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Journal of Chromatography A, 1152 (2007) 138–149
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Reliability of fibres in solid-phase microextraction for routine analysis of the headspace of aromatic and medicinal plants

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Available online 12 February 2007

Abstract

This article evaluates the HS-SPME recovery repeatability, intermediate precision and their performance over time when applied to HS-SPME sampling for quality control of medicinal and aromatic plants. Experiments were carried out on two sets of fibres coated with two different coatings and belonging to different lots (i.e 100 μm polydimethylsyloxane (PDMS) and Carboxen/divinylbenzene/PDMS 50/30 μm, l: 1 cm (CAR/DVB/PDMS)) and on chamomile (*Matricaria chamomilla* L.), sage (*Salvia lavandulifolia* Vahl.) and a standard solution containing 3-hexanol, isoamyl acetate, 1,8-cineole and menthol in diisobutyl phthalate. The performance of each set of fibres was evaluated by determining a group of complementary statistical parameters including: (i) repeatability of the absolute areas of each marker from each matrix with each fibre; (ii) intra-fibre repeatability of the total absolute areas of the markers of each matrix obtained with each fibre of each set; (iii) inter-fibre intermediate precision of the total absolute areas of the markers of each matrix obtained with all fibres of each set; and (iv) analysis of variance by one-way ANOVA with Fisher and Tukey tests. The influence of the number of analyses on fibre effectiveness (fibre life-time) was studied by linear regression analysis (LRA). The results proved that HS-SPME can successfully be used for routine control analysis of aromatic ad medicinal plants since both types of fibres showed good repeatability and intermediate precision of analytes recovery and consistency over time. Unlike data previously reported by other authors, CAR/DVB/PDMS coated fibres gave better results than those coated with PDMS. The fibre-life seemed mainly to be influenced by the number and conditions of samplings and nature of the matrix investigated.

Keywords: Headspace solid phase microextraction; PDMS; CAR/DVB/PDMS; Repeatability; Fibre-life; Chamomile flower heads; Sage leaves

1. Introduction

Solid phase microextraction (SPME) is a well established solventless sample preparation technique introduced by Pawliszyn in 1989 [1]; its use has been extended to headspace sampling in 1993 [2]. HS-SPME has strongly contributed to a renewal in the interest in headspace sampling that has taken place over the last fifteen years and to the success of high concentration capability headspace (HCC-HS) techniques [3]. One of the main reasons of the success of HCC-HS techniques is that in general sampling is simple and easy to automate and highly repeatable so that the volatile fraction profile resulting from a solid or liquid matrix can very often successfully be used to characterize it either by specific markers and/or by adopting the profile itself as a parameter to be submitted to statistical elaboration (e.g., Principal Component Analysis, PCA). HCC-HS GC profiles can therefore be used as an effective analytical decision maker for a quick and routine screening of a set of samples [4]. Recently HS-SPME-GC-PCA was verified to be suitable to replace the conventional time consuming analysis of the essential oil obtained by hydrodistillation by GC for the direct discrimination of the different chemotypes of chamomile flower heads (*Matricaria chamomilla* L.) [5]. This approach requires that all sampling and analysis parameters are rigorously standardised and highly repeatable, in particular when used for quality control. On the other hand, the most obvious but indispensable condition to apply HS-SPME to this field is a very high repeatability of performance of a single fibre over time (i.e. fibre life-time) and reproducibility of fibres within a lot or between different lots. To the best of the authors' knowledge few studies have been published on this topic. Wang et al. [6] compared the life-time of an home-made sol-gel coated polyethylene-glycol (PEG) fibre with that of four commercial fibres (i.e. PDMS 100, PDMS 30, polyacrylate 85, and CW-DVB 65) applied to the analysis of standard mixtures of aromatic hydrocarbons, phenols and pesticides and found a life-time of above 150 samplings compared to 50–100 for those

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doi:10.1016/j.chroma.2007.02.011

commercially available. In a validation study of a method to analyse the aroma compounds of vinegar by HS-SPME, Natera et al. [7] found considerable differences between the response of the three Carboxen-PDMS (CAR-PDMS) fibres employed and concluded that this coating is characterized by a low reproducibility and, as a consequence, each set of experiments should be performed with a single fibre. In the development of sol-gel-coated calyx[4]arene fibres, Li et al. [8] found a life-time of above 170 samplings and an extremely high inter-fibre and inter-batch reproducibility when they were applied to the analyses of aromatic hydrocarbons and amines, PAHs and phthalic acid esters.

This article aims to evaluate the recovery repeatability and intermediate precision of two sets of fibres belonging to different lots and coated with two different coatings (i.e six 100 μm polydimethylsyloxane (PDMS) and three Carboxen/polydivinylbenzene/polydimethylsyloxane 50/30 μm, l: 1 cm (CAR/DVB/PDMS)) and their performance over time when applied to HS-SPME sampling for the quality control of medicinal and aromatic plants [9], in particular chamomile (*Matricaria chamomilla* L.) and sage (*Salvia lavandulifolia* Vahl.) and of a standard solution containing four compounds with different structures, polarity and volatility (i.e. 3-hexanol, isoamyl acetate, 1,8-cineole and menthol in diisobutyl phthalate) widely found in the medicinal and aromatic plant fields.

2. Experimental

2.1. Plant material and chemicals

Two lots of both dried chamomile flower heads and sage leaves from experimental cultivation harvested in two different periods (Lot 1 and Lot 2) were analysed to avoid that the time between collection and assay influenced the volatile fraction composition. They were kindly supplied by Drs Carla Vender and Nicola Ajello, ISAFA (Trento- Italy). A 0.3 mM standard solution of 3-hexanol, isoamyl acetate, 1,8-cineole and menthol in diisobutyl phthalate was also prepared.

2.2. HS-SPME sample preparation

The SPME device was purchased from Supelco Co. (Bellafonte, PA, USA) as well as the fused silica fibres. The PDMS set of fibres (fibres A–F) consisted of six fibres belonging to different lots, five of them were new and one was already used for 100 headspace samplings (fibre A) and in general submitted to thermal desorption steps of 5 min. The CAR/DVB/PDMS set (fibres G-I) consisted of three new fibres belonging to different lots. Before use, all fibres were conditioned as recommended by the manufacturer. A series of experiments were first made for each matrix to choose the most suitable sampling temperature and time.

Sampling was under the following conditions:

Standard solution: 100 μL of the standard solution in diisobutyl phthalate were transferred into a 7.5 mL vial, hermetically sealed, introduced in a thermostatic bath at 30 °C and sampled by SPME for 30 min. HS-SPME sampling was repeated five times for each fibre of each set. The vial was vibrated for 10 s every 10 min with an electric engraver (Vibro-Graver V74, Burgess Vibrocrafters Inc., Brayslake, Ill. USA).

