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Steam distillation–solid-phase microextraction for the detection of *Ephedra sinica* in herbal preparations

Mari R. Tellez [a,*], Ikhlas A. Khan [b], Brian T. Schaneberg [c], Sara L. Crockett [d], Agnes M. Rimando [a], Mozaina Kobaisy [a]

[a] *Natural Products Utilization Research Unit, US Department of Agriculture, Agricultural Research Service, Box 8048, Oxford, MS 38677, USA*
[b] *National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, Department of Pharmacognosy, School of Pharmacy, University of Mississippi, Oxford, MS 38677, USA*
[c] *National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, Oxford, MS 38677, USA*
[d] *Department of Pharmacognosy, School of Pharmacy, University of Mississippi, Oxford, MS 38677, USA*

Abstract

A new method involving concurrent solid-phase microextraction combined with continuous hydrodistillation of essential oil was developed. This new methodology allowed for the detection by GC–MS of very small amounts of a diagnostic peak for the authentication of *Ephedra sinica*, in a short period of time and using only small sample sizes. This diagnostic peak was identified as 4-vinylanisole, and elucidated from the chromatographic profile allowed for the identification of a sample as *E. sinica* among other species investigated in this study. To the best of our knowledge this is the first report on using continuous solid-phase microextraction coupled to hydrodistillation for the investigation of essential oil components, and the first report of 4-vinylanisole as a marker compound for *E. sinica*. A total of 46 collections representing 21 species of *Ephedra* were studied.

Keywords: *Ephedra sinica*; Steam distillation; Extraction methods; Essential oils; Pharmaceutical analysis; Plant materials; Vinylanisole

1. Introduction

Ephedra sinica Stapf. (Chinese ephedra, mahuang, Ma Huang) is an evergreen shrub native to Asia. It is included in a number of pharmacopeias including the Chinese, German, and Japanese pharmacopeias for the treatment of various ailments, and is therefore of considerable economic importance. Recently an excellent monograph has been published detailing the uses and contraindications of *E. sinica* [1]. Use of this plant as a herbal treatment for ailments or as herbal dietary supplement for weight loss or enhanced athletic performance has come under FDA and scientific scrutiny because of many issues concerning its safety [2–19]. Many efforts are currently being expended to find methods that would allow for the identification of *E. sinica* in various forms, especially in situations where the plant material cannot be recognized (e.g. ground material in capsules or other formulations). The aim and objective of this work was to establish a protocol that could authenticate the presence of *E. sinica* in ground plant material and allow for the differentiation of *E. sinica* from other *Ephedra* species that are usually not encountered in commercial formulations. Many studies on the chemical constituents of *E. sinica* and other *Ephedra* species, including their alkaloid, flavonoid, lipid, and essential oil composition, have been previously reported as have methodologies for the screening of their alkaloid contents [20–42].

2. Experimental

2.1. Plant material

Samples of *Ephedra* were obtained commercially from FrontierCoop (Norway, IA, USA), or collected as follows: *E. sinica* (nine collections) from Hebei and Neimeng Provinces, China, *E. alata* (two, male and female) from Egypt,

* Corresponding author. Tel.: +1-662-915-5898; fax: +1-662-915-1035.
E-mail address: matellez@ars.usda.gov (M.R. Tellez).

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E. antisyphilitica (three) from Brewster, Pecos and Terrell Counties, TX, USA, *E. aspera* (four including one *E. aspera* × *trifurca*) from Brewster Co., TX, USA and Riverside Co., CA, USA, *E. californica* (two) from Riverside and San Bernadino Co., CA, USA, *E. coryi* (two) from Socorro Co., NM, USA, *E. distachya* [ssps. helvetica (one) and distachya (two)] from Italy and Sardinia, *E. fasciculata* (three) from Mohave Co., CA, USA, *E. foeminea* (one) from Turkey, *E. likiangensis* (one) from Yunnan Province, China, *E. major* (two) from Pakistan and central Italy, *E. nevadensis* (one), *E. ochreata* (one) from Argentina, *E. pedunculata* (one) from Dimmitt Co., TX, USA, *E. torreyana* (four including one ssp. powelliorum) from Jeff Davis and Brewster Co., TX, USA and Socorro Co., NM, USA, *E. triandra* (one) from Argentina, *E. trifurca* (four) from Brewster and Jeff Davis Co., TX, USA and Otero Co., NM, USA, and *E. viridis* (one) from Kane Co., UT, USA.

