an internal standard for quantitative analysis; repeated analyses of one sample, frequently required in GC-EAD studies, as well as cool on-column injections are also impossible with this technique. However, because of its ease and flexibility, SPME might prove to be a useful tool in GC-EAD analyses of floral scent. Few studies have yet applied the SPME technique to collection of floral volatiles; MacTavish et al. (2000) used a 100-µm polydimethylsiloxane (PDS) fibre to sample volatiles from Brown Boronia flowers. Care must be taken when comparing SPME results with traditional collection techniques. The affinity and selectivity of SPME fibres towards different classes of molecules, and their small volume, which limits their trapping capacities, still need to be carefully evaluated (Bartelt 1997). In a comparative study, Agelopoulos and Pickett (1998) found the ratios of ripe banana volatiles trapped on a 100-µm PDS fibre to differ from Porapak Q and Tenax TA collections, with no differences between the two polymers.

9.3 Gas Chromatography

For a number of reasons, including its simplicity, high resolution and reproducibility of relative retention times, capillary gas chromatography (GC) has become the method of choice for analysing complex blends of volatile organic molecules (Heath and Dueben 1998). The flame ionisation detector (FID), which is most commonly used with GC, is extremely sensitive and detects nearly all classes of compounds. Floral scents, unlike many insect pheromones, typically comprise of a large number of compounds having different carbon chain lengths, functional groups, and isomeric configuration (Knudsen et al. 1993). Furthermore, abundances of individual compounds often differ greatly. The separation of such complex blends can be difficult and requires special attention. The next sections focus on methods for preliminary fractionation and GC separation of complex samples and the coupling of the GC with the electroantennographic detector.

9.3.1 Fractionation of Samples

A method to reduce the number of components in a complex sample is to split it into different fractions (Millar and Sims 1998). This is also useful if it is necessary to remove certain compounds from a sample. For example, low-volatility lipids are often the predominant compounds in flower extracts and can mask minor peaks with possible EAG activity. A sample can be separated into fractions of different polarity using a short bed of chromatographic packing (e.g. silica gel, Waters, Milford, Massachusetts). Fractions can be eluted from the column using solvents of different polarity. Thiéry et al. (1990) used purified pentane and ether to split sunflower extracts on a silica gel column into polar and non-polar fractions. Ômura et al. (2000) separated concentrated *Osmanthus fragrans* flower extract into six fractions using silica gel and hexane, benzene, benzene-chloroform (1:1), chloroform, acetone, and methanol. Andersen and Metcalf (1986) used preparative HPLC with a radial-compression-type silica gel column and a mobile phase of 10% tetrahydrofuran in hexane to collect 13 fractions of *Cucurbita maxima* volatiles. While fractionating can aid GC-EAD analyses, too many fractions will significantly complicate the procedure, since repetitive analyses of one sample type are generally necessary to discriminate reproducible EAD responses from shifts in the baseline due to noise, which may resemble an EAD response.

9.3.2 Injector Types

Two types of GC injectors are widely employed, each offering different advantages. Split/splitless injectors involve flash vaporisation of the sample and solvent, which are then swept onto the column by the carrier gas (Heath and Dueben 1998). The injector is heated to 200–300 °C and compounds that do not volatilise at this temperature will be retained. This is advantageous when crude flower extracts are injected, as these often contain low-volatility compounds that will not elute from the column. The second injector type that has commonly been used in GC-EAD studies with floral volatiles is the cool on-column injector (Thiéry et al. 1990; Wadhams et al. 1994; Blight et al. 1995). With this technique, the sample is directly introduced onto the column at low temperatures, thus avoiding degradation of thermally sensitive compounds. A retention gap (1–20 m of uncoated, deactivated, fused silica column) inserted between the injector and the analytical column is generally recommended, since it traps non-volatile compounds before they reach the analytical column (Heath and Dueben 1998).

9.3.3 Columns

The crucial part for the successful separation of compounds is the type of column used, which should be chosen according to the type of chemicals to be analysed. Most commonly used, and a good starting point for flower volatile analyses, are non-polar columns with dimethylpolysiloxane (e.g. DB-1, J&W Scientific) or 5% phenylmethylpolysiloxane (e.g. DB-5) bonded phases or polar columns with polyethylene glycol (e.g. DB-WAX). For the separation of enantiomers, which frequently differ in their biological activity, columns with chiral phases are often essential (Jones and Oldham 1999). To detect all physiologically active compounds in a heterogeneous sample, GC-EAD recordings with different columns may be necessary (Fig. 9.1). Cork et al. (1990) describe a GC-EAD system with two injectors and two columns connected at their outlet with a Y-tube connector, followed by a short capillary tubing and another Y-tube, which splits the effluent, with one part leading to the FID and one to the antenna. In this elegant setup, one column can be used to analyse a sample, with helium at 1 ml/min as carrier gas, whereas the other column delivers make-up gas at 2 ml/min for better performance of the split (see Sect. 9.3.4.1). The flow rates can be interchanged to use the other column for analysis. For simultaneous analysis of a sample using two types of stationary phases, two columns can also be connected in series (Jones and Oldham 1999). This will, however, lead to longer retention times, which is disadvantageous for GC-EAD analyses, since it is often difficult to maintain a live antennal preparation during the whole time needed for an analysis. This should also be considered when choosing the length of a single column: although longer columns generally lead to better separation, longer retention times may be a problem for electrophysiological recordings. As a good compromise, 30-m columns are commonly used for GC-EAD

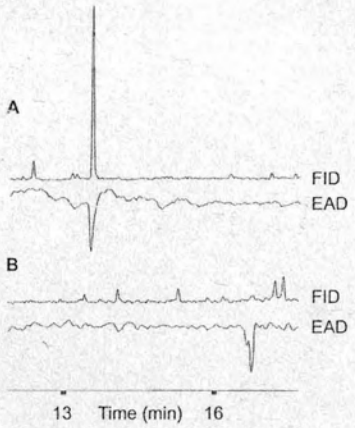

Fig. 9.1. GC-EAD analyses of the same floral odour sample on a non-polar DB-5 column (A) and a polar DB-WAX column (B). In A, the *big peak* seems to be the only active one. The second analysis (B), however, suggests that the real active peak is quite small and underlying the big one in analysis A. Additionally, the polar column resolves two active peaks. (Schiestl, unpubl. data)

analyses. The heating rate also influoftennces retention times and resolving power of a column. Whereas slow heating is often desirable for optimal resolution, in GC-EAD recordings, heating rates above 10 °C/min are generally recommended to shorten retention times and to ensure sharp peaks with a maximum amount of material being delivered onto the antenna at the onset of the peak.