Chamomile flower heads: 200 mg of dried chamomile flower heads were hermetically sealed in a 7.5 ml vial, introduced in a thermostatic bath at 80 °C, equilibrated for 15 min and sampled by HS-SPME for 30 min. Sampling was repeated five times for each fibre of each set. The vial was vibrated for 10 s every 10 min with an electric engraver to favour the diffusion of the analytes from the vapour phase to the polymeric coating.

Sage leaves: 150 mg of dried sage leaves in a hermetically sealed 7.5 mL vial were introduced in a thermostatic bath at 50 °C and submitted to HS-SPME sampling for 30 min. Sampling was repeated five times for each fibre of each set. The vial was vibrated for 10 s every 10 min with an electric engraver.

For all experiments, only the part of the vial with the solid matrix was submerged, to keep the SPME fibre as cool as possible to minimize analyte release from the fibre to the vapour phase as a consequence of the partition equilibrium [10].

After sampling, the SPME device was immediately inserted into the GC injector, and the fibre thermally desorbed. A desorption time of five minutes at 250 °C was used. Before sampling, each fibre was reconditioned for 20 min in the GC injection port at 250 °C.

2.3. Capillary GC and GC/MS analysis

Capillary GC-FID analyses were carried out on a Thermo Electron Trace GC unit (Rodano – Italy). Data processing was by Chromcard (Version 2.3) (Thermo Electron Rodano, Italy). Capillary GC/MS analyses were performed on an Agilent 6890 GC-5973N MS system (Agilent, Little Falls, DE, USA).

2.3.1. Chromatographic conditions

2.3.1.1. Capillary GC analysis–. Injection temperature: 250 °C, mode: splitless; detector temperature: 280 °C; column: FSOT OV-1 (length: 30 m, i.d 0.25 mm, d_f 0.25 μm) (Mega, Legnano (Milano), Italy). Carrier gas: hydrogen; flow rate: 1.5 mL/min in constant flow mode.

Temperature programmes: (a) for the standard solution: from 100 °C (1 min) to 220 °C (5 min) at 5 °C/min; (b) for chamomile flower heads: from 100 °C (1 min) to 220 °C (5 min) at 5 °C/min; (c) for sage leaves: from 50 °C (1 min) to 200 °C (5 min) at 3 °C/min, than to 250 °C at 15 °C/min.

Capillary-GC-MS analyses were carried out under the same conditions reported for GC-FID except that helium was used as carrier gas at a flow-rate of 1.0 mL/min, in constant flow mode. MS was in EI mode at 70 eV. Ion source temperature: 230 °C. Markers were identified by comparison of their mass spectra with those of authentic standards or from an home made data base or from the literature.

2.4. Statistical elaboration

Analysis of variance (ANOVA), Fisher and Tukey tests, regression analysis was by XLSTAT (Version 7.5.1) copyright 1995–2005 Addinsoft SARL.

3. Results and discussion

This study evaluates the reliability of SPME fibres from different lots together with the evolution over time of their effectiveness in recovering analytes from the headspace of different matrices. When HS-SPME reliability has to be evaluated a number of important sources of random errors must first be considered, even when a rigorous standardization is applied:

- the analyte recovery of a fibre is strongly influenced by two steps: (a) the mass transfer from the matrix to the vapour phase that conditions headspace composition; and (b) the mass transfer from the vapour phase to the polymer coating. As a consequence, if reasonable sampling times have to be applied, pre-equilibrium conditions must be applied because the equilibrium for all components of a complex matrix, as a medicinal or aromatic plant, can be very difficult to achieve because of their different volatility and polarity.
- a further source of random variability is the physical state of the matrix. For instance, the results with solid samples such as medicinal and aromatic plants can seldom be normalised *versus* those of a standard matrix because generally it is not available.

Two sets of fibres coated with two of the most effective and used coatings applied to HS-SPME sampling of medicinal and aromatic plant headspace, i.e. PDMS and CAR/DVB/PDMS, were investigated [11]. These coatings have also been adopted because they are representative of the mono- and multi-component fibres as well as of fibres whose analyte recovery is based either on one or more phenomena (i.e. sorption for PDMS and sorption/adsorption for CAR/DVB/PDMS). The PDMS set consisted of six fibres, five of them were new and from different lots and one already used for 100 headspace samplings (fibre A) in order to check if and how the number of analyses could influence fibre performance; CAR/DVB/PDMS set included three new fibres.

The performance of each fibre set was evaluated by determining a group of complementary statistical parameters calculated on the results of five experiments. For several statistical elaborations, the total absolute areas obtained from the sum of the areas of each marker of each matrix was adopted as representative of the extraction yield of a fibre for a given experiment and under the analysis conditions applied: this assumption was justified by the consistency of the resulting peak areas ratios. The following parameters were measured: (i) relative standard deviation (RSD %) of the absolute areas of each single marker of each matrix for each fibre to evaluate its repeatability; (ii) RSD% of the total absolute areas of the markers of each matrix obtained with each fibre of each set for intra-fibre repeatability; (iii) RSD% of the total absolute areas of the markers of each matrix obtained with all fibres of each set for inter-fibre intermediate precision, and iv) analysis of variance by one-way ANOVA with Fisher and Tukey tests.

The influence of the number of samplings on fibre effectiveness (or fibre life-time) was studied by applying linear regression analysis (LRA) to the variation of the total area of all marker components of each matrix investigated as a function of the number of analysis.

Experiments were carried out on a standard solution of 3-hexanol, isoamyl acetate, 1,8-cineole and menthol in diisobutyl phthalate (Table 1), and two medicinal and aromatic plants: chamomile flower heads and sage leaves. Chamomile was chosen because its volatile fraction is quite simple but requires a relatively high headspace sampling temperature (80 °C) to give a significant GC-FID profile because it consists of seven medium volatility sesquiterpenoids (Table 6) and because one of its markers, i.e. chamazulene, is an artefact formed by the thermal degradation of matricine [12]. On the other hand, the volatile fraction from sage is rather complex consisting of several mono- and sesqui-terpenoids but its headspace can be sampled at relatively low temperature (50 °C). Twenty four components with different structures, polarity and volatility were chosen as markers (Table 11). Fig. 1a–d reports the GC-FID profile of the headspace of chamomile flower heads (lot 1) and sage leaves (lot 1) after SPME samplings with both PDMS and CAR/DVB/PDMS fibres.

