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Analysis of Volatile Organic Compounds in Natural Matrices by HS-SPME/GC-MS

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Abstract

This thesis is about the analysis of volatile organic compounds in natural samples. It is focused on the application of preconcentration techniques, extraction techniques (for example, solid-phase microextraction, distillation or supercritical fluid extraction) and the application of analytical separation techniques for the identification and semi-quantification of volatile compounds typically found in natural materials, or products made from them.

The experimental part of the thesis is divided into three main chapters: the extraction of compounds present in rose petals, the analysis of different kinds of alcoholic beverages, and the aroma-profile characterization of volatile compounds found in tonka beans (*Dipteryx odorata*). The theoretical part of the thesis contains the basic information about that which has been studied, and the practical part contains the chemicals, instruments and analytical procedures used. Each chapter contains its own section where the results are discussed.

Abstrakt

Disertační práce je zaměřena na analýzu těkavých organických látek ve vzorcích přírodního materiálu. Zabývá se aplikací prekoncentračních technik, extrakčních technik (mikroextrakce tuhou fází, destilace, extrakce nadkritickou tekutinou) a použitím analytických separačních metod pro identifikaci, příp. semikvantifikaci těkavých sloučenin charakteristických pro některé přírodní materiály či produkty z nich vyrobené.

Experimentální část disertační práce je rozdělena na tři hlavní kapitoly, kterými jsou problematika extrakce těkavých sloučenin přítomných v okvětních lístcích růží, analýza různých druhů alkoholických nápojů a charakterizace profilu těkavých látek tonkových bobů. Základní informace o zkoumaných matricích jsou vypsány v teoretické části práce, v praktické části jsou uvedeny použité chemikálie, přístroje a analytické postupy, včetně jejich optimalizace. Každá kapitola obsahuje vlastní sekci, kde jsou diskutovány dosažené výsledky.

Keywords

HS-SPME/GC-MS, roses, alcoholic beverages, tonka beans, *Dipteryx odorata*

Klíčová slova

HS-SPME/GC-MS, růže, alkoholické nápoje, tonkové boby, *Dipteryx odorata*

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Rose Petals

1. INTRODUCTION

It is generally known that rose flower slices contain mixtures of aromatic compounds which vary among different species and varieties. For example, the *Rosa genus* includes 100-200 species and more than 18 000 cultivars and hybrids [1]. More than 400 volatile compounds have been identified in the rose scent of various rose cultivars. The chemical composition of rose scent is significantly complex due to the presence of several chemical groups: hydrocarbons (e.g. *β*-caryophyllene), alcohols (e.g. phenylethyl alcohol), terpenes (e.g. geraniol, nerol), esters (e.g. hexyl-acetate, geranyl acetate), aromatic ethers (e.g. 3,5-dimethoxytoluene), and other compounds [2, 3] Literary sources report monoterpenes as major components of rose scent (principally citronellol, geraniol, nerol and linalool), 2-phenylethanol and sulphated volatiles, such as dibenzothiophene [4–7].

One of the possibilities how to extract volatile or semi-volatile compounds from plant material is the headspace solid-phase microextraction (HS-SPME) which presents several advantages. Some of these advantages of HS-SPME are easy and fast preparation of sample as well as the elimination of solvents or easily automated sampling [8–10]. SPME can be a fast, sensitive and economical method of sample preparation preceding the analysis using gas chromatography [11].

2. EXPERIMENTAL

2.1 Chemicals

Two *n*-alkane standard mixtures (C_8–C_{20} in *n*-hexane; C_{21}-C_{40} in toluene; 40 mg L^{-1} each component) were purchased from Sigma-Aldrich (Prague, Czech Republic).

Plant Material

Six different types of rose petals (*Rosa genus*) were analysed: *Rosa Mariyo*, *Rosa Rhodos*, *Rosa Sudoku*, *Rosa Tara*, *Rosa Tacazzi* and *Rosa Deep Purple*. All roses have been brought from the Netherlands within the period from October 2014 to March 2015.

2.2 Sample preparation

Roses were air-dried on the filter paper at a room temperature of 20-25 °C to dryness. Residual moisture was determined by a moisture analyzer KERN MLB50-3 from Kern (Balingen, Germany) and dry weight ranged from 82.3 % to 86.1 %. Dried rose petals were milled into a powder using a 5100 Mixter Mill (SPEX SamplePrep, Metuchen, NJ, USA). Extraction by HS-SPME was carried out in 20 mL headspace vials and closed by a cap with a Teflon septum (Supelco, Bellefonte, PA, USA).

2.3 Headspace solid-phase microextraction

SPME experiments were carried out using 50/30μm DVB/CAR/PDMS fiber (divinylbenzene/carboxen/polydimethylsiloxane) (Bellefonte, PA, USA). The fiber was conditioned before use, as the manufacturer recommends. The HS-SPME was

carried out at optimised conditions. 0.7 g of sample was taken into 20-mL glass vials and the extraction procedure was conducted at a temperature of 90 °C for 60 minutes.

2.4 **GC-MS analysis**

A gas chromatograph, model GC-2010 Plus, coupled with mass spectrometry detector TQ-8030 and auto-sampler AOC-5000 Plus (all from Shimadzu, Kyoto, Japan) was used for analysis. Injections have been performed in splitless mode. The GC-MS system has been equipped with a capillary column SLB^{TM}-5ms with 30 m length, 0.25 mm inner diameter and 0.25 μm film thickness (Supelco, Bellefonte, PA, USA). Helium 5.0 (Linde Gas a.s., Prague, Czech Republic) was used as a carrier gas at a constant linear velocity of 30 cm s^{-1}. The injector and the interface temperature were maintained at 230 °C. The column temperature has been programmed as follows: the initial temperature was 40 °C (5 min) then increased at a rate of 3 °C/min up to 280 °C (15 min). The mass spectrometer was operated in the full scan mode over a mass range of *m*/*z* 45–500 and in the electron ionization mode (70 eV).

The mixtures of *n*-alkanes (C_8-C_{20}, C_{21}-C_{40}) were injected using the above temperature program in order to calculate the retention index (RI) for each peak. Identification of the components was done by comparison of mass spectral fragmentation patterns stored in MS data libraries NIST 11, Willey 209 and FFNSC 2 and verified by comparison of retention indices of identified compounds with published index data (NIST Chemical Webbook database) and RIs from MS data library FFNSC 2. The criterion of similarity for the mass spectra was at least 80 %.

2.5 **GC-FID analysis**

GC-FID analysis was performed on Shimadzu GC2010 (Shimadzu, Co., Kyoto, Japan) coupled with a flame ionization detector (FID). The detector temperature was maintained at 300 °C. The other analytical conditions (column type and temperature program, the injector temperature, carrier gas and its linear velocity were the same as those of GC–MS analysis.

2.6 **Optimization of the extraction conditions**

The HS-SPME conditions were optimized using a central composite design (CCD). The statistical analysis and CCD were performed using Statistica CZ software, version 12 (StatSoft CR, Prague, Czech Republic). CCD was based on a 2^3 factorial design plus nine axial points plus three replicates in the center of the design. The independent variables in design were extraction time, extraction temperature and sample weight. Twenty experiments (Table 1) were generated by CCD and executed in the given order. All optimization experiments were performed with milled rose petals of *Rosa Mariyo*.

3. RESULTS AND DISCUSSION

3.1 **Optimization of HS-SPME**

At the beginning of experiment three parameters have been chosen for the optimization of HS-SPME technique: the time of extraction (20-60 min), the temperature of extraction (50-90 °C) and the weight of the sample (0.3-0.7 g). The distribution coefficient between the sample and the HS and between the HS and the fiber is influenced by the extraction temperature [12]. When selecting an SPME

sorbent, the polarity of the sorbent coating should match the polarity of the analyte, and the coating should be resistant to high-temperature conditions [13]. The correct fiber was selected based on recommendations Sigma Aldrich Guide [14]. The bipolar fiber (DVB/CAR/PDMS) has been examined for analysis of compounds in roses. Optimum extraction conditions were determined by the method of response surface modeling. The whole design consists of twenty experimental points as seen in Table 1.

