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Review

Techniques for gas chromatography of volatile terpenoids from a range of matrices

G.B. Lockwood*

School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester M13 9PL, UK

Abstract

The commercial importance of the volatile mono- and sesqui-terpenoids has resulted in a wide range of techiques being used for extraction, concentration, chromatography, and characterisation of constituents. The major chromatographic technique is gas chromatography, and tandem techniques of chromatography linked to further chromatography and spectroscopy, allow much increased resolution, and greater ease of characterisation of terpenes. A wide range of extraction techniques are discussed, and suitability for particular matrices and sample sizes outlined. Chromatography operating conditions and stationary phases, and techniques for solute identification are laid out. A number of applications of terpene analysis in many different matrices are discussed.

Keywords: Review; Terpenoids volatile

Contents

*Tel.: +44-161-275-2399; fax: +44-161-275-2396.
E-mail address: brian.lockwood@man.ac.uk (G.B. Lockwood).

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1. Introduction

Volatile terpenoids consist of two groups of biosynthetically related terpenoids, the monoterpenoids and sesquiterpenoids, both of which often co-occur in the volatile or essential oils. These oils are often used for their flavour or fragrance properties, in a wide selection of products ranging from foods and drinks, medicines and cosmetics. Gas chromatography (GC) has been used since its infancy for separation of numbers of related terpenes in applications as diverse as quality assurance of natural materials and formulated products, characterisation of new essential oils and biotechnological investigations involving these terpenes. A number of these terpenes of natural origin are now being detected in a range of atmospheric and environmental matrices, and have great ecological significance.

In parallel with technological developments in gas chromatographs, there have been dramatic improvements in detection devices and stationary phases, and major developments in attached tandem devices, particularly spectroscopic devices for structure elucidation. Many improvements in sample extraction and concentration have taken place, which have dramatically increased the applications for gas chromatographic analysis.

Although GC is naturally suited to analysis of these terpenes, due to their volatility, by far the most useful information is today obtained using tandem techniques involving prior/further chromatography, or spectroscopy. In certain instances such as analysis of less volatile constituents of essential oils or absolutes, HPLC is the method of choice, and is particularly suited to analysis of terpene glycosides which are not volatile, and whose conversion to volatile compounds suitable for GC involves problems of delays, concentration, and possible losses.

2. Techniques

A wide range of techniques have been used for sample extraction and concentration, chromatographic separation of constituents, and sample detection and constituent characterisation.

2.1. Sample extraction and concentration

The simplest and safest technique is cold expression, but is usually only suitable for citrus peel oils [1]. Extraction of volatile terpenoids from plant material and a wide variety of other matrices is often carried out using steam distillation. Possible problems with this method have recently been reported, where degradation of certain monoterpenes, such as sabinene has been shown to occur, due to acid catalysed hydration [2]. A deviation from these basic methods involved superheated water extraction [3], and in this case solutes can be extracted by partitioning with hexane. Fractional distillation has been used for analysis of oleoresins from five species of conifers [4]. Simultaneous steam distillation and solvent extraction with dichloromethane [5] or hexane [6] has been used. Solid phase microextraction (SPME) has been used [7–10], but recoveries of monoterpenes were not as high as with other methods [7]. An alternative method useful for much smaller sample sizes involves solvent extraction, followed by evaporation of the extract. With low levels of volatile terpenoids, this technique is still widely used. When this technique has been used, volatile solvents such as dichloromethane have been used for solute extraction, followed by concentration under reduced pressure, or flow of nitrogen [11].

Collection of low level terpenoids from growing plants has involved use of special chambers [12,13], bags [14], and frames [15]. Samples can also be collected directly from the atmosphere. Table 1 shows a range of terpenes and matrices which have been adsorbed onto various grades of Tenax, for example Tenax GC [16,17], Tenax TA [18–23], or Tenax GR [24], this latter grade having lower water retention, or charcoal [25], Carbopack B [26], or Carboxen 569 [27]. Low boiling point monoterpenes are by far the most frequently found, in particular the pinenes and carene. Fig. 1 shows some representative monoterpenes frequently found in the environment. However, decomposition of α-pinene and sabinene have been reported on Tenax TA and Carboxen 569 [27]. Constituents are usually removed prior to chromatography using thermal desorption [19,21–23,25], or solvent extraction using hexane [27]. A comparison of the efficacy of these two techniques