9.3.4 Coupling the GC with the Electroantennographic Detector (EAD)

In order to record FID and EAD responses simultaneously, the end of the GC column needs to be split into two branches of equal length. One branch leads to the FID and the other to the EAG preparation through a heated transfer line inserted into the stimulus delivery tube (Fig. 9.2) Fig. 9.3.

9.3.4.1 Split

The use of a simple splitter (press-fit Y-connection, J&W Scientific) without the addition of a make-up gas generally works well (Thiéry et al. 1990; Schiestl et al. 2000). However, to reduce residence time and condensation of compounds in the transfer line, and to maximise FID responses, an addition of 30–60 ml/min nitrogen as make-up gas is advantageous (Fig. 9.2; Arn et al. 1975; Wadhams 1982). This can be achieved by using a four-way X-cross (SGE Australia Pty. Ltd.). The split ratio can be altered by varying the inner diameter of the fused silica transfer line or by using an adjustable microneedle valve effluent splitter (Scientific Glass Engineering Ltd., SGE). This feature might be useful if it is necessary to deliver more material to the antenna to obtain significant EAD responses.

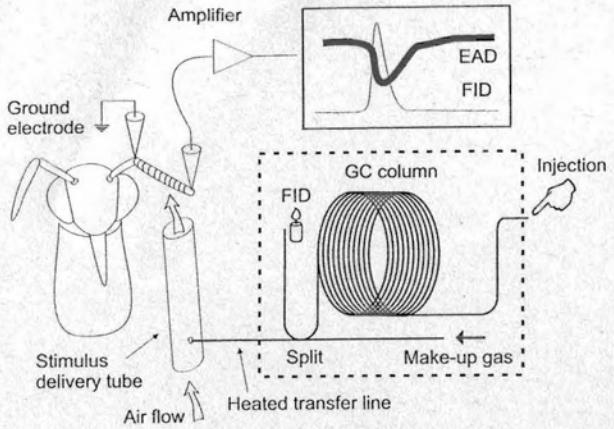

Fig. 9.2. Typical setup of a gas chromatograph with an electroantennographic detector (GC-EAD)

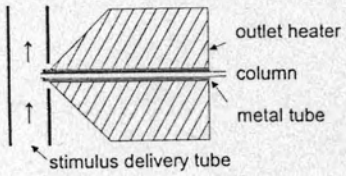

Fig. 9.3. Heating of the transfer line used by Schiestl et al. (2000) to reduce condensation of compounds on the column protruding in the cold air-flow through the stimulus delivery tube

9.3.4.2 Heating of the Transfer Line

To prevent condensation of the effluent in the tip of the split column, the transfer line into the cool air needs to be heated by an auxiliary heating system. The temperature is typically held at the maximum oven temperature or 10–15 °C above. Particularly for detecting EAD reactions to low-volatility compounds, it is crucial to properly heat the column part that leads into the cool airflow. This can be achieved by minimising the insulating air layer between the heater and the column by shielding the column in the stimulus delivery tube with a metal (optimally copper) tube, from which it protrudes only approx. 1 mm into the airflow (Fig. 9.3; Schiestl et al. 2000).

9.3.4.3 Air Flow Over the Antenna

To reduce desiccation of the antenna and avoid unwanted stimulation from contaminants in the laboratory air, the airflow (250–500 ml/min) through the stimulus delivery tube needs to be cleaned and humidified, before the effluent from the

column is directed into it. This is most easily achieved by directing the air through two gas washing bottles, one filled with activated charcoal and the other with distilled water.

9.4 Electrophysiology

To identify biologically significant compounds eluting from a GC column, the coupling of a physical detector (typically FID) with an odourant-sensing organ seems an ideal approach. Two important assumptions are required, however. The first is that only chemicals that are detected by the olfactory systems are potentially relevant: if an insect is "blind" to a compound, it is unlikely that it will influence the behaviour. The second assumption is that, if a chemical has a particular biological significance, it will be detected with more sensitivity than the other components of a sample. Therefore, when presenting a series of compounds to an insect antenna, we should be able to identify the compounds most likely to influence its behaviour. In a natural flower bouquet, some compounds are emitted in large quantities whereas others are less pronounced. However, the compounds that are likely to mirror the effectiveness of the blend are not the most abundant ones, but those that are most detectable to the olfactory receptors. Compounds that are not detected by the olfactory receptor neurons (ORNs), although they might influence factors such as the emission rates of other volatiles, will only indirectly contribute to the biological activity and may be omitted from the analysis. In the following sections we briefly review the insect's olfactory system, describe techniques for recording summed receptor potentials and single sensillum activity, highlight possible solutions for sensitivity problems, and compare different methods.