Table 1 Central composite design-coded independent variables (x_1, x_2, x_3), corresponding experimental conditions (X_1, X_2, X_3) and results represented by the total area in chromatogram for 50/30 μm (DVB/CAR/PDMS) fiber. (C) – central point

Run	Extraction time, min	Extraction temperature, °C	Sample weight, g	Total area Predicted	Total area Observed
	x_1 (X_1)	x_2 (X_2)	x_3 (X_3)		
1	-1 (28)	-1 (58)	-1 (0.38)	3.02×10^8	2.43×10^8
2	-1 (28)	1 (82)	1 (0.62)	8.62×10^8	8.00×10^8
3	1 (52)	-1 (58)	1 (0.62)	4.40×10^8	3.61×10^8
4	1 (52)	1 (82)	-1 (0.38)	8.48×10^8	8.13×10^8
5 (C)	0 (40)	0 (70)	0 (0.50)	4.88×10^8	5.10×10^8
6 (C)	0 (40)	0 (70)	0 (0.50)	5.21×10^8	5.10×10^8
7	-1 (28)	-1 (58)	1 (0.62)	2.59×10^8	2.78×10^8
8	-1 (28)	1 (82)	-1 (0.38)	6.38×10^8	7.00×10^8
9	1 (52)	-1 (58)	-1 (0.38)	2.52×10^8	2.97×10^8
10	1 (52)	1 (82)	1 (0.62)	9.00×10^8	9.42×10^8
11 (C)	0 (40)	0 (70)	0 (0.50)	5.37×10^8	5.10×10^8
12 (C)	0 (40)	0 (70)	0 (0.50)	5.85×10^8	5.10×10^8
13	-α (20)	0 (70)	0 (0.50)	4.03×10^8	4.19×10^8
14	α (60)	0 (70)	0 (0.50)	5.74×10^8	5.82×10^8
15	0 (40)	-α (50)	0 (0.50)	1.82×10^8	2.18×10^8
16	0 (40)	α (90)	0 (0.50)	1.10×10^9	1.08×10^9
17	0 (40)	0 (70)	-α (0.30)	4.47×10^8	4.33×10^8
18	0 (40)	0 (70)	α (0.70)	5.31×10^8	5.70×10^8
19 (C)	0 (40)	0 (70)	0 (0.50)	4.55×10^8	5.10×10^8
20 (C)	0 (40)	0 (70)	0 (0.50)	4.79×10^8	5.10×10^8

The response was based on the sum of the peak areas of all being detected, which belongs to one of the most common parameters for the optimization of the SPME conditions [15, 16]. These values were statistically processed in Statistica 12 program, which allows creating of second-order models. Reliability of the model, which includes linear terms, quadratic terms, and interaction between linear terms was expressed R-squared = 0.96443. The second-ordered polynomial equation, as you can see on equation 1, was constructed for the response variable (the total area of all detected peaks, coded TA) related to the experimental conditions:

$$TA = 1.079\times10^{9} - 3.737\times10^{6} X_1 - 2.330\times10^{4} X_1^2 - 3.742\times10^{7} X_2 + 3.521\times10^{5} X_2^2 - 4.383\times10^{8} X_3 - 2.099\times10^{8} X_3^2 + 1.024\times10^{5} X_1 X_2 + 5.012\times10^{6} X_1 X_3 + 1.131\times10^{7} X_2 X_3 \quad (1)$$

where X_1 (extraction time, in min), X_2 (extraction temperature, in °C), X_3 (sample weight, in g) and are experimental conditions of independent variables, as you can see in Table 1. The significant factor (p-value less than 0.05 at confidence level 95 %) of the equations was extraction temperature. Correlation between values of experimental data and predicted values is shown in Table 1. For this model we have also generated the optimum conditions: the extraction time 60 minutes, the temperature of extraction 90 °C and the sample weight 0.7 g.

3.2 **Analysis of real samples**

Table 2 shows the relative abundance expressed as a relative area in percent of compounds detected in the volatile fraction of all samples. A 211 of volatile compounds were identified in the six samples of the *Rosa genus* (*Rosa Mariyo*, *Rosa Rhodos*, *Rosa Sudoku*, *Rosa Tara*, *Rosa Tacazzi* and *Rosa Deep Purple*). Table 2 is classified according to chemical composition (alcohols, hydrocarbons, carbonyls, terpenes, esters and others). However, relatively large number of the peaks was not identified, often due to the absence of appropriate mass spectrum in libraries or absence of retention indices calculated for given column.

Leffingwell [17] reports the list of compounds present in Rose oil. The main constituent of Rose oil is citronellol (38 %), C14-C16 paraffins (16 %), geraniol (14 %), nerol (7 %), phenethyl alcohol (2.8 %) , eugenol methyl ether (2.4 %), eugenol (1.2 %), farnesol (1.2 %), linalool (1.4 %), Rose oxide (0.46 %), carvone (0.41 %), Rose furan (0.16 %), *β*-damascenone (0.14 %) and *β*-ionone (0.03 %). The relative odor contribution (in rel. % of odor units) provides differences in the content for the minor constituents compared with the major component citronellol. The minor constituents *β*-damascenone (70 %) and *β*-ionone (19.2 %) provide a significant majority of the odor contribution against citronellol (4.3 %).

The characteristic floral rose fragrance is majorly influenced by a few compounds. The main compound is *cis*-rose oxide present in isomer form. Another compound that contributes to the scent of roses is *β*-damascenone known as rose ketones. Other compounds that make minor contributions to the aroma include *trans*-geraniol, nerol, citronellol, farnesol, and linalool [18]. Some substances were not detected in, extracts; those were *cis*-rose oxide, *trans*-geraniol, citronellol, and farnesol.

Analysis of the volatile composition of rose petals showed that alcohols, carbonyls, and hydrocarbons (mainly paraffin), were the most represented classed of compounds in all chromatographic profiles of six samples of rose petals, accounting for up 60 % of the total volatile fraction.

Table 2 Chemical composition of the volatile components of extracts of rose petals *Rosa genus*, contents of individual compounds are expressed as average relative percent peak area of GC-FID after three replicates (n = 3), n.i. = not identified.

	Rel. Area, %					
Chemical species	*Mariyo*	*Rhodos*	*Sudoku*	*Tara*	*Tacazzi*	*D. Purple*
Alcohols	8.29	5.25	4.43	40.89	2.84	2.74
Alicyclic hydrocarbons	n.i.	2.00	n.i.	n.i.	0.17	n.i.
Aliphatic hydrocarbons	13.58	14.13	27.81	17.19	14.96	12.47
Aromatic hydrocarbons	0.01	0.04	0.17	0.02	0.03	0.22
Esters	2.42	2.32	1.57	3.62	0.82	1.25
Aldehydes	1.62	16.2	7.15	2.00	10.22	12.72
Ketones	2.22	3.51	6.64	3.45	9.32	5.90
Acids	0.33	1.46	2.50	1.80	3.25	4.12
Monoterpenes	0.03	0.38	n.i.	0.05	0.06	0.22
Sesquiterpenes	0.02	0.68	n.i.	n.i.	n.i.	n.i.
Oxidated monoterpenes	0.14	0.17	0.38	0.10	n.i.	0.50
Oxidated diterpenes	n.i.	0.12	1.07	0.17	0.11	0.08
Oxidated sesquiterpenes	0.28	n.i.	n.i.	n.i.	0.10	n.i.
Phenolic derivates	0.64	n.i.	0.39	0.51	0.13	2.36
Apocarotenoids	0.80	1.39	0.93	1.01	0.47	3.42
Pyrazines	0.25	0.22	0.09	0.09	0.86	0.41
Furans	0.21	0.59	6.79	0.71	1.07	0.80
Lactones	0.11	0.08	0.14	0.39	0.43	0.26
Sulfides	0.02	n.i.	0.09	0.03	0.14	0.27
Others	n.i.	0.45	0.01	4.58	2.30	0.12

1. INTRODUCTION

Alcoholic beverages, including fruit spirits (FS), are very popular worldwide. Moreover, in Eastern and Central Europe, FS are regarded as a kind of tradition or gastronomic heritage [19]. FS, considered as excellent therapeutic agents, were already produced in alchemical workshops and pharmacies from the Middle Ages, and during the sixteenth century also in distilleries. According to European Community Regulation EC 110/2008, 'Fruit spirit is a spirit drink produced exclusively by the alcoholic fermentation and distillation of fleshy fruit or must of such fruit, berries or vegetables, with or without stones'[20].

Although the Czech Republic is well known especially for beer production, manufacture of FS is also very popular, particularly in its eastern part – the Moravian region. The most common and traditional fruit for such production is undoubtedly plums. The largest producers of plum spirit, apart from the Czech Republic, are Poland, Slovakia, Hungary, Bulgaria, Serbia, and Romania. However, not only plums are used for alcoholic distillates in the Czech Republic. Often, there are spirits made from other kinds of fruits. Those can be separated into three groups: stone fruits (plums, cherries, sour cherries, apricots, peaches, mirabelles, etc.), pome fruits (pears, apples) and small fruits (various kinds of berries). Therefore, the aroma of the final beverage may be very varied. The main constituents of spirits are ethanol and water. However, minor compounds, i.e. aroma-active volatiles, determine the odour and taste of a beverage. Such volatile compounds presented in fresh distillates include alcohols, aldehydes, esters, acids and volatile phenols [21]. The quality of spirits is strongly influenced by the natural aroma of the fruits (primary flavour). The latter is determined by many factors such as the geographical origin, the method of cultivation, storage and time of harvest. Subsequently, fermentation (secondary flavour), distillation (tertiary flavour) and the maturation of the spirits (quaternary flavour) influence the aroma [19, 22].

