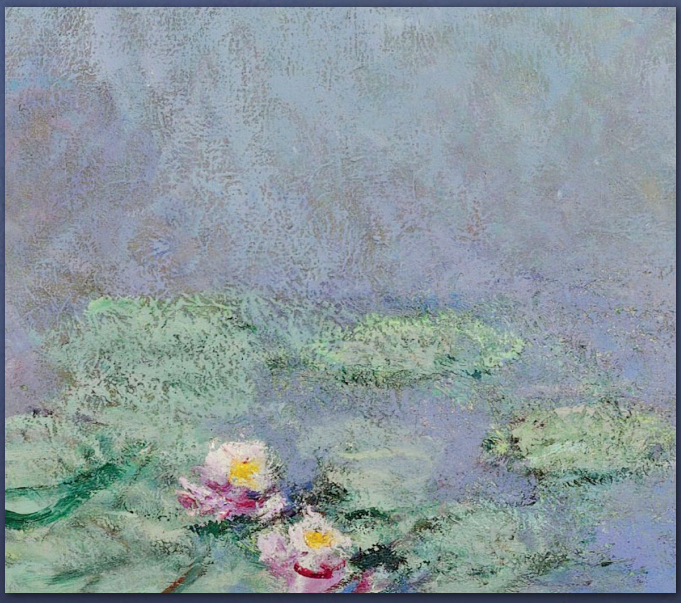
Clinical Pharmacognosy Series

AROMATHERAPY

Basic Mechanisms and Evidence-Based Clinical Use



Edited by
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Aromatherapy: Basic Mechanisms and Evidence-Based Clinical Use

Giacinto Bagetta, Marco Cosentino, and Tsukasa Sakurada

Herbal Medicines: Development and Validation of Plant-Derived Medicines for Human Health

Giacinto Bagetta, Marco Cosentino, Marie Tiziana Corasaniti, and Shinobu Sakurada

Natural Products Interactions on Genomes

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Preface

Aromatherapy is a specialized form of phytotherapy that uses essential oils extracted from diverse parts of aromatic plants more often delivered via inhalation or massage for several minor clinical uses. Essential oils are phytocomplexes made up of several components endowed with a broad spectrum of biological activities, some of which have been deciphered only recently.

A PubMed search on April 13, 2015, using "essential oils" as the keyword phrase resulted in 13,844 articles dating back to 1880, the year of the first article published by H.C. Wood and E.T. Reichut in the prestigious *Journal of Physiology*: "Note on the Action upon the Circulation of Certain Volatile Oils." The reading of this article has an obvious historical meaning, though it anticipates the experimental complexity in studying such phytocomplexes, starting from the important, and not so obvious, qualitative differences in their biological effects in living animals, emerging by changing their route of administration.

In the industrialized countries, the interest in aromatherapy increased a great deal during the second half of the last century for treating stress-induced symptoms of anxiety, mood, and sleep disorders and certain forms of pain, among other disorders. During the last couple of decades, basic research yielded a large body of information in experimental models of diseases, probably not considered enough to provide the rational basis for further research and development of discrete essential oils to be assessed for their efficacy and safety in clinical trials. Instead, controlled clinical trials have been completed in some age-related neurodegenerative diseases where no preclinical data have been generated. Among the most distressing features of dementia are the behavioral and psychological symptoms. Addressing this facet has received particular interest in aromatherapy trials, with a shift in focus from reducing cognitive dysfunction to the reduction of behavioral symptoms in dementia. In fact, some behavioral disorders occurring in demented patients appear to be sensitive to the beneficial action of aromatherapy in a way similar, if not superior, to atypical antipsychotics, at least in some of these trials. Obviously, this is of great importance in view of the lack of major side effects compared to antipsychotics. The notion that olfaction is often dysfunctional in demented people, as well as in other neurodegenerative diseases, is per se a demonstration of the importance of the systemic absorption and distribution of the pharmacologically active components of the phytocomplex for aromatherapy to control disordered behavior, thus minimizing the power of the reported psychological action. For a correct understanding of the latter, however, an entire chapter is dedicated to the transduction mechanisms of odorant signals, immediately after the fascinating historical notes on essential oils and aromatherapy. Along the lines of the previous book in the series, *Herbal Medicines: Development and Validation of Plant-Derived Medicines for Human Health*, (Giacinto Bagetta, Marco Cosentino, Maria Tiziana Corasaniti, Shinobu Sakurada, eds., CRC Press, 2011) in Section I, several chapters are dedicated to botanical, biotechnological, phytochemical, technological, and quality issues concerned with standardization of the natural resource to limit variability of the phytocomplex. Section II reviews the

recent growing literature around the cytotoxic and cytoprotective effects of essential oils, together with the underlying mechanisms dissected *in vitro* using mostly tumor cell lines. The rational basis for further research and development of aromatherapy in therapeutic areas in great demand of innovation, such as infectious and inflammatory diseases and control of diverse forms of pain, is reported in Section III. At variance with the latter, research and development of aromatherapy are far from being granted in the cancer area, where more controlled preclinical studies *in vitro* and *in vivo* are needed. As anticipated above, evidence-based information is scarce and available for controlling symptoms associated with neurodegenerative diseases and some pediatric conditions. The use of aromatherapy in dementia care settings is due to increase a great deal because it is one of the few options attractive to practitioners and families, as patients often have reduced insight and ability to verbally communicate adverse reactions. The book ends with important notes on the epidemiology of essential oil utilization in conjunction with the need for pharmacovigilance and phytovigilance for safer use of aromatherapy. Despite limited regulatory issues, together with fundamental aspects of clinical trial planning, performing and reporting are key aspects for the scientifically correct development of this growing area of phytotherapy.

This book should therefore be available to undergraduate and PhD students of pharmacy and health courses and professionals involved in aromatherapy in the clinic for research, regulatory, or therapeutic purposes. The wealth of references collected in the book make it a good venue from which to search for valuable information by scientists dealing with essential oils. Finally, the complete set of knowledge on essential oils and aromatherapy reported herein should be made available to any individual for rationale counseling and competent marketing and to policy makers in health systems worldwide.

The editors are indebted to Hilary LaFoe, whose professional skill made it possible for our venture to come true. Also, we would like to sincerely thank all the people from Taylor & Francis for their highly qualified technical contributions, and all our collaborators. A special thanks goes to Dr. Damiana Scuteri for valuable collaboration during the whole editorial process of the book.

Giacinto Bagetta
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Editors

Giacinto Bagetta, MD, (1983), (consultant in pharmacology since 1987) has been a full professor of pharmacology since 1994 in the Department of Pharmacy, Health Science and Nutrition, University of Calabria, Rende, Italy. He is the author of approximately 160 papers (see PubMed) in internationally indexed journals (H-index 40, Citations > 5000, ISI Web of Knowledge; Top Italian Scientist and Top Ten Scientist of the University of Calabria established by Via-Academy), and an editor of eight books produced by Pergamon Press, Elsevier plc, and CRC Press. He has been an invited speaker at prestigious international research institutions such as Imperial College London (UK), the University of Tokyo School of Dentistry, the University of Sendai School of Medicine and Tohoku Pharmaceutical University, Sendai, Department of Pharmacology, University School of Medicine of Wakayama (Japan), Burnham Research Institute, La Jolla (USA), the MRC Toxicology Unit, Leicester (UK), and others. He was awarded the International Galien Prize for Young Investigator. He is a member of the Italian Societies of Pharmacology, Neuroscience and of the International Society for Neurochemistry; a member of the editorial board of high-impact-factor journals such as *Current Opinion in Pharmacology* and a past member of the editorial board of the *Journal of Neurochemistry*, *Journal of Neuroscience Methods*, and *Journal of Chemotherapy*; a referee for prestigious, internationally recognized scientific journals and research-funding agencies such as The Wellcome Trust (UK), the National Council for Research (CNR, Italy), the Italian Space Agency (ASI), and the Ministry of Scientific Research of the Austrian Government; and a member of the National Health Research Committee at the Ministry of Health from 1996 to December 2010. He was a member of the ethical committees at the Regional General Hospital of Cosenza and IRCCS Mondino Foundation (PV) from 1996 to 2013.