3.1. Repeatability and intermediate precision

The component repeatability, intra-fibre repeatability and inter-fibre intermediate precision of the two sets of fibres was first determined. This series of experiments were carried out with both chamomile flower heads and sage leaves from Lot 1. Tables 1, 2, 6, 7, 11 and 12 report the repeatability of each fibre of the PDMS and CAR/DVB/PDMS sets, respectively, based on the peak areas of the components of the diisobutyl phthalate standard solution and of the markers of chamomile and sage

Table 1
Statistical elaboration of standard solution results: PDMS marker repeatability and intra-fibre total area repeatability

	PDMS fibre RSD%						
	Compounds	A	B	C	D	E	F
1	3-Hexanol	7.28	3.17	9.18	3.85	6.57	4.58
2	Isoamyl acetate	9.61	6.52	8.69	9.28	7.91	9.24
3	1,8-Cineole	0.88	2.02	2.50	3.89	2.22	1.79
4	Menthol	21.19	7.46	5.39	24.30	22.26	14.16
Intra-fibre repeatability (RSD%)		1.75	0.92	3.39	3.00	1.60	2.96

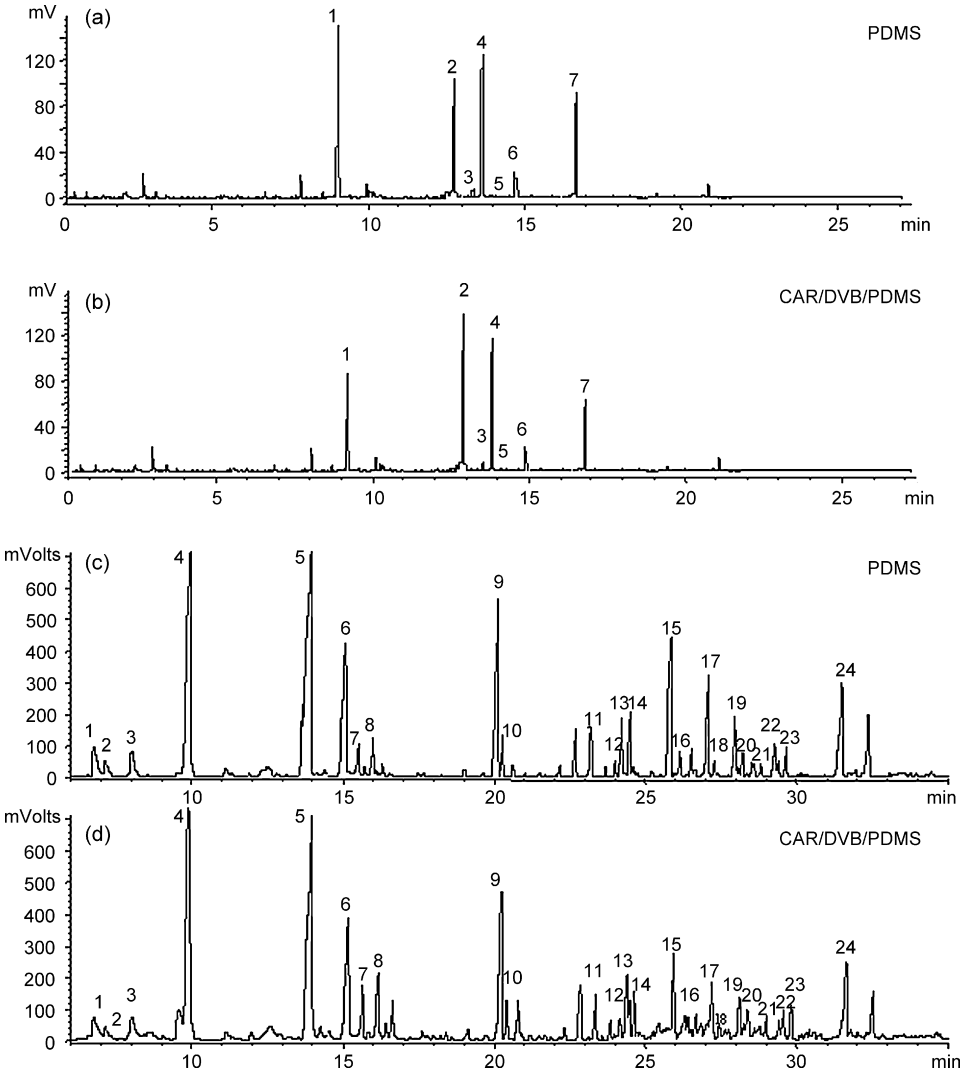

Fig. 1. GC-FID profiles of the headspace of chamomile flower heads (a, b) and sage leaves (c, d) after SPME sampling with PDMS and CAR/DVB/PDMS fibres (for details and identification see text and Tables 2 and 3).

as well as the intra-fibre repeatability determined through the sums of their areas. Results were expressed as RSD% calculated on five analyses. Tables 3, 8 and 13 report the means of marker absolute areas and of the marker repeatability for each set of fibres for each matrix and the inter-fibre intermediate precision within each set expressed as RSD% of the total areas of all marker components of each matrix investigated for each fibre.

Tables 1 and 2 show that for the standard solution the repeatability of each fibre within both sets with 3-hexanol, isoamyl acetate and 1,8-cineole is very high while that of menthol is much lower. These results are confirmed by the average RSD% of each component within each set (Table 3). The intra-fibre RSD% measured on the total area of the four standard components is also very good for both coatings ranging from 0.92 for the fibre B to 3.39 for fibre C with PDMS coating and from 1.22 for fibre G to 3.77 for fibre I with CAR/DVB/PDMS. In spite of the high menthol RSD %, the inter-fibre intermediate precision (i.e. within each set) is still good being RSD% 4.41 for CAR/DVB/PDMS and 9.55 for PDMS. These results are quite surprising because a better RSD% was expected for the set of PDMS fibres in comparison to the CAR/DVB/PDMS set, since it consists of a single component i.e. it involves a lower number of variables. This variability may be due to either the different number of fibres considered for each set or to the fact that

Table 2
Statistical elaboration of standard solution results: CAR/DVB/PDMS marker repeatability and intra-fibre total area repeatability

	CAR/DVB/PDMS fibre RSD%			
	Compounds	G	H	I
1	3-Hexanol	0.88	3.61	5.01
2	Isoamyl acetate	2.76	6.05	5.14
3	1,8-Cineole	3.64	9.07	6.47
4	Menthol	29.05	30.73	30.57
Intra-fibre repeatability (RSD%)		1.22	3.37	3.77

Table 3
Statistical elaboration of standard solution results: mean of marker absolute areas, mean of marker repeatability and inter-fibre total area intermediate precision

	Compounds	PDMS fibre		CAR/DVB/PDMS fibre	
		Average absolute area($\times 10^4$)	Average RSD%	Average absolute area($\times 10^4$)	Average RSD%
1	3-Hexanol	137	5.77	1539	3.16
2	Isoamyl acetate	132	8.54	1358	4.65
3	1,8-Cineole	223	2.21	670	6.39
4	Menthol	79	15.79	150	30.12
Inter-fibre intermediate precision (RSD%)		9.55		4.41	

Table 4
Statistical elaboration of standard solution results: PDMS fibre classification by Tukey test

Categories	Mean	Groupings		
PDMS A	471	A		
PDMS D	545		B	
PDMS B	591			C
PDMS F	600			C
PDMS C	600			C
PDMS E	616			C

fibre A in the PDMS set had already been used for 100 headspace samplings and its recovery capability significantly differed from those of the new fibres.