2. EXPERIMENTAL

2.1 Spirit samples

All examined home-made spirit samples (24) were produced from fruits which were grown in the Czech Republic. The samples, which were obtained from private local producers who guaranteed their authenticity, were not aged in wooden barrels and are listed in Table 3 with additional information. As can be seen, there were six apple spirits, five plum spirits, four pear spirits, four mirabelles spirits, two apricot spirits, two raspberry spirits and one cherry spirit. All samples were analyzed in August 2015.

Table 3 List of fruit spirit's samples

Sample number	Fruit origin	Year of production	Label of sample
1	Plum	2014	3 - Plum 2014
2	Plum	2012	14 - Plum 2012
3	Plum	2013	15 - Plum 2013
4	Plum	2014	25 - Plum 2014
5	Plum	2013	26 - Plum 2013
6	Mirabelle	2014	6 - Mirabelle 2014
7	Mirabelle	2012	12 - Mirabelle 2012
8	Mirabelle	2013	17 - Mirabelle 2013
9	Mirabelle	2014	30 - Mirabelle 2014
10	Cherry	2007	19 - Cherry 2007
11	Apricot	2013	16 - Apricot 2013
12	Apricot	2011	20 - Apricot 2011
13	Apple	2014	4 - Apple 2014
14	Apple	2014	5 - Apple 2014
15	Apple	2014	8 - Apple 2014
16	Apple	2014	9 - Apple 2014
17	Apple	2015	37 - Apple 2015
18	Apple	2015	38 - Apple 2015
19	Pear	2014	7 - Pear 2014
20	Pear	2009	10 - Pear 2009
21	Pear	2012	11 - Pear 2012
22	Pear	2014	18 - Pear 2014
23	Raspberry	2014	2 - Raspberry 2014
24	Raspberry	2014	31 - Raspberry 2014

2.2 **Chemicals and materials**

n-Hexane, and *n*-alkane mixture standard solutions C8–C20, and C21–C40 in concentrations of 40 mg L^{-1} dissolved in *n*-hexane and toluene, respectively, were purchased from Sigma-Aldrich (Prague, Czech Republic). Distilled water was purified using a Milli-Q® Water Purification System (Millipore SAS, Molsheim, France). Sodium chloride (analytical grade) was purchased from Lach-Ner, s.r.o. (Neratovice, Czech Republic). SPME fibers, 100 μm PDMS (polydimethylsiloxane) and 50/30 μm StableFlex DVB/CAR/PDMS (divinylbenzene/carboxene/ polydimethylsiloxane), were purchased from Sigma-Aldrich (Prague, Czech Republic).

2.3 **HS-SPME (optimized method)**

Before each extraction, the samples were diluted in water in a ratio of 1:4 owing to modification of ion power and then properly mixed. Subsequently, 10 mL was transferred into 20mL headspace vials and sodium chloride was added to a final concentration 28.5% (w/v). The vials were then closed by a cap with a Teflon septum. The treated samples were pre-incubated at 45 °C for 20 min to obtain steady-state extraction conditions. The extraction was performed using a 100 μm PDMS fiber at 45 °C for 60 min. After that, volatile compounds were automatically desorbed from the fiber in the GC injector port, set up at 250 °C.

2.4 **Chromatographic analysis**

A gas chromatograph, model GC-2010 Plus, coupled with mass spectrometry detector TQ-8030, was used for the analysis. Autosampler AOC-5000 Plus (Shimadzu, Kyoto, Japan) equipped with an agitator/heater unit was used for automated HS-SPME procedure and thermal desorption of extracts in the injector. Capillary column SLB-5 ms with a length of 30 m, a 0.25 mm inner diameter and a 0.25 μm film thickness (Supelco, Bellefonte, PA, USA) was used for separation. Helium 5.0 (Linde Gas a.s., Prague, Czech Republic) was used as a carrier gas at a constant linear velocity of 30 cm s^{-1}. The injector was maintained at 200 °C and desorption time was 15 s. Injections were done in split mode at a split ratio 1:20. The column temperature programme was set up as follows: the initial conditions were 40 °C for 3min, and then increased by a rate of 2 °C/min up to 250 °C for 12 min. ‘Solvent cut’ time was set up at 5.5 min and therefore all compounds with retention index (RI) <738 could not be observed. The mass spectrometer was operated in the electron ionization mode (70 eV) and in the full-scan mode over a mass range of m/z 33–500. The interface temperature and the ion source temperature were maintained at 200 °C. To avoid carry-over of analytes in subsequent extracts the fiber was desorbed prior to subsequent analysis in the heater unit for 10 min at 250 °C. The mixtures of *n*-alkanes (C8–C20, C21–C40) were injected using the above-mentioned temperature programme in order to calculate the RI for each peak. Identification of the components was done by comparison of mass spectral fragmentation patterns stored in MS data libraries NIST 11 (NIST, Gaithersburg, MD, USA) and FFNSC 2 (Shimadzu, Kyoto, Japan), and verified by comparison of RI of assigned compounds to published data [23–25] and/or RI from MS data library FFNSC 2.

2.5 **Experimental optimization of HS-SPME**

Apple spirit (sample no. 9) was used for the optimization of HS-SPME conditions. Extraction variables for this experiment were: the amount of diluted sample in 20 mL

headspace vial (X_1 with coded levels x_1); the amount of added sodium chloride (X_2 with coded levels x_2); extraction temperature (X_3 with coded levels x_3); and extraction time (X_4 with coded levels x_4).

Table 4 summarizes the whole central composite design consisting of 30 experimental points, which was done to estimate the effects of each factor on the extraction efficiency. A method of response surface modeling was used for evaluation of obtained data.

Table 4 Central composite design-coded independent variables (x_1, x_2, x_3, x_4), corresponding experimental conditions (X_1, X_2, X_3, X_4) and results represented by number of peaks in chromatogram for 100 μm PDMS fibre

Run	Sample volume [mL] x_1 (X_1)	NaCl [% w/V] x_2 (X_2)	Temperature [°C] x_3 (X_3)	Time [min] x_4 (X_4)	Number of peaks	
					Predicted	Observed
1	-1 (2)	-1 (0)	-1 (35)	1 (60)	76	74
2	-1 (2)	-1 (0)	1 (60)	-1 (10)	69	68
3	-1 (2)	1 (30)	-1 (35)	-1 (10)	61	61
4	-1 (2)	1 (30)	1 (60)	1 (60)	70	61
5	1 (10)	-1 (0)	-1 (35)	-1 (10)	64	69
6	1 (10)	-1 (0)	1 (60)	1 (60)	109	105
7	1 (10)	1 (30)	-1 (35)	1 (60)	118	115
8	1 (10)	1 (30)	1 (60)	-1 (10)	98	97
9 (C)	0 (6)	0 (15)	0 (47,5)	0 (35)	107	106
10 (C)	0 (6)	0 (15)	0 (47,5)	0 (35)	107	103
11	-1 (2)	-1 (0)	-1 (35)	-1 (10)	48	47
12	-1 (2)	-1 (0)	1 (60)	1 (60)	85	92
13	-1 (2)	1 (30)	-1 (35)	1 (60)	86	92
14	-1 (2)	1 (30)	1 (60)	-1 (10)	56	60
15	1 (10)	-1 (0)	-1 (35)	1 (60)	91	91
16	1 (10)	-1 (0)	1 (60)	-1 (10)	93	91
17	1 (10)	1 (30)	-1 (35)	-1 (10)	95	92
18	1 (10)	1 (30)	1 (60)	1 (60)	110	115
19 (C)	0 (6)	0 (15)	0 (47,5)	0 (35)	107	106
20 (C)	0 (6)	0 (15)	0 (47,5)	0 (35)	107	109
21	-1 (2)	0 (15)	0 (47,5)	0 (35)	84	80
22	1 (10)	0 (15)	0 (47,5)	0 (35)	112	115
23	0 (6)	-1 (0)	0 (47,5)	0 (35)	99	97
24	0 (6)	1 (30)	0 (47,5)	0 (35)	106	108
25	0 (6)	0 (15)	-1 (35)	0 (35)	97	95
26	0 (6)	0 (15)	1 (60)	0 (35)	103	105
27	0 (6)	0 (15)	0 (47,5)	-1 (10)	95	94
28	0 (6)	0 (15)	0 (47,5)	1 (60)	115	115
29 (C)	0 (6)	0 (15)	0 (47,5)	0 (35)	107	109
30 (C)	0 (6)	0 (15)	0 (47,5)	0 (35)	107	113

2.6 **Principal component analysis**

The principal component analysis was made for the chemometric interpretation of data using the software Statistica 12 (StatSoft CR, Prague, Czech Republic). PCA reduces the dimensionality of a data set by forming linear combinations of original variables called principal components (PCs). Classification of objects using PCA was done by the construction of two-dimensional plots, using PCs chosen by the authors. In this case, the discrimination between samples of different origin was done, based on groups of volatile components.