Marco Cosentino, MD, PhD, a professor of medical pharmacology at the University of Insubria (Varese, Italy), earned his MD degree (cum laude) from the University of Pavia and his PhD in pharmacology and toxicology from the University of Turin. He is the director of the Center for Research in Medical Pharmacology and of the School of Specialization in Medical Pharmacology. He is also a coordinator of the PhD program in experimental and clinical medicine and medical humanities. His main research interests include neuro- and immunopharmacology, clinical pharmacology and pharmacogenetics, pharmacoepidemiology and pharmacovigilance, and pharmacology of herbal medicines. He has published more than 100 full-length papers in international scientific journals indexed by ISI-WoS and Scopus, and has served as a referee for more than 50 indexed journals. He is also a reviewer for several public and private funding agencies and academic institutions, including the National Multiple Sclerosis Society (New York, USA), the Slovak Research and Development Agency, the Research Foundation–Flanders (FWO), The Ohio State University (USA), and the University of Southern California (USA). He has served on the editorial board of *Acta Phytotherapeutica* and *Neuroendocrinology Letters*, and is presently on the

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1 Historical Notes on Essential Oils and Aromatherapy with Special Reference to Bergamot Essential Oil

Alfredo Focà and Maria Carla Liberto

CONTENTS

1.1 HISTORICAL NOTES

Before the advent of medical textbooks, and even the written word, we used plants, roots, leaves, and flowers, not only for nutrition, but also as a protection against disease—particularly infection arising as a result of wounds, traumas, and accidents. This instinctive, or folk, medicine has been around as long as we have walked the earth, and officinal plants are still widely used as natural remedies today, the practice evolving alongside progress in scientific understanding. Indeed, primitive preparations of plant matter for medicinal use involving simple processes such as crushing, mastication, and maceration were largely superseded by more complex methods of preparation, such as infusion, digestion, decoction, percolation, distillation, and enfleurage (enfleurage—both hot and cold—is the most ancient "technical" method of obtaining plant-based remedies), made possible by the discovery of fire.

These more advanced methods, many of which are still in use today, furnished more accurate and effective preparations—essential oils, for example, which were prized not only for their medicinal properties, but also as perfumes, and their scope of application continued to evolve alongside further advances in technology. Distillation in particular represented an early but important technological leap, and we know that the Chinese practiced a rudimentary form of distillation 6000 years BCE; they also made use of techniques such as filtration and sublimation. Similar techniques are also thought to have been used by the ancient Sumerians, and

fragments of what appears to be primitive distillation equipment have been found at archaeological sites in Mesopotamia—the so-called cradle of civilization—dating back to 5000 years BCE. Evidence of the ancient Egyptians' familiarity with home-made remedies comes in the form of inscriptions on the perimeter wall of the Temple of Horus at Edfu (Behedet). Dating back 2000 years, these inscriptions comprise a kind of medical handbook, a phytopharmaceutical recipe book displayed for all the populace to see and make use of. We also know that the ancient Egyptians were using purpose-designed apparatus for distilling wine and cider even earlier than that, 4000 years BCE.

The oldest literature source we have illustrating the use of distillation apparatus is conserved in the Biblioteca Marciana in Venice (Ms. of St. Mark, gr. 299, fol. 88v) and dates back to around the second century BCE. Attributed to the alchemist (the doctors of the age) known to posterity as Cleopatra, the ancient Greek papyrus document *Chrysopoeia* clearly shows a workable distillation setup of the same name comprising a wide-necked double alembic heated over a water bath and featuring two inclined lateral tubes through which the distilled liquid could be collected (Figure 1.1).

Similar, but less detailed, illustrations can be seen in the alchemical text Ms. 2327 of the Bibliothèque Nationale in Paris, and in the Leyden papyrus, purportedly written at the end of the third century CE. Zosimos of Panopolis, another prominent alchemist, who practiced his art around the turn of the fourth century CE and wrote the oldest known books on the subject, also left us with an illustration of distillation apparatus observed in an ancient temple at Memphis, alongside instructions for preparing the so-called divine waters, that is, distilled liquids of various types, including plant-based essential oils (Figure 1.2). As a condensation system was still lacking at this time, the heated vapors produced during the distillation process were

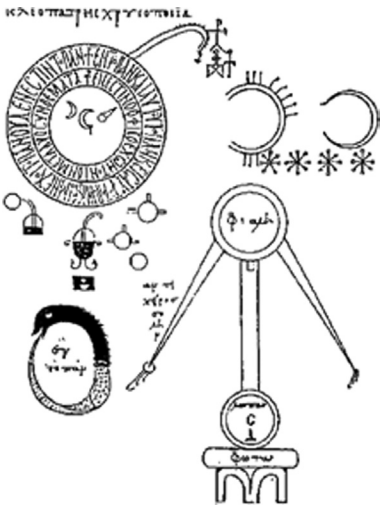

FIGURE 1.1 Cleopatra's *Chrysopoeia*. (From Ms. of St. Mark, gr. 299, fol. 88v.)

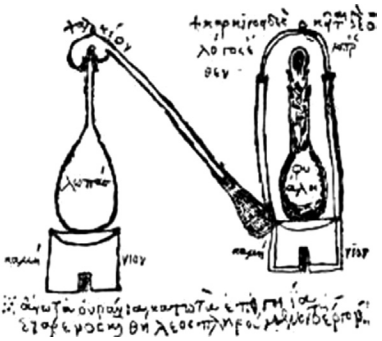

FIGURE 1.2 Distillation apparatus of Zosimos. (From Marcelin Berthelot, *Collection des anciens alchimistes grecs*, 3 vol., Paris, 1887–1888.)

dripped onto fabric pads, which were later squeezed to obtain the few drops of precious liquid produced.

The Arabs, who learned the technique from the ancient Egyptians, became masters of distillation techniques—indeed, the terms *alembic* and *alcohol* are clearly of Arabic origin—and made great strides in the process of extracting essential oils. Khālid al-Qasrī, (?–743 CE), alchemist and governor of Iraq under the Umayyad Caliphate, perfected the distillation technique to produce essential oils from flowers, particularly the rose. Jabir al-Sufi (Geber) (c. 721–c. 815), another Arabic alchemist, classified two extraction techniques, one making use of fire (distillation) and the other without (filtration), which he used to separate two clear liquids by means of a filter. Muhammad ibn Zakariyā Rāzī (854–c. 925), a Persian alchemist, philosopher, and physician, known simply as Razi or Rhazes in the West, and the most eminent chemist of his time, described several methods of distillation: *per ascensum*, *per descensum*, and *per latus*. In his copious works he described the preparation of *aqua vitae*, a concentrated solution of ethanol, and how to concentrate alcohol through distillation, passing alcohol vapors over hot coals or ash, a method he also used to extract perfume from flowers.

The various techniques for obtaining aqua vitae and perfumes developed over the centuries helped scholars to isolate and understand the natural essences derived from plants, and to describe the protective properties of such aromatic substances against infection. The Greek physician, pharmacist, and botanist Dioscorides (Anazarbe, c. 40–c. 90), for example, wrote one of the first written records of the scientific use of therapeutic preparations obtained from plant matter. For distillation, Dioscorides used a piece of equipment that he called the ambic (helmet), from which the Arabic term *alembic* later arose, and his comprehensive encyclopedia of herbal medicine, *De Materia Medica*, was widely circulated throughout the civilized world for centuries after his demise.

Dioscorides himself, not to mention Galen and Hippocrates, is likely to have drawn on the work of Theophrastus (c. 371–c. 287 BCE), a Greek scholar and successor to Aristotle who is today considered the father of botany. Indeed, his *Historia Plantarum*, an encyclopedic work of 10 volumes, classified more than 500 types of plants, focusing on the anatomy and propagation of trees, shrubs, "undershrubs," cereals, legumes, and herbs. The ninth volume of the series described the therapeutic uses of plants and is one of the earliest medicinal herbals to be written. He later

expanded upon this theme in his *On the Causes of Plants*, another weighty series of eight volumes.

These texts continued to be relevant in the tenth century (and beyond), when another Persian polymath and father of early modern medicine, Ibn-Sīnā, more widely known in the West as Avicenna (930–1037), introduced a convoluted cooling tube into the distillation apparatus. He described the use of a heating and cooling system for extracting volatile essential oils, which comprised aromatic liquids and what was considered to be their active, antibacterial principle. The conviction that essential oils, and therefore the plants they were obtained from, had antibacterial properties persisted until the Middle Ages (alongside Dioscorides's "pharmacopoeia") and explains why plague doctors wore the distinctive beak-like masks described by Thomas Bartholin in 1661 (Figure 1.3); these masks contained cotton pads soaked in aromatic liquids to filter, and therefore protect them from, the miasma (putrid air), thought to be the means by which the disease was spread.

Avicenna extracted essence of rose by boiling petals of *Rosa centifolia* in a curved alembic and collecting the resulting vapor. The extraction of rose essential oil evidently became fairly commonplace in the Arab world of the early Middle Ages, being described in a number of Byzantine texts. Considered a valuable commodity and exported as far as India and China, Persian rosewater was recommended as a medicament by Theophanes Nonnus, physician to Emperor Michael VIII. It was also immortalized as a toilet water in Constantine Porphyrogenitus's (emperor of the eastern Roman empire) codex of ceremonies, published in 946 CE. Giovanni Plateario, in his *Circa Instans*, tells us that the technique was adopted by the School of Salerno, one of the most influential schools of medicine in eleventh-century Europe and precursor to the universities of today.

FIGURE 1.3 Thomas Bartholin's plague doctor wearing a "beak" mask filled with aromatic herbs.

Although the use of plant extracts for phytotherapeutic purposes was initially linked to empirical observation, popular customs more likely to be prescribed by shamans and charlatans than by physicians, as the technology evolved, scientific testing has borne out their validity in many cases, granting phytotherapy and aromatherapy a return to respectability, even in the medicine of today. In fact, essential oils from a considerable number of plant species have important applications alongside or as alternatives to better-known natural or synthetic remedies for treating diseases of microbial origin. Although nowadays we can point to the active ingredients and their specific mechanisms of action, the officinal plants from which these essential oils derive are the same used in ancient times, however crude their preparation might first have been.