Comparable results were obtained with chamomile flower heads. RSD% of each marker component sampled with each fibre from the set of PDMS fibres (Table 6) is good ranging from a minimum of 0.47 for α-bisabolol with fibre A to a maximum of 12.0 for farnesene with fibre C (incidentally this is the only RSD% above 10%). The intra-fibre repeatability varies from 1.05 for fibre A to 6.31 for fibre E: the very low RSD% value for fibre A can be explained with the "age" of the fibre that makes its performance highly stable (see part 2). The component repeatability within the CAR/DVB/PDMS set (Table 7) ranged from 1.15 for α-bisabolone oxide A to 15.62 of spiroether both with fibre I, while the intra-fibre repeatability of this set was very similar ranging from 5.25 for fibre H to 6.46 for fibre I. The average RSD% for each component for the PDMS set varied from 2.53 for α-bisabolone oxide A to 5.95 for α-bisabolol while for the CAR/DVB/PDMS set ranged from 1.73 for α-bisabolone oxide A to 12.83 for spiroether (Table 8). In spite of the high spiroether RSD %, the inter-fibre intermediate precision of the CAR/DVB/PDMS set is much better than that for the set of PDMS fibres being then 5.85 and 15.7 respectively.

Sage leaves results were similar to the other samples in this paper. RSD% of each marker component sampled with each fibre from the set of PDMS fibres (Tables 11 and 12) varied from a minimum of 1.99 for camphor with fibre D to a maximum of 15.18 for caryophyllene oxide with fibre A. Fibre A was the only one giving RSD% above 10% for the less volatile markers (γ- cadinene, δ-cadinene and caryophyllene oxide). The RSD% of the CAR/DVB/PDMS set varied from 0.64 for 1,8-cineole to 9.88 for caryophyllene oxide both with fibre G. Intra-fibre repeatability varied from 1.78 for fibre D to 8.80 for fibre F with the PDMS set and between 1.58 for fibre H and 2.32 for fibre G in the CAR/DVB/PDMS set. The average repeatability of each component ranged from 3.62 for caryophyllene to 7.36 for α-pinene for the set of PDMS fibres and from 1.97 for bornyl acetate to 5.53 for caryophyllene oxide for the CAR/DVB/PDMS set (Table 13). Inter-fibre intermediate precision were 4.93 for the CAR/DVB/PDMS and 8.40 for the PDMS set i.e. the three component fibres again gave better results than those coated with a single polymer.

Table 5
Statistical elaboration of standard solution results: CAR/DVB/PDMS fibre classification by Tukey test

Categories	Mean	Groupings
CAR/DVB/PDMS H	3623	A
CAR/DVB/PDMS I	3641	A
CAR/DVB/PDMS G	3812	A

Table 6
Statistical elaboration of chamomile flower heads results: PDMS marker repeatability and intra-fibre total area repeatability

	Compounds	PDMS fibre RSD%					
		A	B	C	D	E	F
1	*trans*-β-Farnesene	2.43	7.40	12.0	5.43	5.09	9.70
2	Bisabolol oxide B	1.77	2.60	4.80	4.30	7.04	5.17
3	α-Bisabolone oxide A	0.87	0.90	6.10	2.03	3.09	2.17
4	α-Bisabolol	0.47	9.80	5.70	6.19	6.57	6.97
5	Chamazulene	4.09	7.70	5.50	5.65	4.78	5.32
6	Bisabolol oxide A	2.00	6.90	7.30	3.02	7.75	6.91
7	Spiroether	1.03	3.60	9.40	5.73	7.78	6.79
Intra-fibre repeatability (RSD%)		1.05	3.87	4.18	4.06	6.31	4.91

Table 7
Statistical elaboration of chamomile flower heads results: CAR/DVB/PDMS marker repeatability and intra-fibre total area repeatability

	Compounds	CAR/DVB/PDMS fibre RSD%		
		G	H	I
1	*trans*-β-Farnesene	2.80	6.91	5.37
2	Bisabolol oxide B	5.13	3.84	4.44
3	α-Bisabolone oxide A	1.30	2.74	1.15
4	α-Bisabolol	7.51	8.42	6.03
5	Chamazulene	3.94	3.57	3.04
6	Bisabolol oxide A	8.75	5.20	7.48
7	Spiroether	12.52	10.34	15.62
Intra-fibre repeatability (RSD%)		5.59	5.25	6.46

Table 8
Statistical elaboration of chamomile flower heads results: mean of marker absolute areas, mean of marker repeatability and inter-fibre total area intermediate precision

	Compounds	PDMS fibre		CAR/DVB/PDMS fibre	
		Average absolute area($\times 10^4$)	Average RSD%	Average absolute area($\times 10^4$)	Average RSD%
1	*trans*-β-Farnesene	295	7.01	156	5.03
2	Bisabolol oxide B	294	4.28	276	4.47
3	α-Bisabolone oxide A	13	2.53	12	1.73
4	α-Bisabolol	311	5.95	230	7.32
5	Chamazulene	5	5.51	5	3.52
6	Bisabolol oxide A	70	5.65	58	7.14
7	Spiroether	314	5.72	156	12.83
Inter-fibre intermediate precision (RSD%)		15.7		5.85	

Table 9
Statistical elaboration of chamomile flower heads results: PDMS fibre classification by Tukey test

Categories	Mean	Groupings			
PDMS F	972	A			
PDMS E	1132		B		
PDMS C	1149		B		
PDMS D	1202		B		
PDMS B	1373			C	
PDMS A	1986				D

Table 10
Statistical elaboration of chamomile flower heads results: CAR/DVB/PDMS fibre classification by Tukey test

Categories	Mean	Groupings	
CAR/DVB/PDMS I	835	A	
CAR/DVB/PDMS H	912		B
CAR/DVB/PDMS G	935		B

