Journal of Chromatography A, 1514 (2017) 110–119

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Fast, sensitive, and selective gas chromatography tandem mass spectrometry method for the target analysis of chemical secretions from femoral glands in lizards

Jorge Sáiz [a,*], Roberto García-Roa [b], José Martín [b], Belén Gómara [a]

[a] Institute of General Organic Chemistry, Spanish National Research Council (CSIC), Calle Juan de la Cierva, 3, 28006 Madrid, Spain
[b] Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (MNCN-CSIC), Calle José Gutiérrez Abascal 2, 28006 Madrid, Spain

ARTICLE INFO

Article history:
Received 24 April 2017
Received in revised form 27 June 2017
Accepted 18 July 2017
Available online 21 July 2017

Keywords:
Lizards
Chemical secretions
GC–MS/MS
Target analysis

ABSTRACT

Chemical signaling is a widespread mode of communication among living organisms that is used to establish social organization, territoriality and/or for mate choice. In lizards, femoral and precloacal glands are important sources of chemical signals. These glands protrude chemical secretions used to mark territories and also, to provide valuable information from the bearer to other individuals. Ecologists have studied these chemical secretions for decades in order to increase the knowledge of chemical communication in lizards. Although several studies have focused on the chemical analysis of these secretions, there is a lack of faster, more sensitive and more selective analytical methodologies for their study. In this work a new GC coupled to tandem triple quadrupole MS (GC-QqQ (MS/MS)) methodology is developed and proposed for the target study of 12 relevant compounds often found in lizard secretions (*i.e.* 1-hexadecanol, palmitic acid, 1-octadecanol, oleic acid, stearic acid, 1-tetracosanol, squalene, cholesta-3,5-diene, α-tocopherol, cholesterol, ergosterol and campesterol). The method baseline-separated the analytes in less than 7 min, with instrumental limits of detection ranging from 0.04 to 6.0 ng/mL. It was possible to identify differences in the composition of the samples from the lizards analyzed, which depended on the species, the habitat occupied and the diet of the individuals. Moreover, α-tocopherol has been determined for the first time in a lizard species, which was thought to lack its expression in chemical secretions. Globally, the methodology has been proven to be a valuable alternative to other published methods with important improvements in terms of analysis time, sensitivity, and selectivity.

1. Introduction

Animals have diverse sensory modes to communicate (*e.g.*, visual, acoustic, chemical, tactile, *etc.*) [1]. Visual and acoustic mechanisms are relatively well-known forms of communication and a considerable amount of literature addressing animal ecology and evolution by using these types of traits is available [2–5]. However, the understanding of alternative modes of communications, as chemical communication, remains relatively elusive [6]. Chemical communication is actually considered the oldest channel of communication, based in scents to convey information [7,8]. Overall, chemical signaling allows for interspecific recognition, the establishment of social structures, sexual interactions or/and territoriality [9–11], which play key roles for understanding the chemical ecology of many animals. This communication channel has mainly been studied in insects based on their pheromones [6], while the studies addressing chemical signals of vertebrates, such as birds, reptiles, or mammals, have recently grown in the past decade [10]. In particular, there has been an increasing interest to study the role of chemical signals in vertebrates, using reptiles as a model system [10,12], since their scents engage in survival and reproduction of many reptile species [11,13]. Chemical signals are especially relevant in some species of lizards because of their use for prey and predator recognition [14,15], territoriality [16], social recognition [17,18], and/or mate choice [19–21]. Multiple sources of chemical signals can be found in lizards, such as feces and skin [12], although most of the works are based on those signals from chemical secretions protruded from follicular glands located in the ventral side of the thighs [22], as shown in Fig. 1.

Pioneer works assessing the chemosensory abilities of lizards were conducted mainly based on behavioral approaches [23]. The number of tongue flicks (*i.e.*, lingual protrusion) in response to a

* Corresponding author.
E-mail address: jorge.saizg@gmail.com (J. Sáiz).

http://dx.doi.org/10.1016/j.chroma.2017.07.068
0021-9673/

Fig. 1. Ventral view of the hindlimbs of a lizard. The highlighted area corresponds to the femoral pores in the thighs, from which the chemical secretions are protruded.

