Determination of α-/β-Thujone and Related Terpenes in Absinthe using Solid Phase Extraction and Gas Chromatography

Joachim Emmert[1#], Günter Sartor[1], Frank Sporer[2] and Joachim Gummersbach[3]

[1]Fluka Production GmbH, Industriestr. 25, CH-9471 Buchs, Switzerland
[2]Pharmaceutical Biology (IPMB), Ruprecht-Karls University, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany
[3]Thermo Electron Corporation, Im Steingrund 4–6, D-63303 Dreieich, Germany

Summary

A solid-phase extraction method (SPE) is proposed as a novel sample preparation method for the simultaneous determination of α-, β-thujone, and anethole in absinthe. It is followed by capillary gas chromatography (GC) with flame ionisation detection (FID) or mass spectrometry (MS) for final quantitation of the terpenes. Anethole was quantitated with external standard calibration, whereas α-thujone was evaluated by standard addition and finally confirmed by MS. In many samples a terpene with almost identical retention time in GC interfered with α-thujone, which was revealed in MS as linalool (M = 154.2). This compound was also found in extract of wormwood (*Artemisia absinthium* L.) and is very likely responsible for formerly reported high values of thujone in absinthe. The use of MS for quantitation or at least for identification of the compounds is therefore highly recommended.

Zusammenfassung

Eine Festphasen-Extraktion wird als neue Probenvorbereitungs-Methode zur simultanen Bestimmung von α-, β-Thujon, und Anethol in Absinth vorgeschlagen. Zur quantitativen Bestimmung der Terpene wird danach eine gaschromatographische Trennung (GC) durchgeführt mit anschliessender Detektion entweder mit Flammenionisationsdetektor (FID) oder Massenspektrometer (MS). Die Quantifizierung des Anethols erfolgt über Externe Standard Kalibrierung, die von α-Thujon mittels Standard-Addition. In vielen Proben wurde ein Terpen beobachtet, das nahezu die gleiche Retentionszeit wie α-Thujon hatte und dessen Bestimmung störte. Es wurde im MS schließlich als Linalool identifiziert (M = 154,2). Dieses Terpen wurde auch im Extrakt von Wermutkraut (*Artemisia absinthium* L.) gefunden und ist sehr wahrscheinlich die Ursache für die früher berichteten hohen Thujonwerte in Absinth. Es wird daher empfohlen zur Quantifizierung oder zumindest Identifizierung der Inhaltsstoffe unbedingt ein MS einzusetzen.

Keywords: absinthe, thujone, anethole, linalool, *Artemisia absinthium* L., GC/FID, GC/MS / Absinth, Thujon, Anethol, Linalool, *Artemisia absinthum* L., GC/FID, GC/MS

Introduction

A controversial discussion is going on about the amount of thujone in absinthe, a traditional bitter spirit, which is legally back on the market in Europe[1)], after having been banned in many countries for about 70 years. The subject is often treated very speculatively and based on historical anecdotes and mystification, but only few analytical data have been published recently[2,3)]. Analytical methods at the end of the 19th century, when absinthe was invented as a herbal extract spirit, were neither able to determine the small amounts of thujone, a monoterpene, which is blamed to cause severe health problems, nor to distinguish between the two isomers α- and β-thujone. Some modern official methods for food control use distillation and packed or capillary gas chromatography (GC) columns with flame ionisation detection (FID)[4,5)]. Other approaches for sample preparation propose liquid/liquid extraction[6,7)] or headspace sampling combined with solid-phase microextraction[8)].

A separation of α- and β-thujone is important, because the α-isomer is said to have higher toxicity[9)]. Some papers discuss the impact of α-thujone on the central nervous system, and, in more detail, which acceptors are blocked[9–11)], or the health hazard in general[12)]. Nevertheless, from an analytical point of view, too less emphasis has been put on sample preparation and the detection after chromatographic separation. An overview of history, toxicity and analytics of absinthe is recently given in Ref.[13)].

This work presents for the first time a sample preparation method with solid-phase extraction (SPE) for simultaneous determination of thujone and anethole, and compares separation methods using capillary GC with either FID or MS detection. Retention index (RI) and comparison of MS data with library entries are used for identification of compounds, external standard calibration and standard addition are used for quantitation of anethole and α-/β-thujone respectively.