Modern-day scientific literature contains numerous examples of natural substances with antimicrobial properties (antibacterial, antiviral, antifungal, and antiprotozoal) [1–5], and phytopharmaceuticals also possess certain other advantages, including low toxicity, ease of handling, pleasant smell, and collateral properties, that enhance their effectiveness [6,7] and make them much sought-after remedies even in these enlightened times. Among the substances derived from nature, essential oils are particularly useful for healing purposes, as they possess marked antiseptic and antimicrobial properties, among others. They also possess great evocative power in being able to stimulate the olfactory system (volatile fraction) via a complex synaesthesic process that, although largely unconscious, is exquisitely perceptive and closely linked to memory. Our sense of smell is regulated by a specific area of the brain that has close anatomical ties to the limbic system, specifically the hippocampus, and the olfactory cortex.

Odorant molecules, for example the volatile products of essential oils, are detected by receptors on the olfactory sensory neurons in the mucous lining of the nasal cavity. These sensory neurons project axons to the brain through the olfactory nerve, a cranial nerve that carries electrical signals through the cribiform plate of the ethmoid bone and toward the olfactory bulb, which in turn projects to the olfactory cortex. The olfactory bulb is the site of the so-called olfactive memory, the ability to decipher and discriminate the various aromatic compounds present in the volatile fraction of essential oils. Olfaction develops and is modulated differently in different individuals, but in all cases it enables us to form immediate connections between a particular smell and our own specific memories and emotions. Through its connections to the hypothalamus, the olfactory bulb can stimulate the release of powerful psychoactive mediators and hormones, such as serotonin and noradrenaline, thereby explaining the various sedative, relaxant, euphoric, and stimulant properties of essential oils.

Essential oils are complex substances secreted by plants for various purposes: to attract pollinators, for cell-to-cell communication, and to protect against pests and predators, to name but a few. They are stored in secretory structures located either inside (secretory cavities, ducts, or cells) or on the surface (glandular trichomes) of the plant. Their organoleptic, phytotherapeutic, and psychoactive properties are conferred not only by each of the various chemical constituents, but also by the strong synergistic action they exert. Hence, their characteristics will vary in function depending on the plant species they are extracted from (there is considerable variation between species of the same genus), the part of the plant they are extracted from

(fruit, leaf, flower, etc.), the microclimate of the region their parent plants inhabit, the changing of the seasons, and the technique by which they are extracted. There is a wide variety of chemical constituents in the different essential oils [8], and even those from related plant species will exhibit markedly different characteristics. Taking sage, for example, *Salvia sclarea* produces an essential oil of low toxicity and excellent therapeutic properties, while *Salvia officinalis*, common sage, is far more toxic, due to the presence of thujone—a ketone and monoterpene with a menthol odor, also found in wormwood—among its constituents.

At room temperature, essential oils generally form liquids of various densities, although some have a resinous consistency. They are generally pale orange to yellow in color, although those that contain azulene may be green or blue (*Matricaria chamomilla*, *Helichrysum italicum*). Essential oils tend to darken with age via a process of oxidation, and as the name suggests, they are insoluble in water but soluble in alcohol, organic solvents, and vegetable oils.

Until very recently, the branch of medicine that studies the properties of essential oils, aromatherapy, has been considered a niche speciality, and very few articles on such substances have found their way into the mainstream scientific publications. Gattefossé [9] was the first (1926) to introduce the concept of aromatherapy and indicate some specific healthcare applications for essential oils [10,11]. Since then, however, the majority of studies into the phytotherapeutic benefits of officinal plant extracts, including the essential oils, have been regarded as something of a curiosity and relegated to the status of empirical or alternative medicine. Nevertheless, as laboratory methods advance, more and more repeatable, recognized, and above all, scientific research has been carried out and is beginning to find a wider audience among the readership of accredited journals. Initially such research was focused on globally known (and highly fashionable) plants such as the tea tree (*Melaleuca alternifolia*), but nowadays the scope of aromatherapy has broadened considerably to include a wide variety of plant sources (Table 1.1).

As mentioned, the specific properties of an essential oil will depend on its chemical composition, and therefore the part of the plant it is obtained from and the variety of plant itself. The activity of citrus oils from the leaves of *Citrus aurantium*—the Seville orange—for instance, are high in esters (linalyl acetate), whereas their flowers have a greater preponderance of alcohols (linalool). To give another example, the bark of the cinnamon tree yields an oil rich in cinnaminic aldehyde, while oil from the leaves of the same plant has a high phenol content.

The antiseptic properties of many plants have been recognized empirically for millennia, but the effects of essential oils from common plants used for such purposes are now being confirmed by science. Chamomile (*Chamaemelum nobile*), for example, has long been used to cleanse wounds, burns, and boils, and many other common European plants, including the red fir, silver fir, birch, angelica seeds, basil, and sweet flag (*Acorus calamus*), are also used to produce oils of antiseptic action (Table 1.1). Other, rarer and more exotic, sources of antiseptic oils include sandalwood, myrrh, and the round leaf buchu (*Agathosma betulina*). Cardamom, cinnamon, and caraway oils are antiparasitic as well as antiseptic, and oil of cloves has long been used in dentistry as an analgesic and antiseptic. Rockrose (*Cistus*) yields another powerful antiseptic oil and is one of the first aromatic herbs to be mentioned

TABLE 1.1
Antimicrobial Activities of Essential Oils

Binomial Name	Common Name	AB	AM	AV
Abelmoschus moschatus	Ambrette (seeds)			
Abies alba	White spruce			
Abies balsamea	Balsam fir (needles)	+		
Abies sibirica	Siberian fir			
Achillea millefolium	Yarrow			
Acorus calamus	Sweet flag			
Allium sativum	Garlic			
Amyris balsamifera	Sandalwood			
Anethum gravolens	Dill			
Angelica archangelica	Angelica (seeds)			
Aniba rosaeodora	Brazilian rosewood			+–
Anthemis nobilis	Roman chamomile	+–		
Apium graveolens	Celery			
Aquilaria malaccensis	Agarwood			
Artemisia dracunculus	Tarragon	++	+	
Artemisia pallens	Dhavanam			
Artemisia vulgare	Wormwood			
Barosma betulina	Buchu			
Betula alba	Birch			
Boswellia carterii	Frankincense	++		
Boswellia rivae	Frankincense			
Brassica nigra	Black mustard			
Cananga odorata	Ylang-ylang			
Canarium luzonicum	Elemi			
Carum carvi	Caraway			
Chamaemelum nobile	Roman chamomile	+–		
Cinnamomum canphora	Camphor			
Cinnamomum cassia	Chinese cinnamon			
Cinnamomum verum	Ceylon cinnamon (bark)	+++^	++	+–
Cinnamomun zeylanicum	Ceylon cinnamon (bark)			
Cistus ladaniferus	Brown-eyed rockrose	+	+–	+
Citrus aurantifolia	Lime		+–	+
Citrus aurantium am. (*flos*)	Neroli	++	+–	
Citrus aurantium am. (*fol*)	Seville orange	+	+++*	
Citrus aurantium am. (*per*)	Bitter orange		+–	
Citrus aurantium am. (*sinensis*)	Sweet orange		+–	
Citrus aurantium dulcis	Citrus dulcis			

(*Continued*)

TABLE 1.1 (CONTINUED)
Antimicrobial Activities of Essential Oils

Binomial Name	Common Name	AB	AM	AV
Citrus bergamia	Bergamot	+++		++
Citrus cedra	Citron			
Citrus grandis	Shaddock/Pomelo			
Citrus limon	Lemon			+
Citrus paradisi	Grapefruit	++		
Citrus reticulata	Mandarin			
Commiphora erythraea	African myrrh			
Commiphora myrrha	Myrrh			+
Copaifera officinalis	Copaiba			
Coriandrum sativum	Coriander	++	+−−	
Corymbia citriodora	Spotted gum			
Cuminum cyminum	Cumin		+−	+−
Cupressus sempervirens	Cypress	+		+−
Curcuma longa	Turmeric			
Cymbopogon citratus	Lemongrass	+−	+−	
Cymbopogon martinii	Palmarosa	+−	+−	+
Cymbopogon nardus	Citronella			
Daucus carota	Wild carrot (seeds)			
Elettaria cardamomum	Cardamom		+−	
Eucalyptus citriodora	Lemon-scented gum	++	+−	
Eucalyptus globulus	Tasmanian blue gum	+++^^	+−−	
Eucalyptus dives	Broad-leaved peppermint	+	+−−	+
Eucalyptus radiata	Narrow-leaved peppermint	+	+−−	+−
Eucalyptus smithii	Gully gum	+−		+−
Eucaria spicata	Australian sandalwood	+	+++*	
Evernia prunastri	Oak moss			
Foeniculum vulgare dulce	Fennel	+	+—	
Gaultheria procumbens	Wintergreen			
Helichrysum angustifolium	Curry plant	++		
Hyssopus officinalis	Hyssop	++	+−−	+
Illicium verum	Star anise		+−−	
Inula heleinium	Elecampane/horse-heal		+	+−
Kunzea ericoides	Kanuka		+−	
Juniperus communis	Common juniper			
Juniperus virginiana	Red cedar			
Laurus nobilis	Bay			+−
Lavandula angustifolia	Lavendar	+++^^^	++*	
Lavandula intermedia super	Lavandin		+−−	+−