9.4.1 Olfactory System

To identify which odours are detected by the olfactory system of insects, one can use electrophysiological methods and record the electrical activity from ORNs. Fortunately, most of the ORNs are grouped on the antenna, within individual sensing units called sensilla which each houses a small number of neurons. All sensilla consists of one or several ORNs, three modified epithelial cells called accessory cells, and a cuticular structure, which may differ to a great extent between insects (Kaissling 1986). The receptor cells extend their odourant-sensitive dendrites (branched or unbranched) into the sensillum lymph, while their axons project directly into the first synaptic relay (the antennal lobe) within the central nervous system. Combined anatomical and physiological observations have led to the separation of sensilla into several types. In moths, most hair-like sensilla trichodea house two or three ORNs (Steinbrecht 1987). In honeybees,

pore plates (called sensilla placodea) house between 15 and 30 ORNs (Esslen and Kaissling 1976). On one antenna of a worker honeybee, there can be as many as 3,000 sensilla placodea (Lacher 1964). In all insects studied so far, the ORNs project into the antennal lobe, which is a central nervous structure, specialised to process this information (Hansson 1995). In the worker honeybee, one antenna bears about 65,000 ORNs that converge onto about 7,000 interneurons (Esslen and Kaissling 1976). The olfactory information thus encoded and processed is then sent to higher centres, mainly the mushroom bodies, where it is integrated with signals from other modalities and eventually memorised (Menzel 1993).

9.4.2 EAG

Two approaches are available to record an electrical signal from the antennae: single sensillum recording (SSR) and whole antenna electroantennography (EAG), with the latter being the most frequently used (Roelofs 1984). EAG records variations of potentials between the two ends of an insect antenna. During stimulation with an appropriate odourant molecule, this potential drops by a few millivolts. The shape of this response is similar to a receptor potential and it is claimed to be the sum of individual receptor potentials (Schneider 1962). Additional factors (air speed, temperature, variations in airflow), however, contribute to an EAG, and its true origins are still controversial (De Kramer and Hemberger 1987). The amplitude of the deflection is large when a significant number of olfactory receptors are simultaneously activated. There seems to be a good agreement between the intensity of the EAG response and the behavioural effectiveness of a given odourant. However, the converse is not true, i.e. the absence of a response does not imply that a substance is not detected. For example, a given odourant might stimulate, very efficiently, specialised neurons that are too few to induce a measurable EAG response. EAG has been used extensively in pheromone research on a number of insects, especially moths, to screen for effective pheromonal molecules (Roelofs 1984). The technique is simple and experience with different insect species has accumulated over the years. Used as an "electroantennographic detector (EAD)" it is suitable to detect ORNs reactions to compounds eluting from a GC column (Arn et al. 1975). Complete devices, including software, for EAG, SSR, GC-EAD, or GC-SSR are available from Syntech (Hilversum, The Netherlands; syntech@knoware.nl).

9.4.2.1 EAG Preparations

An adequate EAG signal can be obtained by different techniques. The simplest technique consists of maintaining a cut antenna between two silver electrodes, with the help of an electrocardiogram gel. This is adequate for large antennae and avoids the use of an expensive micromanipulator (antennal holders are available from

Peter Ockenfels, Freiburg, Germany, ockenfel@uni-freiburg.de). The gel prevents the desiccation of the antennal tissues and is convenient for a number of insects. The ends of a cut antenna can also be inserted into two glass capillaries mounted on a micromanipulator and filled with either insect's Ringer's solution or indeed any electrolyte solution, for example 0.1 M KCl. In case of problems with rapidly declining EAG responses, Bjostad (1998) recommends careful matching of ion concentration, osmolarity, and pH in the chemoreceptor cells and gives some recipes for electrolyte solutions. Arrangements with micromanipulators are quite convenient for small antennae and allow a great flexibility in the design of the recording setup.

Some insects are simply too small or too fragile to allow such approaches. Here, leaving the head connected to the antenna can be a possibility to get a good EAG signal (Cossé et al. 1995). In that case, the excised head can be mounted on the indifferent electrode, and the recording electrode placed at the end of one antenna. In some insects it is not necessary to cut the end of the antenna. A setup like this is good for recording EAG from insects as small as thrips (Pow et al. 1998) or aphids (Birkett et al. 2000). Some authors record the EAG from both antennae (Pow et al. 1998; Zhang et al. 1999). In general, the indifferent electrode can be inserted either at the base of one antenna, in the head, or even covering the other antenna. However, the best preparations are obtained by using undissected insects (Cork et al. 1990). In these cases, one has to design a system to immobilise the insect and choose an appropriate place to insert the indifferent electrode so that muscle potentials do not contaminate the EAG signal. Although the electrophysiological signal is generally better and the preparation more viable than with the simpler methods, one needs a more elaborate setup with several micromanipulators and a stereomicroscope placed close to the GC.

9.4.2.2 Recording an EAG

Recording an EAG signal is generally straightforward. The electrodes used for this purpose are of low impedance (around 1–10 MOhms), which means that EAG recordings are less prone to contamination by electrical interference than are SSR recordings. Shielding the preparation with a Faraday cage is usually not necessary, but may improve recordings in case of noise introduced by electrostatically charged air. In addition, this signal has a slow kinetic property and one can afford to sample it at a low frequency (1–10 Hz) provided that it is correctly filtered by pass-band filters (0.1–5 Hz, for example). Although many laboratories use standard electrophysiological amplifiers (with high-input impedance, typically 10^{12} Ohms) and filters, several authors have proposed custom-made amplifiers that perform reasonably well (Bjostad and Roelofs 1980; Bjostad 1998). In addition to amplifier and filters, an oscilloscope or a suitable computer with a data acquisition program is necessary to record and check the signal before and during an experiment. The EAG might be sampled along with the FID signal on a two-channel integrator. More integrated approaches (see, for example, Syntech) sample and store the electro-

physiological signal along with the FID signal on a computer file (Thiéry and Marion-Poll 1998).