Materials and Methods

Chemicals

All solvents (water, methanol, hexane) were of analytical grade (*Fluka*, Buchs, Switzerland), terpene standards (anethole, α-thujone, α-/β-thujone, fenchone, linalool) were also from Fluka, solid-phase extraction columns (DSC-18, DPA-6S, Envi-carb) from *Supelco* (Bellefonte PA, USA). Samples were dissolved in methanol after processing, oils and ex-

corresponding author: Dr. Joachim Emmert, e-mail: JEmmert@sial.com

tracts, measured for comparison purposes, were diluted 1 : 200 in hexane.

Instruments

GC/FID separations were performed on a Mega2 HRGC (*Carlo Erba, Thermo Electron*, Dreieich, Germany), equipped with a 30m BGB-5 (5 % diphenyl-, 95 % dimethylpolysiloxane) capillary column, 0.25 mm ID, 0.25 µm film (*BGB Analytik*, Anwil, Switzerland). The temperature program was 50–250 °C rising with 5 °/min, and the carrier gas was helium; 1µl-samples were injected with a split ratio of 1 : 20 on a split/splitless injector.

In addition, GC/MS runs were performed on a Polaris Q ion trap or a DSQ single quadrupole mass spectrometer each coupled with a Trace 2000 GC (*Thermo Electron*, Dreieich, Germany). For separation a 30 m J&W DB-5 (5 % diphenyl-, 95 % dimethylpolysiloxane) capillary column, 0.25 mm ID, 0.25 µm film (*Agilent Technologies*, Waldbronn, Germany) was used. Split, injection volume, carrier gas and flow, as well as the temperature program were identical to the GC/FID.

MS conditions: fullscan mode 50–500 dalton, total ion current (TIC), to obtain full scan spectra for subsequent library search.

Tab. 1 Concentrations of thujone and anethole in different absinthe samples obtained with GC/FID (values in brackets are revised results after MS detection, n.d. = not determined)

Sample No.	Sample Name	α-Thujone [mg l^{-1}]	β-Thujone [mg l^{-1}]	Anethole [mg l^{-1}]
101	Mata Hari	1.9	9.9	11 (0)
102	Grüne Fee	2.4	9.0	7 (6)
103	Versinthe Blanche	24.7 (5.2)	4.8	3220
104	La Fee 68°	0.0	0.0	1184
105	Pernod 68°	0.0	0.0	1056
106	Francois Guy	4.8	20.0	1334
107	Emile Pernot 68°	3.2	0.9	412
108	Oxygenee	2.2	0.0	1025
109	Segarra	0.0	0.0	1208
110	Candela	33.3 (0.0)	7.9	2669
111	Rote Fee Anis	19.2 (0.0)	0.0	740
112	Absente	2.6	1.6	1365
113	Prohibido	10.3 (0.0)	3.0	337
114	Martini rosso	0.0	14.4 (0.0)	0
115	Fuchs Absinth	8.7 (0.0)	0.0	434
116	Pernod Anis	0.0	0.0	2484
117	Pernod Tarragona	0.8	0.5	n.d.

Sample Preparation

The sample preparation with solid-phase extraction was performed with 1 ml SPE-tubes, filled with C18 material (DSC-18) to separate the lipophilic terpenes from the alcoholic spirit matrix. In a first step, the tube was activated with 1 ml methanol, followed by conditioning with 1 ml water. Then exactly 1 ml of sample or spiked sample was applied, to be washed next with 1 ml water to dryness. After this cleaning step, the sample was eluted with exactly 1 ml methanol directly into the sample vial.

Results and Discussion

Different SPE materials were evaluated to obtain both a good separation from the matrix and a high recovery of the analytes. But only C18 material yielded in acceptable results. With other materials, e.g. polyamide (DPA-6S) or carbon (Envicarb), no useful experimental set-up could be found to achieve sufficient recovery of both anethole and α-thujone. With C18 material a breakthrough of colour in step 3 and 4 (application of sample and washing) was observed for many samples and could be correlated with the presence of artificial food dye. Absinthes with natural colouring did not show this effect, but this had no further impact on subsequent analysis steps. Recovery of anethole was found within 95–100 % in multiple runs. Accordingly, quantitation in sample spirits was done with external standard calibration. The recovery of α-thujone was not as good and varied depending on the

