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Development of a gas chromatography–mass spectrometry method to monitor in a single run, mono- to triterpenoid compounds distribution in resinous plant materials

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ABSTRACT

A new procedure based on gas chromatography coupled to mass spectrometry (GC–MS) was developed for the simultaneous determination of mono- to triterpenoid compounds in resinous materials. Given the difference of volatility and polarity of the studied compounds some critical steps in this methodology had to be identified and investigated. The recovery of volatile compounds after sample extraction was studied. A recovery range from 30% to 100% from the more volatile monoterpene to the least one was observed. Then the mandatory derivatization step for the analysis of pentacyclic triterpenes bearing hydroxyl and carboxyl groups was optimized. Results showed that derivatization using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) in pyridine (22:13:65 v/v/v) for 2 h at 30 °C was the most efficient method of derivatizing all the hydroxyl and carboxylic acid groups contained in the triterpene structures. After choosing the best injection parameters for these compounds, the selectivity of the GC column towards the separation of these terpenoids was investigated using statistical tools (principal component analysis and desirability functions). A separation with a good resolution was achieved on an HP-5ms column using a programmed temperature vaporizing injector (PTV). The method was pre-validated in terms of detection limits (LOD from 100 $\mu g\,L^{-1}$ to 200 $\mu g\,L^{-1}$ depending on the compound), linearity and repeatability using seven compounds representative of mono- and triterpenoid classes. An exhaustive characterization of various types of resins (di-, triterpenic and oleo-gum resins) was achieved.

1. Introduction

Resins are hydrocarbon secretions of many plants and well known for their protective benefits [1]. They have been used as raw materials and over the course of history for a wide range of applications from cosmetic and pharmaceutical fields to painting varnishes in artworks [2]. For example, oleo-gum resins from *Boswellia* species are used in traditional medicine in India and African countries for the treatment of a variety of diseases [1,3] and the *Commiphora* species have emerged as a good source for the treatment of inflammation and diseases caused by blood stagnation in traditional Chinese medicine [4]. Moreover, the mastic (*Pistacia lentiscus*) extracts is commonly employed in cosmetic products [5].

Plant resins are complex mixture of organic substances mainly terpenoid compounds while gum resins contain also a fraction of water-soluble gum. Terpenes, which constitute the most abundant and structurally diverse group of plant secondary metabolites, play an important role in plant interactions with its environment [6,7]. Terpenes, which are obtained biosynthetically from units of isoprene, are synthesized by many plant organs and stored in secretory cells, cavities, canals, or glands. They are classified according to the number of carbons and the presence, position and number of acidic, hydroxyl or ketone moieties on their structure. Monoterpenes (C10) and sesquiterpenes (C15) constitute the most volatile fraction of terpenoids and are implicated in plant defense against herbivores and phytopathogens [8,9]. Diterpenes (C20) and triterpenes (C30) constitute the least volatile fraction and are well-known for their anti-inflammatory, anti-arthritic, anti-proliferative and analgesic activities [10–12]. Most often a vegetal could synthetize resin containing either di- or triterpenoids. However one resin family exception to this rule: *Burseraceae* resins like *Myrrh*

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(*Commiphora*) and Olibanum (*Boswellia*) resins that can synthetize these two classes of terpenes simultaneously [13,14].

It is well established that plant material may vary depending on the know-how and place of harvest. Thus, there is a true need for exhaustive methods allowing rapid qualitative analysis in order to check batch to batch variability, certify origin, and ensure quality assurance and control, using the most common equipment. However, the chemical characterization of resinous materials is a complex task. The wide range of volatility and polarity of terpenoids results in long and difficult separation. In addition, overlapping peaks require a selective method. Furthermore, in these complex mixtures, some terpenoids are major compounds whereas some others are present in trace amounts. Then, it was necessary to develop a highly sensitive analytical technique to obtain the most exhaustive composition for these samples from mono to triterpenoids.

For the analysis of terpenoids present in resinous material, various separation techniques were used. Depending on the terpene class studied, these techniques could be associated to different methodologies for sample pre-treatment. Liquid chromatography (LC) has been performed especially for identification of naturally non-volatile compounds (especially pentacyclic triterpenes). As most compounds are lacking chromophore groups, LC–MS was investigated with atmospheric pressure photoionization (APPI) or atmospheric pressure chemical ionization (APCI) [15–18]. Alternatively, thin layer chromatography (TLC) has been investigated as a suitable method to simultaneously screen numerous samples directly from plant extracts especially boswellic acids in gum resins of frankincense [19,20]. With the emergence of high performance thin layer chromatography (HPTLC), a number of enhancements were made to the basic method of TLC in the automatization of the different steps leading to an increase of the resolution achieved [21–23]. However, the chemical structure of most of the isolated substances was at best only partially characterized. For these reasons, gas chromatography (GC) remains a powerful alternative technique for the analysis of natural components present in resin. Separation of terpenoids in complex matrices could be performed using GC with flame ionization (FID) or mass detection (MS) which is a useful tool for obtaining structural and chemical information about compounds. In literature, to our knowledge, there is no single protocol to analyze exhaustively and simultaneously from mono- to triterpenoids present in resinous materials. GC–MS was applied to analyze specifically on one hand volatile compounds [24–26], and on the other hand, non-volatile compounds after a derivatization step [11,27,28]. Thus, it was thought of interest to develop a methodology to analyze terpenoids as a whole in resin extract regardless of its nature. In this objective and even if mono- and sesquiterpenes are volatile compounds and can be analyzed easily by GC, a prior derivatization step is necessary to increase volatility of di- and triterpenoids compounds before their analysis. In these conditions, all compounds that contain hydroxyl functions (alcohol, diol and acid) will be derivatized.