(*Continued*)

TABLE 1.1 (CONTINUED)
Antimicrobial Activities of Essential Oils

Binomial Name	Common Name	AB	AM	AV
Lavandula latifolia	Spike lavander	+	+−−	+
Leptospermum scoparium	Manuka	+−	+−	+
Levisticum officinalis	Lovage			++
Lippia citriodora	Verbena			
Litsea cubeba	May Chang			
Matricaria recrutita	German chamomile	+−	+	
Melaleuca alternifolia	Tea tree	+++^	++*	
Melaleuca leucadendron	Cajeput	+++^	++*	++
Melaleuca viridiflora	Broad-leaved paperbark	++	+−−	++
Melissa officinalis	Lemon balm/Melissa		+−−	+
Mentha arvensis	Field mint		+−−	
Mentha piperita	Peppermint	++	+−	+
Mentha spicata	Spearmint		+−	
Mentha suaveolens	Apple mint			
Myristica fragrans	Nutmeg	+		
Myrtus communis	Myrtle	+	+−	
Nardostachys jatamansi	Spikenard		+	
Nepeta cataria	Catnip		++*	++°
Ocimum basilicum	Basil	+	+	+
Origanum heracleoticum	Greek oregano		++	
Origanum majorana	Sweet marjoram	+++	+−−	+
Origanum vulgare	Oregano	++		
Ormenis multicaulis	Moroccan chamomile	+		
Pelargonium graveolens	Geranium	++		+−
Pelargonium asperum	Geranium	++	+−−	+−
Petroselinum sativum	Parsley			
Picea abies	Norway spruce			
Pimenta dioica	Allspice			++
Pimenta racemosa	West Indian bay			+
Pimpinella anisum	Aniseed	+−	++*	
Pinus cembra	Swiss stone pine			
Pinus pinaster	Maritime/cluster pine			
Pinus sylvestris	Scots pine			
Piper nigrum	Black pepper	+−		++
Pistacia lentiscus	Mastic			
Pogostemon cablin	Patchouli		+	
Ravensara aromatica	Clove nutmeg	+−		++°°
Rosa damascena	Damask rose	+		
Rosmarinus officinalis	Rosemary	+	+−	+
Rosmarinus officinalis verbenone	Rosemary verbenone	+	+−	++

(*Continued*)

TABLE 1.1 (CONTINUED)
Antimicrobial Activities of Essential Oils

Binomial Name	Common Name	AB	AM	AV
Salvia officinalis	Sage		+	++
Salvia sclarea	Clary sage			
Santalum album	Indian sandalwood			
Santalum spicatum	Australian sandalwood		+–	
Satureja hortensis	Summer savory	+++^		+–
Satureja montana	Winter savory	+++^	+––	+–
Syzygium aromaticum	Clove	+++^	++	++°
Tagetes marigold (patula)	Marigold	+	+–	
Tagetes minuta	Southern cone marigold			
Thuya occidentalis	Northern white cedar			
Thymus capitatus	Spanish oregano	+++^	+++*	
Thymus mastichina	Spanish majoram	+	+–	
Thymus serpyllum	Wild thyme	+	+–	+–
Thymus vulgaris	Thyme	+++^	+–	++
Valeriana officinalis	Valerian			
Vetiveria zizanioides	Vetiver			
Zingiber officinalis	Ginger			

Source: Adapted from Price, S., and Price, L., *Aromatherapy for Health Professionals*, Churchill Livingstone Elsevier, London, 2012.

Note: AB: Antibacterial activity; AM: Antimycotic activity; AV: Antiviral activity; ^, Gram-positive and Gram-negative; ^^, *Diplococcus pneumoniae*; ^^^, betahemolytic *Streptococcus*. *, *Candida* species; °, herpes simplex; °°, herpes zoster.

in writing. The tribes of the Amazon have long relied on the antiseptic, fungicidal, and anti-inflammatory properties of Copaiba oil, which is rich in oleic and linoleic acids. Closer to home, the various populations that inhabit the Mediterranean region have an ancient tradition of exploiting the fungicidal and antibacterial properties of coriander oil, and coriander seeds were even found in the tomb of Egyptian pharaoh Ramses the Great. Antibacterial action has also been documented for the essential oils of garlic and Seville orange (obtained by cold-pressing the fresh peel), which is also fungicidal. Other antifungal antibacterial essential oils include sweet orange, lavender, lemon, laurel, rose geranium, and hyssop. The antiseptic properties of cajeput oil have been attributed to a specific antiviral action, and essential oils from the cedar and spotted gum can be used to treat cold sores (herpes simplex). Eucalyptus oil is also effective against the herpes virus, and this, as well as its antiseptic action, makes it useful in the treatment of respiratory infections.

The antimicrobial and antiseptic actions summarily described above are potentiated by the many other benefits of essential oils, such as anti-inflammatory, cicatrizing, stimulant, and fluidificant properties. Little wonder then that, over the centuries,

in some parts of the world the cultivation of the source plants for particular essential oils has shaped the geographical surroundings and culture of certain populations, who have wisely preserved and transmitted the relative know-how down through the generations, despite the waxing and waning of the interest in such products.

1.2 BERGAMOT: A REGIONAL TREASURE

The *Citrus bergamia* (bergamot), cultivated for centuries in a certain region of Calabria, southern Italy, produces an essential oil with certain biochemical characteristics that has long been undervalued and used predominantly as a stabilizer for the luxury fragrance market. However, oil of bergamot has recently been proven to possess a powerful antimycotic antimicrobial action [12], and its volatile products are antiseptic, cicatrizing, and psychoactive [13]. Its antimicrobial properties have attracted interest from researchers from all over the globe, and it has been the subject of numerous articles recently published in major scientific journals [14], testament to their scientific rigor. This has thrust bergamot oil from its humble origins as a local product firmly onto the international stage.

There are several varieties of the bergamot plant (*Citrus bergamia*, family Rutaceae) grown in Calabria, namely "*castagnaro*", "*femminello*", and "*fantastico*". Its branches are irregular and its leaves dark green, and it grows best in sunny areas close to the sea. Its flowers, which appear at the end of March in its preferred climate, are highly fragrant and known in the local dialect as *zagara* (from the Arabic *zahara*, "flower"). The fruit, the bergamot orange, is rounded in shape and has a peel (epicarp) that turns from green to yellow as it ripens. The peel is covered with the utricles that contain the essential oil. The endocarp or pulp, which makes up 65%–70% of the fruit, contains juice that is used in medicine to combat high cholesterol, and the spongy mesocarp is rich in pectin (Figure 1.4).

FIGURE 1.4 *Citrus bergamia*.

The fruits are harvested from October to January, as soon as they are ripe enough for the utricles to open and permit the ready extraction of their essential oil. Until the advent of the industrial revolution, bergamot oranges were picked by hand, and their clear greenish yellow oil was extracted with the aid of a sharp knife and collected in natural sponges. In 1844, however, Nicola Barilla invented "the Calabrian machine," which was specifically designed to extract the essence of bergamot. Nowadays, however, this has been superseded, and the process considerably accelerated, by hydraulic peeling machines with rotating rollers or plates.

1.3 BERGAMOT ESSENCE: A HISTORY OF THE RESEARCH

Empirical observations on the antimicrobial, cicatrizing, and balsamic properties of the essence of bergamot were first published in 1800 by Francesco Calabrò, a physician from Reggio Calabria [15]. According to Calabrò, bergamot had been grown commercially in Calabria since before the mid-1700s. This was disputed by several sources, which stated that the first commercial crop was planted in 1750 in the countryside close to Reggio Calabria, but in reality, Gian Paolo Feminis from Santa Maria Maggiore, near Novara, had begun to market his bergamot-based "Admirabilis Aqua" as a painkiller as early as 1660, presumably after the essential oil had become available. Later, in 1676, Feminis relocated to Cologne, and in 1727 he patented this "miraculous" water as "Eau de Cologne," whereupon it was promptly pirated in Italy and elsewhere.

Calabrò, thanks to his medical training, was the first to commission a detailed chemical analysis of bergamot extract [16] in the attempt to provide scientific support for the empirical evidence. Although he did make accurate reference to the possible deposition of gelatin or gluten products that may promote wound healing, the chemist that Calabrò entrusted with this analysis was unable to characterize the volatile products of the essence, to which the latter ascribed its "heroic virtues," leading him to conclude that "given its physical qualities, I would like to believe that, in addition to its excitatory and stimulatory action, it must possess other singular properties in the treatment of wounds, the nature of which we are unaware, whose action depends on its principal components."

