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RESEARCH ARTICLE

Assessment of headspace solid-phase microextraction (HS-SPME) for control of asymmetric bioreduction of ketones by *Alternaria alternata*

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ABSTRACT

The aim of this study was to assess the effectiveness of headspace solid-phase microextraction (HS-SPME) compared to liquid–liquid extractions using with methylene chloride (CH_2Cl_2) for control of fungal biotransformation of ketones of varying volatility. The proposed method was successfully applied. The best way to extract all the components of the mixture (alcohols, aldehydes) in quantities similar to the extraction of methylene chloride was the use of fibres coated with a combination of nonpolar material. SPME fibre assembly polydimethylsiloxane/divinylbenzene (PDMS/DVB) was most suitable for the extraction of the products mixture obtained after biotransformation of acetylcyclohexane and acetophenone. On the other hand, the best results were obtained for 2-acetylthiophene, α,α,α-trifluoroacetophenone and their derivatives using divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre. In addition, our study showed that *Alternaria alternata* is a good biocatalyst for bioreduction of ketones to alcohols according to Prelog's rule.

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KEYWORDS
HS-SPME; liquid–liquid extractions; fungal biotransformation; ketones; *Alternaria alternate*

Introduction

Extraction is one of the most critical steps in the chemical analysis of samples (Chen et al. 1981). Since many years, a standard method used for sample preparation after biotransformation is liquid–liquid extractions using with intera alia methylene chloride (CH_2Cl_2) (Parshikov et al. 2000; Skrobiszewski et al. 2013). This solvent will form two layers in contact with aqueous solutions if they are used in sufficient quantities, and it is also used in pharmaceutical, environmental, food, and chemical industries (Chen et al. 1981; Mahmud and Kales 1999; Ogórek and Jarosz 2013).

Methylene chloride is a common and an effective extraction solvent, but this chemical agent is very harmful to the environment and to human health, among others, it may lead to poisoning, death or cancer (Leikin et al. 1990; Mahmud and Kales 1999). For example, once inhaled or absorbed through the skin, is converted to carbon monoxide, which interferes with oxygen delivery. Therefore, methylene chloride can make angina and other heart symptoms worse in people with heart disease. People with lung conditions, smokers, and people who are overweight or pregnant also may be more sensitive to methylene chloride. Additionally, this chemical agent can reach the developing foetus through the placenta, it also can enter breast milk, and it affects the nervous system (brain) and can cause headaches, dizziness, nausea, clumsiness, drowsiness, and other effects like those of being drunk. However, unlike the poisonings, cancer in people caused by methylene chloride can take years to develop and is more difficult to document (Hall and Rumack 1990; Soden 1993; Mahmud and Kales 1999). Therefore, it is necessary to search for new sensitive analytical methods that do not require the use of organic solvent. One of the ways may be solid-phase microextraction (SPME).

SPME was developed by Pawliszyn et al. (1990) and it is an organic solvent-free sample preparation followed by desorption inter alia into a gas chromatograph (GC) or high-performance liquid chromatograph (HPLC) for subsequent analysis (Arthur and Pawliszyn 1990; Górecki and Pawliszyn 1995). This method is an efficient and sensitive for the extraction and pre-concentration of samples, mainly because of the lack of organic solvents and reduction in associated interferences (Ouyang and Pawliszyn 2006; Zhuo-Min et al. 2006). Most commonly used SPME analysis are direct

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immersion (DI-SPME) mode and headspace (HS-SPME) mode. DI-SPME mode it used for extraction of analytes (absorbent-type fibres), mainly non-volatile compounds from aqueous matrices, and HS-SPME mode for the analysis of volatile and semi-volatile compounds available in air or in the headspace volume above liquid or solid matrices (adsorbent-type fibres) (Górecki and Pawliszyn 1995; Górecki et al. 1999; Lord and Pawliszyn 2000). Literature report also about various factors affecting the efficiency of extraction of the analyte in each mode (Risticevic et al. 2009; Gura et al. 2010).

Currently, SPME has been successfully used to rapidly concentrate volatile and semi-volatile organic compounds and a wide variety of other organic compounds occurring in aqueous and other matrixes and used to elucidate the biotransformation pathways of various chemicals by microorganism (Ghiasvand et al. 2011; Bocato et al. 2012; Kotowska and Bieńczyk 2013). Literature reported also about comparison of SPME and liquid–liquid extraction for the analysis of various chemical compounds (Bonadio et al. 2008; Thompson-Witrick et al. 2015). Nevertheless, there are no reports about comparison of HS-SPME and liquid–liquid extraction using methylene chloride for the analysis of biotransformation of varying volatility compounds.