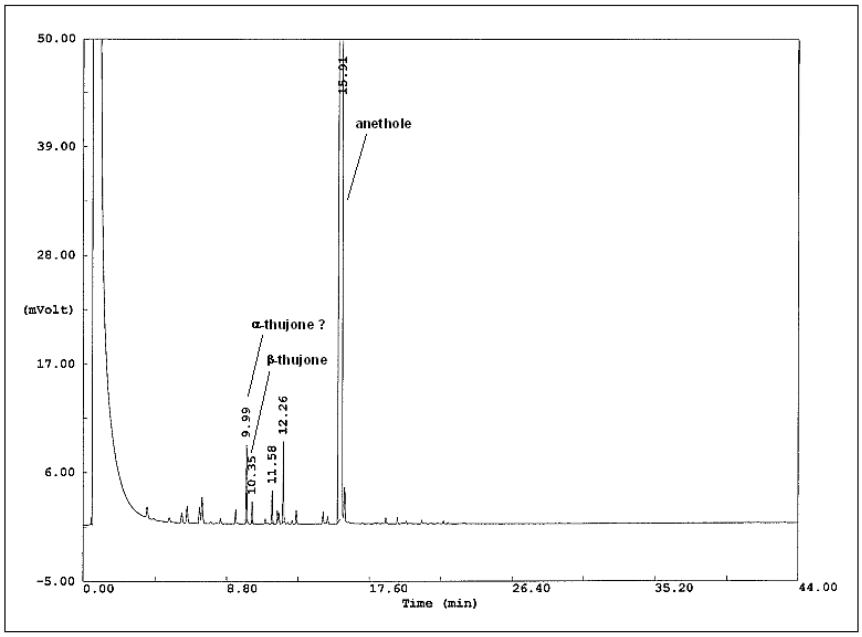


Fig. 1 GC/FID chromatogram of sample 110, Candela; it implies the presence of α- and β-thujone

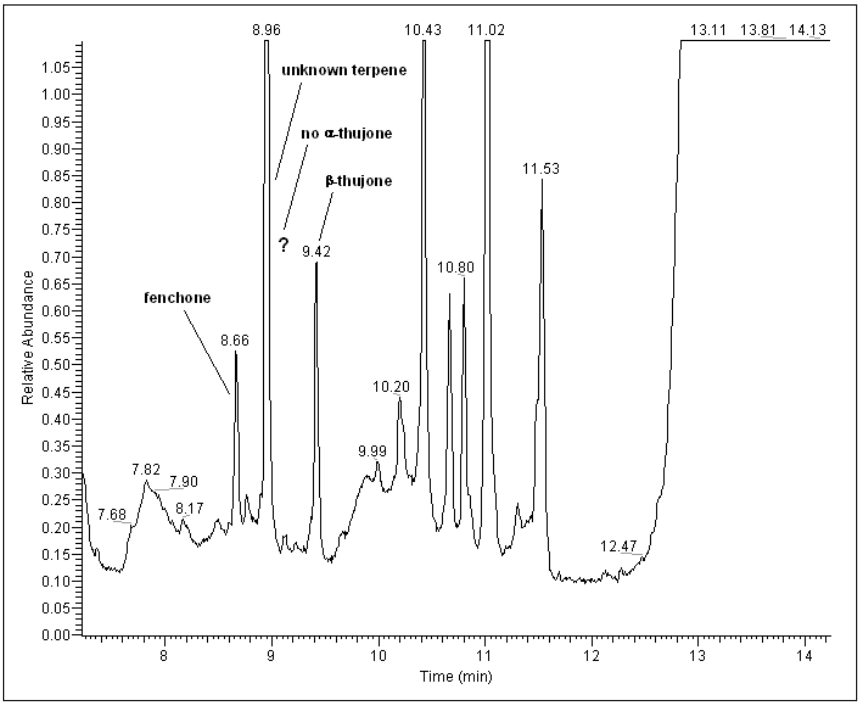


Fig. 2 GC/MS chromatogram (TIC) of sample 110, Candela (no α-thujone, but another (first unknown) terpene and fenchone)

matrix of the different absinthes between 40 and 70 %. Therefore, the amount of α-thujone was calculated with standard addition to each sample; β-thujone, where no high purity standard is available, was calculated later with the response factor of α-thujone of the respective sample. With this procedure, a detection limit of 0.1 mg l^{-1} and a precision of 5 % could be achieved. For anethole the precison was better than 2 %, but with much higher absolute values.

