Solid-Phase Microextraction of Volatile Components from Natural Grassland Plants

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The volatile components from nine plants growing on natural grasslands in Auvergne, central France, selected for the broad qualitative and quantitative diversity of their terpenoid fractions, were analyzed by high-resolution gas-phase chromatography and mass spectrometry (HRGC−MS) after static headspace solid-phase microextraction (SHS−SPME). SHS−SPME allowed all the plant material to be analyzed under the same conditions despite its wide-ranging composition. This is not always possible with other extraction methods. Using an apolar poly(dimethylsiloxane) (PDMS) phase, numerous terpenoid hydrocarbons, together with alcohols, cyclic ethers, and esters, were extracted. Its ease of use and the high resolution of the chromatographic profiles obtained make SHS−SPME well suited to the rapid characterization of the main components of the volatile fraction of plants. Of the nine plants studied, four (*Meum athamanticum*, *Pimpinella saxifraga*, *Achillea millefolium*, and *Thymus pulegioides*) exhaled more than 60 different volatile components. Certain terpenes present in large amounts in these plants might help link dairy products to grazing pasture, thus improving food traceability.

Keywords: *Headspace; SPME; PDMS; GC−MS; terpene; pasture; Achillea millefolium; Meum athamanticum; Pimpinella saxifraga; Thymus pulegioides*

INTRODUCTION

In the plant kingdom the qualitative and quantitative distribution of terpenes is highly variable, but it is species-specific (*1*). Many researchers have suggested that the analysis of such substances might provide a basis for the characterization of plant ecosystems such as grasslands (natural or cultivated), and thus improve the traceability of dairy products or meat originating from animals raised in specific geographical areas (*2*−*6*). The transfer of terpenes from grass into milk is rapid, and it leaves a specific mark on the dairy products obtained that is linked to the plant species ingested (*6*, *7*). Various methods currently exist for the extraction of these substances from plants: steam distillation, use of supercritical fluids (*8*), extraction under very low pressure (*9*), dynamic headspace extraction (*3*, *6*), and solid-phase microextraction or SPME (*10*−*12*).

Our aim was to determine whether SPME could be used to compare, under identical operating conditions, different plants displaying a broad range of terpenoid compositions. To be considered efficient this extraction method must at least allow us to identify the most abundant terpenes desorbed from the plants. These are the substances that are most likely to be detected after ingestion by the animals and dilution in their various physiological compartments (digestive tract, blood, and tissues). This method was chosen for its flexibility in use (e.g. the possibility of collecting plant samples in the field in sealed flasks if required) and high sensitivity toward terpene (*11*). Experimentally, nine plants representative of a natural Auvergne grassland (Marcenat, Cantal, France) and harvested in July and September were analyzed. These plants were chosen for their wide range of terpenoid compositions and concentrations (*13*).

MATERIALS AND METHODS

Collection, Sampling, and Storage of Plant Materials. The plants studied were yarrow (*Achillea millefolium*, Asteraceae); cocksfoot (*Dactylis glomerata*, Poaceae); spignel (*Meum athamanticum*, Apiaceae); sweet vernal (*Anthoxanthum odoratum*, Poaceae); yellow gentian (*Gentiana lutea*, Gentianaceae); burnet saxifrage (*Pimpinella saxifraga*, Apiaceae); dandelion (*Taraxacum officinale*, Asteraceae); ribwort (*Plantago lanceolata*, Plantaginaceae); and wild thyme (*Thymus pulegioides -sectio serpyllum-*, Lamiaceae). Aerial parts of each plant species (about 1 kg) were collected in July (time VII) and September (time IX) 1998 (except for sweet vernal and yellow gentian which were collected only in July), from a field used as summer pasture for many years, located at an altitude of 1200 m, on a dry acidic filtering volcanic soil. As soon as they were collected, the plants were barn-dried for 6 days at ambient temperature (about 18 °C) in the dark (residual moisture between 9 and 17%). After they were dried, the plants were cut into 2-cm lengths, wrapped in aluminum foil, bagged under moderately reduced pressure, and stored at −20 °C.