2.2. Essential oil isolation and chemical characterization

Steam distillation and analyses of the essential oil of *Ephedra sinica* (90.370 g) and *E. cutleri* (113.348 g) were conducted as previously described [50–52]. Analyses were performed by GC–MS [electron impact ionization (EI), 70 eV] with a DB-5 column (30 m × 0.25 mm fused-silica capillary column, film thickness 0.25 μm) using He as carrier gas (1 ml/min), 1 μl injection size and a programmed (injector temperature 220 °C, transfer line temperature 240 °C, initial column temperature 60 °C, final column temperature 240 °C, 3 °C/min) temperature run [52,53]. A shorter method was developed to screen for the presence of 4-vinylanisole by modifying the oven temperature program so that after 13.33 min the temperature gradient was changed to a rate of 90 °C/min and a 5.11 min isotherm added at the final temperature of 240 °C (total program time 20 min). Identification of oil components was performed by comparison of mass spectra with literature data, and by comparison of their relative retention times with those of authentic compounds, or by comparison of their retention indices with those in the literature [53,54]. The relative amounts (RAs) of individual components of the oil are expressed as percent peak area relative to total peak area. Clear yellow oils were obtained in yields of 69.6 mg (0.08% dry mass) and 34.84 mg (0.03% dry mass) for *E. sinica* and *E. cutleri*, respectively. Furfural, *p*-anisaldehyde, benzaldehyde, β-ionone, 2,3,5-trimethylpirazine, 2,3-dimethylpirazine, 2-acetylfuran, and 4-vinylanisole used as standards were purchased from Aldrich (Milwaukee, WI, USA).

2.3. Isolation, identification and quantitation of 4-vinylanisole

Column chromatography of the essential oil of *E. sinica* (58 mg) was carried out on silica gel 60 (EM Science, 0.063–0.200 mm) (10 cm × 2 cm) first equilibrated with 400 ml of pentane. Elution was performed first with 1 l of pentane, then 1 l of dichloromethane and 1 l of methanol collecting 100-ml fractions. Solvents from each fraction were reduced to 10-ml volumes. The peak at 738 s was found in the third through the sixth pentane fractions. This peak was identified as 4-vinylanisole by mass spectrometry and NMR. A commercial standard of 4-vinylanisole (Aldrich) was identical in all respects with the isolated compound.

2.4. Extractions

Room temperature in the absence of added water: *E. sinica* (500 mg) was ground in the presence of pentane (20 ml) with a mortar and pestle for 5 min. Solids were filtered and the filtrate analyzed “as is” by GC–MS or after further concentration to 1 ml and to 100 μl.

Room temperature in the presence of water: *E. sinica* (500 mg) was ground in the presence of water (20 ml) with a mortar and pestle for 5 min. The mixture was extracted with pentane (20 ml) and the pentane extract examined by GC–MS without further work up or after further concentration to 1 ml and to 100 μl.

Reflux in water: *E. sinica* (1.1 g) was allowed to reflux in water (25 ml) for either 4 h or 30 min. The mixture was allowed to cool to room temperature and then extracted with pentane (20 ml) which was then examined by GC–MS without further work up.

2.5. SD–SPME of Ephedra and 4-vinylanisole

SD–SPME was performed in the apparatus shown in Fig. 1, using a 50-ml round bottom flask as the refluxing vessel along with 25 ml of water. Heating was achieved with a heating mantle and a magnetic stir bar was used for stirring. SPME fibers (Supelco, Bellefonte, PA, USA; part 57341-U) consisted of a 100 μm polydimethylsiloxane coating on a 23 gauge needle. Loaded SPME fibers were stored at −20 °C in capped test tubes until GC–MS analyses were performed. Injections were performed manually in an unmodified GC injector with desorption times of ∼2 s. Fibers were reconditioned between runs in a second injector set at 260 °C for 30 min with a helium flow (1 ml/min). Fibers were then stored in a vacuum desiccator for at least 15 min or until used. Blank injections with reconditioned fibers were performed at least once a week to ensure fiber integrity and absence of analyte build up.