Therefore, the main goal of this study was to assess the effectiveness of headspace solid-phase microextraction (HS-SPME) compare to liquid–liquid extractions using with methylene chloride for control of microbial biotransformation of ketones of varying volatility. Additionally, *Alternaria alternata* 18570 as a potential biocatalyst for bioreduction reaction of carbonyl group was also assessed.

Materials and methods

Materials

The filamentous fungus strain *A. alternata* 18570 was obtained from the collection of the Department of Forest Pathology, Mycology and Tree Physiology, University of Agriculture in Krakow. It was cultivated at 27 °C on Sabouraud agar slants (Biocorp, Poland) and stored at 4 °C. Biotransformations were conducted in the medium containing 3 g of glucose (The Industrial and Trading Enterprise "Stanlab" Co. Ltd., Lublin, Poland) and 1 g of peptobac (BTL sp. z o.o., Warszawa, Poland) in water (100 mL), pH 6.8.

The substrates acetylcyclohexane (**1**), acetophenone (**2**), 2-acetylthiophene (**3**) and α,α,α-trifluoroacetophe none (**4**) were used in this study and they were selected on the basis of screening tests from other substrates such as 2-acetylpyridine, 3-acetylpyridine, 4-acetylpyridine, 2-bromoacetophenone, 4-bromoacetophenone, 3-nitroacetophenone, 4-nitroacetophenone. All the substrates were purchased from Fluka. Screening tests were aimed at identifying substrates that were biotransformed by the fungus (*A. alternata* 18570) and determining the times in which the substrates were not completely converted to products.

Biotransformation procedure

Erlenmeyer flasks (250 mL), each containing 50 mL of the culture medium described earlier, were inoculated with *A. alternata* 18570 and incubated for 4 days at 27 °C on a rotary shaker (190 rpm). Then, 20 mg of a suitable substrate dissolved in 1 mL of isopropanol was added and incubated for 24 h (acetylcyclohexane), 30 h (α,α,α-trifluoroacetophenone) or 48 h (acetophenone, 2-acetylthiophene) – the times established on the basis of screening tests. After incubation, the sample was extracted by using headspace HS-SPME or by using liquid–liquid extractions with methylene chloride (CH_2Cl_2).

Analytical methods

The composition of biotransformation mixtures was established by GC on Agilent 6890 N GC instrument, fitted with a flame ionization detector (FID) and a chiral column Chirasil-Dex CB 25 m × 0.25 mm × 0.25 μm. Reference samples of the racemic alcohols were prepared by reducing the ketones with sodium borohydride ($NaBH_4$) in methanol. All the experiments were repeated three times.

The following temperature programmes and helium carrier gas were used to determine the composition and enantiomeric excesses of product mixtures, for acetylcyclohexane (**1**): injector and detector (FID) 330°C, column temperature: 85–200 °C (rate 5 °C min^{-1}), 85 °C (hold 5 min), flow 2 ml · min^{-1}, split vent 180 mL · min^{-1}; for acetophenone (**2**): injector and detector (FID) 230 °C, column temperature: 90–200 °C (rate 6 °C · min^{-1}), 90 °C (hold 9 min), flow 2 ml · min^{-1}, split vent 180 mL · min^{-1}; for 2-acetylthiophene (**3**): injector 200 °C, detector (FID) 230 °C, column temperature: 90–200 °C (rate 6 °C min^{-1}), 90 °C (hold 5 min), flow 2 ml min^{-1}, split vent 180 mL · min^{-1}; for α,α,α-trifluoroacetophenone (**4**): injector and detector (FID) 230 °C, column temperature: 80–150 °C (rate 3 °C min^{-1}), 80 °C (hold 3 min), flow 2 ml· min^{-1}, split vent 180 mL min^{-1}. In addition, during the analysis of HS-SPME was used 1 min desorption of SPME fibre (Table 1), and in the case of methylene chloride

Table 1. Characteristics of SPME fibres used in the experiment for headspace solid-phase microextraction (HS-SPME).