Extraction and Separation of Constituents. An SPME fiber coated with 1 μm of nongrafted poly(dimethylsiloxane) (PDMS) phase (Supelco 5-7300, mounted on a Supelco 5-7330 support) was conditioned for 1 h at 250 °C in a stream of

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helium. A single fiber was used for the complete study. A blank run was performed after the analysis of each volatile-rich plant in order to confirm that no residual compound was polluting the fiber or the column.

The plant samples were brought to ambient temperature overnight before the bags were opened. For each plant species the headspace was generated from 1 g of sample cut up roughly with scissors and placed in a 20-mL flask. The flasks were sealed and heated for 15 min in an aluminum block maintained at 44 °C, which afforded a temperature of 40 °C at the bottom of the flask and 36 °C near the septum. The volatile components were then adsorbed on the fiber for 15 min. This duration was chosen after testing durations of 1, 15, and 25 min with a terpene-rich plant, burnet saxifrage.

The fiber was then introduced into the injection port of a GC 8000 chromatograph coupled to an MD 800 mass detector (Fisons Instruments, Milan, Italy). The injection port, equipped with a glass insert (internal diameter 0.75 mm), operated at 230 °C in splitless mode. The injection of the components desorbed from the fiber was stopped after 2 min by opening a septum purge with a flow rate of 2 mL·min^{-1} and the fiber was removed.

The volatile components were separated in an SPB5 column 60 m long, of internal diameter 0.32 mm and film thickness of 100 μm (SUPELCO Inc., Bellefonte, PA). The carrier gas was helium N55 (purity 99.9995%) injected at the column head at a pressure of 0.6 bar (linear velocity 1.2 mL·min^{-1}). The initial oven temperature was maintained at 40 °C for 5 min and then increased at 3 °C·min^{-1} up to 220 °C, and the final temperature was maintained for 15 min. The mass detector operated in EI+ mode at 70 eV in a range of 15 to 210 amu.

Processing of Results. A single analysis was performed for each plant. The chromatograms obtained from the total ion current (TIC) were integrated without any correction for coelutions and the results were expressed in arbitrary surface units (asu). Area sums were calculated separately for groups of peaks corresponding to monoterpenes and derivatives (10 to 37.5 min) and sesquiterpenes (37.5 to 50 min), for the construction of bar charts (Figure 1a and b).

Identification of Main Volatile Components. The most important peaks for the four richest plants were identified from their mass spectra using the NIST/EPA/NIH Mass spectral Library (1997), and their relative retention index (*14*) measured with a series of added alkanes (*15*). The identification of several peaks was confirmed by Fourier transform infrared (FTIR) spectrometry under analogous chromatographic conditions. The gas-phase chromatograph (DI700, Delsi Instruments) was equipped with a desorption concentration injection (DCI) unit (Delsi Instruments, F-92150 Suresnes, France) and coupled to a Fourier transform infrared spectrometer (Magna 550 source with GC−IR Nicolet interface) operated at a scan rate of 1.4 spectrum·sec^{-1} with a resolution of 8 cm^{-1}. The compounds were identified using the Nicolet/Aldrich vapor phase database (Nicolet Instrument Corp., Les Ulis, France).

RESULTS AND DISCUSSION

SPME is an extraction method involving adsorption of analytes on a solid-phase deposited on a silica fiber (*16*). The extraction of the volatile components is achieved either by immersing the fiber into the liquid to be analyzed (L-SPME), or by simple contact with its headspace under static conditions (SHS−SPME). This method obviates the classical steam distillation, which is liable to modify unstable constituents. The quantity of analytes adsorbed on the fiber results from two simultaneous mechanisms: release of the volatile components from their matrix, and their adsorption on the SPME fiber. The release rate depends, among other factors, on the exchange surface area between the sample and the headspace, as well as on the affinity of the volatile components for the matrix. The adsorption on the fiber depends on the concentration and on the affinity of each molecule for the phase and on the presence of substances with a high affinity for the fiber phase that can compete with the analytes. The presence of water vapor can also modify the adsorption of volatile components.