SD–SPME was conducted on 1.14 g of *E. sinica* (ES1) with 30 min reflux, and on 1.03 g *E. sinica* (ES1) and 1.07 g *E. sinica* (ES2) with 10 min reflux each. SD–SPME was also carried out for *E. sinica* (ES1) with 30 min reflux and 108, 11, 1, and 0 mg of plant material. Three additional runs using 1 mg and 10 min reflux were also performed.

All *Ephedra* samples were analyzed in triplicate by SD–SPME with a 30-min reflux and 112 ± 13 mg of plant material. We chose 30 min because this maximized time of fiber exposure while keeping it within sampling time frames limited by the GC–MS runs. One SD–SPME of

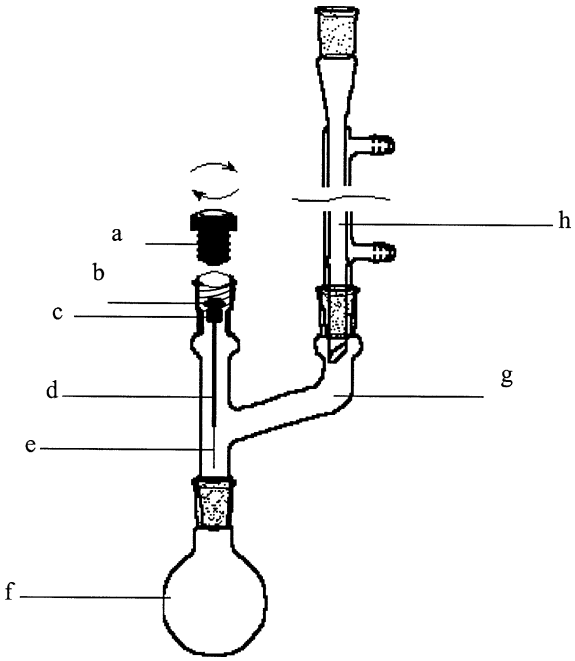

Fig. 1. SD–SPME assembly. a, Compression nut or cap; b, O-ring; c, threaded plug (hub) of SPME fiber assembly; d, protecting needle of SPME fiber assembly; e, SPME fiber; f, round bottom flask; g, Claisen distillation head; h, condenser.

E. sinica, which had already been analyzed to ensure the presence of 4-vinylanisole, was conducted first thing each day to establish that the marker peak could be detected.

To establish the Kovat's index, necessary to characterize compounds by GC–MS, the homolo- gous series of *n*-hydrocarbons (10 ng of C_8–C_{25} each in 1 μl pentane) were loaded onto the SPME fiber using the same set-up (50-ml round bottom flask, 25 ml water). Standards of 4-vinylanisole were introduced in the same way and SD–SPME conducted as above. The limit of detection (LOD) was calculated from these results based on three times the signal over noise with help of program software. The experiment was also performed with 1 μg of 4-vinylanisole in 3 l of water using the same set-up with the exception that a 55/50 to 24/40 ground glass adapter was also used to match the SPME assembly described in Fig. 1 with the larger distilling flask.

2.6. Automated SPME of E. sinica

SPME autosampler runs on *E. sinica* (ES1) were performed with 100 mg ground material in 20-ml vials with water. Samples were incubated for 1 min and sampled (absorption time) for 10.5 min with shaking at 300 rev./min (10 s on, 4 s off), before injection (3-s desorption). Sampling and incubation temperatures tested were 65 °C (10 ml water), 105 °C (10 ml water), 200 °C (10 ml water), and 140 °C [2 ml with and without NaCl (100 mg)].

3. Results and discussion

Preliminary steam distillation of 100 g of *E. sinica* afforded volatile profiles (Table 1) similar to that reported in the literature [26,30]. Steam distillations of this type however are time consuming and need large amounts of sample, typically in the ten to hundreds of grams scale. Direct extraction of *E. sinica* (500 mg) with pentane (20 ml) failed to provide any volatiles observable by GC–MS under our conditions. Similar results were obtained from the pentane extraction of an aqueous mixture of plant material ground in water. Pentane extraction of an aqueous (25 ml water) plant mixture (1.1 g ground *E. sinica*) that had been subjected to 4 h reflux, on the other hand, afforded a profile of volatiles similar to that obtained by steam distillation. Shortening the reflux time to 30 min also afforded the same profile. We were unable to scale down this procedure since smaller amounts of plant material led to extracts that were too dilute for our purposes. Though we were able to compare volatile profiles of the essential oil of *E. sinica* and that of *E. cutleri*, we wanted to devise a method that would be amenable to routine analysis of small sample sizes, and in shorter periods than that required for steam distillation. Excellent results were obtained using a new technique we developed which combines continuous hydrodistilla-tion (steam distillation) of plant material with concurrent solid-phase microextraction (SD–SPME).