Table 1
Adsorbents used for concentration of volatile monoterpenes

Adsorbent	Matrix	Monoterpenes [Reference]
Tenax GC	Atmosphere	Pinenes, carene, limonene [16]
	Flue gasses	Pinenes, camphene, limonene, carene [17]
Tenax TA	Atmosphere of pine forests	Pinenes, carene, camphene [18]
	Atmosphere	Pinenes,carene [19]
	18 Mediterranean plants	Pinenes, sabinene, linalool [20]
	Atmosphere of pine forests	Pinenes, carene, limonene [23]
Tenax GR	Eucalyptus volatiles	13 Monoterpenes [24]
Charcoal	Pine processing plant air	Birch/spruce monoterpenes [25]
Carbopack	Forest air	Mono- and sesquiterpenes [26]
Carboxen 569	Monoterpene standards	α-Pinene, sabinene [27]

was carried out on seventeen blackcurrant leaf volatiles [2], and although levels were roughly similar using the two elution systems, consistently lower levels of Δ^3-carene were found in thermally desorbed samples. Alternatively, adsorbtion onto XAD-2 ion-exchange resin followed by elution with Freon-11, has been employed for wine flavour components, and the results obtained were comparable with results obtained using liquid–liquid extraction using Freon. In both extraction systems, there was high recoveries of longer chain alcohols, esters, aldehydes, ketones and monoterpenes, and lower recoveries for low-molecular-mass alcohols and organic acids [28].

Microwave assisted extraction has been used for volatile extraction from a number of plant materials [29,30]. In work comparing the relative recoveries of steam distillation, simultaneous distillation and dichloromethane solvent extraction, microwave assisted extraction with dichloromethane, and supercritical fluid extraction (SFE) using CO_2, the greatest number of compounds were collected using the SFE system — 79, compared to 67 for the microwave assisted technique, 61 for distillation–solvent extraction, and only 43 using steam distillation [29].

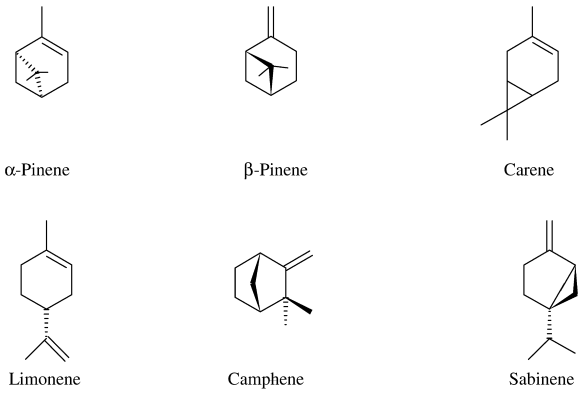

Fig. 1. Representative monoterpenes frequently found in the environment.

Cryotrap concentration of atmospheric extracts is frequently carried out after adsorption of solutes, and has been used for the quantitation of monoterpenes in humid and ozone-rich air [31], and mono- and sesquiterpenes collected from tropical rainforest [26].

Small amounts of constituents in either plant materials, or complex matrices such as formulated medicines [32,33], have been collected using the headspace technique, which involves volatilising the terpenoids in the sample in a closely confined space, and then analysing the constituents in this gaseous phase.

Both liquid CO_2 [34] and supercritical fluid extraction, SFE [35,36] have been employed for extraction of essential oils, both in analytical and commercial scale. The latter technique is claimed to produce a superior quality product to that produced by steam distillation [5,36]. Simultaneous steam distillation–solvent extraction has been compared with SFE during extraction of *Spilanthus americana* volatiles, and the former was found to produce a

generally higher proportion of oxygenated compounds and monoterpenes than the latter technique [5].