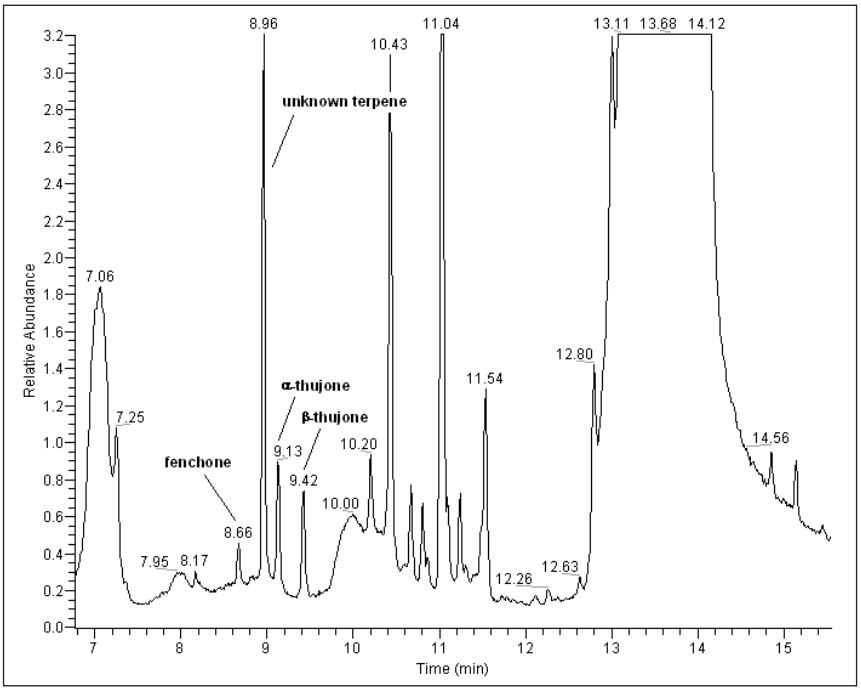


Fig. 3 GC/MS chromatogram (TIC) of sample 103, Versinthe Blanche (α-thujone and the unknown terpene are present)

The detection limit of anethole was not determined in detail, but in the same range as for thujone. After a brief check for linearity we worked with one-point calibration, because anethole was not the main focus of this work. All quantitative work was done by GC/FID, MS measurements were complementary. Further investigations of some parameters may be needed, before using the method in routine work.

Table 1 summarizes all quantitative results of α-/β-thujone and anethole for 17 different spirits obtained with GC. These values were double-checked with MS, some additional substances were only identified with MS, without quantitation. Obviously, some results had to be revised after MS.

The main differences between different recipes of absinthes include extraction procedure of the herb *Artemisia absinthium* L. and subsequent steps, e.g. alcoholic maceration and distillation. The plants contain many chemically diverse compounds; the composition may vary between different species within the family, and is depending from the region and the time of growth[14–20]. The used solvent is of great influence on the result of the extraction. Martini i.e., which was investigated for comparing purposes, is a bitter wine (no absinthe) and contains only the water-soluble bitter compounds of *Artemisia absinthium* L., but no thujone, because it is extracted with water. A compound eluting at the same retention time as β-thujone was identified as phenyl ethanol by MS.

Some absinthes showed rather high concentrations of α-thujone in GC/FID, i.e. sample 110, Candela (Fig. 1), which could be confirmed in a first step with standard addition. Further investigation with GC/MS however uncovered, that no α-thujone was present, but an additional unknown compound (Fig. 2), which could not be identified at first glance, and fenchone. In sample 103, Versinthe Blanche, both the unknown compound and α-thujone are present (Fig. 3). The quantitative result for α-thujone had to be corrected to a much lower amount than originally measured with GC. The unknown terpene, which could be identified by MS later, is relatively constant in most samples, whereas the amount of α-thujone varies quite dramatically, but at a rather low concentration level. These

results show the importance of a very good separation on the GC column and a doubtless identification of the measured analyte, e.g. by MS.