Undeterred by the technological limitations of the age, Calabrò widened his research and proposed the use of compresses soaked in a moderate quantity of bergamot essence to heal lacerations, contusions, and knife wounds. He also recommended its use in fever and reported an interesting anecdote regarding its antimalarial properties: "In 1760, Colonel Bernardo Scasanto used the essence as an antimalarial. In fact, having been given 5–6 kilos of essence of bergamot from a nephew from Reggio Calabria, Salvadore Pandari, he used it to treat the soldiers of his regiment affected with tertian or quartan fever."

Calabrò's observations were subsequently published in the *Annales de Thérapeutique* [17], a French medical journal, and commented upon in detail by another Calabrian physician, Francesco Rognetta. Rognetta noted that the workers employed to extract bergamot essence using very sharp knives often cut themselves, but due to the fact that their hands were soaked in bergamot oil, they healed rapidly without medical intervention; he therefore concluded that essence of bergamot promoted cicatrization and prevented suppuration.

Another physician from Reggio Calabria, Vincenzo De Domenico, also studied the medicinal properties of bergamot, describing several cases of malarial fever that he treated with bergamot essential oil, and noted its dynamic hypotensive action [18]. He also used it to bring down fever, with consistently encouraging results, and as a vermicide. Being a liquid, according to De Domenico, bergamot oil could readily be administered using any type of beverage as a vehicle. De Domenico also decided to test this potion in its topical form for its effectiveness against scabies. Once again, he reported very encouraging results, which led him to recommend it to Don Ferdinando Bergamo, surgeon to the 12th regiment, stationed at that time in Reggio Calabria. Bergamo appears to have used this "ointment" to cure many cases of scabies, noting that the essence "heals even the scabs." He also went so far as to state that bergamot essence was a more efficacious treatment than sulfur, as it "acts on the mood of the patient" [19]. To provide experimental proof of his observations, De Domenico conducted a series of trials, first testing the essence of bergamot on dogs, and then, to verify its effect on "healthy men," he decided to self-administer, describing its effects with extreme precision [18].

These initial empirical observations were soon followed by more scientific investigations into the benefits of bergamot essential oil, with specific focus on its antiseptic and antibacterial properties. In 1869, the Scottish chemist Robert Angus Smith, taking up where his predecessor, David MacBride, left off, confirmed the antiseptic efficacy of the volatile oils of various essences, bergamot included [20,21], and Charles Chamberland, celebrated French microbiologist and friend to Louis Pasteur, demonstrated the inhibitory effect of several on the growth of anthrax bacteria (*Bacillus anthracis*) [22]. In 1894, Francesco Maltese, a lecturer in dentistry at the University of Naples, investigated the use of a bergamot-based preparation in his field [23], inspiring Guido Bracchetti to use a 10% solution of bergamot essence to sterilize root canals and fully heal infected periapical and periradicular tissues, demonstrating its superiority with respect to other essences, like oil of cloves and thyme, as well as carbolic acid [24,25].

Giuseppe Sergi, in an article published in Reggio Calabria in 1925, described the properties of bergamot essence as follows: "This essence rapidly moderates and heals wounds, impeding their suppuration; if applied promptly, it impedes phlogosis and cyanosis in surgical lesions, fortifies and vivifies healthy tissue, and, finally, encompasses the virtue of a strongly aromatic analgesic; it is necessary to apply it always to the affected parts by means of a little cotton impregnated with it in the quantities deemed to be sufficient" [26,27].

Around the same time, Arturo Sabatini, military physician, found that in its raw state the essence of bergamot was an excellent antiseptic and rapidly cicatrized the alveolus and gum after dental extraction, as well as sterilizing dental caries and curing various disorders of the scalp. Among its advantages, he highlighted the fact that it did not irritate damaged tissue, even in high doses. Bursting with tables and experimental data, Sabatini's paper also compared the effects of bergamot essence with those of other essences and their vapors, calculating the time to its onset of action on various bacterial species *in vitro*. His paper also contained the results of his *in vivo* experiments, conducted on guinea pigs and frogs, which he used to calculate the lethal dose [28]. Sabatini also repeated research performed by Albert Morel and

Anthelme Rochaix, who, in his series of works on bergamot essence, had obtained very satisfactory results in treating meningitis, diphtheria, typhus, and pustules [29].

In the decade that followed, Prof. Antonino Spinelli, head surgeon at Reggio Calabria hospital, experimented with bergamot essence in his operating theater. He successfully used it for disinfecting small wounds, and in the treatment of contused lacerations and anfractuous lesions, concluding that it possessed evident bactericidal action against both cutaneous and dermal infections. He added that it neither stained the skin nor caused irritation or toxicity upon absorption, and was particularly useful for treating putrid, malodorous wounds, due to its pleasing fragrance. Spinelli confirmed his deductions regarding the efficacy of the preparation via a battery of *in vivo* tests on rabbits, guinea pigs, and rats [30].

In 1933, Fulvio Pulcher, from the University of Genoa, published the results of his research demonstrating the disinfectant action of a bergamot essence soap emulsion, with which he successfully overcame the difficulties in obtaining a stable hydroalcoholic preparation of the essential oil [7], citing studies on its antiseptic action by Robert Koch, C. Cadéac, and A. Meunier (bactericidal effect on the glanders bacillus), C. Guargena, W. Collier, and Y. Nitta, and others. Also in 1933, Prof. Giovanni Carossini, head surgeon at Reggio Calabria hospital, published his observations on a disinfectant called Sabeol, invented and patented by a certain Dr. Usellini of Milan, which was made from bergamot essence "purified and subjected to a particular chemical treatment." Carossini was enthusiastic about its excellent disinfectant properties and its pronounced detersive and anaesthetic effects on wounds and sores, and he also noted that it produced marked keratinization of ulcers [6]. The effectiveness of bergamot as a surgical disinfectant was also confirmed 2 years later by Prof. S. Puglisi-Allegra, who used a 15% alcoholic solution of the essence in surgery and in the treatment of suppurating lesions [31].

One of the milestones in the rich history of publications on the properties of the essence of bergamot is the study by the man considered the father of aromatherapy, Prof. René-Maurice Gattefossé. In his laboratory at the University of Lyon Faculty of Medicine, he tested various formulations of the essence (lotions, irrigations, powders, ointments, tablets, syrups, paints, etc.) in numerous infective processes of the skin and respiratory and urinary systems and on wounds, with excellent results [9,32]. Another luminary, Giuseppe Sanarelli, hygiene lecturer for the Universities of Bologna, Siena, and Rome, also conducted a wide-ranging study on the properties of bergamot. Published in 1936, his memoirs recounted a series of tests conducted on an aqueous solution of the essence called Bergamon, which had been prepared by means of a process developed by another Calabrian son, Dr. F. Romeo. Sanarelli described two preparations, Bergamon-alpha and Bergamon-beta, the latter being more concentrated, and demonstrated the bactericidal action of both on typhus and diphtheria, as well as pyogenic *Staphylococcus* species and *Vibrio cholerae*. He concluded that this bergamot-based disinfectant was of comparable efficacy to better-known, more powerful antiseptics, but did not possess their disadvantages; like the essential oil it was based on, Bergamon had a pleasant smell, did not stain, irritate, or corrode, and above all, was nontoxic, making its field of experimentation and application almost limitless [33]. Citing an article that had appeared in the *Journal of the American Association of Medical Research* in November 1935, Romeo described

specific cases in which an aromatic disinfectant like Bergamon would be particularly beneficial to patients, namely childbearing women, neurasthenics, convalescents, and those affected by insomnia and respiratory disorders.

Bergamon was also popular with Antonino Spinelli [34], who, like Dr. Attilio Anedda of the University of Cagliari, used it in surgical settings. In 1940, the latter also reported his observations on its usefulness in the treatment of scabies [35]. The same topic was discussed by Lt. Col. Dr. Dogalino Maimone of Rome's military hospital [36] and many other Italian civilian and military researchers. In ophthalmology, Bergamon was used with excellent results by G. Gandolfi and G. Boari of Parma University [37], as well as Carlo Gandolfi of the same university's eye clinic [38]. Prof. Pompeo Scoto from the University of Cagliari used it in obstetrics [39] and described it as a "powerful antiseptic at various dilutions, and a rapid cicatrizing agent."

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2 The Olfactory System
From Odorant Molecules to Perception

Simone Pifferi and Anna Menini

CONTENTS

2.1 INTRODUCTION

The process leading to olfactory perception begins in the nasal cavity, where odorant molecules reaching the olfactory epithelium bind to a large number of different odorant receptors located in the cilia of olfactory sensory neurons. The number of odorant receptors varies between about 300 in humans and 1200 in mice, representing about 1%–4% of proteins encoded by the entire genome. However, each olfactory sensory neuron expresses only one odorant receptor type that can bind different odorant molecules. Vice versa, each odorant molecule can bind to several odorant receptors according to a unique combinatorial code. Axons of olfactory sensory neurons send information to second-order neurons (mitral and tufted cells) in the olfactory bulb, which in turn project to several cortical areas. Sensory coding in the

olfactory bulb is based on a high level of convergence of projections from neurons of the olfactory epithelium. Indeed, all olfactory sensory neurons expressing a given odorant receptor project to specific synaptic units, called glomeruli, in the olfactory bulb. Thus, an individual glomerulus represents a single odorant receptor, and each odorant produces the activation of a unique combination of spatially invariant glomeruli in the olfactory bulb.