Czerwinski et al. (*11*) used SHS−SPME to analyze terpenes in plant-based pharmaceutical preparations. They quantified β-pinene, β-myrcene, limonene, and menthol by measuring the response factors of authentic standards. However, these pharmaceutical preparations were homogeneous, which cannot be the case of whole or cut plant samples. Plant matrix is naturally very heterogeneous, both structurally and chemically. It is built of different organs, tissues, and cell types which themselves are composed of cytoplasm containing lipid droplets and cell walls made up of hydrophilic (polysaccharide) and hydrophobic (lignin) elements.

The exact quantification of substances in the sample and not in the headspace would only be possible by choosing conditions (sample quantity, temperature) such as virtually all the analytes are completely in the gas phase (*10*). Owing to our prerequisite to handle all the samples in the same conditions, a precise quantification of volatile compounds would have necessitated a prohibitive mass of approximations for example, about the reproducibility of exchange surfaces from one sample to another and about the values of terpenoid response factors. It was therefore not attempted to standardize particle size or residual water content. It was chosen to describe plant headspaces on an arbitrary surface unit basis, with their SPME images obtained under identical analytical conditions and a sample treatment (harvest, drying, and cutting) analogous to animal feeding.

Burnet saxifrage was used to choose the duration of the extraction. Czerwinski et al. (*11*) have shown that the SPME extraction rates of three different terpenes from homogeneous liquid preparations were different, but all three of the terpenes tested reached their extraction maximum in 10 min on a PDMS phase 100 μm thick. The total peak areas obtained in our tests after 1, 15, and 25 min were respectively 3.6, 2.8, and 6.2 (10^9 asu) for monoterpenes and 2.7, 12.7, and 24.6 (10^9 asu) for sesquiterpenes. An increased adsorption duration especially favored the binding of the sesquiterpenes on the fiber. These rate differences are probably linked to the higher molecular weight of the sesquiterpenes (higher molecular weight being generally associated with lower volatility), and hence, to lower rates of transport from plant matrix to SPME fiber. These kinetics did not enlighten a clear competition among the components. Moreover, the three durations tested showed no steady state indicating an equilibrium or a limit of adsorption capacity of the fiber. Hence, it is important to work under strictly defined conditions for a valid comparison of plants. To analyze plants with widely different levels of terpenes a duration of 15 min offers a good compromise between a manageable number of peaks and the emergence of coelutions between terpenoids. Compared with dynamic headspace extraction (DHS), SPME affords cleaner chromatograms with much fewer coelutions (results not given). The trap concentration step makes it possible to use DHS to analyze samples containing traces of volatile compounds, for which the SPME chromatographic profiles are practically flat. However, DHS requires adjusting trapping durations according to the levels of volatile