Volatile terpenoids can exist in the form of glycosides in most plant material, and this non-volatile form is the biosynthetic precursor of the free terpene. For extraction, hydrolysis of the glycoside is carried out either by enzymic hydrolysis with β-glucosidase [37,38], or by acid hydrolysis [38]. The resultant free aglycone, the volatile terpene, can then be collected, and analysed by GC, and in some instances, more than ten aglycones have been identified in one wine [38]. Compared to analysis of free terpenes in essential oils, separation of terpenes is easier due to the presence of less types, but concentrations are usually very much lower than in essential oils. An additional problem is that large scale extraction by hydrodistillation after enzyme inactivation with hot water has been shown to cause production of degradation products, for example linaloyl glucoside was shown to have been degraded to geraniol, nerol, and α-terpineol in addition to linalol [39]. Enzymic maceration with pectolase has been used for release of volatile terpenoids of mango pulp [40]. A comparison of the levels of terpenes hydrolysed from grape juice after treatment with one of seven enzyme preparations showed wide quantitative differences, but generally higher levels occurred after treatment with Klerzyme 200 [38]. Apart from analysis of the glycosides by HPLC, GC of trimethylsilyl and trifluoroacetyl derivatives has been undertaken, and found to give complementary results to HPLC methods [38].

2.2. Chromatographic separation of constituents

Since its invention in the 1950s, GC has been the method of choice for the separation, identification, and quantification of these volatile terpenoids. Up to the 1980s packed column (large diameter, short column) separation of constituents using a range of stationary phases, was the best method available, and still finds applications in analysis of essential oils and terpene extracts, for example with marjoram and aniseed [35], and also in preparative GC. The advent of fused-silica capillary columns containing bonded stationary phases produced vastly higher resolution of these complex mixtures, and it has been estimated that 85% of all reported GC analyses were on capillary columns by 1986 [41]. The stationary phases used for separation of mono- and sesquiterpenes include DB-1, Carbowax, OV-1, OV-101, PEG 20M, BP5, DB-5, which cover a range of polarities, and are the most widely used. Column lengths range from 25 to 100 m, and stationary phase film thicknesses range from 0.2 to 0.7 μm. Operating temperatures are usually in the range of 50°(70)–280°, at 5°/min. In the 1990s the discovery of chiral phases allowed resolution of enantiomers of volatile terpenoids. A wide range of commercial cyclodextrin phases have been developed, and used for separation of enantiomeric mono- and sesquiterpenes [42,43]. In a comparative study of chiral phases, Betts found that relative retention time increases on an α-cyclodextrin phase were greater than on β- and γ-phases, and dependent on monoterpene structure, acyclic monoterpene retention times increased by 60%, whereas bicyclic monoterpene retention increased by 150% [42]. A wide range of differently esterified cyclodextrins have been produced, for example butyryl, propionyl and trifluoroacetyl esters of γ-cyclodextrins, giving changed elution sequences for both monoterpenes and sesquiterpenes. Liquid crystal phases, such as 4,4′-azoxydiphenetole, have also been developed and used for separation of monoterpenes [44]. This latter stationary phase yielded different elution sequences for a range of eight terpenes after melting and supercooling, and provided a distinct advantage in identification due to large changes in solute relative retention times.

A number of tandem techniques has been developed, including GC–GC, which is the coupling of two columns or two chromatographs and HPLC–GC, which provides increased resolution. Both these techniques allow sequential use of techniques with different separating abilities, combining chiral and non-chiral columns, polar and non-polar, or reversed-phase HPLC with temperature programmed GC. GC–GC analysis has been used to separate complex mixtures of enantiomeric monoterpene hydrocarbons and alcohols in lemon oils [45]. A butyldiethylsilyl-β-cyclodextrin stationary phase was able to evaluate the enantiomeric contents of limonene, linalool, β-pinene, sabinene, α-terpineol and terpinen-4-ol in four lime oils [46]. Comparison with single column separation showed greatly increased numbers of

resolved constituents [47]. The increased resolution of HPLC–GC allowed for differences to be noted for oils from the same material, but prepared differently, to be distinguished. This fully automatic system enabled detection of linalool and terpin-4-ol enantiomers from lemon and mandarin oils without interference [48]. Tandem GC with droplet counter current chromatography (GC–DCCC) [49], has been used for chromatography of 27 monoterpenes, and identification of a number of novel mono- and disaccharide aglycones of Reisling wine, particularly the ρ-menth-1-ene-8,9-diols.