From the olfactory bulb, mitral and tufted cells transmit signals from glomeruli to cortical neurons in several anatomically distinct areas collectively named the primary olfactory cortex, including the piriform cortex and the cortical amygdala. It is of interest to note that, different from other sensory systems, the olfactory system sends signals directly to the cortex without first connecting to the thalamus. Two different patterns of projections have been found from the olfactory bulb to the piriform cortex and the cortical amygdala. The topographical organization of odorant information of the olfactory bulb is not maintained in the piriform cortex, where axons of mitral and tufted cells from a given glomerulus project diffusely without apparent spatial order. Moreover, individual odorants activate a sparse ensemble of piriform neurons without spatial preference. In the cortical amygdala, projections from different glomeruli are organized in broad but anatomically distinct regions, suggesting that the cortical amygdala may be critical in processing information related to innate behaviors.

Each area of the olfactory cortex sends information to other regions of the brain and also has extensive projections back to the olfactory bulb, allowing top-down modulation of the olfactory bulb activity. Neurons of the primary olfactory cortex directly project to the orbitofrontal cortex and also to the hippocampus, hypothalamus, and mediodorsal nucleus of the thalamus. Moreover, several of these regions are reciprocally connected. This complex neuroanatomical organization is at the basis of olfactory perception, a process that depends not only on the specific odorant molecules binding to odorant receptors, but also on how the brain filters, organizes, and interprets the olfactory information according to several other factors, including learning, experience, expectations, attention, memory, and emotion.

This chapter presents an overview of the current knowledge of the organization and function of the olfactory system in mammals, from the binding of odorant molecules to odorant receptors to human olfactory perception.

2.2 ODORANT MOLECULES

Odorant molecules are small volatile organic compounds with a molecular mass of <300 Da that activate odorant receptors (see Section IV) and the subsequent components of the olfactory system. Odorant molecules include aliphatic and aromatic compounds with various functional groups, such as esters, alkanes, cyanides, imines, thiols, halides, formates, amines, ketones, carboxylic acids, alcohols, and aldehydes, as shown in Figure 2.1a. In general, an olfactory stimulus is composed of a complex mixture of dozens of different types of odorant molecules. For example, it has been estimated that a rose emits more than 200 types of volatile molecules (Ohloff 1994), although not all of them bind to odorant receptors. A recent study investigated the

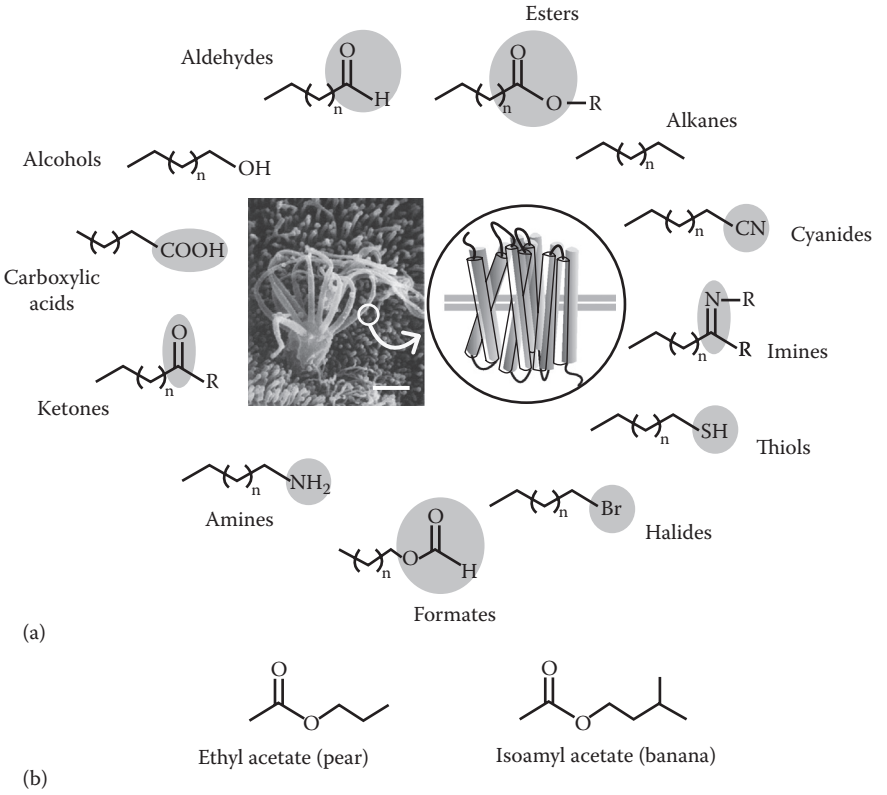

FIGURE 2.1 (a) Chemical structures of several functional groups in some odorant molecules. Center, left: Scanning electron micrograph of the knob of a human olfactory sensory neuron with several cilia. (Modified from Morrison, E.E., and Costanzo, R.M., *J Comp Neurol* 297: 1–13, 1990. With permission.) Center, right: Schematic representation of an odorant receptor showing seven-transmembrane domains typical of GPCRs. (b) Chemical structures of molecules that smell like a pear and a banana.

number of discriminable olfactory stimuli and estimated that humans can discriminate more than 1 trillion olfactory stimuli (Bushdid et al. 2014), whereas previous estimates indicated that humans could discriminate about 10,000 olfactory stimuli. As the human visual system seems to be able to discriminate among 2.3 million and 7.5 million colors and the auditory system can distinguish about 340,000 tones (Nickerson and Newhall 1943; Pointer and Attridge 1998; Stevens and Davis 1983), the human olfactory system outperforms other sensory systems in the number of different stimuli it can discriminate (Bushdid et al. 2014).

The olfactory system has very high detection capabilities; indeed, some odorants have a very low detection threshold. For example, ethyl mercaptan (ethanethiol) can be perceived by humans at a concentration as low as 1 part in 2.5 billion parts of air (Whisman et al. 1978). For this reason, ethyl mercaptan is added to natural gas, which is odorless, as a warning for escaping gas.

The olfactory system also has a high power of discrimination among small changes in the chemical structure of an odorant molecule. For example, ethyl acetate is the main component of the odor of pear, while the structurally similar isoamyl acetate mediates the odor of banana (Figure 2.1b). Moreover, some pairs of enantiomers can elicit different odors. For example, R-carvone smells like spearmint, while its mirror image, S-carvone, smells like caraway (Laska and Teubner 1999).

2.3 NOSE AND OLFACTORY EPITHELIUM

During inspiration through the nostrils, odorant molecules reach the olfactory epithelium, located in the upper part of the nasal cavity (Figure 2.2). The nasal cavity is largely occupied by turbinates that determine the direction of the airflow, and during normal respiration, only 5%–10% of the inhaled air reaches the olfactory epithelium. However, many animals actively explore the presence of odorant molecules in the external environment by sniffing. A sniff consists of taking air into the nose in short breaths to increase the percentage of air reaching the olfactory epithelium, without carrying the air deep into the lung. In humans, a typical sniff lasts on average 1.6 s, with an inhalation velocity of 27 L/min and a volume of 500 cm^3 (for review, see Mainland and Sobel 2006).

Odorant molecules reach the olfactory epithelium not only with the air passing through the nostrils (orthonasal pathway), but also through the nasopharynx

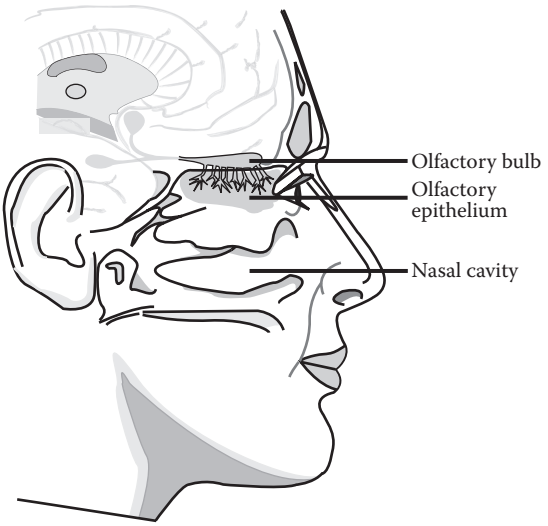

FIGURE 2.2 Anatomical organization of the olfactory system. The olfactory epithelium is located in the upper part of the nasal cavity. Odorant molecules can reach the olfactory epithelium both with the air inspired through the nostrils (orthonasal pathway) and with the air expired through the nasopharynx when food or drinks are ingested (retronasal pathway). Axons of olfactory sensory neurons in the olfactory epithelium directly project to the olfactory bulb passing through the cribriform plate of the ethmoid bone.

(retronasal pathway) during mastication or drinking. The retronasal pathway plays a fundamental role in the generation of food or drink flavor, as volatile molecules released in the mouth during mastication reach the olfactory epithelium through the nasopharynx during expiration (for review, see Shepherd 2006).