scent and preferences for using scent-marked areas were some of the first tools used to measure the response of lizards to chemical stimuli [24]. Although these methods are still used in behavioral experiments [25], chemical ecologists have studied the chemical profiles of scents to broad the knowledge of the chemical signaling process. To the best of our knowledge, the first analytical study of lizard secretions was carried out by Chauhan three decades ago [26]. The researcher used TLC for the determination of lipid components of the precloacal secretion of two lizard species. This work constituted a preliminary report describing the composition of lizard secretions, in which phospholipids, cholesterol, and cholesterol esters were the main components and triglycerides, fatty acids, and wax esters were found in trace amounts. TLC was also used by Weldon et al. [27] for the analysis of femoral secretions in green iguanas, expanding the chemical analysis of secretions to other species of lizards. At the same time, Alberts et al. [28,29] used gel electrophoresis for the determination of proteins in 16 lizard species. In 2001, Escobar et al. [30] performed new studies on *Liolaemus* lizard species analyzing the chemical profile of precloacal secretions by GC–MS. The authors of this work found a total of 49 compounds distributed in the 20 species studied. This was the first time that GC–MS was used for the analysis of lizard secretions. Later on, this technique was repeatedly used, to the extent that it has become the technique of choice for chemical ecologists in the analysis of this type of samples. GC–MS has been used by different groups, such as Niemeyer's group [30,31], Martin's group [32–36], Khannoon's group [37,38], Heathcote's group [39], Preest's group [40] or Schulz's group [41]. Relevant compounds belonging to different classes were found at different amounts in lizard secretions, such as alcohols (*e.g.* 1-hexadecanol, 1 octadecanol and 1-tetracosanol), fatty acids (palmitic acid, stearic acid and oleic acid), terpenoids (squalene) or steroids (cholesterol, campesterol, ergosterol, cholesta-3,5-diene) or tocopherols, among others [35,38]. In fact, in view of this literature, the study of the chemical communication in lizards by analyzing their gland secretions has attracted the interest of several ecologists in the recent years. All these works made use of long chromatographic separations, operating the mass spectrometer in scan mode for fingerprinting (untargeted) analysis, with the aim of listing large numbers of compounds present in the secretions. However, it is well known that scan mode in mass spectrometry does not offer as good sensitivity or selectivity as other modes, such as SIM or multiple reaction monitoring (MRM). Moreover, the quantitation of components might be difficult in scan mode, since co-elution of compounds might occur. Other groups have performed quantitative studies in lizard secretions in GC–MS operated in scan mode [42] or in GC with flame ionization detection [43,44]. However, these studies did not perform adequate quantitation procedures, such as the use of calibration curves, while they only considered the relative response to the amount of internal standard added [44] which is a well-known non-accurate approach in analytical chemistry.

In this framework, the aim of this work was to develop, for the first time, a GC-QqQ (MS/MS) methodology for the targeted study and quantification of 12 relevant compounds often found in lizard secretions (1-hexadecanol, palmitic acid, 1-octadecanol, oleic acid, stearic acid, 1-tetracosanol, squalene, cholesta-3,5-diene, α-tocopherol, cholesterol, ergosterol and campesterol) and that have been suggested to a have a role as signals in chemical communication in different lizard species [46].

2. Material and methods

2.1. Reagents

All reagents used for the analyses were of analytical grade. *n*-Hexane (99%) for organic residue analysis was obtained from J.T. Baker (Deventer, The Netherlands). Methanol ($\geq$99.9%), chloroform ($\geq$99.9%), dichloromethane (DCM, $\geq$99.9%), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, $\geq$98.5%) for GC derivatization, 1-hexadecanol ($\geq$99%), palmitic acid ($\geq$99%), 1-octadecanol ($\geq$99%), oleic acid ($\geq$99%), stearic acid ($\geq$98.5%), 1-tetracosanol ($\geq$99%), squalene ($\geq$98%), cholesta-3,5-diene ($\geq$93%), ($\pm$)-α-tocopherol ($\geq$96%), cholesterol ($\geq$99%), ergosterol ($\geq$95%), and campesterol ($\sim$65%) were from Sigma-Aldrich (St Louis, MO). Stock solution of 20 mg/mL of all the analytes were prepared in the adequate solvent for their complete solution, *i.e.* n-hexane for 1-hexadecanol, palmitic acid, 1-octadecanol, oleic acid, stearic acid, squalene, cholesta-3,5-diene, tocopherol and cholesterol, methanol for 1-tetracosanol, and chloroform for ergosterol and campesterol, which were stored at $-20\,^{\circ}$C. In the case of 1-tetracosanol and cholesterol, the application of heat (60 $^{\circ}$C) was needed in order to allow the complete dissolution of the compounds in the solvent. Subsequently, stock solutions of 1 mg/mL and 100 μg/mL were prepared in *n*-hexane and stored at $-20\,^{\circ}$C.

2.2. Selection of the analytes of interest

The chromatographic separation was optimized in terms of analysis time and signal intensity. Fig. 2 shows the chemical structures of the 12 compounds selected for this work. We focused on a subset of chemicals, which are representative of the major classes of lipophilic compounds found in secretions of lizards, given their abundance and frequency of appearance and their putative role in intraspecific communication in different lizard species (see reviews in [12,22,48]): (1) 1-hexadecanol and (2) 1-octadecanol, are two of the alcohols more often found in lizards secretions, and have been suggested to signal male social dominance in some lizards [49]; (3), palmitic acid and (4), stearic acid are the two most common fatty acids in lizard secretions [12], and they have been associated signaling health state in some lizards [50]; (5), oleic acid, is a fatty acid which proportions in secretions are related to the body fat reserves and thus suggested to signal body condition [51]; (6) 1-tetracosanol is a waxy alcohol particularly abundant in lizards of the genus Acanthodactylus, where it could signal age and dominance [39,52]; (7) squalene, a terpenoid suggested to have antioxidant properties in secretions of species living in wet environments [53], which could also signal sex and be used in male competition [54,55]; (8) cholesta-3,5-diene, a common steroid found in secretions of most lizards [12]; (9) α-tocopherol (*i.e.*, vitamin E), usually found in secretions of some lizard species (*e.g.* Lacerta, Timon) in very high proportions [56,57]. It is believed that, because of their antioxidant properties [56], it may protect other compounds in secretions. Also, high levels of α-tocopherol in secretions are related to the