9.4.3 GC-SSR (GC-SCR)

GC-SSR (GC-SCR) (SSR, single sensillum recording; SCR, single cell recording) uses the same principle as GC-EAD, but recordings are made from individual sensilla (Boeckh 1962; Wadhams 1982). SSR gives a good indication of the sensitivity of individual neurons. It is particularly useful when the number of stimulated neurons is small and gives only a minute EAG. For example, one active compound from the aggregation pheromone of the bark beetle *Scolytus scolytus* was detected by GC-SSR but not by GC-EAD (Wadhams 1982, 1990).

9.4.3.1 Technique

Recordings can be made from intact ORNs by inserting finely etched tungsten electrodes into the cuticle (or at the surface of it) near the target sensilla. For some insects, it is feasible to use fine glass capillary electrodes filled with an electrolyte and to record the electrical signal just through the cuticle. If one wishes to record from sensilla trichodea, it may be simpler to cut the end of the hair and cap its tip with a capillary electrode filled with sensillum saline (Kaissling and Thorson 1980). These techniques are noticeably more difficult than EAG. They require a skilled operator and more complex electrophysiological equipment. When physical space is limited, one could make use of a portable SSR system, such as the one designed by Syntech, which can be used with the cut sensillum technique.

9.4.3.2 Signal Measurement

The electrical signal recorded with these techniques has two components: fast action potentials and slow potential changes during the stimulation. Slow potential changes are usually described as receptor potentials. A receptor potential is a depolarisation affecting the ORN's membrane when its acceptor sites are stimulated by an odour. The amplitude of this potential is directly linked to the number of sites occupied and will ultimately trigger action potentials. This term is misleading, however, since the electrodes are extracellular, and this potential possibly includes potentials from several ORNs and an active potential (called a transepithelial potential) generated by the accessory cells. Nonetheless, when the ORNs are stimulated by an odour, their membrane depolarises and one can record a downward-going potential deflection that has many of the characteristics of a true receptor potential. To our knowledge, this potential has never been used in the context of GC-SSR studies.

Most studies concentrate on action potentials (APs) since they carry the information that is conveyed to the central nervous system. An AP is a rapid event (2–3 ms) which usually has the shape of a small upward-trending hyperpolarisation (500 µV–3 mV) that quickly returns to the baseline. It should be stressed that APs are generated in the membranes of the individual neurons. If the electrodes were placed intracellularly, their amplitude would be much larger. An extracellular electrode picks up only a portion of the currents generated in the neurons, currents that follow different paths in the sensillum.

When a neuron is stimulated it may be excited or inhibited. With an exciting response, it discharges APs at a high rate, up to 200–300/s. When it is not stimulated, it usually remains silent or maintains a discharge rate of 1–3 APs per second. Since several ORNs may be housed within a given sensillum, one is never sure which neuron is firing. In many experimental situations, however, it is possible to discriminate APs of different neurons on the basis of their amplitude and shape. Actually, the neurons within a sensillum have slightly different sizes, leading to the differences in the signals recorded extracellularly from them.

9.4.4 Overcoming Problems of Low Sensitivity

Electroantennograms record sums of potentials from receptor cells located in the antenna. Therefore, the number of ORNs activated by a given compound is likely to influence the detectability of these reactions. The number of ORNs selective for floral odours may be lower than receptors sensitive for pheromones. In the honeybee, receptors for queen substance are the most frequent types in drones (Vareschi 1971). The worker's antenna seems to be less specialised for detecting pheromone signals (Brockman and Brückner 1998); however, Akers and Getz (1993) identified more ORNs sensitive to citral than to geraniol > linalool > limonene; citral and geraniol are constituents of the honeybee's Nasonov pheromone (Free 1987). Given that less ORNs react to a floral odour compound than to a pheromone substance, the former may be more difficult to detect in a summed recording since the signal-to-noise ratio will be poorer.

One possibility for increasing sensitivity in GC-EAD recordings is the use of more than one antenna. Antennae can be connected in series (Moore 1981) or parallel. Antennal holders for EAG recordings from more than one antenna are available from Syntech. In the honeybee, conditioning to the odour of interest prior to EAG analysis increased the EAG responses (De Jong and Pham-Delègue 1991; Wadhams et al. 1994), which might be useful in other insects as well. It should be stressed that olfactory receptors do not perform optimally when stimulated with odour compounds eluting from a GC. GC peaks are often quite long (10–20 s) and asymmetrical. Olfactory receptors respond best to fast-changing stimuli and to the logarithm of the concentration, i.e. they encode fluctuations well at low concentration but less accurately changes at higher concentrations (Marion-Poll and Thiéry 1996). In this respect, systems that use pulsed stimuli are more appropriate for EAG recordings. This has been considered by systems that collect the effluent

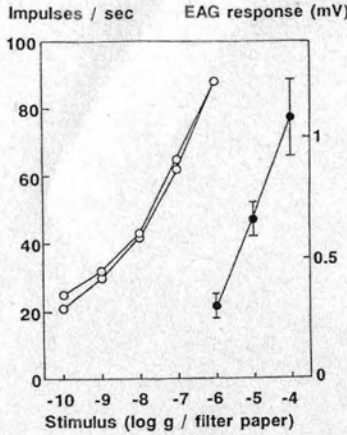

Fig. 9.4. Comparison of SCR (*open circles*) and EAG (*solid circles*) responses of honeybee antennae stimulated with 2-phenylethanol in various concentrations, demonstrating the lower threshold concentration in SSR. (Wadhams et al. 1994; C.M. Woodcock, unpubl. SSR data)

from the column and flush it over the antenna every 2–3 s (Cork et al. 1990; Gouinguené et al. 1998). These methods increase the detection threshold since the effluent is concentrated (10–100 times less molecules are needed) and reduce adaptation of antennal receptors, however, at the cost of losing resolution of the analysis.

SSRs are generally more sensitive than summed recordings, since the detection threshold for a compound is not dependent upon the number of receptors sensitive to it, and threshold concentrations for olfactory neurons have been shown to be similar, irrespective of their specificity (Fig. 9.4; Wadhams 1990). Indeed, studies employing this technique have detected ORNs sensitive for specific compounds even when no EAG reaction could be recorded (Wadhams 1984; Blight et al. 1995).