	Supelco SPME fibres			
Compound	Name	Matrix active group	Diameter (d_f)	Compatibility
3*	PA	Polyacrylate	85 μm (partially crosslinked phase)	For analyte group polar semivolatiles (MW 80–300)
1, 2	CAR/PDMS	Carboxen/Polydimethylsiloxane	65 μm (partially crosslinked phase)	For analyte group volatiles, amines, and nitroaromatic compounds (MW 50–300)
1, 2	PDMS/DVB	Polydimethylsiloxane/Divinylbenzene	65 μm (partially crosslinked phase)	For analyte group volatiles, amines, and nitroaromatic compounds (MW 50–300)
2, 3, 4	DVB/CAR/PDMS	Divinylbenzene/Carboxen/ Polydimethylsiloxane	50/30 μm	For analyte group flavours (volatiles and semivolatiles, C3-20) (MW 40–275)

*Acetylcyclohexane (**1**), acetophenone (**2**), 2-acetylthiophene (**3**), α,α,α-trifluoroacetophenone (**4**).

(CH_2Cl_2) extraction was used liners for injection port, split 50:1, and Split Flow 100 mL min^{-1}.

The commercial SPME device (Supelco) consist of a 1 cm length fused silica fibre of ca. 100 μm diameter coated on the outer surface with a stationary phase fixed to a stainless steel plunger and a holder similar to a microliter syringe, and four types of SPME fibre coated with different stationary phases (Supelco) were used for HS-SPME – Table 1. After biotransformation, the SPME device was installed on Erlenmeyer flasks (250 mL) and needle with the SPME fibre was inserted into the stopper. Then, the SPME fibre was exposed to headspace for 15 min at 27 °C, and Erlenmeyer flasks was still shaken on a rotary shaker (90 rpm).

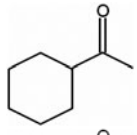
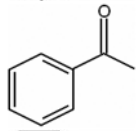
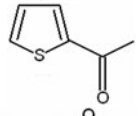
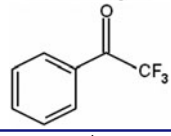
In the case of using methylene chloride (CH_2Cl_2), the samples were withdrawn (ca. 50 mL) and extracted three times (50 mL). The extracts were dried over $MgSO_4$ and concentrated *in vacuo*. The crude mixture obtained this way was analysed by GC as described earlier.

Screening procedure was similar regarding the conditions for GC, but it used only methylene chloride (CH_2Cl_2) procedure for the extraction of the products mixture after biotransformation. Absolute configurations of the products were determined by comparison of their optical rotation values with our previous research (Ogórek and Jarosz 2013) – shorter reaction times were determined in (R)-enantiomers of products obtained from reduction of acetylcyclohexane (**1**), acetophenone (**2**), and 2-acetylthiophene (**3**), and (S)-enantiomers of reduction of α,α,α-trifluoroacetophenone (**4**) (Table 2).

Statistical analysis

The results were analysed by ANOVA, using Statistica 12.0 package. Means were compared using Tukey HSD (Honest Significant Differences) test at $\alpha \leq 0.01$. Percentage data, before analysis of variance were transformed to Bliss (1934) angular degrees by the formula $y = \arcsin(\text{value}\%)^{-0.5}$. After transformation, the variance is approximately constant, allowing analysis of variance to compare particular components.

Table 2. The retention times of acetylcyclohexane (1), acetophenone (2), 2-acetylthiophene (3) and α,α,α-trifluoroacetophenone (4) and their products (alcohols) after bioreduction obtained by using GC and a column Chirasil-Dex CB 25 m × 0.25 mm × 0.25 μm.

Compound			Products t_R[a] (min)	
Number	Structure	Substrate t_R[a] (min)	Enantiomer 1[b]	Enantiomer 2[c]
1		9.42	12.25	12.43
2		6.78	10.80	11.14
3		8.87	11.92	12.23
4		2.41	18.00	18.41

[a]t_R – retention time; [b](R)-enantiomer for compounds **1**, **2** and **3**, and (S)-enantiomer for compound **4**
[c](S)-enantiomer for compounds **1**, **2** and **3**, and (R)-enantiomer for compound **4**.

Results and discussion

SPME is an innovative, solvent-free sample preparation technique that is fast, relatively inexpensive, versatile, and compatible with analytical separation systems inter alia GC (Lord and Pawliszyn 2000). Our research confirmed this; however, analyte properties, and the type of fibre coating are one of the most important aspects of this technique (Górecki et al. 1999). Therefore, we used four types of SPME fibre coated with different stationary phases and liquid–liquid extractions in our study (Table 1). Generally, we found significant differences between types of extraction in the case of the products mixture after biotransformation of acetophenone (**2**) and 2-acetylthiophene (**3**) by *A. alternata*. Whereas, in other cases, there no of such differences (Table 3).