These results show that fibres from both sets can be used reliably for routine sampling of the headspace of a high number of samples in terms of the repeatability of marker recovery, intra-fibre repeatability and inter-fibre intermediate precision. Although some articles in the literature reported that fibres containing Carboxen are more effective in the extraction of different classes of compounds but their repeatability is relatively low [7,13–15], these results show that the CAR/DVB/PDMS fibres are more repeatable than those coated with PDMS for all matrices. However, PDMS results are partly affected by the "age" of

Table 11
Statistical elaboration of sage leaves results: PDMS marker repeatability and intra-fibre total area repeatability

	Compounds	PDMS fibre RSD%					
		A	B	C	D	E	F
1	α-Pinene	9.13	9.21	6.49	4.65	3.32	11.39
2	Camphene	7.91	8.13	5.81	4.19	3.18	9.82
3	β-Pinene	7.01	6.68	5.12	3.54	3.10	9.43
4	1,8-Cineole	4.51	6.02	4.07	2.23	5.73	9.45
5	Camphor	2.84	4.75	3.48	1.99	8.02	9.50
6	Borneol	3.36	6.77	4.24	2.80	8.21	10.32
7	4-Terpineol	7.81	6.20	2.74	3.53	6.62	9.96
8	α-Terpineol	4.08	5.82	2.94	2.19	7.10	9.87
9	Bornyl acetate	3.36	6.55	3.77	2.18	7.57	9.85
10	Sabinyl acetate	3.48	6.64	3.93	2.26	7.13	9.67
11	α-Cubenene	2.99	7.23	3.84	2.55	6.84	9.19
12	α-Ylangene	4.24	7.75	3.64	2.78	6.57	9.19
13	α-Copaene	3.40	7.24	3.89	2.65	6.52	9.17
14	β-Bourbonene	3.33	6.45	4.28	2.78	6.26	8.88
15	β-Caryophyllene	3.96	6.83	3.95	3.21	5.57	8.44
16	MW 204	4.01	7.43	3.71	3.47	5.83	8.45
17	α-Humulene	4.96	7.86	4.40	3.42	6.18	8.04
18	Allo-aromadendrene	5.49	7.19	3.80	3.94	5.72	8.22
19	γ-Muurolene	6.98	7.16	3.96	4.44	5.53	7.58
20	β-Selinene	6.74	8.27	3.80	4.57	6.18	7.46
21	α-Muurolene	8.49	6.91	3.43	4.71	6.14	7.09
22	γ-Cadinene	10.01	5.32	2.94	5.32	6.44	5.83
23	δ-Cadinene	10.23	7.39	4.63	5.07	6.48	7.43
24	Caryophyllene oxide	15.18	4.47	2.24	6.78	8.72	5.41
Intra-fibre repeatability (RSD%)		3.26	6.45	3.39	1.78	5.78	8.80

Table 12
Statistical elaboration of sage leaves results: CAR/DVB/PDMS marker repeatability and intra-fibre total area repeatability

	Compounds	CAR/DVB/PDMS fibre RSD%		
		G	H	I
1	α-Pinene	3.09	9.70	3.19
2	Camphene	3.26	9.57	3.46
3	β-Pinene	1.88	5.68	2.71
4	1,8-Cineole	0.64	5.00	2.99
5	Camphor	1.28	2.43	2.70
6	Borneol	3.32	1.31	4.32
7	4-Terpineol	4.32	1.83	3.59
8	α-Terpineol	3.49	1.89	2.81
9	Bornyl acetate	2.24	0.86	2.80
10	Sabinyl acetate	5.85	3.21	1.43
11	α-Cubenene	3.80	1.69	1.80
12	α-Ylangene	4.86	0.99	2.84
13	α-Copaene	4.02	0.84	1.83
14	β-Bourbonene	4.14	1.42	2.23
15	β-Caryophyllene	5.43	2.64	1.94
16	MW 204	4.52	2.20	2.60
17	α-Humulene	7.59	4.18	2.05
18	Allo-aromadendrene	6.55	1.88	5.50
19	γ-Muurolene	7.63	3.44	2.45
20	β-Selinene	6.75	2.08	4.44
21	α-Muurolene	7.31	1.59	3.48
22	γ-Cadinene	7.82	0.95	4.39
23	δ-Cadinene	8.22	2.98	4.64
24	Caryophyllene oxide	9.88	2.97	3.74
Intra-fibre repeatability (RSD%)		2.32	1.58	2.24

fibre A, whose performance is highly repeatable but different from that of the new fibres of the same set.

3.1.1. One way ANOVA

Analysis of variance was applied here to evaluate the contribution that each fibre gives to the variability of the results and to discriminate between the variations due to the intrinsic fibre variability and those due to random effects. The experimental data resulting from each set of fibres for each of the three matrices were first submitted to the test to verify whether their distribution were normal in order to apply ANOVA correctly. This test showed that all experimental data sets were congruent with a normal distribution with a significance level $\alpha = 0.05$. The following statistical approaches were then applied:

a) analysis of variance of each experimental data set by one way ANOVA using the Fisher test. *F*-test was used to compare the mean of the sums of the marker peak areas (μ) of each matrix obtained with each fibre of each set. This approach was based on the null hypothesis (H_0):

$$\text{i.e. } H_{0\,\text{PDMS}} = \mu_{\text{PDMS A}} = \mu_{\text{PDMS B}} = \mu_{\text{PDMS C}} = \mu_{\text{PDMS D}} = \mu_{\text{PDMS E}} = \mu_{\text{PDMS F}}$$

$$\text{and } H_{0\,\text{CAR/DVB/PDMS}} = \mu_{\text{CAR/DVB/PDMS G}} = \mu_{\text{CAR/DVB/PDMS H}} = \mu_{\text{CAR/DVB/PDMS I}}.$$

Table 13
Statistical elaboration of sage leaves results: mean of marker absolute areas, mean of marker repeatability and inter-fibre total area intermediate precision