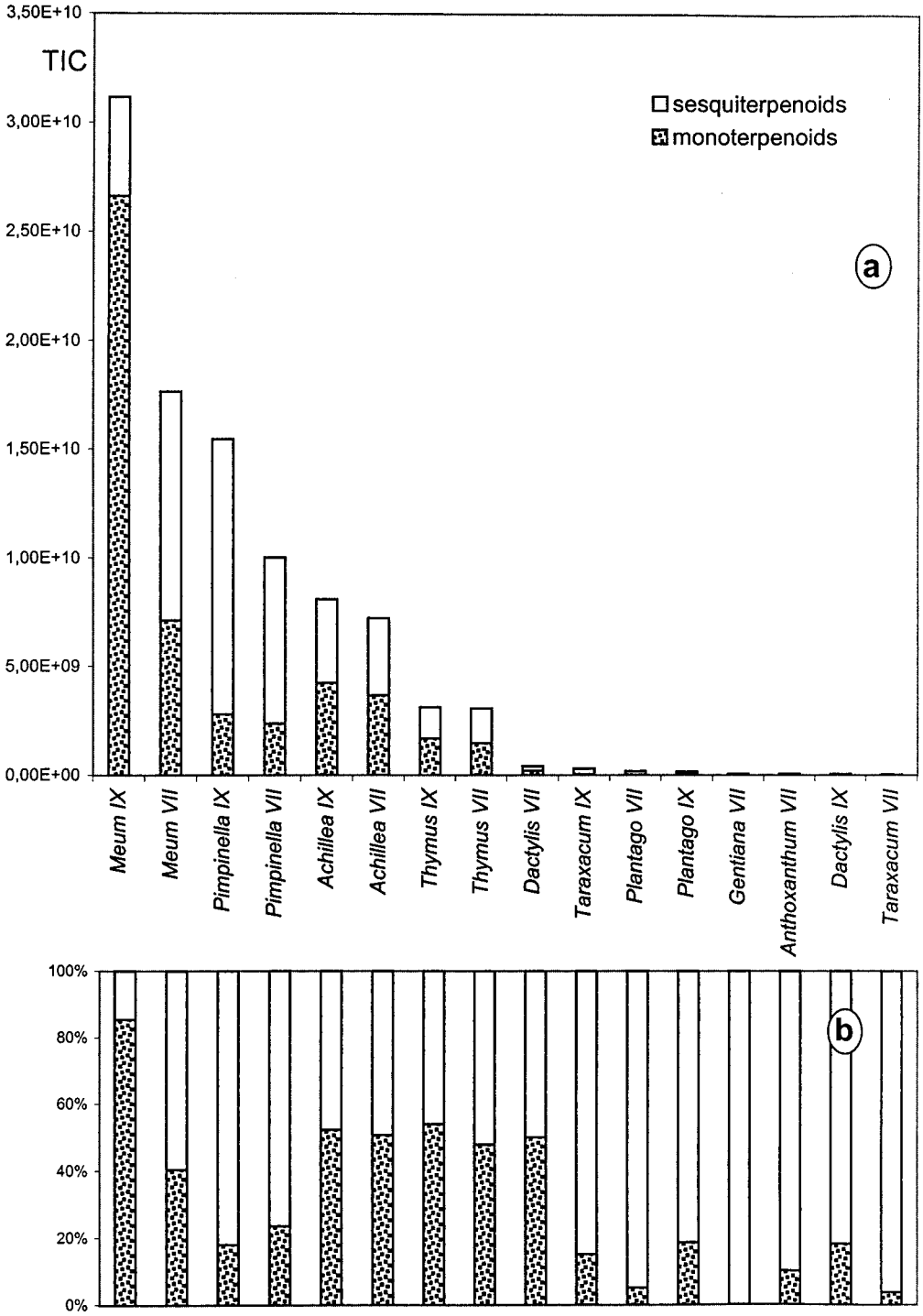

Figure 1. (a) Cumulative histograms of peak areas (in arbitrary units) of mono- and sesquiterpenes from plants harvested in July (VII) and September (IX). (b) Proportions of mono- and sesquiterpenes.

constituents in the plants. In preliminary tests, using DHS, the analysis of a particularly rich plant such as spignel caused the safety failure of the mass detector on several occasions (vacuum broken or signal too strong). In this case split mode injection with a high leak rate does not afford a solution because too-rich samples generate memory effects in both adsorbent and transfer lines. SPME generated no such memory effect in the analysis of spignel even when the main components saturated the chromatography column.

The results of integration of the TIC chromatograms are presented as bar charts representing total areas (Figure 1a) and ratios (Figure 1b) of mono- and sesquiterpenes extracted from the different plants at the two harvest times. Spignel (*Meum*) was by far the richest in terpenes, followed by burnet saxifrage (*Pimpinella*), yarrow (*Achillea*), and wild thyme (*Thymus*). The terpene-poor plants included cocksfoot (*Dactylis*), dandelion (*Taraxacum*), and sweet vernal grass (*Anthoxanthum*). According to Ashton and Jones (*17*), the scent of sweet vernal is due to coumarin, which was not detected here. The harvesting time did not modify the sorting out of the plants according to the amount of volatile compounds released. *Meum* and *Dactylis* exhibited the widest variations in composition; September *Meum* was the only plant in which monoterpenes were predomi-