9.4.5 Comparison of EAG, GC-EAD, and GC-SSR

Although EAG has been used for the same purpose as GC-EAD by testing single synthetic compounds identified from flowers, the use of a coupled GC-EAD system has several advantages. Trace compounds with physiological activity are more likely to be detected in a coupled system, since it screens the whole sample and avoids a preselection of compounds to be tested. A GC provides a means to quantify compounds in a sample; therefore, the amounts actually being delivered onto the antenna are easier to estimate. Olfactory receptors often react very unspecifically when stimulated with large amounts of compounds (Hansson 1995). This might be one reason why many studies using EAG found all tested compounds to be physiologically active to some degree, whereas in GC-EAD investigations, typically only a subset of compounds elicited EAD reactions. When testing synthetic compounds, the GC provides a powerful device for purifying compounds, with

even enantiomers being separable. Therefore, contamination, which can also contribute to an EAG signal, can usually be detected. However, in GC-EAD analyses, multiple compounds cannot be tested simultaneously, which is necessary when investigating the detection of whole blends versus single compounds; additionally, the whole setup is considerably more expensive than an EAG device only.

The GC-SSR technique generally provides lower detection thresholds for active compounds. Additionally, information on the specificity and abundance of receptor neurons can be obtained. Recording from single sensilla is considerably more difficult, however, and hence requires more training of the experimenter. Moderately expensive electrophysiological and microscopic equipment is necessary. The method is also more time consuming, since the recorded electrophysiological signal usually needs additional processing before being used (for example to count and discriminate spikes into classes). The approach requires the sampling of many sensilla before one gets a good image of the potentialities of the olfactory system.

9.5 Behavioural Tests

In order to evaluate the behavioural activity of specific odour compounds, they need to be tested in bioassays, since electroantennographic reactions are not necessarily correlated with behavioural responses. A problem in behavioural tests with floral volatiles is that reactions to these compounds may not be hard wired, i.e. they may not elicit innate reactions in the insects, as with sex pheromone compounds. Instead, associative learning as well as other signals emitted from flowers (e.g. colours or heat) are often involved and mediate behavioural responses, thus complicating the interpretation of a test result. To complete this chapter, we briefly overview behavioural tests that have been used in connection with EAG and GC-EAD studies.

9.5.1 Attraction Tests

In some systems, behavioural responses can be elicited by providing an odour stimulus only. Synthetic, physiologically active compounds were used to trap chrysomelid beetles, *Diabrotica virgifera virgifera* (Andersen and Metcalf 1986), and the cabbage seed weevil *Ceutorhynchus assimilis* (Blight et al. 1992) in the field. Gabel et al. (1992) trapped European gravevine moths, *Lobesia botrana*, in field cages. In other insects it may be important to also present appropriate visual cues, taking into account that UV reflection can be significant. In attraction tests with the butterfly *Pieris rapae*, Honda et al. (1998) used an Erlenmeyer flask covered with green paper and a doughnut-shaped, yellow filter-paper disk dyed with 0.1% Tartrazine on top of it. Stimulus chemicals were dispersed in water in the flask or

applied on a white paper towel inserted within the flask. Schiestl et al. (2000) used dried, odourless, female bees (*Andrena nigroaenea*) onto which a blend of synthetic, pheromone-mimicking compounds identified in the early spider orchid (*Ophrys sphegodes*) was applied. These dummies were offered to male bees in the field.

9.5.2 Proboscis Extension

A commonly used bioassay involves recording the extension of the mouthparts in response to an odour stimulus, which can be interpreted as a feeding response. Such tests can be done either with unconditioned insects (proboscis extension reflex, PER) or with insects that had previously been conditioned to an odour stimulus (conditioned proboscis extension, CPE). In combined PER and attraction tests in *Pieris rapae* butterflies, EAG-active compounds with high PER activity stimulated visiting of artificial flowers more than compounds with low PER activity (Honda et al. 1998; Ômura et al. 1999b). PER was also shown to predict the deterrency potential of certain compounds in *P. rapae* better than EAG reactions (Ômura et al. 2000). In honeybees, however, individuals that were not previously conditioned to a stimulus showed no proboscis extension reaction to physiologically active odour compounds (Wadhams et al. 1994). Using the conditioned proboscis extension bioassay coupled with EAG and GC, these authors showed that most honeybees extended their proboscis only in response to three compounds out of a blend of six EAG active compounds, to which they had been conditioned (Fig. 9.5).

9.6 Compilation of Results

Tables 9.1–9.3 summarise studies on floral volatiles using GC-EAD or GC-SSR (Table 9.1), and EAG or SSR (Table 9.2). In Table 9.3, physiologically active floral odour compounds that have been identified in these studies are listed.

9.7 Concluding Remarks

Gas chromatography with electroantennographic detection provides an extremely useful tool for identification of physiologically active volatiles from complex blends of compounds. Although it has been used extensively in studies dealing with pheromones and general plant volatiles, surprisingly few investigations applied this technique to study the detection of floral odour by insects. The great diversity of plant–pollinator interactions, from generalised to extremely specialised systems,