	Compounds	PDMS fibre		CAR/DVB/PDMS fibre	
		Average absolute area($\times 10^4$)	Average RSD%	Average absolute area($\times 10^4$)	Average RSD%
1	α-Pinene	1113	7.36	875	5.33
2	Camphene	607	6.51	435	5.43
3	β-Pinene	868	5.81	890	3.42
4	1,8-Cineole	9975	5.33	6652	2.88
5	Camphor	11225	5.10	9775	2.14
6	Borneol	2694	5.95	3171	2.98
7	4-Terpineol	436	6.14	988	3.25
8	α-Terpineol	492	5.33	1330	2.73
9	Bornyl acetate	3243	5.54	3107	1.97
10	Sabinyl acetate	473	5.52	540	3.50
11	α-Cubenene	650	5.44	670	2.43
12	α-Ylangene	229	5.70	347	2.90
13	α-Copaene	790	5.48	1081	2.23
14	β-Bourbonene	853	5.33	635	2.60
15	β-Caryophyllene	2692	3.62	1612	3.33
16	MW 204	322	5.48	535	3.10
17	α-Humulene	1735	5.81	1046	4.60
18	Allo-aromadendrene	220	5.73	290	4.64
19	γ-Muurolene	932	5.94	753	4.50
20	β-Selinene	330	6.17	591	4.42
21	α-Muurolene	205	6.12	324	4.12
22	γ-Cadinene	471	5.98	511	4.38
23	δ-Cadinene	431	6.87	458	5.28
24	Caryophyllene oxide	2540	7.13	1636	5.53
Inter-fibre intermediate precision (RSD%)		8.40		4.93	

b) Fisher test (F-Test). It was used to evaluate whether the variances s^2_B between the fibres of a given set and the variances s^2_W within a fibre of the same set in recovering marker components from the headspace of each matrix investigated was significant, i.e. if the variance was due to their different performance.

$$F = \frac{s^2_B}{s^2_W}$$

c) Tukey test. It was used to identify the fibres in a set inducing the rejection of the null hypothesis i.e. those showing significant difference from the others at 95% confidence interval. Tukey test is a post hoc test giving a new critical value that can be used to evaluate whether differences between any two pairs of means are significant ($\mu_{PDMSA} - \mu_{PDMSB}$, $\mu_{PDMSA} - \mu_{PDMSC}$ etc.). Each difference is then compared to the Tukey critical value. When the difference exceeds the Tukey value, the comparison is significant.

$$Q' = Q_{\alpha,c,n-c}\sqrt{\frac{s^2_W}{n_j}}$$

where Q' is the critical variation field, that depends on the confidence level (α), number of fibres set (c), total number of experiments (n), number of observation of the j group (n_j) and of the variance between the groups (s^2_W).

The F-test was applied to the total areas obtained from the analysis of the markers of the standard solution, sage leaves and chamomile flower heads with both sets of fibres to verify the null hypothesis gave different results. The value of F-calculated (F_c) for the PDMS set of fibres was higher than that of F-tabulated (F_t) with all three matrices investigated meaning that the null hypothesis was not verified at a 95% confidence interval and the PDMS fibre performance significantly varied with all matrices, although to a different extent. With the CAR/DVB/PDMS set, F_c was lower than F_t with the standard solution and sage meaning that the results were in agreement with the null hypothesis, i.e. no significant difference occurred between the fibre performance. With chamomile, F_c was higher than F_t showing that with this matrix a significant variability also occurred in CAR/DVB/PDMS fibre performance. The data were therefore submitted to the Tukey test to identify those fibres within each set that were responsible for the rejection of the null hypothesis. Tables 4, 5, 9, 10, 14 and 15 report how the fibres of each set are grouped and discriminated in function of the similarity of their performance. Within the PDMS set, the Tukey test results were significantly different depending on the matrices:

a) for the standard solution the fibres were divided into three groups: fibres B, C, E, and F whose performance were similar at 95% of the confidence interval, while fibre D and A differed, although to a different extent.
b) for chamomile, four groups were formed: fibres C, D, and E with showed performance not statistically distinguishable, F and B were in different groups but closed to the previous one and A.
c) for sage, the performance of fibre A was distinguished from those of the group including B, C, D, E, and F.

Table 14
Statistical elaboration of sage leaves results: PDMS fibre classification by Tukey test

Categories	Mean	Groupings	
PDMS A	37111	A	
PDMS D	42187		B
PDMS C	43630		B
PDMS B	44705		B
PDMS E	45876		B
PDMS F	46724		B

These results show that fibre A has a different performance when compared to the new fibres probably because the high number of samplings (100) run before submitting it to this series of experiments produced a variation of the PDMS coating performance (see part 2). The variability of fibre performance with chamomile was probably also related to the low volatility of the markers that require long times to achieve the equilibrium and to the formation of chamazulene that is difficult to control quantitatively.

Within the CAR/DVB/PDMS set, the results were more homogenous since the fibres were all in a single group for the standard solution and sage, while for chamomile two groups were created the first one including fibres G and H and the second fibre I.

The reported results on fibre reliability are very positive keeping in mind (a) the dual equilibrium that conditions fibre recovery in HS-SPME and makes sampling of complex mixtures of analytes with different volatility in reasonable times a non-equilibrium process; (b) the limited homogeneity of the solid matrices and in particular of vegetable natural products; (c) the "instability" of chamomile as matrix that influence the headspace composition as it is clear when its results are compared to those of sage; and (d) the high level of confidence (95%) adopted in consideration of the points a), b) and c).

3.2. Influence of the number of samplings on fibre recovery effectiveness ("fibre life-time")

The second part of this study aimed to evaluate the short and long term life-time of a fibre or more precisely the number of samplings where a single fibre applied to the routine HS-SPME analysis of vegetable matrices keep reliable performance, as well

Table 15
Statistical elaboration of sage leaves results: CAR/DVB/PDMS fibre classification by Tukey test

Categories	Mean	Groupings
CAR/DVB/PDMS H	37289	A
CAR/DVB/PDMS G	38920	A
CAR/DVB/PDMS I	40014	A

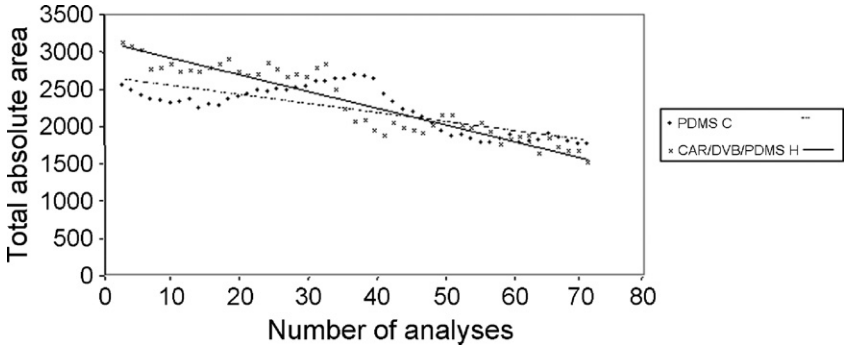

Fig. 2. Variation of the total areas of the chamomile flower heads markers *vs.* the number of samplings with the PDMS C and CAR/DVB/PDMS H fibres.

as to detect if and how matrix effects affected fibre performance in headspace sampling. These experiments were carried out with both chamomile flower heads and sage leaves from Lot 2. The fibre life-time was monitored by determining the variation of the total areas of the marker components of sage and chamomile sampled by HS-SPME as a function of the number of analyses while the diisobutyl phthalate standard solution was taken as a reference arbitrarily assuming it to be free from matrix effect. Chamomile flower heads and sage leaves were chosen because of their different composition, complexity and nature of the solid matrix in order to evaluate the influence of matrix effect not only on recovery as already reported by other authors that showed that SPME results are affected by the overall volatile compounds present in the matrix [7,16,17], but also on fibre life-time. These experiments were run with fibres belonging to the same groups identified by the above Tukey test.