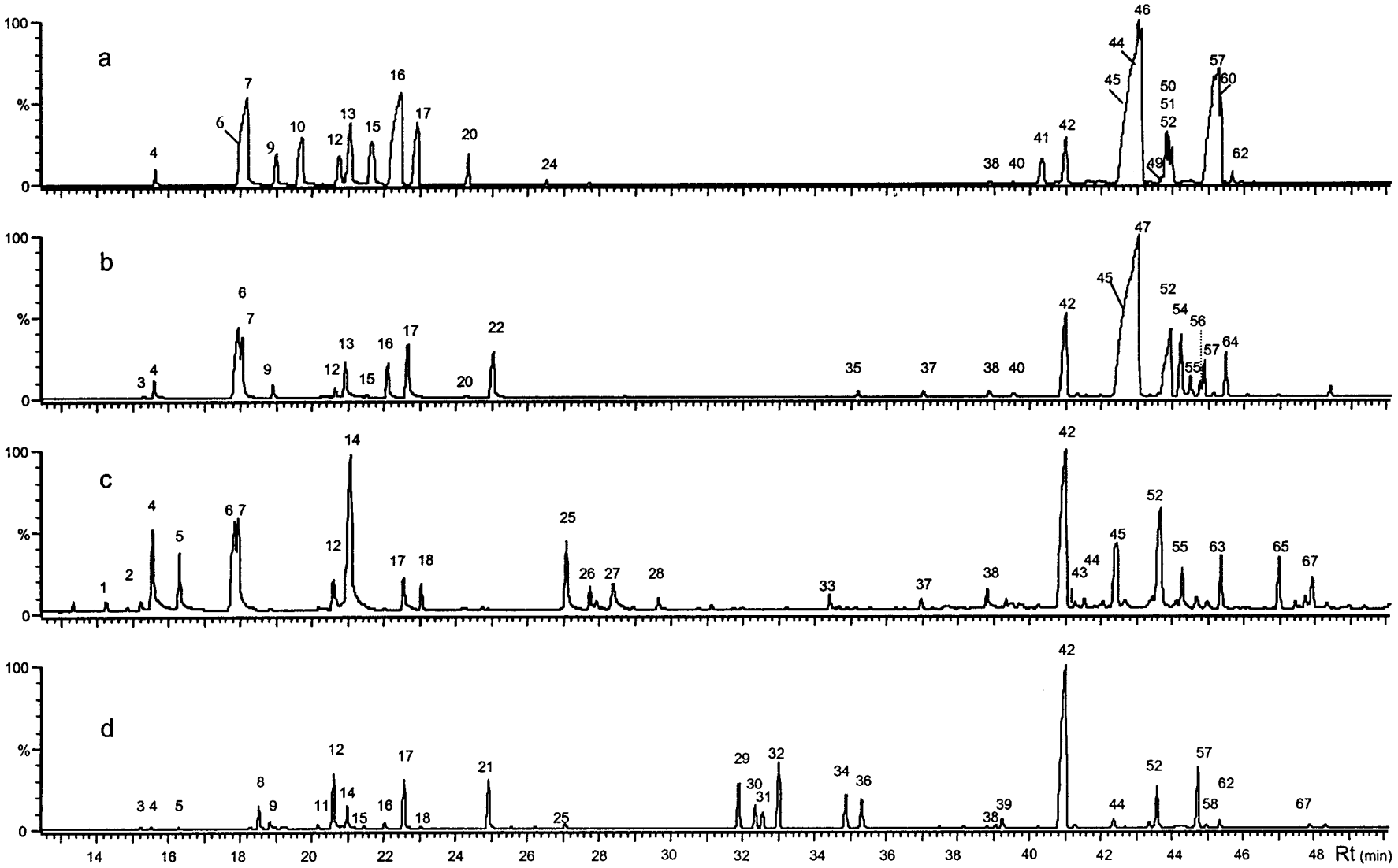

Figure 2. Chromatograms obtained from four plants rich in volatile components harvested in July. Extraction by SPME on a PDMS fiber, 15 min at 40 °C. Peak numbers correspond to identifications presented in Table 1. (a) Spignel (*Meum athamanticum*), 100% = 1.48 10^8; (b) burnet saxifrage (*Pimpinella saxifraga*), 100% = 1.19 10^8; (c) yarrow (*Achillea millefolium*), 100% = 7.69 10^7; (d) wild thyme (*Thymus pulegioides*), 100% = 6.71 10^7.