3. RESULTS AND DISCUSSION

3.1 **Extraction process**

Firstly, the HS-SPME method was optimized. The optimization was composed of SPME fiber selection and then evaluation of suitable extraction conditions.

In this study, two different fibers were compared for evaluation of their suitability and efficiency for the extraction of fruit spirit volatiles. Those were 100 μm PDMS and 50/30 μm StableFlex DVB/CAR/PDMS. The fibers were chosen according to the producer‘s recommendations (20). The comparison was performed in preliminary studies at various extraction conditions Table 5 Total ion chromatogram area (TIC) and a number of peaks (NoP) in chromatograms were used for determination of the suitability of individual fibers.

As can be deduced from Table 5, analysis using 100 μm PDMS fiber gave a higher total signal and a simultaneous higher number of peaks in chromatograms than the second tested fiber (DVB/ CAR/PDMS). Therefore, PDMS fiber was chosen for the extraction of the volatile compounds from the real samples mentioned in Table 3. Suitable conditions for extraction of volatiles by PDMS fiber were determined by the method of response surface modeling. The design of experiments was formed from 30 experiments for PDMS fibre with the following examined parameters: sample volume in 20 mL headspace vial, 2–10 mL; amount of added NaCl, 0–30% w/v; extraction temperature, 35–60 °C; and extraction time, 10–60 min. Pre-incubation time was maintained constantly at the temperature of the extraction for 20 min. Chromatographic analysis of SPME extracts was performed as is stated above. Evaluation of the whole experiment was done according to NoP in individual chromatograms using statistical software Statistica 12. This software allows the creation of different polynomial models which refer to a linear modeling and second-order modeling. Second-order modeling provides models which have the advantage of better predictions of response. Thus, the second-ordered polynomial equation (2) was constructed for the response variable (NoP) related to the experimental conditions.

Table 5 Results of HS-SPME/GC–MS analysis with use of two different SPME fibers and different extraction parameters, expressed as NoP (number of peaks) and total ion chromatogram area (TIC) area

Extraction parameters (final volume of sample, conc. of NaCl in the sample, extraction temp. and time)	100 µm PDMS NoP/TIC area	50/30 µm DVB/CAR/PDMS NoP/TIC area
2 ml, 0 % w/V, 60 °C, 60 min	60 / 0,9E+07	23 / 0,1E+07
2 ml, 30 % w/V, 60 °C, 10 min	74 / 1,1E+07	28 / 1,7E+07
6 ml, 15 % w/V, 48 °C, 35 min	109 / 3,6E+07	65 / 1,2E+07
10 ml, 0 % w/V, 60 °C, 60 min	105 / 4,9E+07	96 / 2,9E+07
10 ml, 30 % w/V, 60 °C, 10 min	109 / 3,6E+07	65 / 1,2E+07

$$PP = -102.705 + 7.843X_1 - 0.607X_1^2 + 2.135X_2 - 0.021X_2^2 + 5.219X_3 - 0.046X_3^2 + 1.200X_4 - 0.004X_4^2 + 0.073X_1X_2 + 0.043X_1X_3 - 0.004X_1X_4 - 0.034X_2X_3 - 0.002X_2X_4 - 0.009X_3X_4 \quad (2)$$

where X_1, X_2, X_3, and X_4 are independent variables (Table 4) The square root of the determination coefficient of the model (R^2) with linear terms, quadratic terms, and two-factorial interaction between linear terms were 0.9674. This means that <4% of variations could not be described by the model. It is necessary to include all terms to the model because the R^2 of the model without two-factorial interaction between linear terms decreases to 0.8589, and the R^2 of the model considering only linear terms decreases to 0.5419. The significant factors (p-value <0.05 at confidence level 95 %) of the equation are X_1, $X_{1\,2}$, X_2, X_3, $X_{3\,2}$, X_4, X_1X_2, X_2X_3, X_3X_4. The values predicted by the model correlate well with the experimental values (shown in Table 4). Critical values of independent variables were in the maximum of the respond surface of the model. The values were as follows: sample volume, 9.4 mL; amount of added NaCl, 28.5% w/v; extraction temperature, 42.2 °C; and extraction time, 81.9 min. Under these conditions, the value of NoP is 124. However, the critical value of the extraction time is located outside of the examined area. Taking into account this fact, a shorter extraction time (60 min; it was the highest tested level of extraction time within the optimization process) was chosen, and values of other independent variables were set using equation (1) to reach the value of NoP as high as possible. In such a way the adjusted values of extraction parameters were 10 mL of sample with the addition of 28.5% of NaCl (w/V), extraction temperature was 45 °C and extraction time was 60 min, resulting in the value of NoP 122. The difference between NoP values calculated from the critical values of the model and adjusted values was 2, which is an acceptable change.

3.2 **Analysis of real samples and comparison of volatile profiles**

In total, 271 of the volatile compounds were assigned at least in one of the samples. All of the discussed results were applied to the chemical composition of volatile profiles obtained by extraction using 100 μm PDMS fiber and evaluated by percentage of TIC area. Groups of assigned volatiles (see Table 6) were input parameters for PCA. The PCA score plot of the FS is depicted in Figure 1. The first PC (factor 1) explained 32.8 % of the data variation and mainly showed separation between pome FS and other analyzed spirits. Factor 2 described 20.1 % of the data variation. In total, the first four factors explained 76.4 % of the data variation. Factor 1 showed high correlation with oxidated sesquiterpenes (-0.841), sesquiterpenes (-0.821), oxidated monoterpenes (0.781) and lactones (0.723). Factor 2 correlated with acetals (0.825) and apocarotenoids (0.784). Factors 3 and 4 correlated with esters (-0.739) and the group of phenols and phenolic ethers (0.595), respectively.

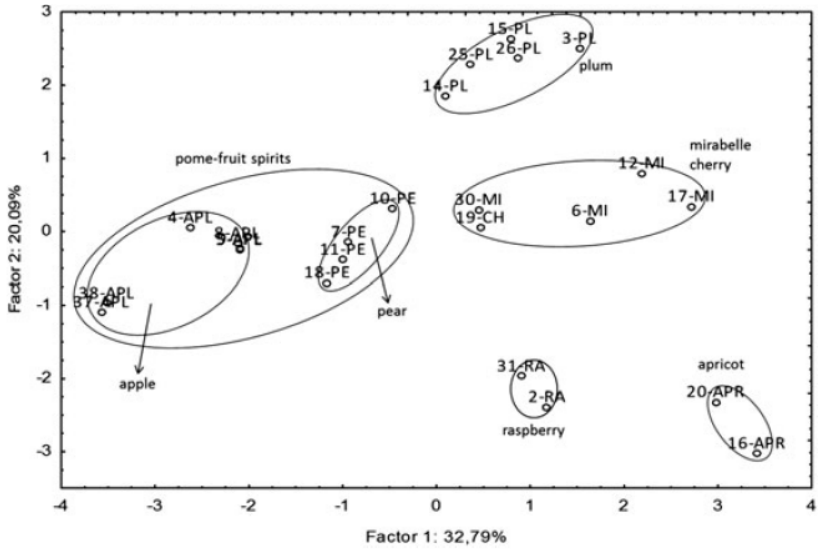

Figure 1 Principal components analysis score plot of the investigated fruit spirits. APL, Apple spirits; PE, pear spirits; PL, plum spirits; MI, mirabelle spirits; CH, cherry spirit; RA, raspberry spirits; APR, apricot spirits.

As is obvious in Table 6, the esters, mainly ethyl esters such as ethyl caprate, RI = 1395 or ethyl laurate, RI = 1594, generated the majority of the aroma profiles of all analyzed samples. A high proportion of these two compounds in volatile profiles of analyzed plum spirits is in agreement with the research of Tesevic [26], who analyzed volatiles in old plum brandy samples of Serbian origin. Ethyl esters, as typical representatives of FS aroma, have been published in many reports dealing with the aroma profiles of FS [26–29].