2.3. Detection and characterisation of constituents

The flame ionisation detector is the most commonly fitted to chromatographs, and is the most widely used for detection and quantification of these terpenoids. Other detectors reported to have been used include the photoionisation detector [50], which has been used for investigation of α- and β-pinene in the atmosphere, and the surface ionisation detector [51] has been successfully used for detection of a mixture of terpenes.

Separated constituents can be identified by co-chromatography with standards if they are well separated and characterised, but usually with GC of essential oils, identification is carried out by comparison with Kovats retention indices (RI). Using this type of retention data derived from two GC columns of different polarity, allows highly reliable identification of large numbers of terpenes in a particular sample.

Probably the most widely used system for characterisation of eluted terpenoids is GC linked to mass spectroscopy (GC–MS), but other tandem systems include GC–IR, or GC–FTIR [52]. GC–FTIR has been commercially available since 1980, and differs from GC–MS in applications in this field, as it can provide solute identification in cases where the molecular ion is not apparent in the mass spectrum.

GC–MS can involve either quodrupole MS or use of the relatively cheap ion trap MS detector [23]. GC–IRMS involves use of the isotope ratio mass spectrometer, and is capable of measuring the ratios of stable carbon isotopes $^{13}C/^{12}C$ for each eluted peak. This last system can be used to determine the origin of enantiomeric pairs [41].

Sniff testing, or GC–O, is widely used to detect potent odorants in foods and perfumes, and gives invaluable details concerning the actual applications for which the terpenoids are to be used. More sophisticated aroma extract dilution analysis (AEDA) gives even more detailed information concerning the sensory qualities of particular volatile terpenoids [1].

3. Applications

3.1. Analysis of terpenoids in essential oils

Widescale GC screening of essential oils in Australian plant species has been carried out by Brophy and co-workers over the last 15 years, a most recent example of their work has been the analysis of the essential oils of a large number of Australian members of the genus *Leptospermum* [53]. The extensive data produced include the identities and concentrations of 112 different terpenes present in 15 species and subspecies of the genus *Leptospermum,* and required a (85 m×0.5 mm) SP 1000 capillary column, programmed at 65–225°, 3°/min. Similar mass screening in food products has been published by Buttery and co-workers since 1965, ranging from materials as diverse as Bartlett pear leaves [54] to corn tortilla chips [55], and identifying large numbers of volatile mono- and sesquiterpenes involved in the flavour profiles of the materials. Much research has been published on oils of commercial importance, particularly those of *Mentha spp*. Rohloff [56] recently published data on the composition of essential oils from mint leaves and flowers from different positions on plants, and identified 19 different compounds. Older parts of the plants contained higher concentrations of menthol, menthyl acetate and neomenthol than the younger plant parts, whereas younger plant parts contained higher concentrations of menthone and isomenthone. SPME sampling had resulted in relatively higher amounts of high-volatile monoterpenes and lower detection of less volatile compounds, such as the menthone and isomenthone. Use of achiral stationary phases in conjunction with retention indices, MS data, and possibly other chromatographic techniques is often suited to separation of closely related species of essential oil containing plants, and also good comparison between samples

of material from different geographical sources, for example in the case of related species of Achillea from Iran [57,58].

Mondello and co-workers reported on the enantiomeric distribution of monoterpenes in a range of both lime and lemon oils [45,46]. Techniques and equipment are becoming increasingly sophisticated, with the result that recent publications list far greater numbers of identified constituents, 89 were recently identified in one sample of peppermint essential oil. This number was identified using tandem GC–GC, whereas only 30 were identified using GC–MS, and such improved levels of resolution mean that even closely related species of plant genera are now easily identified [47]. Song et al. also detected 89 terpenes in cold pressed essential oil of *Citrus junos* and identified the oxygenated terpenes to be mainly responsible for the characteristic flavour [1].

3.2. Analysis of free terpenoids in plant material

Monoterpenes from a number of commercially important plant species have been identified, notably pine needles [7,6], and in one report Orav found 60 components in Estonian Spruce [6]. In this latter report, the constituents of Estonian spruce and pine needles were identified using a combination of four stationary phases, and while spruce oil was shown to contain up to 70% oxygenated monoterpenes, and more limonene and 1,8-cineole than oils from other regions, pine oil contained up to 85% monoterpenes [6].