SD–SPME works essentially on the same concept as that used in concurrent steam distillation–extraction set-ups (i.e. using Likens–Nickersen apparatus) except that the oil is concentrated into the SPME fiber instead of being diluted in a recovering solvent. The set-up is as shown in Fig. 1 and uses standard laboratory equipment. In short, a round bottom flask containing the plant material and water is fitted with a simple distillation head and a condenser set up for reflux rather than distillation. A regular SPME fiber assembly, as normally used with an autosampler, is placed where a thermometer is usually situated. This is possible because the O-ring that normally holds and seals the thermometer in place is exactly the right size to hold the plastic plug (hub) situated on one end of the fiber assembly (the hub is designed to thread the SPME assembly onto the autosampler's holder). The O-ring is placed around the threaded part of the plastic hub and sealed in place by the nut that is used to seal the thermometer. The SPME fiber, which is extended from its protecting needle casing, sits right below the junction to the condenser and is therefore in place to trap volatiles while at the same time not being affected by the returning condensate that drips back to the distilling flask via the sides of the apparatus. In this location the fiber does not sample the solid plant material nor the liquid aqueous phase, but rather the vapors as they are refluxing throughout the continuous distillation. The SPME fibers were reused multiple times (10–20 times per fiber) and, as long as proper reconditioning was performed (see Section 2), no carry over was observed (as monitored by a weekly GC–MS of the recon-

Table 1
Constituents of the oil of *Ephedra sinica*

Compound	t_R (s)	*I*	%RA
3-Methyl-2-buten-1-ol	140	772	5.2
Furfural[a]	181	830	0.1
Styrene (MS only)	241	889	0.1
Heptanal	250	896	t
2-Acetylfuran[a]	262	906	t
2,3-Dimethylpirazine[a]	273	915	t
Cumene	283	923	t
α-Thujene	288	927	t
α-Pinene	300	936	0.1
Camphene	321	950	t
Benzaldehyde[a]	333	958	1.5
trans-*m*-Mentha-2,8-diene	365	977	0.8
2,3,5-Trimethylpirazine[a]	405	997	0.1
1,4-Cineole	432	1014	0.6
α-Terpinene	438	1015	0.2
p-Cymene	451	1023	3.1
Limonene	461	1028	1.5
1,8-Cineole	465	1030	1.0
Benzene acetaldehyde	484	1040	0.1
α-Methylbenzenemethanol	519	1057	t
γ-Terpinene	525	1060	0.4
Acetophenone	532	1063	0.1
cis-Linalool oxide	553	1072	0.2
Tetramethylpirazine[a]	583	1084	7.4
Terpinolene	591	1087	1.8
Methyl benzoate	599	1090	0.1
Linalool	615	1097	0.3
α-Thujone	623	1100	0.2
p-Menthatriene	641	1108	t
cis-Rose oxide	642	1109	0.1
cis-*p*-Menth-2-en-1-ol	665	1119	0.1
Terpinen-1-ol	696	1133	0.8
trans-*p*-Menth-2-en-1-ol	709	1138	0.1
cis-β-Terpineol	721	1143	1.7
p-Vinylanisole[a]	738	1150	3.3
Isoborneol	751	1155	t
trans-β-Terpineol	769	1162	0.5
Ethyl benzoate	776	1165	1.6
Terpinen-4-ol	803	1175	0.8
p-Cymen-8-ol	824	1183	1.3
α-Terpineol	845	1190	13.2
Dihydrocarveol	858	1192	2.6
cis-Dihydrocarvone	866	1193	0.1
trans-Piperitol	881	1205	t
Cumin aldehyde	960	1236	0.8
Carvone	971	1240	0.1
p-Anisaldehyde[a]	992	1248	0.1
Piperitone	997	1250	t
Chavicol	1000	1251	t
trans-*p*-Menth-2-en-7-ol[b] (MS only)	1008	1254	t
cis-*p*-Menth-2-en-7-ol[b] (MS only)	1011	1255	1.8
Perrilla aldehyde	1045	1268	0.2
Dihydrolinalool acetate	1055	1271	4.1
α-Terpinen-7-al	1076	1279	0.2
trans-Anethole	1081	1281	0.1
p-Cymen-7-ol	1096	1286	1.2
Thymol	1103	1288	t
Perrilla alcohol	1116	1293	0.1
Carvacrol	1127	1296	0.3
4′-Methoxyacetophenone	1250	1345	0.1
Eugenol	1269	1353	0.1
α-Copaene	1324	1373	0.1
Longifolene	1393	1397	t
Methyl eugenol	1395	1398	t
(*E*)-β-Caryophyllene	1435	1414	0.2
Coumarin	1458	1424	0.2
α-Himachalene	1507	1444	0.4
Seychellene	1540	1457	1.2
γ-Muurolene	1582	1473	0.3
β-Ionone[a]	1603	1481	0.9
cis-β-Guaiene or Valencene	1619	1487	0.4
Bicyclogermacrene	1628	1490	0.2
β-Dihydroagarafuran	1638	1493	0.4
GermacreneA	1656	1500	0.1
Myristicin	1688	1514	0.1
cis-Calamene	1694	1517	t
δ-Cadinene	1701	1520	t
Methyldodecanoate	1705	1521	0.1
trans-Calamene	1720	1528	t
Selina-3,7(11)-diene	1742	1537	0.2
α-Agarofuran	1755	1543	t
Dodecanoic acid	1827	1572	2.0
Viridiflorol	1862	1586	0.3
Guaiol	1878	1592	0.1
10-*epi*-γ-Eudesmol	1927	1613	0.7
γ-Eudesmol	1957	1628	1.0
β-Eudesmol	1998	1647	0.1
α-Eudesmol	2005	1650	0.3
7-*epi*-α-Eudesmol	2015	1655	0.2
Cadalene	2051	1671	0.1
Juniper camphor	2094	1690	0.1
(*E*,*E*)-Farnesol	2154	1717	1.2
Methyltetradecanoate	2163	1722	0.1
1-Octadecene	2315	1791	0.1
Octadecane	2331	1798	0.2