In order to obtain some more information about the unknown compound, a distillation of wormwood herb (*Artemisia absinthium* L.) was performed in our own laboratory. The substance could be found in rather high concentration in the extract, thus confirming, that it originates from the plant and is no artefact. Additional runs were performed with this sample on a quadrupole GC/MS system to obtain comparable mass spectra for a library search, because terpene spectra from the ion trap MS detector, which we used first, were not compatible with common library entries. In this run α- and β-thujone could be identified easily (Fig. 4) and finally also the unknown terpene as linalool (Fig. 5). The molecular mass is 154.2 D, which can hardly be seen as molecular ion, because of a very easy loss of 18 dalton (H_2O). The retention index (RI) is 1100 on a DB-5 column, which is very close to α-thujone.

Using GC/MS, none of the analyzed absinthe samples exceeded the maximum limit of 35 mg l^{-1} for thujone in bitter spirits[1]. However surprisingly, five absinthes did not contain thujone at all. According to Ref.[4] thujone concentrations below 2 mg l^{-1} disprove the use of wormwood as an ingredient, suggesting careful evaluation of absinthe spirits by official food control. Furthermore, the natural relation of the thujone isomers in wormwood shows normally β-thujone as the more abundant isomer. Higher values for α-thujone indicate either unnaturally added α-thujone or an interference with another compound.

The analysis of sample 117, a vintage Pernod absinthe from Tarragona (ca. 1930) showed only a relatively low thujone concentration of 1.3 mg l^{-1} (sum of α- and β-thujone). This confirms the results of *Hutton*[3], who also found only 6 mg l^{-1} thujone in a vintage Pernod absinthe (ca. 1900). Thujone concentrations as high as 260 mg l^{-1}, reported in the 19th century[3], may therefore have been the result of inadequate analytical techniques.

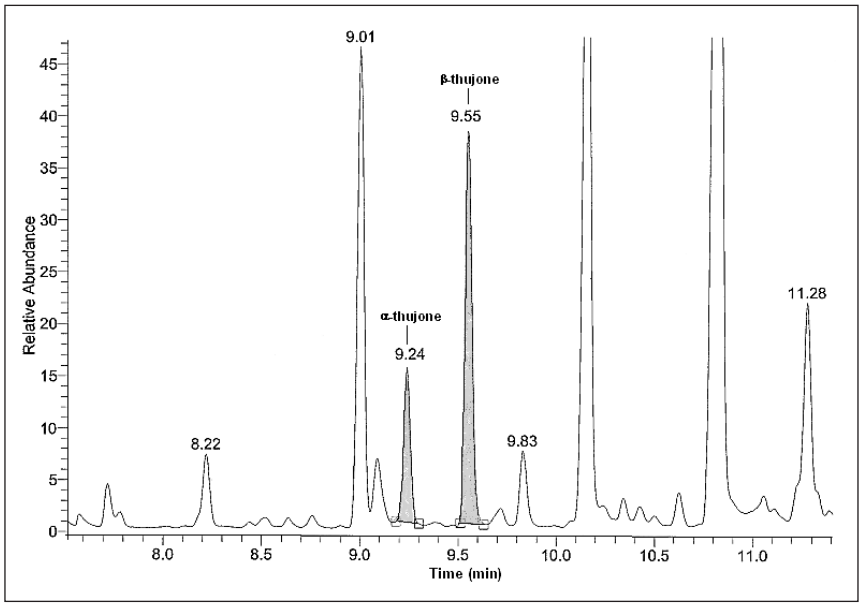


Fig. 4a Single quad GC/MS chromatogram (TIC) of an *Artemisia absinthium* L. extract (α- and β-thujone are present)