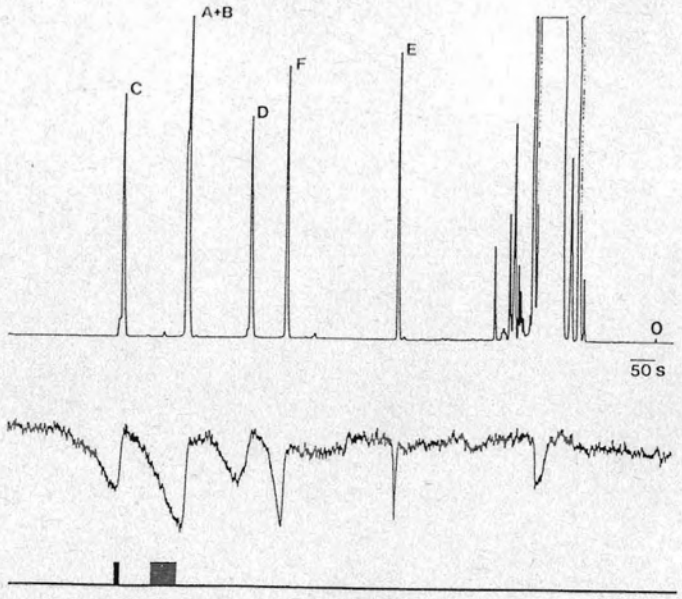

Fig. 9.5. Coupled GC-EAD-CPE recording with a six-component mixture using a conditioned honeybee. *Upper trace* represents the FID, *middle trace* EAD, and *lower trace* CPE responses. Tested compounds are *A* linalool, *B* 2-phenylethanol, *C* methyl salicylate, *D* benzyl alcohol, *E* (E)-2-hexenal, and *F* 1-octen-3-ol. (Wadhams et al. 1994; with kind permission from Kluwer Academic Publisher)

including mutual benefit and exploitation, is likely to be based on a variety of chemical communication systems. Knowledge about these mechanisms may therefore contribute to our general understanding of odour detection, insect behaviour, and the evolution of plant–insect interactions. We hope this review will stimulate GC-EAD research on floral volatiles, combined with chemical and behavioural studies.

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Table 9.1. GC-EAD and GC-SSR studies on floral odour compounds: list of investigated plant and insect species

No.	Plant sp. (Family)	Insect sp. (Order)	Motivation for flower visit	Odour collection[a]	Electrophysiology[b] Behavioural assay	Reference
1	*Brassica napus* (Brassicaceae)	*Ceutorhynchus assimilis* (Coleoptera)	Herbivory	HS	GC-EAD+GC-SCR, field trapping	Blight et al. (1992, 1995)
2	*Chrysanthemum* (*Dendranthema morifolium*, Asteraceae)	*Frankliniella occidentalis* (Thysanoptera)	Herbivory	HS	Olfactometer, glasshouse trapping	Pow et al. (1998)
3	*Cirsium arvense* (Asteraceae)	*Autographa gamma* (Lepidoptera)	Food reward	HS	Wind tunnel	Plepys (unpubl.); Plepys et al. (2000)
4	*Centaurea scabiosa* (Asteraceae)	*A. gamma*	Food reward	HS	Wind tunnel	Plepys (unpubl.)
5	*Helianthus annuus* (Asteraceae)	*Apis mellifera* (Hymenoptera)	Food reward	FE	–	Thiéry et al. (1990)
6	*Malus* x *domestica* (Rosaceae)	*Anthonomus pomorum* (Coleoptera)	Herbivory	HS	–	Kalinová et al. (2000)
7	*Medicago sativa* (Fabaceae)	*A. mellifera*	Food reward	Synthetics	–	Henning and Teuber (1992)
8	*Nepeta faasseni* (Lamiaceae)	*A. gamma*	Food reward	HS	Wind tunnel	Plepys (unpubl.)-
9	*Ophrys sphegodes* (Orchidaceae)	*Andrena nigroaenea* (Hymenoptera)	Mating behaviour	FE	Field tests	Schiestl et al. (2000)
10	*Platanthera bifolia* (Orchidaceae)	*A. gamma*	Food reward	HS	Wind tunnel	Plepys (unpubl.)-; Plepys et al. (2000)
11	*Saponaria officinalis* (Caryophyllaceae)	*A. gamma*	Food reward	HS	Wind tunnel	Plepys (unpubl.)-; Plepys et al. (2000)
12	*Tanacetum vulgare* (Asteraceae)	*Lobesia botrana* (Lepidoptera)	Unknown	FE	Attraction in field cage	Gabel et al. (1992)
13	*Trifolium pratense* (Fabaceae)	*A. gamma*	Food reward	HS	Wind tunnel	Plepys (unpubl.); Plepys et al. (2000)
14	General floral odour	*A. mellifera*	–	–	CPE	Wadhams et al. (1994); Pham-Delègue et al. (1997)

[a] HS: Headspace collection, FE: flower extracts
[b] GC-EAD if not otherwise mentioned
CPE: Conditioned proboscis extension

Table 9.2. EAG and SSR studies on floral odour compounds: list of investigated plant and insect species. *HS* Headspace collection; *FE* flower extracts