The second most abundant group in volatile profiles of analysed samples is organic acids (particularly capric acid, RI= 1373; and lauric acid, RI = 1568), which are a natural component of fruit and are formed by the activities of bacterium and yeasts during the fermentation process. A substantial amount of high-molecular-weight acid remains in distillation residue, and only a small portion passes into the distillate. In the presence of ethanol, they are converted into esters. A comparison of individual volatile profiles in term of assigned compounds shows several interesting results.

The content of higher alcohols in the aroma was similar in all analyzed spirits made from the same kind of fruit. These compounds constitute a substantial part of the fusel, which is one of the characteristic properties of distillates. That is the reason why fusel is not completely removed from the spirits during distillation, but its content is effectively controlled by the setting of the distillation process.

Sesquiterpenes were found in high quantities in pome-fruit spirits, compared with other analyzed samples. Some 3.7–16.6% of apple spirits volatile profile and 2.6–6.4% of pear spirits volatile profile corresponded to sesquiterpenes. (E, E)-α-Farnesene had the highest response of all assigned sesquiterpenes, 2.5–15.2% in pome-fruit spirits and 0.01–0.7% in other analyzed samples. From the group of oxidated sesquiterpenes, the most common assigned compounds were (E)-nerolidol, (E)-2,3-dihydrofarnesol and (2E,6E)-farnesol.

Apocarotenoids, which are formed from carotenoids by oxidative cleavage during ripening or food processing [30, 31], appeared mainly in spirits produced from fruit with a significant scent, such as raspberry or apricot spirits. To name a few, vitispirane was assigned in 19 of 24 samples. All of the assigned compounds of this group were C13 apocarotenoids with the exception of β-cyclocitral (C10 apocarotenoid).

Most carbonyl compounds assigned in the analysed samples were higher aldehydes such as benzaldehyde, pelargonaldehyde or (7Z,10Z)-hexadecadienal, to name a few. Aldehydes are passed to spirits from the fruit (particularly from overripe fruit), and moreover, they are formed during the fermentation process. Benzaldehyde, as a common natural constituent of stone FS, because of its formation by enzymatic hydrolysis of amygdalin contained in the kernels [32], was observed also in pome FS.

More precisely, it was found in apple spirits (0.05–0.16%) and also in raspberry spirits (0.05–0.17%). From the above, it is obvious that apple seeds in comparison to the kernels of pears most likely contain significantly higher amounts of amygdalin. Ethyl benzoate was observed in all samples. This could be given by the fact that aging of distillates on-air leads to oxidation of benzaldehyde to benzoic acid, which is subsequently esterified by present alcohols, mostly ethanol. Pelargonaldehyde was assigned in the majority of samples (except sample no. 10, 12, 19, 24), particularly in plum and mirabelle spirits. (7Z,10Z)-Hexadecadienal was found in the volatile profile of mirabelle spirits at quite high levels (0.80–1.87%). In contrast, in the other analysed samples in which it was assigned [one plum (no. 3) and three apples (no. 4, 8, 9)

spirits], it did not generate a relative response higher than 0.14%, and in one sample of raspberry it was found to have a relative response of 0.46%.

Acetals, which are characteristic by their very pleasant fragrance, were also assigned in almost all samples [except one sample of apple spirit (no. 8)]. The richest volatile profiles regarding the acetals content were plum spirits (see Table 6), which contained higher levels of nonanal diethyl acetal than the other analysed samples.

Numerous lactones are found in food. Some of the representatives belong among the typical aroma substances of various fruits. Since the aroma of lactones is very pleasant, these substances are also of interest for commercial aromatization of food. Among lactones with very low odour threshold are γ-decalactone and γ-dodecalactone [33]. In around half of the samples γ-dodecalactone was assigned (with the exception of samples 30 and 38, and all pear and raspberry spirits). γ-Dodecalactone is a common compound identified in various kinds of fruit spirits [plum brandy [26], Calvados [34], mirabelle brandy [34, 35] or apricot distillate [36] The highest total response of lactones was observed in the volatile profiles of apricot spirits (see Table 6). Unfortunately, no δ-lactone was assigned, although these are discussed as contributors to the aroma of apricots [33, 37, 38].

Table 6 Proportion of groups of volatile compounds in the aroma profile of the fruit spirit samples (relative percentage of TIC area)

	Σ Relative percentage of TIC area											
Spirits from	Acetals	Acids	Higher alcohols	Aldehydes and ketones	Apocarotenoids	Esters	Lactones	Monoterpenes	Oxidated monoterpenes	Sesquiterpenes	Oxidated sesquiterpenes	Phenols and phenolic derivates
Plum	3,9 - 4,2	0,7 - 12,9	1,3 - 3,7	0,6 - 1,7	n.i. - 0,2	61,8 – 88,0	0,1 - 0,7	n.i. - 0,1	0,1 - 0,3	0,01 - 0,8	0,1 - 2,5	0,1 - 2,4
Apple	n.i. - 1,1	9,5 - 20,3	1,6 - 3,4	0,2 - 0,4	0,2 - 0,6	54,0 - 67,0	n.i. - 0,1	n.i. - 0,1	n.i. - 0,1	3,7 - 16,6	3,1 - 12,1	0,1 - 0,5
Pear	0,3 - 0,4	2,8 - 13,3	1,6 - 4,7	0,1 - 0,4	<0,01 - 0,7	65,1 - 79,1	n.i.	n.i. - 1,7	n.i. - 0,1	2,6 - 6,4	1,8 - 5,9	0,4 - 1,6
Mirabelle	0,3 - 3,0	0,7 - 13,7	2,4 - 4,6	1,8 - 2,9	0,1 - 0,4	59,8 - 66,2	n.i. - 1,0	n.i. - 4,0	0,2 - 1,6	n.i. - 0,2	0,3 - 1,6	0,3 - 1,6
Raspberry	0,2 - 0,3	n.i. - 7,2	1,6 - 3,6	0,4 - 0,8	1,6 - 1,8	64,1 - 81,7	n.i.	n.i. - 2,3	0,3 - 0,4	n.i. - 0,5	0,4 - 2,3	0,1 - 2,1
Apricot	0,2 - 0,5	4,2 - 5,6	1,3 - 1,7	0,4	0,7 - 1,3	78,1 - 82,1	1,7 - 2,0	0,1	1,7 - 2,0	0,1 - 0,5	0,2 - 0,5	0,2 - 0,4
Cherry	0,2	11,1	0,4	1,1	n.i.	78,1	0,1	n.i.	0,2	0,1	0,5	0,5

3.3 **Differences between spirits according to fruit origin**

Based on cluster analysis of each group affecting factors 1 and 2 of PCA, non-uniform compounds in dendrograms were assigned and their statistical significance was confirmed by ANOVA. This approach permits some characteristic marker compounds for various groups of spirits to be revealed. The main difference between the volatile profiles of spirits produced from pome fruits and other spirits was the relative content of sesquiterpenes, particularly (E,E)-*α*-farnesene. This compound created 2.5–15.2 % of the total response in chromatograms of pome FS samples, while in chromatograms of stone FS samples its representation was only up to 0.74 %. Furthermore, in the sample of raspberry spirit (sample no. 2), (E,E)-α-farnesene was assigned merely in a trace amount. Moreover, only in pome FS samples were α-zingiberene (0.08–1.16% of TIC area) and (E)-*α*-bisabolene (0.03–0.18% of TIC area) assigned. The difference between tested pome-fruit spirits (pear spirits and apple spirits) was composed of benzaldehyde, *γ*-dodecalactone [both also identified in calvados by Ledauphin et al. [34, 35] and (E)-β-farnesene (all assigned in volatile profiles of tested apple spirits), and isovaleraldehyde diethyl acetal (observed in pear spirits). Cis- and trans-isomers of linalool furanoxide were together observed in all pear spirits samples; only in one sample of apple spirit was there found cis isomer with a very low response.

In contrast, only in stone FS were propyl decanoate (0.02–0.70 %) and ethyl salicylate (0.01 0.19%) assigned [both compounds were also identified in plum brandies by Tesevic et al. [26]. Stone-fruit spirits and raspberry spirits were characterized by the presence of monoterpenoid *α*-terpineol in volatile profile, which was assigned in the entire set of analysed samples except for pome-fruit spirits. Propyl decanoate and ethyl salicylate were observed only in volatile profiles of all stone-fruit spirits, with a higher relative response of propyl decanoate in volatile profiles of apricot spirits (0.37–0.70 %) than in others (0.02–0.50 %). (E)-Anhydrolinalool oxide, (E)-ocimenol, nerol and (Z)-*β*-ocimene were found only in apricot spirits, but these compounds were observed with a relatively low response. More than these monoterpenoids, *γ*-decalactone was typical of the volatile profile of apricot spirits [as well as was published in work of Genovese et al. [36].