Table 1 (*Continued*)

t_R, retention time on a DB-5 column; *I*, Kovat's indices as determined on a DB-5 column using the homologous series of *n*-hydrocarbons. RA, relative area (peak area relative to total peak area); t, trace (<0.05%).

[a] Standard used for identification was purchased from Aldrich.

[b] Tentative assignments by MS only and reported by Miyazawa et al. [26] to be present in the essential oil of *E. sinica*.

ditioned fibers) even when sampling the largest amounts of standard or plant material. Method reproducibility was good with total area counts for 4-vinylanisole in *E. sinica* ranging from 736 692 to 880 961 (eight samples of 100 mg of plant material subjected to SD–SPME over a period of 2 months).

When the SD–SPME technique was applied to plant samples, specifically 46 collections representing 21 species of *Ephedra* used in this study, good profiles were obtained with as little as 10 mg of plant material with 10 min of reflux. From these profiles, several peaks (i.e. tetramethylpirazine, α-terpineol, and dihydrolinalyl acetate) were observed to be more prominent in *E. sinica* than in non-*sinica* species. In general these early eluting peaks tend to predominate in the volatile profile of *E. sinica* compared to non-*sinica* species where sesquiterpenoids and other late eluting peaks appeared as the predominant volatile components. A peak showing at 738 s (Kovat's retention index $I = 1150$), however, was found to be qualitatively present in all