A number of oxygenated and non-oxygenated terpenes were reported in angelica root [34], following extraction with supercritical carbon dioxide. Addition of water as co-solvent increased the percentage of both groups of compounds. Fourteen volatile terpenoids from blackcurrant cultivars were identified in leaf surface extracts, in an attempt to determine their effect on midge resistance [2].

3.3. Analysis of free terpenoids in plant products

Commercial interest in grapes and wine has resulted in much literature on the monoterpene contents. Mateo and Jimenez [38] recently reviewed the importance of monoterpenes on varietal flavour of wines and grape juice, and DelaCalleGarcia [8] has reported on the monoterpene aroma components of wine bouquet in US products. This latter group were able to detect 0.1–0.5 μg/l from simulated wine matrix.

3.4. Analysis of glycosidically bound terpenoids in plant material

The monoterpene glycosides of a number of plant parts and species have been investigated and identified by VandenDries and BaerheimSvendsen [37]. Stahl-Biskup has published a number of papers on these terpenes, including analysis of *Thymus× citriodora*, in which levels of glucosidically bound geraniol were the highest of the bound terpenes, and the major free component in the oil, indication a biosynthetic relationship between the two [59]. About 50 monoterpenes have by now been identified in grape must and wine, and glycosidically bound forms predominate over free forms [38].

3.5. Analysis of terpenoids in plant tissue cultures

The analysis of volatile terpenoids and volatile biotransformation products in plant cell cultures has been widely reported. A number of reports [60,61] have highlighted the low levels of constituents reported, and complete absence of constituents in cultures derived from certain plants. Often the more volatile monoterpenes are absent from cultures, even if sesquiterpenes are still present. Work with parsley cultures identified only one terpenoid amongst the volatile constituents, although these cultures retained the biosynthetic ability for biotransformation of exogenous terpene substrates [62,63]. However, a number of workers have identified volatile terpenoids, albeit at lower levels than in intact plants, but also different constituents were found to predominate, as exemplified by Berlin et al. [64] in suspension cultures of *Thuja occidentalis*. Recent work using cultures of *Peganum harmala* shows that loss of terpenes by evaporation is severely limiting ability of cultures to accumulate these volatile components, but incorporation of controlled release polymers for substrate storage has produced dramatic increases in product levels, particularly of geraniol and linalol [11].

3.6. Analysis of atmospheric/airborne terpenoids in natural habitats

In the example of *Fagus sylvatica* [12], adsorbed atmospheric terpenoids were thermodesorbed and analysed at 37–80°C, at 5°/min on a 50-m BP 5 column, and identified by co-chromatography with standards.

The monoterpene emissions in pine forests have been extensively studied using adsorption of air samples onto Tenax TA adsorbent. α-Pinene and β-pinene were detected in all cases, and sampling height [23], ambient temperature [18] and tapping for resin, affected atmospheric composition [65]. Enantiomeric composition of monoterpenes has recently been reported for samples of eucalyptus, cedar and pine grown in Algiers, using a β-cyclodextrin column [66]. The terpenoid emissions from other trees has been reported; light, temperature, and season affected the composition in deciduous trees [15], and wind speed affected composition in forest air in the Black Forest [67]. A number of monoterpenes were also found to suffer atmospheric degradation [67,22]. Large scale screening of emissions from both Mediterranean flora [20] and Australian *Eucalyptus spp*. [24] revealed the widespread presence of atmospheric terpenes. These terpenes differed, depending on the topographical position of the trees [20]. Herbivory by weevils on Norway spruce was shown to cause a several-fold increase in emission of monoterpenes, mainly α-pinene, β-pinene and camphene [13]. A number of species of bacteria and fungi were shown to be able to degrade emitted monoterpenes from southern yellow pinewood, and reduce total monoterpenes by at least 94% [68]. Bioconcentration of atmospheric terpenoids was reported when leaves of nine different species were found to take up the terpenes, and notably higher levels were produced by plants producing volatile terpenes, namely juniper, rosemary and pine [51].