The fibre life-time was monitored by the Linear Regression Analysis (LRA). LRA was adopted because it is the simplest approach to study the relationship between two variables: it was used to evaluate the trend of the recovery of the markers of a matrix from its headspace by a given fibre as a function of the number of samplings and not to correlate two sets of data. In LRA, the distance of a point from the line is due to two factors: (i) the explained variation, which is due to the total area (y)/number of samplings (x) relationship, and (ii) the unexplained variation, which depends on variables different from the above x–y relationship. The parameters characterising LRA assume specific meanings: the slope in the regression line equation resulting from LRA represents the loss of total recovery with the number of samplings, the y-intercept is the effectiveness of the total recovery and the determination coefficient r^2 gives the proportion of variation of total areas that can be explained with the increase of the number of analysis [18]. This means that high r^2 values have not necessarily to be achieved because analyte recovery are influenced by several other factors than the nature of the fibre coating and the number of analysis, one of the most important of them being the non-equilibrium sampling conditions for most analytes.

Long term fibre-life was monitored on chamomile flower heads by analysing seventy samples from the same lot analysed by HS-SPME both with the PDMS C and CAR/DVB/PDMS H fibres and GC-FID under the same operative conditions reported above. LRA applied to the total areas of the seven chamomile markers in function of the number of samplings gave the following equations:

-PDMS C fibre :

$$y = -15.14\,x + 2683.2 \qquad r^2 = 0.5698 \tag{1}$$

-CAR/DVB/PDMS H fibre :

$$y = -28.15x + 3102.1 \qquad r^2 = 0.8462 \tag{2}$$

Fig. 2 reports the diagrams of the variation of the total areas of the chamomile markers *versus* the number of samplings with the PDMS C and CAR/DVB/PDMS H fibres respectively. The slopes in the line Eqs. (1) and (2) indicate that the loss of efficiency over 70 samplings is higher for CAR/DVB/PDMS H than for the PDMS C fibres and that after 70 samplings it can indica-

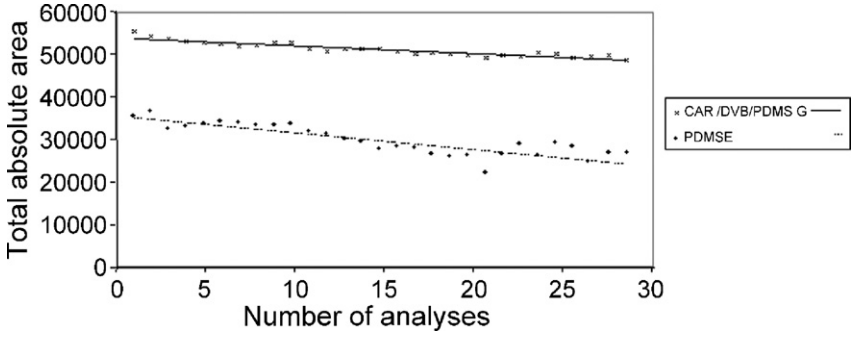

Fig. 3. Variation of the total areas of the sage leaves markers *vs.* the number of samplings with the PDMS E and CAR/DVB/PDMS G fibres.

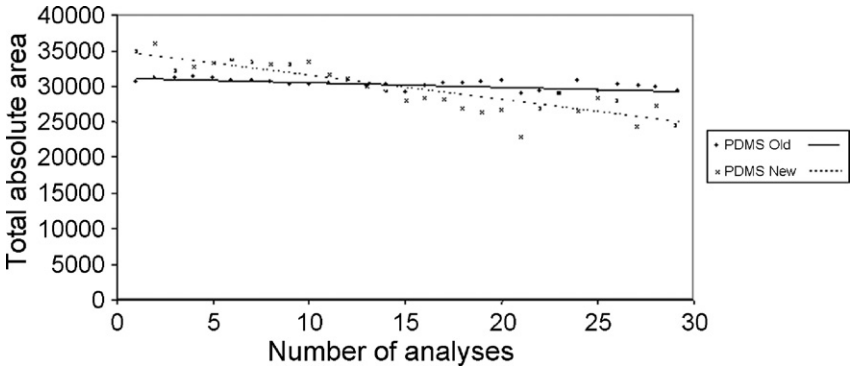

Fig. 4. Variation of the total areas of the sage leaves markers with the number of analyses with a new and an old PDMS fibre (for detail see text).

tively be estimated at about 25% for PDMS C and 34% for CAR/DVB/PDMS H. A possible explanation of the difference in slopes between the two coatings can be the change in recovery effectiveness of the fibre components operating in adsorption (i.e. CAR and DVB) with the number of analysis. The *y* intercepts of the two equations show that the CAR/DVB/PDMS H fibre recovery is quite higher than that of the PDMS C as expected [11], because of its better affinity to analytes with different polarity and volatility due to the different recovery approaches of its components.

Short term fibre-life was monitored by analysing thirty samples of sage leaves from the same lot analysed by HS-SPME with both the PDMS E and CAR/DVB/PDMS G fibres and GC-FID under the same operative conditions reported above.

The statistical elaboration of the experimental total areas of the twenty four markers gave the following regression equations:

-PDMS E fibre :

$$y = -343.8x + 34858.28 \qquad r^2 = 0.6843 \tag{3}$$

-CAR/DVB/PDMS G fibre :

$$y = -161.03x + 52352.50 \qquad r^2 = 0.8635 \tag{4}$$

Fig. 3 reports the comparison between the variation of the total areas of the sage markers *versus* the number of samplings with the PDMS E and CAR/DVB/PDMS G fibres. The difference in the slope of the two lines showed that the loss of efficiency over 30 samplings was about 12% for the PDMS E fibre and 8% with CAR/DVB/PDMS G. As for chamomile also for sage, the *y* intercepts of the two Eqs. (3) and (4) show an higher extraction yield for the CAR/DVB/PDMS G fibre. For both matrices the determination coefficients r^2 related to the two sets were different but similar for each fibre coating indicating a similar proportion of explained variability. These results will also be confirmed by those of the next experiments.