Table 1. Main Constituents Identified in Spignel, Burnet Saxifrage, Yarrow, and Wild Thyme Collected in July and September[a]

		KI			plant							
					Meum athamanticum		*Pimpinella saxifraga*		*Achillea millefolium*		*Thymus pulegioides*	
	compound	lit.	exp.	id	July	Sept.	July	Sept.	July	Sept.	July	Sept.
1	heptatriene-1,3,6-trimethyl			(1)					0.3	1.5		
2	tricyclene	922	911	(1)					0.4	0.5		
3	3-thujene	925	934	(2)			0.1	0.1			t	t
4	α-pinene	934	939	(3)	1.0	0.9	4.3	4.1	t	t		
5	camphene	946	951	(2)					2.7	3.1	t	t
6	sabinene	971	974	(3)	*	*	6.4	5.3	6.3	5.2	t	t
7	*β*-pinene	976	976	(3)	10*	14*	3.0	1.0	4.1	4.9	t	t
8	3-octanone	986	986	(2)							2.1	4.7
9	*β*-mircene	990	991	(3)	0.5		0.5	0.1	0.1	t	t	
10	α-phellandrene	1005	1003	(2)	3.5	5.3			1.6			
11	α-terpinene	1016	1014	(3)							t	1.1
12	*p*-cymene	1026	1022	(3)	0.4	0.4	1.6	2.8	6.5	12		
13	limonene	1031	1029	(3)	1.7	2.9						
14	cineole	1029	1030	(3)					14	7.0	2.0	1.1
15	*β*-ocimene (Z)	1035	1041	(2)		0.1	0.1			t	t	
16	*β*-ocimene (E)	1045	1051	(2)	11	*	1.8	1.2			t	t
17	*γ*-terpinene	1057	1060	(3)	3.8	3.6*	3.6	2.7	1.5	2.2	5.4	13
18	*p*-menth-2-en-1-ol	1064	1068	(2)					1.1	1.1	t	t
19	*p*-mentadiene n.i.		1075	(1)		9.1*						
20	terpinolene	1087	1089	(3)	1.0	7.1	t	t	0.3	0.2		
21	α-linalool	1101	1099	(3)					(IR)	(IR)	6.1	t
22	undecane	1100	1100	(2)			3.4	1.4				
23	3-thujanone	1102	1114	(1)						1.8		
24	allo-ocimene	1129	1128	(2)	0.1	1.8						
25	camphor	1140	1141	(3)					3.5	7.2	t	t
26	isoborneol	1152	1157	(2)					0.9	0.4		
27	endo-borneol	1162	1166	(3)					2.2	0.9	t	t
28	α-terpineol	1188	1190	(3)					0.4	0.4	t	1.3
29	thymol methyl ether	1235	1232	(2)			y	0.1			5.4	2.8
30	carvacrol methyl ether	1242	1241	(2)							2.7	5.2
31	thymoquinone	1249	1245	(2)							1.6	1.5
32	linalyl acetate	1255	1254	(2)							7.5	
33	bornyl acetate	1284	1284	(3)					0.6	0.8	t	t
34	thymol	1290	1295	(2)							4.0	1.5
35	tridecane	1300	1300	(2)			0.3	0.1				
36	carvacrol	1299	1307	(2)							3.3	7.1
37	*δ*-elemene	1337	1338	(2)	t	41	0.3	0.3	0.5	0.5		
38	α-copaene	1375	1377	(2)	t	0.4	0.3	0.3	0.9	0.6	t	t
39	*β*-bourbonene	1383	1385	(2)							0.9	t
40	*β*-cubebene	1390	1390	(2)	t	0.3	t	0.4				
41	bergamotene (Z,α,cis)	1415	1407	(2)	1.5							
42	*β*-caryophyllene	1418	1424	(2)	7.7	6.9	16	9.8	39	26		
43	bergamotene (Z,α,trans)	1435	1438	(2)					0.4	0.3		
44	α-humulene	1452	1457	(2)		0.1	0.3			0.3	0.9	1.1
45	*β*-farnesene (E)	1458	1458	(2)	*	1.5	*	*	5.0	2.0		
46	α-acoradiene	1463	1471	(2)	32*							
47	9-epicaryophyllene	1467	1470	(2)			44*	37*				
48	allo-aromadendrene	1460	1481	(1)					0.8	0.6		
49	*β*-chamigrene	1474	1478	(2)	0.2	0.6						
50	*γ*-curcumene	1479	1481	(2)	1.9							
51	α-curcumene	1482	1483	(2)	1.3							
52	germacrene D	1479	1487	(2)	1.4	4.2	8.9	5.9	8.0	9.1	4.8	6.4
53	sesquiterpene n.i.		1493	(1)	0.3							
54	α-zingiberene	1495	1495	(2)			3.9	4.7				
55	bicyclogermacrene	1494	1500	(2)		0.8	1.0	0.3	2.0	1.4		
56	α-farnesene (E)	1505	1509	(2)		0.9	0.6		0.7	1.3		
57	*β*-bisabolene	1509	1511	(2)	16		1.7	14			7.2	12
58	*γ*-cadinene	1513	1516	(2)		0.2			0.4		t	
59	sesquicineole	1514	1520	(2)						10		
60	*β*-curcumene	1512	1513	(2)	2.2	*						
61	calamenene (1-S,cis)	1521	1526	(2)		0.4*						
62	*δ*-cadinene	1524	1526	(2)	0.3						t	
63	*β*-farnesene (E)	1523	1527	(2)					2.5	1.7		
63	*β*-sesquiphellandrene		1527	(1)			2.2	3.1				
65	n trans-nerolidol	1563	1566	(2)					2.3	1.0		
66	spathulenol	1575	1585	(2)					0.5	0.2		
67	caryophyllene oxide b	1580	1588	(2)					1.3	0.5	t	