No.	Plant sp. (Family)	Insect sp. (Order)	Motivation for flower visit	Odour collection	Electrophysiology[a] Behavioural assay	Reference
1	*Brassica napus*	*Apis mellifera* (Hymenoptera)	Food reward	HS	CPE	Pham-Delègue et al. (1992)
1.1	(Brassicaceae)	*Ceutorhynchus assimilis* (Coleoptera)	Herbivory	HS	–	Evans and Allen-Williams (1992)
2	*B. rapa* (Brassicaceae)	*Pieris rapae* (Lepidoptera)	Food reward	HS, FE	PER, attraction test	Ômura et al. (1999b)
3	*Clarika breweri*	*Sphinx elegans* (Lepidoptera)	Food reward	HS[b]	–	Raguso and Light (1998)
3.1	(Onagraceae)	*Hyles lineata* (Lepidoptera)				Raguso et al. (1996)
4	*Cucurbita maxima* (Cucurbitaceae)	*Diabrotica undecimpunctata howardi* (Coleoptera)	Food reward	HS	Field trapping	Andersen and Metcalf (1986)
5	*Ligustrum japonicum* (Oleaceae)	*P. rapae*	Food reward	FE	PER, attraction tests	Honda et al. (1998)
6	*Ophrys insectifera* (Orchidaceae)	*Argogorytes mystaceus* *A. fargei*	Mating behaviour	HS, FE	–	Ågren and Borg-Karlson (1984)
6.1	O. insectifera					
6.2	O. insectifera	*Gorytes quinquecinctus*				
6.3	O. insectifera	*G. quadrifasciatus* (all Hymenoptera)				
7	*Ophrys* spp.	*Eucera tuberculata* (Hymenoptera)	Mating behaviour	FE	–	Priesner (1973)
8	*Osmanthus fragrans* (Oleaceae)	*P. rapae*	Repelled	FE	PER, attraction tests	Ômura et al. (2000)
9	*Prunus yedoensis* (Rosaceae)	*Luehdorfia japonica* (Lepidoptera)	Food reward	FE	PER	Ômura et al. (1999a)
10	General floral odour	*Bombus hypnorum*, *B. terrestris* (Hymenoptera)	–	–	–	Fonta and Masson (1984)
11	General floral odour	*Spodoptera littoralis* (Lepidoptera)	–	–	SCR	Anderson et al. (1995)
12	General floral odour	*S. elegans*	–	–		Raguso and Light (1998)
13	General floral odour	*H. lineata*	–	–		Raguso et al. (1996)

[a] EAG if not otherwise mentioned

Table 9.3. Physiologically active floral odour compounds, which have been identified by GC-EAD, GC-SSR, EAG, and SSR. *Superscript numbers* refer to numbers of references listed in Tables 9.1 and 9.2

GC-EAD and GC-SSR	EAG and SSR
Fatty acid derivatives	
Alkanes	**Alkanes**
Octadecane[13]	Undecane[6,6.1]
Heneicosane[9]	Tridecane[6,6.1]
Docosane[9]	Pentadecane[6,6.1]
Tricosane[9]	Nonadecane[6]
Tetracosane[9]	
Pentacosane[9]	**Alkenes**
Hexacosane[9]	1-Heptadecene[6,6.1]
Heptacosane[9]	
	Aldehydes
Alkenes	(*E*)-2-Hexenal[5]
(*Z*)-9-Pentacosene[9]	
(*Z*)-12+(*Z*)-11-Heptacosene[9]	**Ketones**
(*Z*)-9-Heptacosene[9]	(*Z*)-Jasmone[12,13]
(*Z*)-12+(*Z*)-11-Nonacosene[9]	
(*Z*)-9-Nonacosene[9]	**Alcohols**
	Butanol[10]
Acids	(*E*)-2-Hexen-1-ol[5]
Hexadecanoic acid[5]	(*Z*)-3-Hexen-1-ol[1.1,5]
	Octan-1-ol[5,6,6.1,6.2,6.3]
Aldehydes	1-Octen-3-ol[5]
Hexanal[1]	
(*E*)-2-Hexenal[1,5,14]	**Esters**
Octadecanal[9]	3-Methylbutyl acetate (Isoamyl acetate)[10]
Eicosanal[9]	(*Z*)-3-Hexen-1-yl acetate[1.1]
	Methyl jasmonate[13]
Ketones	γ-Decalactone[8]
3-Octanone[7]	
Alcohols	
1-Penten-3-ol[5]	
1-Pentanol[1]	
2-Methylbutanol[7]	
3-Methyl-1-butanol[5]	
1-Hexanol[1,5]	
(*Z*)-3-Hexen-1-ol[1,4]	
1-Octen-3-ol[1,14]	
Tetradecanol[5]	
Esters	
Decyl acetate[7]	
Dodecyl acetate[7]	
(*Z*)-3-Hexenyl acetate[1,2,7]	
Methyl (D)-lactate[4]	
Methyl hexadecanoate[13]	
2-Nonyl tetradecanoate[9]	
2-Nonyl hexadecanoate[9]	
Dodecyl dodecanoate[9]	
Dodecyl tetradecanoate[9]	

Table 9.3. *Continued*

GC-EAD and GC-SSR	EAG and SSR
Aromatic compounds	
Aldehydes	**Acids**
Benzaldehyde[1,3,10,13]	Benzoic acid[9]
4-Methoxybenzaldehyde (p-Anisaldehyde)[1]	
Phenylacetaldehyde[1,11]	**Aldehydes**
Vanillin (4-Hydroxy-3-methoxybenzaldehyde)[5]	4-Methoxybenzaldehyde[9]
4-Hydroxy-2-methoxy cinnamaldehyde[5]	Benzaldehyde[2,5,9,12,13]
	Phenylacetaldehyde[2,5,9,11,12,13]
Alcohols	Vanillin (4-Hydroxy-3-methoxy benzaldehyde)[3,3.1,10]
Benzyl alcohol[1,10,11,14]	Veratraldehyde[3,3.1]
2-Methoxyphenol (guaiacol)[1]	(*E*)-Cinnamic aldehyde[12,13]
2-Phenylethanol[1,14]	
Cinnamic alcohol[10]	**Alcohols**
	Benzyl alcohol[1,2,5,9,12,13]
Esters	4-Methoxybenzylalcohol[9]
Benzyl acetate[10]	2-Phenylethanol[1,2,5,8,9,12,13]
Benzyl benzoate[3,10,11,13]	Eugenol[3,3.1,5,10]
Benzyl salicylate[10]	Methyl isoeugenol[3,3.1]
Methyl benzoate[10,11]	
Methyl 2-methoxybenzoate[3]	**Esters**
Methyl salicylate[1,2,3,7,10,14]	Amyl salicylate[12,13]
	Benzyl acetate[3,3.1]
	Benzyl benzoate[3,3.1]
	Benzyl salicylate[12,13]
	Ethyl phenylacetate[5,9]
	Methoxy-2-methyl benzoate[12,13]
	Methyl benzoate[5,12,13]
	Methyl phenylacetate[5]
	Methyl salicylate[1,3,3.1,5]
	Methyl 4-methoxybenzoate[9]
	Methyl cinnamate[12,13]
	2-Phenylethyl acetate[5]
Isoprenoids	
Monoterpenes	**Monoterpenes**
Bornyl acetate[5]	1,8-Cineole[12,13]
3-Carene[6]	Citronellol[6,6.1,6.2]
Chrysanthenone[2]	Geranial[10]
1,8-Cineole[1,5]	Geraniol[5,10,11,12,13]
p-Cymene[2,12]	Geranyl + neryl acetone[6,6.1]
d-Limonene[12]; Limonene[7]	Linalool[1,1.1,5,6.1,8,9,12,13]
Linalool oxide[3,13]	Linalool oxides (*E*/*Z*-Furanoid)[3,3.1,8]
(±) Linalool[1,3]; Linalool[7,14]	Linalool oxide (*Z*-Pyranoid)[3.1,8]
Myrcene[7]	Linalool oxide (*E*-Pyranoid)[8]
Myrtenal[5]	d-Limonene[5]; Limonene[1.1,6,6.1,10,12,13]
Myrtenol[5]	β-Myrcene[5]; Myrcene[1.1,6,6.2,12,13]
Ocimene[7,8]	Nerol[10]
Perillene[6]	(*E*)-β-Ocimene[12,13]
Piperitone[12]	allo-Ocimene[13]
Sabinene[1]	α-Pinene[1.1]