From the group of alcohols, (Z)-9-tetradecen-1-ol was observed only in the volatile profile of mirabelle spirits. Some of the assigned apocarotenoids were characteristic of raspberry spirits; concretely dihydro-*β*-ionone, (E)-*α*-ionone and 3,4-dehydroionene were observed only in volatile profiles of raspberry spirits.

Tonka beans

1. INTRODUCTION

Dipteryx odorata (Aubl.) Willd. (Fabaceae) is a large tree native to the tropical rainforests of Central and Northern South America. In total, the genus Dipteryx involves 14 species. Ten of them are typical to the Amazon region; the other two are found in the Northeast and Central Brazil and the last two might be found in Central America [39]. D. odorata, which is also commonly known as the "tonka bean tree", is the most studied species of the genus. It produces seeds commonly called "tonka beans", which have been already examined for the content of coumarin [40–42]. This compound is liberated from the glycoside melilotoside (an ether of glucose bonded with an ester bond to coumarin) [43] by fermentation during the drying of the seeds which have been soaked in alcohol for 24 h. Its content is variable and usually moves in the range of 1–3% in fermented tonka beans [40].

As reported by Givel [44], "numerous studies, beginning in 1855, have indicated that coumarin has toxic effects on the nervous system, heart, blood vessels, and liver of animals as well as inducing cancerous tumours and toxic conditions in humans. In 1954, the US Food and Drug Administration (FDA) banned coumarin in food, but not tobacco products in the USA based on the results of animal research. Also, since 1954, many European countries have either banned or greatly restricted coumarin because of its toxic properties". In 2004, the European Food Safety Authority (EFSA) established present Tolerable Daily Intake (TDI) of 0–0.1 mg coumarin/kg body weight [45], which was confirmed by EFSA in 2008 [46] on the base of toxicity and clinical studies that have become available since 2004. Due to the various scents, which are vanilla, cinnamon, saffron, almond or cloves, the extracts of tonka beans have a widespread use, particularly as additives in flavouring snuff, cigarettes, cigars, and also in perfumes or liquors [47].

2. EXPERIMENTAL

2.1 Chemicals and materials

Tonka beans (origin South America) were purchased in a local shop in Mallorca (Balearic Islands, Spain). Prior to the analysis, beans were grated and sieved (Mesh size 16 (Standard Mesh, US)). *n*-Alkane mixture standard solution (C8-C40) was purchased from Restek (Bellefonte, PA, USA) in concentrations of 500 μg mL−1 dissolved in carbon disulfide/dichloromethane (3/1, V/V). Carbon dioxide (purity 4.5 and 2.8), and nitrogen (purity 4.0) were purchased from Linde Gas a.s. (Prague, Czech Republic). *n*-Hexane and *n*-heptane were purchased from Sigma-Aldrich (Prague, Czech Republic). Fragrance Material Test Mix (ethyl butyrate, limonene, eucalyptol,

geraniol, benzoic acid, (E)-cinnamaldehyde, hydroxycitronellal, thymol, cinnamyl alcohol, cinnamyl acetate, vanillin, benzyl salicylate) was purchased from Restek (Bellefonte, PA, USA). Menthol, α-terpineol, carvone, p-anisaldehyde were purchased from Sigma-Aldrich (Prague, Czech Republic). SPME fibers 100 μm PDMS (polydimethylsiloxane), 85 μm Carboxen/PDMS and 50/30 μm StableFlex DVB/CAR/PDMS (divinylbenzene/ carboxene/polydimethylsiloxane) were purchased from Sigma-Aldrich (Prague, Czech Republic).

2.2 **Head-space solid phase microextraction procedure**

HS-SPME at constant temperature 100 mg of the sample was placed into a 20 mL headspace vial and closed by a cap with a Teflon septum, and conditioned at an initial extraction temperature for 20 min. HS-SPME was carried-out according to the following conditions: 85 μm Carboxen/PDMS fiber, extraction temperature 62 °C, and extraction time 39 min. After that, volatile compounds were desorbed from the fiber in the GC injector port, set up at 200 °C.

2.3 **Optimisation of HS-SPME at a constant temperature**

The selection of a suitable fiber coating is one of the most crucial steps in the developing of an SPME method. In the present work, three different SPME fibers (100 μm PDMS; 50/30 μm DVB/CAR/PDMS and 85 μm Carboxen/PDMS) were simply tested for the suitability of isolation of volatile compounds from tonka beans. The selection procedure was carried out based on a sum of peaks detected after incubation and extraction steps (20+30 min) at three different temperatures (40, 60, 80 °C). The optimization procedure was contented from 12 experiments for the chosen fiber (85 μm Carboxen/PDMS). The extraction factors observed were extraction temperature in the range from 35 to 95 °C and extraction time that ranged from 10 to 60 min. The incubation time was 20 min for each analysis, while the temperature of the incubation was kept at the same level as the extraction. The evaluation of the whole experiment was done according to the number of peaks (NoP) in individual chromatograms using the method of response surface modeling in STATISTICA data analysis software, version 12 (StatSoft, Inc., www.statsoft.com). Critical values of independent variables were in the maximum of the response surface of the model, and the optimum extraction conditions were found to be 62 °C for 39 min.

2.4 **HS-SPME at decreasing temperature**

100 mg of the sample was placed into a 20 mL headspace vial and closed by a cap with a Teflon septum, and conditioned at an initial extraction temperature for 20 min. Extraction of volatile compounds was carried-out according to the following conditions: 85 μm Carboxen/PDMS fiber, at temperatures decreasing spontaneously from 100 °C to 30 °C. After that, volatile compounds were desorbed from the fiber in the GC injector port set at 200 °C.

2.5 **Supercritical fluid extraction**

Supercritical fluid extractions were performed on an SE-1 instrument from SEKO-K (Brno, Czech Republic). All extractions were performed with supercritical CO2 in dynamic mode. The stainless steel extraction vessel (4.5 mL) was packed with a mixture of 100 mg of sample and glass sand. A silica tube restrictor (15 cm, i.d. 50 mm) was used to collect the extracted analytes. The restrictor outlet was immersed into a liquid n-hexane trap. Components of the sample were extracted using the dynamic extraction mode by 5.2 L of carbon dioxide at 31 MPa and 103 °C. The obtained extract was transferred into a 2 mL volumetric flask and filled up to the mark with n-hexane. The sample was extracted in triplicate.

2.6 **Simultaneous distillation-extraction**

SDE was performed using apparatus of Clevenger type (Kavalierglass a.s., Prague, Czech Republic). 10 g of grated and sieved tonka beans were distilled with 500 mL of

water for 5 h. Volatile compounds were extracted using 1 mL of n-hexane in a separator. The SDE of the sample was performed three times.

2.7 **GC instrumentation**

For the analyses, a gas chromatograph, model GC-2010 Plus coupled to mass spectrometry detector TQ-8030 and auto-sampler AOC-5000 Plus (all from Shimadzu, Kyoto, Japan) was used. A capillary column SLB–5 ms (30m×0.25 mm; 0.25 μm) from Supelco (Bellefonte, PA, USA) was employed for separation. As a carrier gas, helium 5.0 (Linde Gas a.s., Prague, Czech Republic) was used at a constant linear velocity of 30 cm/s (column flow rate was 0.69 mL/min at initial conditions of separation). The temperature of the injector was maintained at 200 °C. The temperature gradient was programmed as follows: the initial temperature at 40 °C was held for 3 min and then increased by a rate of 2 °C/min up to 250 °C (10 min). The mass spectrometer was operated in the electron ionization (EI) mode (70 eV) and in the full scan mode over a mass range of m/z 33–500. The interface temperature and the ion source temperature were maintained at 200 °C. The mixture of n-alkanes was injected using the above-mentioned temperature program in order to calculate the retention index (RI) for each peak. Identification of the components was done by the comparison of the mass spectra of standards (if available, see Section 2.1) or mass spectral fragmentation patterns stored in MS data libraries NIST 11 (Gaithersburg, MD, USA) and FFNSC 2 (Shimadzu, Kyoto, Japan), and verified by the comparison of the RI of identified compounds to published data [24, 25] and/or RI from MS data library FFNSC 2. A GC/FID analysis for the semiquantitative evaluation of the content of individual constituents was performed using the Shimadzu GC 2010 gas chromatograph with a flame ionization detector (FID). The detector temperature was set on 220 °C. Conditions of measurements, including the column type and column temperature, the injector temperature, split ratio, carrier gas and the linear velocity, were set the same as those of GC/MS analysis.