[a] Results are presented in percentage of the total peak area integrated without correction for coelutions. t, peaks representing less than 2% of the largest peak area; *, coelutions; id., identification quality: (1) = mass spectrum (tentative), (2) mass spectrum + retention index, (3) mass spectrum + retention index + infrared spectrum. KI, retention indices: exp, mean of experimental values and lit., literature values in Adams (*19*) and Kondjoyan and Berdagué (*14*).

nant. The ratios of mono- and sesquiterpenes were equivalent in *Meum* VII, *Achillea*, *Thymus*, and *Dactylis* VII. Sesquiterpenes predominated in *Pimpinella*, *Taraxacum*, *Anthoxanthum*, *Plantago*, and *Dactylis* IX. The total ion current of the chromatograms ranged between 3.1×10^{10} (*Meum* IX) and 1.7×10^{7} (*Plantago* VII), which corresponds to an intensity ratio of about 1800. The chromatograms with the most peaks (Figure 2a and b) displayed asymmetrical peaks indicating saturation of the capillary column phase. Nevertheless, using SPME, the lowest and the highest amounts of terpene extracted from the plants both fall into the range of the GCMS analysis capacity. This may be due to the nonlinearity of response of SPME at high concentrations. (*18*).

The main constituents of the four terpene-richest plants (Figure 2) were identified (Table 1). With a few exceptions, the volatile components were eluted within three main groups: the apolar monoterpenes eluted between 10 and 25 min (peaks 1 to 20), their oxygen-containing derivatives eluted between 25 and 37.5 min (peaks 21 to 36), and the sesquiterpenes eluted between 37.5 and 46 min (peaks 37 to 64). Some oxygen-containing sesquiterpenes were detected beyond 46 min (peaks 65 to 67). Comparison of our results with those of the literature is difficult for several reasons. First, the biological variability of the plants (season, place of harvest, growth stage, etc.) makes plants of the same species but analyzed separately difficult to compare. Second, the available compositional data have often been obtained from essential oils extracted by steam distillation. Also, the knowledge that allows most sesquiterpenes to be identified is recent (*19*); researchers had previously focused mainly on the identification of monoterpenes. Unlike many grassland plants, yarrow (*20* (review) and *21*) and wild thyme (*22* and *23*) are well-known because their essential oils possess aromatic and medicinal properties. For burnet saxifrage (*24*) and spignel (*25*), the terpenes in the aerial parts are less well-known than those in the roots.