Table 9.3. *Continued*

GC-EAD and GC-SSR	EAG and SSR
(*E*)-Sabinene hydrate[5]	β-Pinene[1,1]
Terpinene-4-ol[12]	Sabinene[1,1]
(*Z*)-Thujenol[5]	Terpinene-4-ol[6,6,1]
α-Thujene[12]	
α-Thujone[12]	**Sesquiterpenes**
β-Thujone + Thujyl alcohol[12]	γ-Cadinene[7]
(*Z*)-Verbenol[12]; verbenone, verbenol[1]	δ-Cadinene[7]
	Caryophyllene[1,1]
Sesquiterpenes	α-Cedrene[1,1]
(±)-Caryophyllene[4,6,13], β-Caryphyllene[2]	α-Copaene[7]
Caryophyllen oxide[5]	Cyclosativene + cyclocopacamphene[6,6,1]
β-Elemene[5]	α-Farnesene[1,1]
(*E*,*E*)-α-Farnesene[1,2,6]; α-Farnesene[3,11,13]	Farnesol[6,12,13]
(*E*)-β-Farnesene[2]	Germacrene D[12]
Farnesol[9]	γ-Muurolene[7]
Farnesyl acetone[13]	Nerolidol[3,6]
Farnesyl hexanoate[9]	(*E*)-Nerolidol[5]
Germacrene D[5]	α-Ylangene[7]
	Irregular terpenoids
	α-Ionone[8]
	β-Ionone[8]
	6-Methyl-5-hepten-2-one[5]
Nitrogen-containing compounds	
Allyl isothiocyanate[1]	2,5-Dimethyl-3-isoamylpyrazine[6,6,1,6,2,6,3]
Benzyl cyanide (Phenylacetonitrile)[1]	2,6-Dimethyl-3-isoamylpyrazine[6,6,2]
3-Butenyl isothiocyanate[1]	5,6-Dimethyl-2-isoamylpyrazine[6,6,1,6,2,6,3]
Indole[1,13]	Indole[2,4,12,13]
Goitrin[1]	2-Methyl-butyraldoxime[12,13]
4-Pentenyl isothiocyanate[1]	Phenylacetonitrile[2]
2-Phenylethyl isothiocyanate[1]	Methyl anthranylate[12,13]
Others	
2,3,3-Trimethyl-epoxy cyclopentyl acetaldehyde[5]	
Naphtalene[13]	
Propiovallinone[5]	

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10 Analysis of Rhythmic Emission of Volatile Compounds of Rose Flowers

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10.1 Introduction: Rhythmicity in Emission of Volatile Compounds, How and Why

Many phenomena in plants and animals show rhythmic patterns in activity and/or appearance. This rhythmicity may be controlled directly by periodic changes in environmental conditions such as temperature or light, e.g. generation of electrons by photosynthetic pigments in relation to daily changes in exposure to light. Many other processes, which are dependent on this activity, will show rhythmicity with the same interval but with a delay from which the length of time is dependent on intermediary reactions. When there is no further regulatory mechanism other than the abundance or intensity of the external regulatory signal, the rhythmicity of the phenomena will disappear when the signal loses its rhythmicity, e.g. when the plant is kept in constant dark or light.

Some phenomena, which follow a rhythmically changing environment, continue to show rhythmicity even when the external signal becomes constant. Such endogenous or "circadian" rhythms were first discovered for plants by De Mairan (1729) and later more extensively described by Bünning and Stern (1930) who observed that movements of bean leaves continued under constant light or dark. Jones and Mansfield (1975) formulated two additional characteristics which are accepted as typical for circadian rhythmicity. These additional requirements are: (1) a cycle time upon exposure to constant environmental conditions, the so-called free-running period, which is slightly different – mostly longer – than 24 h, and (2) the possibility to induce a phase shift in rhythmicity by changing the periodicity of the signal that controls the cycle, e.g. by introduction of a single 24-h period of constant darkness into a 12-h light /12-h dark regime.

One of the phenomena that is strongly regulated by light–dark cycles is the emission of volatile compounds by flowers. Rhythmicity in emission is related to the activity pattern of pollinating insects (Gimenes et al. 1996). Therefore, plant species that are pollinated by night-flying insects such as moths show nocturnal rhythmicity, with maximum emission during the dark period, while others that are pollinated by bumble bees or honey bees exhibit a diurnal pattern with maximum

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