The goal of this study was to develop a new GC–MS methodology to analyze and characterize as many terpenoids as possible in resinous complex mixture. This task was accomplished by the development of a step by step GC–MS methodology. This study includes: compounds recovery after sample extraction step, optimization of the derivatization step and of the injection conditions, selection of the most appropriate GC stationary phase and optimization of the temperature gradient. The methodology was then pre-validated in terms of detection limits, linearity and repeatability by the analysis of terpenoid standards. Finally, the optimized methodology was used to characterize qualitatively various resinous extracts (Copal, *P. lentiscus*, *Boswellia serrata* and *Commiphora wildii* resins) from mono- to triterpenoids.

2. Materials and methods

2.1. Reagents and chemicals

2.1.1. Terpenoid standards

α-Pinene, camphrene, β-pinene, limonene, thujone, camphre (+), camphre (−), linalool, linalyl acetate, bornyl acetate, β-caryophyllene, menthol, borneol, geraniol, sclareol, cafestol (purity 99%) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). α-Amyrin, β-amyrin, lupeol, erythrodiol, betulin, uvaol, oleanolic acid, betulinic acid and ursolic acid (purity 99%) were purchased from Extra Synthese (Geney, France) and corosolic acid and maslinic acid (purity 99%) from Glentham Sciences (London, United Kingdom). Hexane, chloroform and methyl *tert*-butyl-ether (MTBE) were purchased from Carlo-Erba (Val de Reuil, France), and pyridine from Sigma-Aldrich (Saint Quentin Fallavier, France). Derivatization reagents such as *N*,*O*-bis-(trimehylsilyl)trifluoroacetamide, (BSTFA), trimethylchlorosilane (TMCS) were obtained from Sigma Aldrich (Saint Quentin Fallavier, France).

2.1.2. Four natural resins were studied

(a) Copal: a diterpenic resin from the *Copal* tree (Araucariaceae). (b) Mastic: a triterpenic resin secreted from *P. lentiscus* (Anacardiaceae) that grows almost exclusively in the island of Chios (c) *B. serrata*: an oleo-gum resin from *Boswellia* tree (Burseraceae—Olibanum) and (d) *C. wildii*: a gum-resin from the Burseraceae family (Burseraceae—Myrrh). The four resins were supplied by LVMH-Research (Saint-Jean de Braye, France).

2.2. Stationary phases

Eight columns from a broad range of polarity were tested (Table 1): Rtx-1ms from Restek (Lisses, France), CP-Sil 24 and VF-200ms from Varian (Courtaboeuf, France), DB-5ms and DB-35ms from J&W (Courtaboeuf, France), Optima delta 6 from Macherey-Nagel (Hœrdt, France), ZB-multi residue from Phenomenex (Le Percq, France) and HP-5ms from Agilent (Courtaboeuf, France). All columns were 30 m × 0.25 mm × 0.25 μm. State of columns were not the same, some of them were newly bought (DB-5ms and Optima δ 6) and others were previously used in our lab.

For each column, Mc-Reynolds constant was calculated from the retention factors of five standard solutes (benzene, *n*-butanol, 2-pentanone, nitropropane and pyridine) in order to characterize its polarity [29,30]. Columns were classified in Table 1 by increasing number of Mc-Reynolds (the lower the value, the more apolar the column).

2.3. Sample and standard preparations

2.3.1. Standard preparation

27 stock standard solutions (1000 mg L^{-1}) are prepared independently by weighting and dissolving the adequate amount of each solute in hexane (mono-, sesqui- or diterpenoids) or in MTBE (triterpenoids) and stored at 4 °C. Hexane and MTBE were chosen as solvent for their good solubility toward terpenoids.

2.3.2. Resin preparation

Resins were reduced in fine powder by cryogrinding in liquid nitrogen and then using an electric grinder. For extraction, an ASE 150 system from Dionex (Voisins le Bretonneux, France) with 5 mL stainless steel ASE vessels was used for the extraction. The conditions used are adapted from previous work [31]. 200 mg of resin was mixed with 200 mg of diatomaceous earth and extracted with chloroform using three static cycles for 5 min each, a flush volume of 65% and a 100 s nitrogen gas purge at the end of each cycle. Extractions were carried out at 40 °C and under a pressure of 100 bar. About