The 67 components identified comprise 18 monoterpenes, 15 oxygenated monoterpenes (seven alcohols, three cyclic or aliphatic ethers, two ketones, two esters, and one quinone), 27 sesquiterpenes, four oxygenated sesquiterpenes (two cyclic ethers and two alcohols), two alkanes, and one aliphatic ketone. Headspace SPME is particularly efficient for the extraction of sesquiterpenes and their derivatives. Numerically these accounted for half the volatile components detected in the four plants. Quantitatively the largest peak for most of the plants was a sesquiterpene. The remarkable efficiency of the PDMS fiber for the extraction of β-caryophyllene (Figure 2 peak 46) and sesquiterpenes in general has already been noted in the analysis of the volatile components of cinnamon (*12*). β-caryophyllene predominated in wild thyme and yarrow collected in July. In burnet saxifrage the main component was a sesquiterpene with a closely related structure, 9-epicaryophyllene (peak 47). In spignel α-acoradiene (peak 46) predominated in July and a monoterpene, γ-terpinene, predominated in September (peak 17).

The most marked differences between our results and those of the literature concern the oxygenated compounds. In September yarrow the predominant constituent was an oxygenated derivative, sesquicineole (peak 59). This compound is not described as characteristic of yarrow. It has been found as a minor peak in the SHS−SPME analysis of yarrow essential oils (data not shown). In July, the second-largest peak corresponded to a related structure, cineole (peak 14). According to the review of Lawrence (*20*), this cyclic ether can account for more than 34% of the essential oil of yarrow flowers.

The proportion of polar derivatives (alcohols, aldehydes, ketones, and esters) seems lower in SPME extracts than in the essential oils described in the literature. This discrepancy can have several explanations. First, the PDMS phase used was apolar and thus its affinity for polar substances should be lower than that for terpenes. Nevertheless, some polar components were found in large amounts, such as thymol and carvacrol in wild thyme and cineole in yarrow. Second, essential oils may become enriched in oxygenated derivatives through artifacts arising from steam distillation. Thus chamazulene, which is abundant in the essential oils of certain yarrow samples (*21*), did not appear in our chromatograms. This substance is known to be an artifact, forming during steam distillation from unstable precursors that vary according to the plant. (*26*). The same may be the case for geijerene and pregeijerene, which we failed to find in burnet saxifrage (*26*).

In a study by Mariaca et al. (*1*) using DHS−GC−MS analysis, trans-β-ocimene, limonene, and trans-β-caryophyllene, present in a large proportion of grazed plants analyzed, are proposed as measurable biochemical markers for dairy products. In our study, α-pinene, sabinene, β-pinene, β-myrcene, *p*-cymene, γ-terpinene, α-copaene, β-caryophyllene, and germacrene D were present in the four plants at both growth stages, and were often among the predominant components. In fact, α- and β-pinene, *p*-cymene, and γ-terpinene are among the substances identified in forage plants that are detected in Saint-Nectaire cheese (*6*).

SPME proved to be a simple and fast method to obtain fingerprints of plant headspace. The procedure is flexible, and the material may be transported in the field. In the case of studies on plant mixtures such as hays, test samples of more than one gram should be used in order to improve sample representativity. In natural grasslands, there is a broad diversity of terpenes that can subsequently be found in milk, cheese, and meat, and which might be used as food tracers or markers of the geographical origin. A further study involving cows fed on the same pasture is currently being performed.

ABBREVIATIONS USED:

asu, arbitrary surface unit; DCI, desorption-concentration-injection; IK, retention index; PDMS, poly(dimethylsiloxane); RT, retention time; SHS, static headspace; SPME, solid-phase microextraction; TIC, total ion current.

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