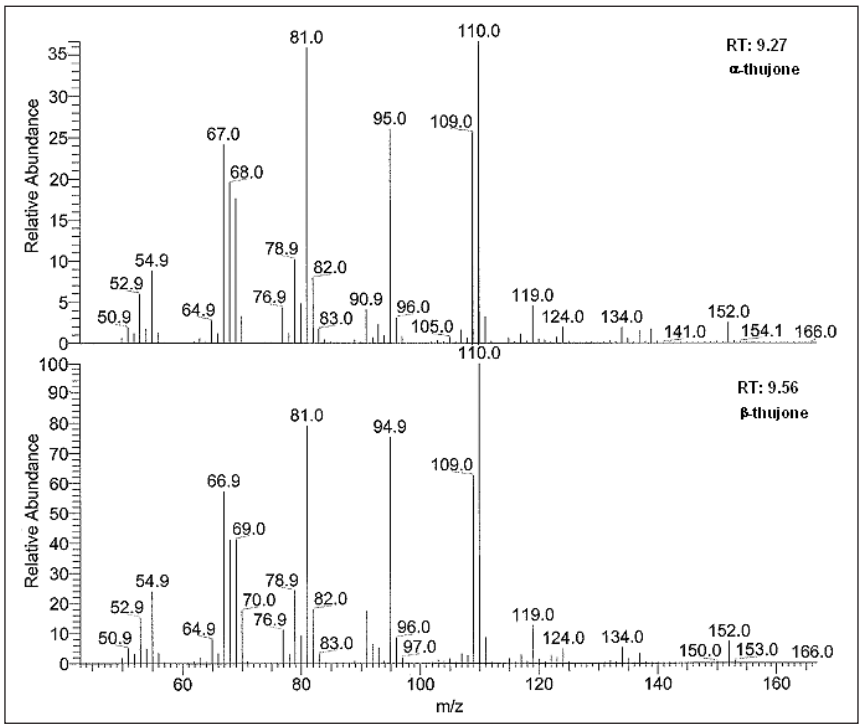
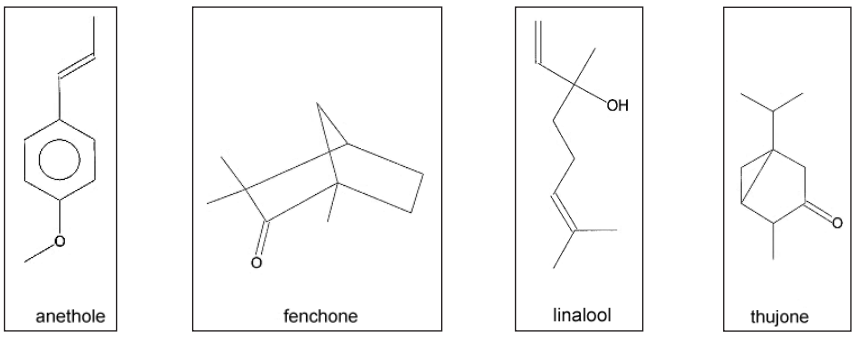


Fig. 4b Single quad mass spectra of α- and β-thujone



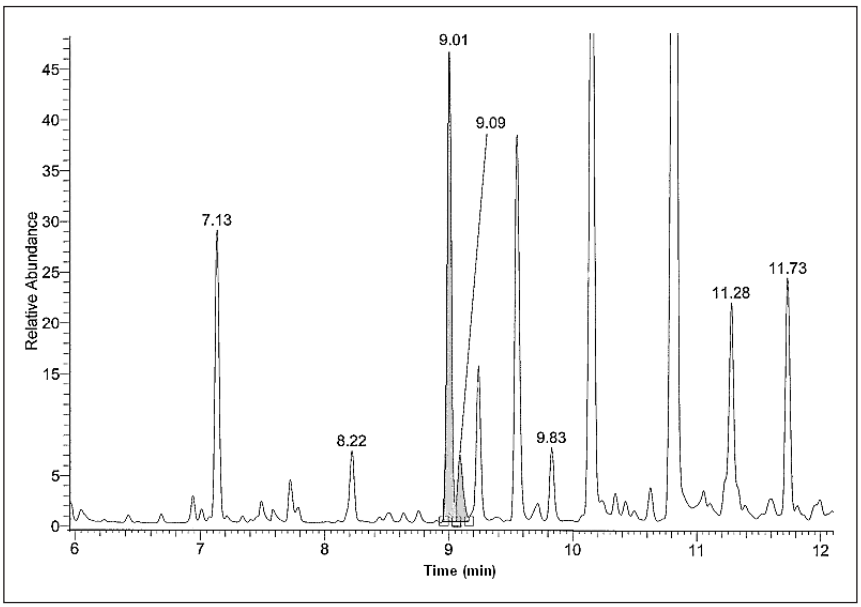


Fig. 5a Single quad GC/MS chromatogram (TIC) of an *Artemisia absinthium* L. extract (the unknown terpene at 9.01 min could be identified as linalool)