The olfactory epithelium is a specialized pseudostratified epithelium mainly composed of three types of cells: olfactory sensory neurons, sustentacular cells, and basal cells (Figure 2.3). In humans, the olfactory epithelium has an average surface of 2–4 cm^2 (Leopold et al. 2000; Moran et al. 1982) and contains about 6 million olfactory sensory neurons in each nasal cavity (Moran et al. 1982; Rowley et al. 1989).

Olfactory sensory neurons are responsible for the detection of odorant molecules and the generation of the electrical response that is transmitted to the brain (Kleene 2008; Pifferi et al. 2010; Schild and Restrepo 1998). They have a bipolar morphology, with a single dendrite that terminates in a knob with several cilia exposing their membrane to the external environment to contact odorant molecules, and a single axon projecting directly to the olfactory bulb of the brain.

In the human olfactory epithelium, the typical diameter of the cell body of an olfactory sensory neuron is about 4–6 μm, while that of the dendritic knob is about 1–2 μm, and the axon has a diameter of 0.1–0.4 μm. Each neuron bears a variable number of cilia, up to about 30, each with a diameter of 0.1–0.3 μm. Cilia have different lengths, varying from 1–5 μm up to 30 μm (Morrison and Costanzo 1990). Cilia of different neurons are tightly intermingled and are embedded in a thick layer of mucus produced by Bowman's glands (Getchell and Getchell 1992). The presence of numerous cilia allows a large increase of the membrane area available for interaction with odorant molecules.

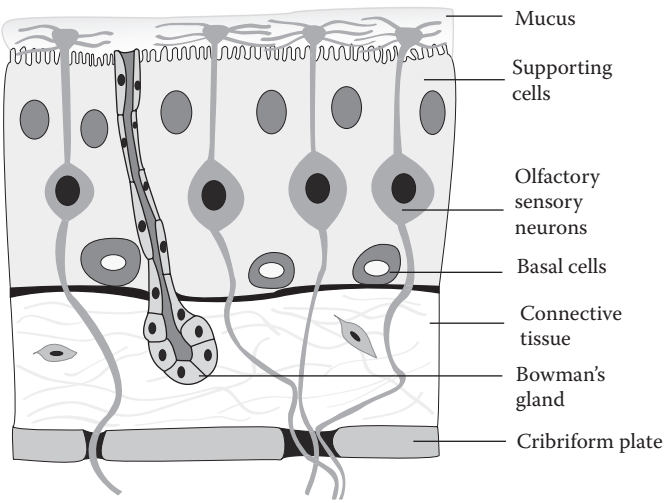

FIGURE 2.3 Organization of the olfactory epithelium. The olfactory epithelium is composed of olfactory sensory neurons, supporting cells, and basal cells. Bowman's glands are responsible for the production of mucus covering the surface of the epithelium.

2.4 ODORANT RECEPTORS

The molecular era of olfactory research began in 1991 with the cloning of odorant receptors by Linda Buck and Richard Axel (1991), who subsequently received the Nobel Prize in Physiology or Medicine in 2004 for their discoveries of odorant receptors and the organization of the olfactory system. This breakthrough discovery opened the possibility to understand the mechanisms of olfactory coding at the molecular level.

Odorant receptors belong to the superfamily of G protein–coupled receptors (GPCRs) and have a typical general structure comprising seven transmembrane domains, as shown in Figure 2.1a (Katritch et al. 2013). Mammals have about 1000 odorant receptor genes, but each species has a different number of functional odorant receptor genes. Primates, including humans, have about 300–400 functional odorant receptor genes, while mice and rats have about 1000–1200 intact genes (Gilad et al. 2004; Niimura and Nei 2005, 2007; Quignon et al. 2005).

To understand how the olfactory system discriminates among odorants, it is important to know how many types of odorant receptors are expressed in each olfactory sensory neuron and to determine the ligands of each odorant receptor.

It has been well established that each olfactory sensory neuron expresses only one odorant receptor gene (for review, see Magklara and Lomvardas 2013; Rodriguez 2013). Moreover, the pattern of expression of odorant receptors in the olfactory epithelium has been extensively studied in rodents (for review, see Malnic et al. 1999). Recent data have been reported in macaques (Horowitz et al. 2014), while data from humans are still missing. Based on the expression pattern of odorant receptors, the olfactory epithelium can be divided into four (mouse and rat) or two (macaque) zones. Inside a given zone, olfactory sensory neurons expressing a given odorant receptor are randomly distributed (Horowitz et al. 2014; Mombaerts et al. 1996; Ressler et al. 1993; Vassar et al. 1993; for review, see Mombaerts 2004).

Several studies have attempted to determine the ligands of odorant receptors, but unfortunately, it has been very difficult to express odorant receptors in heterologous systems suitable for high-throughput screening (Mombaerts 2004; Peterlin et al. 2014). Thus, at present, our knowledge of the pairing between odorant receptors and their odorant ligands is very limited, and about 90% of human odorant receptors are still orphan receptors (for review, see Peterlin et al. 2014).

Despite these limitations, it has been well established that the odorant receptor family uses a combinatorial code to discriminate among odorant molecules. Each odorant receptor can be activated by several types of odorant molecules, and a given type of odorant molecule can activate many odorant receptors (Araneda et al. 2000; Malnic et al. 1999). However, each odorant is encoded by the activation of a unique combination of odorant receptors (Figure 2.4). One great advantage of this combinatorial code is the possibility to detect and discriminate a very large number of odorant molecules.

In addition to the classical odorant receptors described above, a second class of chemoreceptors has been reported to be expressed in the olfactory epithelium of mice and macaques (Horowitz et al. 2014; Liberles and Buck 2006). These receptors

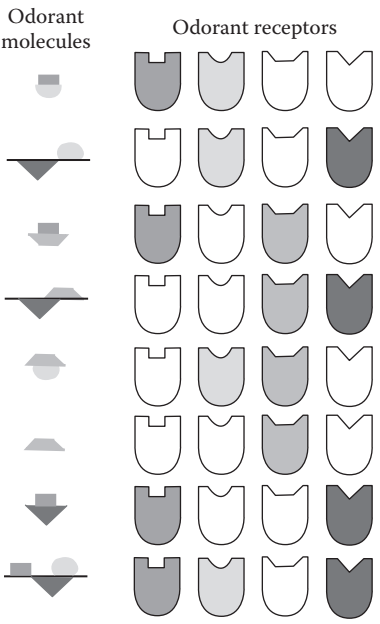

FIGURE 2.4 Combinatorial code used by odorant receptors to discriminate different odorant molecules. Four odorant receptors are schematically drawn on the right. The highlighted receptors can be activated by the corresponding odorant molecules depicted in the left column. A given odorant molecule activates a particular combination of odorant receptors. One odorant receptor can be activated by several odorant molecules.

are unrelated to the previously discovered odorant receptors and belong to the trace amine–associated receptor (TAAR) family (for review, see Sotnikova et al. 2009).

The mouse genome has 15 intact genes encoding for TAARs, while macaques and humans have only 6 functional TAAR genes (Liberles and Buck 2006; Niimura and Nei 2007). Horowitz et al. (2014) have recently shown that five TAARs are expressed by olfactory sensory neurons of the macaque, a high primate, and suggested that these receptors may also be expressed in the human olfactory epithelium. Olfactory sensory neurons expressing TAARs do not express the canonical odorant receptors. Moreover, each olfactory sensory neuron is likely to express only a single TAAR gene, in both mice and macaques (Horowitz et al. 2014; Liberles and Buck 2006). Heterologous expression of some human and mouse TAARs showed activation by volatile amines. For example, mouse, macaque, and human TAAR5 are activated by trimethylamine (Horowitz et al. 2014; Wallrabenstein et al. 2013; Zhang et al. 2013). Moreover, human TAAR5 responds to extracts from rotten salmon, whereas it does not respond to extracts from fresh salmon (Horowitz et al. 2014). As trimethylamine is produced by bacteria during spoilage of food and is responsible for the typical unpleasant and repulsive smell of rotten fish (Gram and Dalgaard 2002), it has been suggested that human TAAR5 may be used to elicit innate responses of avoidance toward spoiled foods that may be dangerous if ingested (Horowitz et al. 2014).

2.5 ELECTRICAL RESPONSES OF OLFACTORY SENSORY NEURONS

The binding of odorant molecules to odorant receptors is converted into an electrical signal in olfactory sensory neurons. Electrophysiological recordings from individual sensory neurons have shown that they respond to odorants in different ways (Figure 2.5). Some olfactory sensory neurons can detect several odorants, and a given odorant can activate neurons with various odorant specificities (Firestein et al. 1993; Ma et al. 1999; Reisert et al. 2005).