3. RESULTS AND DISCUSSION

3.1 **HS-SPME at a constant temperature**

The type of fiber coating was the first parameter to be evaluated. Three types of fiber coating were tested with the same amount of sample (100 mg) at various conditions (see Table 7). From the tested fibers, the most appropriate fiber (Carboxen/PDMS) was chosen according to the highest number of detected peaks in chromatograms. The extraction efficiency of the three tested fibers was in the following order: 85 μm Carboxen/PDMS > 50/30 μm DVB/CAR/PDMS > 100 μm PDMS. After selecting the type of fiber coating, the influence of extraction temperature and time was evaluated through central composite design using the method of response surface modeling, and critical parameters (optimal extraction conditions) were determined.

Table 7 Results of HS-SPME/GC–MS analysis with use of three different SPME fibers and different extraction temperature, expressed as NoP (number of peaks)

Extraction temp. [°C]	NoP		
	100 µm PDMS	50/30 µm DVB/CAR/PDMS	85µm Carboxen/PDMS
40	52	106	93
60	47	96	144
80	33	96	127

3.2 **SFE optimisation**

Carbon dioxide was used as an extraction solvent. Extraction pressure and temperature, and volume of the extraction solvent were optimised. Optimisation of extraction conditions was performed by central composite design consists from 17 runs which covered the following ranges of extraction parameters: pressure 20–40 MPa, temperature 40–120 °C and volume of CO_2 1.0–6.0 L. Extracts from each run were analysed by GC/MS . Evaluation of the responses from the chromatograms was done according to the total peak area (TPA) by the method of response surface modeling in software STATISTICA, version 12. Critical values (optimum extraction conditions) of individual parameters were as follows: extraction temperature 103 °C, extraction pressure 31 MPa, and volume of CO_2 5.2 L.

3.3 **Mechanisms of extraction methods**

The cell wall could be broken using different mechanical methods. Before starting all extractions, tonka beans were grated and sieved and so a certain level of cell disruption by the preparation of the sample was achieved. Each extraction method used has its own substance release mechanisms, but none are based on disruption of cell walls. In HS-SPME, substances are released from the sample by the effect of higher temperature. In SDE substances are released from the sample by the effect of boiling water. Both processes are known as hydrodiffusion. In HS-SPME, volatile compounds are dissolved in water (presented in glands) at a higher temperature and this solution permeates through cell membranes to the surface of plant material. The concentration of substances in the headspace is given by vapour pressures of individual substances.

In SDE volatile compounds are, after passing through the cell membrane, vaporized by boiling water/steam and are carried by steam into the condenser. Supercritical fluid possesses gas-like properties of diffusion, viscosity, and surface tension, and liquid-like density and solvation power [48]. It is stated, that the favourable transport properties of fluids near their critical points allow deeper penetration into solid plant matrix and more efficient and faster extraction than with conventional organic solvents[49].

3.4 **Volatile constituents of tonka beans**

In total, 191 compounds were assigned in chromatograms of extracts obtained by all the used extraction techniques (36 carbonyl compounds, 27 esters, 27 terpenes and terpenoids, 26 aliphatic hydrocarbons, 23 alcohols, 20 acids, 10 lactones, 7 aromatic hydrocarbons and 14 other compounds). Only five compounds were identified in all

chromatograms (coumarin, 3,4-dihydrocoumarin, limonene, pelargonaldehyde, and capraldehyde).

156 compounds were identified in HS-SPME extracts (103 compounds in extracts obtained at a fixed temperature, and 89 compounds in extracts obtained by extraction during decreasing temperature). 77 compounds were identified in SDE extracts, and 36 in SFE extracts. 91 of all assigned compounds were extracted by only one from four used extraction methods.

The categorization of assigned compounds in various extracts is in Figure 2. The majority of bioactive compounds belong to one of a number of families, each of which has particular structural characteristics arising from the way in which they are built up in nature (biosynthesis). There are four major pathways for the synthesis of secondary metabolites or bioactive compounds: shikimic acid pathway, malonic acid pathway, mevalonic acid pathway and non-mevalonate pathway. Phenolic compounds are synthesized through shikimic acid pathway and malonic acid pathway. Terpenes are produced through mevalonic acid pathway and non-mevalonate pathway [48].

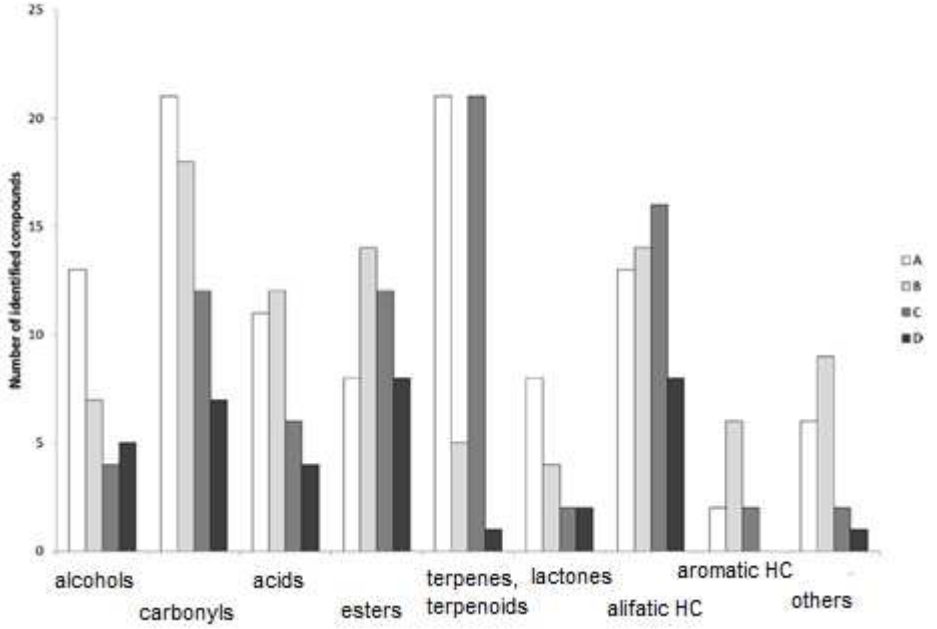

Figure 2 Groups of identified volatile compounds in extracts obtained using (A) HS-SPME at 62 °C, (B) HS-SPME at decreasing temperatures, (C) SDE (D) SFE.

The main differences among used extraction methods are in profiles of individual groups of compounds. SDE and SFE were more effective techniques (in term of the number of identified compounds) for compounds of higher retention indices (RI). It can be seen in Figure 3, which shows the comparison of the chemical composition of tonka beans extracts using bubbles whose sizes correspond with the representation of the individual compounds. For example in the case of identified acids, all of them have RI > 1500 when extracted by SDE or SFE. On the other hand, all identified acids in HS-SPME extracts (except one) have RI < 1500. Compounds of RI < 700 were not observed in chromatograms of SDE and SFE extracts due to solvent cut during chromatographic analysis. We can observe the differences between both SPME methods in profiles of esters, when all esters assigned by HS-SPME at 62 °C have RI < 1300, and esters assigned by HS-SPME at decreasing temperatures have RI > 1700

(except traces of benzyl formate and diethyl phthalate). From the total numbers of assigned compounds, it can be deducted, that HS-SPME was more effective than other used extraction techniques. Moreover, 100 from the total number of 188 compounds were assigned only by using HS-SPME methods. And only 35 compounds were not confirmed in HS-SPME extracts. It seems that HS-SPME methods are good tools for the qualitative characterization of the volatile profile of tonka beans. If we consider the number of assigned compounds, the most represented group was carbonyl compounds (22 aldehydes and 14 ketones). Moreover, one more ketone (carvone) was included in terpenoids.

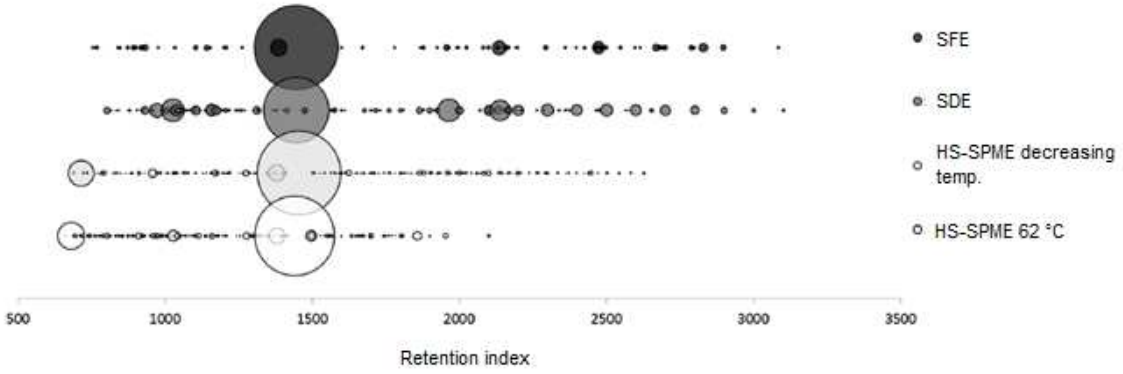

Figure 3 Comparison of chromatograms of extracts obtained using various extraction methods.