3.7. Analysis of terpenoids in urban and industrial environments

Semiurban air in the US was analysed for volatiles, and monoterpenes were identified amongst the pollutants [69], and air sampled in industrial environments, a pine processing plant [25], and industrial flue gas from a decorative ceramic kiln [17], both revealed the presence of monoterpenes. Finnish water samples were also shown to be contaminated by monoterpenes [33], and leachates from three Swedish landfills also showed presence of volatile terpenes [70]. Monitoring of indoor air samples showed living areas to have become increasingly polluted due to increased use of timber and varnishes, causing the worry that this could induce allergic contact dermatitis, especially when air levels of α-pinene were found to reach up to 10 $\mu g/m^3$ [71]. After such exposure, the major metabolite of α-pinene, *trans*-verbenol could be detected in the urine of individuals.

An important ecological and environmental property of volatile terpenoids was discovered after GC analysis of a range of plants showed that those containing essential oils (particularly seven monoterpenes) had uptake levels of environmental pollutants up to ten times higher than those devoid of volatile terpenoids [51].

3.8. Analysis of terpenoids in microorganisms

Volatile terpenoids have been reported to have been produced by a large number of species of fungi. Environmental factors and substrate seem to have a great effect on level and type of terpene. A large number of these terpenoids were found when screening 47 *Penicillium* taxa [72], including 11 mono- and 37 sesqui-terpenes identified by retention indices and MS data. Volatile terpenoids released during culture, were adsorbed onto carbon black for 2 weeks, prior to solvent extraction and identification. Citronelline, limonene, linalool and methyl isoborneol were monoterpenes most frequently identified in different species, whilst geosmin, β-elemene, γ-elemene, and β-caryophyllene were the most frequently represented sesquiterpenes.

3.9. Analysis of terpenoids in miscellaneous products

Although the sesquiterpene parthenolide was not shown to be emitted by growing feverfew plants, a number of potential monoterpene allergens were identified [73]. From the 8 mg emitted daily per

plant, 88% of the total was composed of α-pinene, camphene, limonene, γ-terpinene, (*E*)-β-ocimene, linalol, ρ-cymene, (*E*)-chrysanthenol, camphor and (*E*)-chrysanthenyl acetate.

Carton-board packaging was shown to produce both taint and odour, caused by presence of monoterpenes [74], and depositions found on the surfaces of 13th–17th century Italian buildings have been found to contain a number of monoterpenes, including camphene, cymene, limonene, and camphor, and it is thought that these depositions indicate atmospheric transport of plant terpenes to the sites [10]. Using tandem GC with both DB wax and a chiral cyclodextrin phase, 23 monoterpene hydrocarbons including enantiomers, were identified in xylem and needles from 41 *Picea abies* trees. In the xylem, (−)-α-pinene and (−)-β-phellandrene, and in the needle samples (−)-α-pinene and (−)-limonene and (−)-camphene dominated over their (+)-enantiomers [74].

4. Problems and perspectives

The major qualitative problem with use of modern GC equipment, is operator reliance on structures predicted by computerised data libraries, in particular the use of mass spectral libraries. With over 230 naturally occurring sesquiterpenes with a molecular mass of 204, it would be very difficult to guarantee unequivocal identity, as many would exhibit the major fragment ions. When structural identification is backed up with retention indices, confirmation is more likely, but recently reproducibility of RIs has been questioned under temperature programming conditions [75]. Unequivocal identity in these situations would require corroborative data, preferably other spectroscopic data.

Quantitative data are also widely available from computerised integration software, and this is also liable to calculation of varying concentrations of constituents of an essential oil, due to differing degrees of resolution of constituents on different stationary phases, on columns of different dimensions, of different ages, and with a variety of different injection systems. Both these problems can of course occur with any class of compounds, but the mono- and sesquiterpenes are large groups of compounds, frequently present in combinations with large numbers of either or both of these groups.

5. Summary

Due to the high volatility of the mono- and sesquiterpenes, gas chromatography has been the analytical method of choice for some time. With the use of a range of adsorption concentration techniques, very sensitive detectors and tandem systems, it is now possible to quantify and characterise large numbers of small amounts of these materials.

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