In many animal models the physiological response of olfactory sensory neurons to odorant stimuli has been well characterized with electrophysiological techniques. The current response of isolated olfactory sensory neurons to odorant stimulation has been recorded with the whole-cell patch-clamp technique in the voltage-clamp mode (Figure 2.5a). At the membrane potential of –55 mV, a brief application of odorants induced a rapid development of an inward current that returned to the basal level after the removal of the stimulus (Figure 2.5b). Different olfactory sensory neurons can have very different patterns of responses to odorants. For example, the

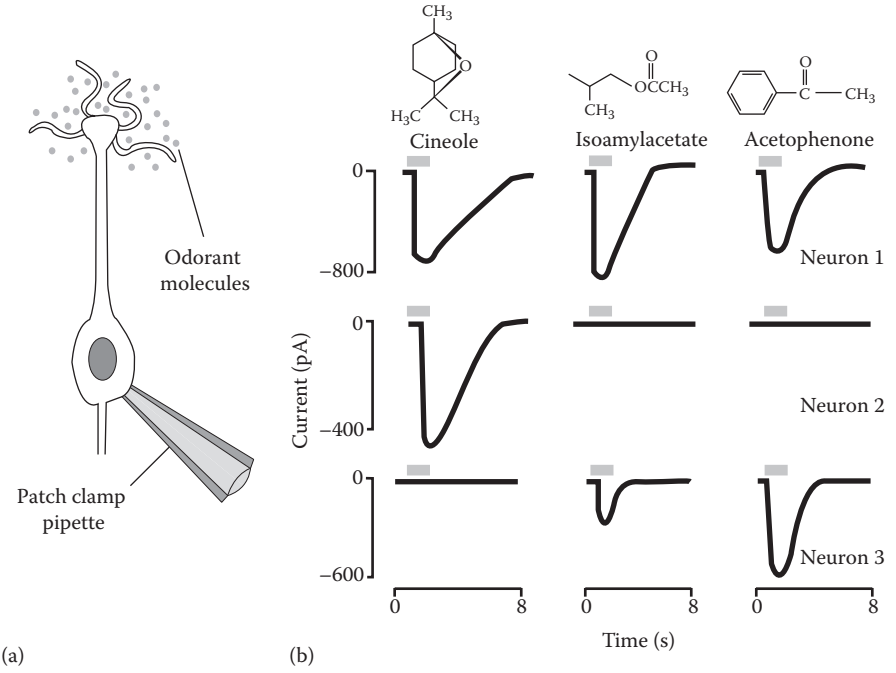

FIGURE 2.5 Electrical responses of olfactory sensory neurons to odorant stimulations. (a) Schematic representation of the experimental approach used to record odorant responses from dissociated olfactory sensory neurons by the whole-cell patch-clamp technique. (b) Responses of three different neurons to three structurally unrelated odorant molecules shown at the top. The holding potential was –55 mV and the odorant was applied for 1.2 s. In the first neuron, each odorant evoked a similar response. Neuron 2 responded to one odorant only, while neuron 3 responded to two of the three odorants with different amplitudes. (Modified from Firestein, S. et al., *J Physiol* 468: 1–10, 1993.)

first neuron in Figure 2.5b was activated by each of three structurally unrelated odorants, while the second neuron was activated by just one odorant, and the third neuron responded to two of the three odorants with responses of different amplitudes (Firestein et al. 1993). This general scheme was confirmed in various animal models using different experimental approaches and reflects the combinatorial code used by odorant receptors illustrated in Figure 2.4 (Araneda et al. 2000; Ma et al. 1999; Reisert and Restrepo 2009; Reisert et al. 2005).

2.5.1 Molecular Mechanisms of Olfactory Transduction

Olfactory transduction is the cellular process that converts the information carried by odorant molecules into a bioelectric signal (Figure 2.6). Transduction takes place in the cilia of olfactory sensory neurons, which, despite expressing different odorant receptors, largely share the same molecular transduction mechanisms (see reviews in Kleene 2008; Pifferi et al. 2010; Tirindelli et al. 2009). The binding of odorant molecules to an odorant receptor causes the activation of a trimeric G protein composed of $G\alpha_{olf}$ and $\beta\gamma$ subunits. In turn, the G protein stimulates the enzymatic activity of a specific adenylyl cyclase (ACIII) causing an increase in the concentration of cyclic AMP (Bakalyar and Reed 1990). The ciliary membrane expresses cyclic-nucleotide gated (CNG) channels that are directly gated by cyclic AMP. CNG channels are nonselective cation channels, and therefore the activation of odorant receptors causes a depolarizing influx of Na^+ and Ca^{2+} (Kaupp and Seifert 2002). The increase of intracellular Ca^{2+} concentration causes the activation of a second type of ion channel, named TMEM16B or anoctamin2, which is selective for Cl^- (Pifferi et al. 2009, 2012; Ponissery Saidu et al. 2013; Stephan et al. 2009). Since olfactory sensory neurons maintain an unusually high intracellular Cl^- concentration, the activation

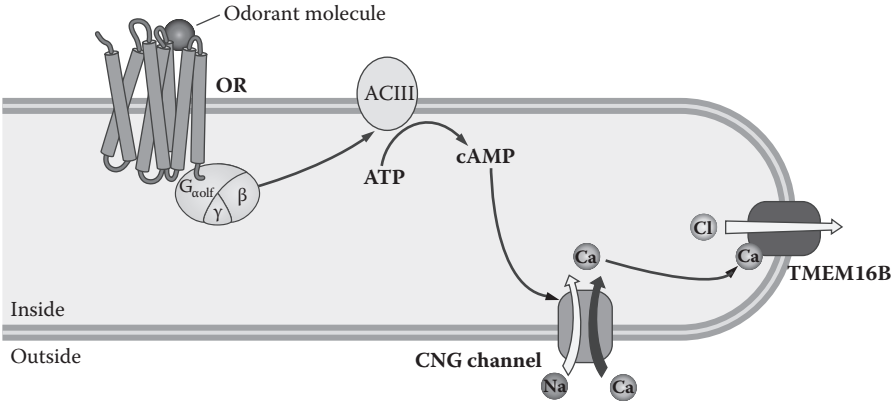

FIGURE 2.6 Molecular mechanisms of olfactory transduction. Schematic representation of olfactory transduction taking place in the cilia. The binding of odorant molecules to an odorant receptor (OR) activates a G protein, which in turn activates adenylyl cyclase (ACIII) producing cyclic AMP (cAMP), which opens cyclic nucleotide-gated (CNG) channels. Ca^{2+} entry causes the activation of TMEM16B generating a depolarizing efflux of Cl^-.

of TMEM16B causes an efflux of Cl^- and the consequent amplification of the odorant response (Kaneko et al. 2004; Reisert et al. 2005). Although the transduction current is composed of up to 90% by the Cl^- current (Boccaccio and Menini 2007; Reisert et al. 2005), the precise role of this current for normal olfaction is unclear. Indeed, the olfactory function of knockout mice for TMEM16B and wild-type mice was not significantly different (Billig et al. 2011). In addition, the olfactory function of human patients having TMEM16B partially deleted was not significantly impaired (Cenedese et al. 2015).

The depolarization generated by the binding of odorants in the cilia is passively transmitted along the dendrite and soma of the neuron and is converted in trains of action potentials that are conducted along the axon to the olfactory bulb (Schild and Restrepo 1998).

Several mechanisms are involved in the termination of the response. The most important are (1) intrinsic GTP-ase activity of $G\alpha_{olf}$ that stops the stimulation of ACIII (Firestein et al. 1991), (2) degradation of cyclic AMP by phosphodiesterase (Cygnar and Zhao 2009; Yan et al. 1995), (3) Ca^{2+}-calmodulin-dependent inhibition of CNG channels (Song et al. 2008), and (4) extrusion of Ca^{2+} through a Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase (Antolin et al. 2010; Stephan et al. 2012).

2.5.2 Odorant Adaptation

It is a general experience that the continuous or repeated exposure to an odorant causes a reduction in the perception of that odorant. For example, in a recent study in humans, Stuck et al. (2014) found a complete absence of perception after exposure for about 60 s to 4 parts per million of hydrogen sulfide (which smells like rotten eggs), while 10% (v/v) of phenylethyl alcohol (which smells like roses) needed about 160 s to induce a complete adaptation.

The entire olfactory system is involved in adaptation, including olfactory sensory neurons. Indeed, electrophysiological recordings obtained with electro-olfactograms, field recordings of the electrical activity of the olfactory epithelium, showed a reduction of about 20% of the response to repeated odorant stimulations (Hummel et al. 1996). The molecular mechanisms of adaptation in olfactory sensory neurons are still not completely understood. However, experiments in several animal models showed that the main player in this process is the modulation of CNG channels by Ca^{2+}-dependent mechanisms. Indeed, the increase of intracellular Ca^{2+} concentration during odorant stimulation causes a shift of the sensitivity of CNG channels toward higher concentrations of cyclic AMP and therefore increases the detection threshold of olfactory sensory neurons. This mechanism will allow neurons to discriminate higher odorant concentrations without saturating the transduction process (Kurahashi and Menini 1997; Song et al. 2008). Indeed, it is important to note that adaptation is not a mere reduction of response to a continuous or repeated stimulus, but an active process that allows the olfactory system to respond over a broad range of stimuli.

2.6 OLFACTORY BULB

The olfactory bulb is the first part of the encephalon devoted to process the information transmitted by olfactory sensory neurons about odorant molecules present in