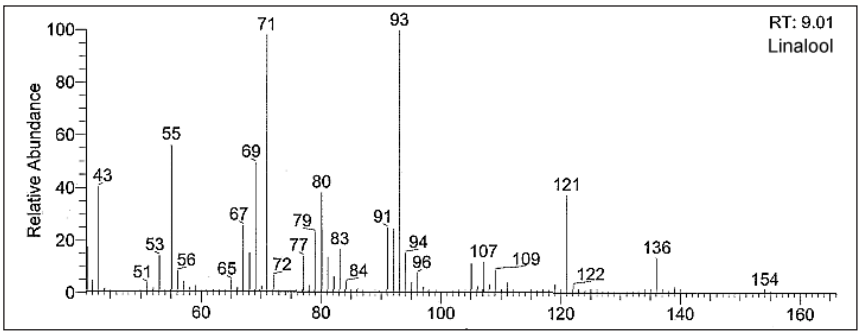


Fig. 5b Single quad mass spectrum of linalool

In conclusion, SPE has been proven as a quick and useful sampling preparation technique for the determination of terpenes in absinthe, especially α-thujone. Methods used in food control often use distillation or liquid/liquid extraction, which are both time consuming and error prone. In addition, this method offers the ability for simultaneous determination of several ingredients, e.g. thujone and anethole. Furthermore, it has been shown, that a powerful separation method is neccessary to avoid interference of α-thujone with linalool, which is present in the plants extracted for absinthe production. The use of MS for qualification of the quantitated peaks is highly recommended. Historically reported high results for α-thujone in absinthes may be caused by analytical methods, which were not able to differentiate between the above-discussed terpene species.

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References

1) Council of the European Communities: Off. J. Europ. Comm. **L184**, 61–66 (1988).
2) *Lang M., C. Fauhl* und *R. Wittkowski*: Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, BgVV-Hefte 08/2002, Berlin.
3) *Hutton, I.*: Curr. Drug Discov. **9**, 62–64 (2002).
4) Bundesamt für Gesundheit: Schweizerisches Lebensmittelbuch 32/13, 1–3. Bern, Schweiz (2000).
5) International Organization of the Flavor Industry (I.O.F.I.) Recommended methods: Z. Lebensm. Unters. Forsch. **186**, 36–38 (1988).
6) *Przyborski, H.* und *F. Bandion*: Mitt. Klosterneuburg 42, 171–178 (1992).
7) *Rapp, A., H. Hastrich, I. Yavas* und *H. Ullemeyer*: Branntweinwirtsch. 134, 286–289 (1994).
8) *Kröner, L. U., S. A. Padosch, M. S. Brückner, D. W. Lachenmeier, F. Mußhoff* und *B. Madea*: Lebensmittelchemie **57**, 78 (2003).
9) *Höld, K. M., N. S. Sirisoma, T. Ikeda, T. Narahashi* and *J. E. Casida*: Proc. Natl. Acad. Sci. USA **97**, 3826–3831 (2000).
10) *Olsen, R. W.*: Proc. Natl. Acad. Sci. USA **97**, 4417–4418 (2000).
11) *Meschler, J. P.* and *A. C. Howlett*: Pharm. Biochem. Behav. **62**, 473–480 (1999).
12) *Hein, J., L. Lobbedey* und *K. J. Neumärker*: Dt. Ärzteblatt **98**, A2716–2724 (2001).
13) *Lachenmeier, D. W., W. Frank, C. Athanasakis, S. A. Padosch, B. Madea, M. A. Rothschild* und *L. U. Kröner*: Deut. Lebensm.-Rundsch. **100**, 117–129 (2004).
14) *El-Shazly, A., G. Dorai* und *M. Wink*: Z. Naturforsch. **57**c, 620–623 (2002).
15) *Pala-Paul, J., A. Velasco-Negueruela, M. J. Perez-Alonso* and *J. Sanz*: J. Chromatogr. A **923**, 295–298 (2001).
16) *Chialva, F., P. A. P. Liddle* und *G. Doglia*: Z. Lebensm. Unters. Forsch. **176**, 363–366 (1983).
17) Sacco, T. and F. Chialva: Planta Med. **54**, 93 (1988).
18) *Nin, S., P. Arfaioli* and *M. Bosetto*: J. Essent. Oil Res. **7**, 271–277 (1995).
19) *Arino, A., I. Arberas, G. Renobales, S. Arriaga* and *J. B. Dominguez*: J. Essent. Oil Res. **11**, 182–184 (1999).
20) *Pino, J. A., A. Rosado* and *V. Fuentes*: J. Essent. Oil Res. **9**, 87–89 (1997)