Nevertheless, the main compound of all extracts was coumarin, belonging to the group of lactones. Coumarin is liberated from the glycoside melilotoside (an ether of glucose bonded with an ester bond to coumarin) by drying coumarin-containing herb material [43]. Its peak area ranged from 51% to 85% of total peak area according to the used extraction method. Therefore, lactones were the most represented group of volatile compounds. In the work of Wörner et al. [50], authors identified 138 compounds in extracts from dry tonka beans. Almost half of these compounds (concretely 61) were assigned in our work, too. The next 129 compounds were assigned "extra". It is mainly caused by using different extraction techniques. Moreover, one of two compounds with the highest content in the work of Wörner, (E)-anethol, was not confirmed in our work. Unfortunately, neither the presence of 2-undecylfuran proposed by Wörner as a suitable indicator of tonka beans failed to prove. It indicates that seed treatment, as well as growth conditions, has a large influence on the volatile profile of tonka beans.

Adrade et al. [51] identified 32 volatile constituents of the flowers of *Dipteryx odorata*, mainly sesquiterpenes, and sesquiterpenoids (17). In our work, only 12 of the mentioned 32 compounds were assigned too (only 4 sesquiterpenes from 17 mentioned), and in the work of Wörner, 9 of the mentioned 32 compounds were identified too (only 4 sesquiterpenes from 17 mentioned). Thus, most terpenic compounds identified by Andrade were characteristic for the flowers only. Many of these substances could also be present as a result of damage of the flowers during collection, because it is known that the release of volatiles can be induced by various effects, like herbivore damage [52].

3.5 **Concluding remarks**

Different extraction methods gave extracts with different profiles of compounds given by different extraction conditions. In the case of both HS-SPME modifications, different results are given by different initial extraction temperature. In modification with decreased temperature, the beginning of the extraction (at a temperature of 100 °C) is in the sense of a sorption of compounds with higher boiling point (i.e. substances with higher retention indices), and a portion of the extraction fiber capacity is exhausted by these substances. Therefore, there is lower sorption capacity for compounds extractable at lower temperatures. In SDE, the plant material is immersed in boiling water, and some oil components like esters are sensitive to hydrolysis (only 12 esters were identified in SDE extracts, while in HS-SPME extracts 22 esters were identified). Some oxygenated components have a tendency of dissolving in water, so their complete removal by distillation is not possible. This may be a reason of the lower number of identified carbonyl compounds in SDE extracts (12 carbonyls) compared to HS-SPME extracts (total of 22 carbonyls). In SFE, the use of higher pressures and temperatures led to the coextraction of heavy compounds with large retention indices [53]. In case of tonka beans extraction conditions of SFE resulted in the highest number of components with large retention indices (47 % of all peaks in chromatogram are of RI > 2000, in SDE it was 33% and in HS-SPME it was not higher than 27 %). Preferred extraction of heavier components can be seen in the chemical composition of profiles of acids, esters, and aliphatic hydrocarbons when compounds with RI < 1850 were not assigned in SFE extracts.

3.6 **Possible application**

The main compound of tonka bean extracts is coumarin belonging to a class of simple coumarins. It is a naturally occurring toxin, but this compound also exhibits anti-inflammatory properties. Other similar compounds belonging to simple coumarins (e.g. 3,4-dihydrocoumarine, etc.) class have anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant, and neuroprotective properties. Therefore, natural coumarins are of great interest due to their widespread pharmacological properties, and this attracts many medicinal chemists for further backbone derivatization and screening them as several novel therapeutic agents [54]. The group of fatty acids is the next relatively abundant class of compounds (more than 10 % of total peak area in HS-SPME and SDE extracts). These compounds are used in the production of soaps, and taking special fragrance into account leads to the use of tonka bean extracts in the cosmetic industry.

4. CONCLUSION

The dissertation deals with the analysis of components in plant material or material prepared from it. The work was divided into three main sections - extraction and analysis of compounds in rose petals, analysis of alcoholic beverages and extraction and analysis of volatile substances in tonka beans. Experimental data, optimization of extraction conditions, data evaluation in Statistics 12 (StatSoft, Inc.), images, graphs and tables are presented in each section.

The main objectives of the work were the use of HS-SPME/GC-MS for the extraction of volatile compounds, to find optimal extraction conditions and subsequently to apply them to real samples. These goals were met within the dissertation work. In the experimental part, other methods were used, such as simultaneous distillation-extraction, supercritical fluid extraction, or an electronic nose based on ultra-fast gas chromatography. In addition to using GC-MS analysis, a combination of gas chromatography with a flame ionization detector (GC-FID) was used.

In the first part of the thesis, attention was paid to the analysis of volatile compounds in dried and milled rose petals. Due to the varied chemical composition of roses, two SPME fibers - 50/30 μm DVB / CAR / PDMS and 100 μm PDMS were selected at the beginning of the work. The efficiency of extraction of both fibers was examined from the viewpoint of two variables - the number of peaks and the total peak area. The DVB / CAR / PDMS fiber was more suitable for the extraction of volatile compounds, while PDMS fibers were more suitable for less volatile substances. However, if the fibers were compared in terms of the total peak area in the chromatogram, larger areas were observed using the DVB / CAR / PDMS fiber. Variant analysis and Paret's effect graph were also used to select a more suitable SPME fiber. Based on the results, 50/30 μm DVB / CAR / PDMS for extraction of volatile organic compounds (VOCs) in real samples were selected. In total, six roses have been analyzed; Rosa Mariyo, Rosa Rhodos; Rosa Sudoku; Rosa Tara; Rosa Tacazzi and Rosa Deep Purple. As an optimal extraction condition extraction parameters were found, the extraction temperature was 90 ° C, the extraction time was 60 min, and the dried sample weighed 0.7 g. In total, 211 compounds were identified which were classified into individual chemical groups.

The second part of the thesis was focused on the analysis of alcoholic beverages. This section contained two parts. In the first part is the analysis of alcoholic beverages, more precisely the analysis of fruit spirits. As in the previous chapter, 2 types of SPME fibers were selected - 50/30 μm DVB / CAR / PDMS and 100 μm PDMS. In order to select a more suitable fiber, the records were compared from the point of view of the total peak area and the number of peaks. On the basis of a higher number of peaks, 100 μm of PDMS fiber was selected, which was applied for the extraction of VOCs in real samples of fruit spirits. To increase the detector response, the dilution factor and salt addition were also investigated. By analyzing the central composite plan, extraction parameters were selected for 10 mL sample, 28.5% w/v, extraction temperature 45 ° C and extraction time 60 min. A total of 24 fruit spirits were analyzed and 271 compounds were identified. Part of the work was to create an aroma-profile and to find a statistically significant change between the factors using the statistical component analysis tool (PCA) and factor analysis (FA). The second part dealing with alcoholic beverages was dealt with using an electronic nose based on ultra-fast gas

chromatography as a tool for rapid classification of species and groups, eg alcoholic beverages. Multidimensional statistical analysis (PCA, DFA and SIMCA) classified 13 types of alcoholic beverages.

In the last part of the dissertation, extraction and analysis of VOCs in tonka beans are presented. Extraction methods for simultaneous distillation extraction (SDE) and supercritical fluid extraction (SFE) were used to prepare extracts for the HS-SPME / GC-MS analysis. SDE and SFE extracts were dispensed into GC-MS as a liquid and also extracted with HS-SPME. Extraction of volatile compounds from SDE and SFE extracts was carried out by HS-SPME in two ways. In the first case, the substances were extracted at a constant temperature of 62 ° C for 39 minutes. In the latter case, gradually decreasing temperatures were used up to 30 ° C, when the temperature of 30° C was reached, the sorption of the volatile compounds was terminated. In both HS-SPME processes, 85 μm Carboxen / PDMS fiber was used, based on the total number of peaks, from three tested SPME fibers (100 μm PDMS, 50/30 μm DVB / CAR / PDMS and 85 μm CAR / PDMS). The optimal extraction conditions were an extraction temperature of 62 ° C and an extraction time of 39 minutes. The extraction temperature was determined by optimal extraction conditions, temperature 103 ° C, pressure 31 MPa and the total volume of CO2 5.2 l. In the obtained extracts 191 compounds were identified using all applied extraction techniques. Seven compounds were identified in SDE extracts and only 36 substances were identified in SFE-extracted extracts.

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