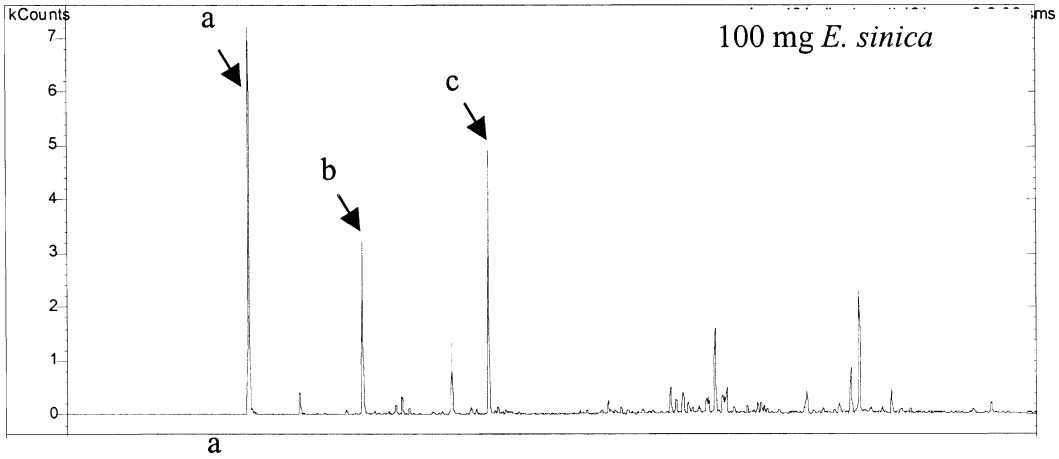

Fig. 2. Extracted chromatograms (*m/z* 134 ion) of SD–SPME of 100 mg of *E. sinica*; (a) *p*-cymene, (b) 4-vinylanisole, (c) dihydrolinalool acetate.

E. sinica specimens tested and absent from all non-*E. sinica* specimens tested with the exception of *E. major* and *E. likiangensis*, which could nevertheless be distinguished from *E. sinica* through other criteria (see below). The peak at 738 s was isolated and identified as 4-vinylanisole and was identical in retention time, index, and mass spectral signature with that of a commercially obtained standard. Interestingly, a compound identified by Miyazawa et al. [26] which has an almost identical mass spectral signature, namely 2,3-dihydro-2-methylbenzofuran, was absent from our samples. The presence of 4-vinylanisole has been reported among the steam distilled volatiles of *E. sinica* [30]. By extracting the more prominent ion of the mass spectrum on 4-vinylanisole, namely the *m/z* 134 ion, from the total reconstructed ion chromatograms, the presence of this peak in *E. sinica*, and its absence in most non-*sinica* species, could easily be established (Fig. 2) from as little as 1 mg of plant material. The limit of detection for 4-vinylanisole was calculated to be 160 fg/ml using this set-up, with concentrations as low as 4 pg/ml still giving excellent signal-to-noise ratio [average *S/N* (RMS) = 76, $n = 3$]. In addition, the presence of *p*-cymene ($I = 1023$) and dihydrolinalool acetate ($I = 1271$) serve as additional markers since these two compounds are always present along with 4-vinylanisole in *E. sinica* and are major components of the 134 ion extracted chromatograms (Fig. 2). These two additional peaks also allow for the distinction of *E. sinica* from the 4-vinylanisole containing *E. major* and *E. likiangensis* species. In the 134 ion extracted chromatograms of *E. major*, dihydrolinalool acetate is absent, while in those of *E. likiangensis*, all three peaks are present in only trace amounts along with larger, late eluting, peaks.

Use of 4-vinylanisole for the identification of *E. sinica* has the advantage that it cannot be detected in the plant or processed (i.e. ground) samples, unless the sample is subjected to aqueous reflux. The presence of 4-vinylanisole (i.e. in a pentane extract) of a sample not subjected to aqueous reflux could therefore be used as an indication of sample contamination or tampering. Naturally occurring 4-vinylanisole has previously been reported in studies of *Thymus* [43], *Ramulus* [44], *Sambucus* [45–47], *Hedyotis* [48], and from degradation of lignin by mushroom [49]; none of these studies reported the presence of dihydrolinalool acetate.

As our results have shown, SD–SPME is an excellent technique that compliments existing methodology in the investigation of volatile components in *E. sinica*, and for any plant sample in general. The method should also be ideal for the analysis of trace amounts of volatile components from large volumes of aqueous matrices, with 1 mg of 4-vinylanisole in 3 l of water (333 pg/ml) easily detected by GC–MS. We also found the SD–SPME set-up to be superior to currently available automated SPME protocols. Several experiments with an automated SPME autosampler at temperatures ranging from 65 to 200 °C with varying amounts of water (2 to 10 ml) led to an optimized protocol that used 2 ml of water and 140 °C. However, even after optimizing, this method afforded signals for 4-vinylanisole that were about an order of magnitude smaller than using SD–SPME. SD–SPME suffers from the fact that there are currently no available systems that allow for automated sampling.

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