The difference of the above results on sage and chamomile also emphasized the importance of the role that the matrix can play on the life of the fibre [6,7]. This effect is also evident when the results from the standard solution analysed every ten samples of chamomile are processed by LRA. The slope in the regression line equations of the standard solution after 70 analyses with both PDMS C and CAR/DVB/PDMS H fibres reported below (5 and 6) show that the total areas with PDMS C are almost unvaried while those obtained with CAR/DVB/PDMS H significantly change

-PDMS C fibre :

$$y = -2.3968x + 543.26 \qquad r^2 = 0.537 \tag{5}$$

-CAR/DVB/PDMS H fibre :

$$y = -46.563x + 3933.3 \qquad r^2 = 0.8844 \tag{6}$$

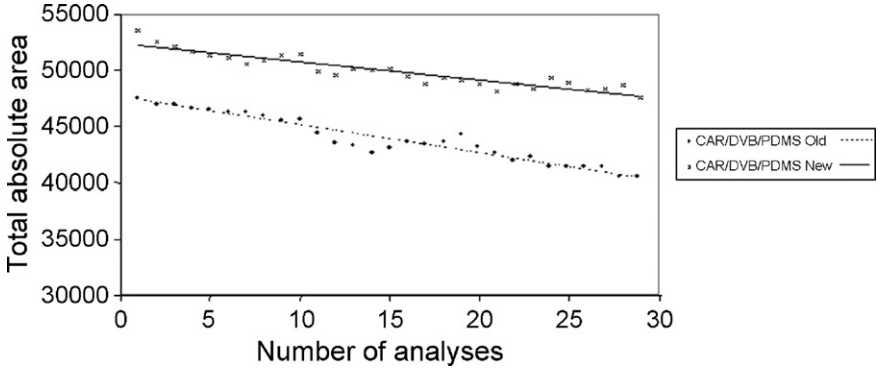

Fig. 5. Variation of the total areas of the sage leaves markers with the number of analyses with a new and an old CAR/DVB/PDMS fibre (for detail see text).

These results confirm that the life of the two types of fibre coatings is also conditioned by the matrix: PDMS fibre-life seems to be more matrix sensitive and showed a higher loss of effectiveness with a complex matrix as sage leaves while CAR/DVB/PDMS fibre-life seems to depend more on the sampling conditions with a higher loss of performance with chamomile flower heads.

The last part of this study evaluated how the total number of analyses may influence the fibre performance: these experiments compared the results of 30 samplings of sage leaves headspace run with two new fibres to those of the PDMS C and CAR/DVB/PDMS H fibres already used for the above 70 samplings of chamomile (for short they are here identified as new and old fibres respectively). Comparison was again by LRA applied to the total areas of the sage marker components.

The regression equation resulting from the new and the old PDMS fibres are:

-New PDMS fibre :

$$y = -343.8x + 34858.28 \qquad r^2 = 0.6843 \tag{7}$$

-Old PMDS fibre :

$$y = -67.344x + 31036 \qquad r^2 = 0.4192 \tag{8}$$

Fig. 4 reports the variation of the total areas of the 24 sage markers in function of the number of analyses with the new and the old PDMS fibres. From both the diagrams and Eqs. (7) and (8), it is clear that the two fibres behave differently: the performance of the old fibre is more stable than the new one as it is also evident from their slopes, while, as expected, the new fibre gave higher recovery (about 10%) in the first analyses.

The CAR/DVB/PDMS fibres behaved differently. The following regression equations for 30 sage samplings were obtained:

-New CAR/DVB/PDMS fibre :

$$y = -161.03x + 52352.50 \qquad r^2 = 0.8635 \tag{9}$$

-Old CAR/DVB/PDMS fibre :

$$y = -257.61x + 47613 \qquad r^2 = 0.8164 \tag{10}$$

Fig. 5 reports the variations of the marker total areas *versus* the number of analysis with the new and the old CAR/DVB/PDMS fibres. These results and in particular r^2 show that the variation of performance of these fibres over the number of analysis is mainly influenced by the effectiveness of the components of the fibre coating.

Lastly, the intra-day repeatability of the total areas of the sage markers was also measured in order to evaluate whether the number of analysis per day could influence the fibre performance. Table 16 shows that mean intra-day RSD% calculated on eight analyses per day for four fibres (a new and an old fibres for each coating) were always below 7% for each day of investigation showing again a good repeatability of the technique.

Table 16
Intra-day repeatability of the total areas of the sage markers expressed as RSD% calculated on eight analyses per day for four fibres (a new and an old fibres for each coating)

	PDMS		CAR/DVB/PDMS	
	Fibre C (RSD%)	Fibre E (RSD%)	Fibre H (RSD%)	Fibre G (RSD%)
Day 1	4.39	4.22	2.85	4.71
Day 2	2.87	1.27	3.31	1.23
Day 3	1.85	2.85	5.77	6.38
Day 4	6.27	4.93	4.58	2.54
Day 5	2.66	3.37	1.58	3.92
Day 6	4.47	3.89	5.18	6.31

4. Conclusions

The reported results show that HS-SPME can successfully be applied to routine control analysis of aromatic and medicinal plants since both types of fibres have shown reliable performance in terms of both repeatability and intermediate precision of analytes recovery and consistency over time also with real-world samples. These results are particularly positive in view of the non-equilibrium sampling conditions that are usually applied to complex mixtures and of the heterogeneity of the solid matrices and in particular of vegetable natural products. Fibres from different lots within each set have shown to have comparable performance in terms of the consistency of analyte recovery making them interchangeable. The reliability of both types of fibre coatings (PDMS and CAR/DVB/PDMS) was comparable and, quite unexpectedly, CAR/DVB/PDMS showed a better performance than PDMS in consideration that coatings containing Carboxen were reported to present a relatively low repeatability [7,13–15]. The fibre-life seems mainly to be influenced by the number of samplings to which it has been submitted and by both the nature of the matrix under investigation and sampling conditions. As a consequence, reliable HS-SPME routine analysis of a high number of solid samples requires the fibre performance to be controlled at regular intervals with a reference material or solution and the results to normalised through correction factors such as the concentration factors *versus* static headspace of some of the target analytes [11] or the fibre concentration capability index (F_{ij}) [19,20] where the number of different fibre coatings compared must be replaced with that of periodical samplings.

Acknowledgements

The authors are indebted with Sigma Aldrich, Milano (Italy) for fibre supplying.

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