Table 3. Comparison of composition (in % according to GC) of the products mixture obtained using different extraction methods (liquid–liquid and HS-SPME), after 24 h biotransformation of acetylcyclohexane (**1**), 30 h of α,α,α-trifluoroacetophenone (**4**), and 48 h of acetophenone (**2**) and 2-acetylthiophene (**3**) by *Alternaria alternata* 18570.

				Products (alcohols)					
Compound	Types of extraction	Substrate		(R)-enantiomer		(S)-enantiomer		Substrate conversion	
1	CH_2Cl_2	3.3 ± 1.7[1]	a[2]	0.0 ± 0.0[1]	a[2]	96.7 ± 2.8[1]	a[2]	96.7	a[2]
	CAR/PDMS fibre	9.6 ± 1.6	a	0.0 ± 0.0	a	90.4 ± 3.4	a	90.4	A
	PDMS/DVB fibre	4.1 ± 1.9	a	0.0 ± 0.0	a	95.9 ± 3.0	a	95.9	A
2	CH_2Cl_2	4.1 ± 0.2[1]	b[2]	0.9 ± 0.1[1]	a[2]	95.0 ± 0.2[1]	a[2]	95.9	a[2]
	CAR/PDMS fibre	28.8 ± 0.4	a	0.9 ± 0.1	a	70.3 ± 1.5	c	71.2	C
	PDMS/DVB fibre	10.1 ± 1.1	b	0.0 ± 0.0	b	89.9 ± 1.9	ab	89.9	ab
	DVB/CAR/PDMS fibre	21.5 ± 6.5	a	0.0 ± 0.0	b	78.5 ± 7.1	bc	78.5	bc
3	CH_2Cl_2	6.3 ± 1.6[1]	b[2]	5.6 ± 0.7[1]	a[2]	88.0 ± 1.0[1]	a[2]	93.6	a[2]
	PA fibre	18.2 ± 1.9	a	4.7 ± 0.6	a	77.1 ± 1.2	b	81.8	b
	DVB/CAR/PDMS fibre	7.0 ± 2.1	b	5.5 ± 0.7	a	87.5 ± 1.4	a	93.0	a
4	CH_2Cl_2	1.4 ± 0.1[1]	a[2]	76.4 ± 1.1[1]	a[2]	22.2 ± 1.4[1]	a[2]	98.9	a[2]
	DVB/CAR/PDMS fibre	2.0 ± 0.2	a	73.3 ± 0.8	a	24.7 ± 0.9	a	98.0	a

[1] SD (standard deviation); [2]For each types of extraction, means followed by the same letter are not statistically different at the $\alpha \leq 0.05$ level according to Tukey HSD test; others are. Letters indicate the effect of extraction types on composition of individual components of the mixture and substrate conversion.

The fibres (CAR/PDMS, PDMS/DVB) and methylene chloride extraction as control were used to evaluate the biotransformation of compound acetylcyclohexane (**1**). All types of extraction were at a similar level and the obtained values were not statistically significant. However, the results of HS-SPME obtained with PDMS/DVB fibre were most similar to the control (Table 3). Additionally, DVB/CAR/PDMS fibre was used for acetophenone (**2**), but also in this case, the results from PDMS/DVB fibre were most similar to the control. However, the values obtained using the two other fibres were exhibited significant differences as compared to control (Table 3). On the other hand, the results of products mixture after biotransformation of substrates 2-acetylthiophene (**3**) and α,α,α-trifluoro acetophenone (**4**) obtained using DVB/CAR/PDMS fibre were most similar to the control – the values of these variants were not statistically significant (Table 3).

Literature report that, if all the analytes to be extracted are polar, a polar fibre coating like polyacrylate (PA) could be used. However, in the case of extracting a mixture of compounds with different polarities (alcohols, aldehydes, ketones etc.) fibres coated with a combination of nonpolar material like polydimethylsiloxane (PDMS) and a more polar material like divinylbenzene (DVB) could be used (Górecki et al. 1999). Our research confirmed this. Therefore, in the case of HS-SPME, the best way to extract all the components of the mixture in quantities similar to the extraction of methylene chloride was the use of fibres coated with a combination of polar and nonpolar material. On the other hand, the use of fibre coated with polar material as polyacrylate (PA) was the cause of excessive extraction inter alia of a polar alcohols (Table 3). However, extraction efficiency of individual fibres coated with a combination of nonpolar and polar materials can be different for substrances within the same chemical group as demonstrated by the results of this study and other researchers (Górecki et al. 1999; Vichi et al. 2003).

In the recent years, so called "green chemistry" enjoys great interest and the production of various enantiopure alcohols catalysed by enzymes, microorganisms and plant cells has attracted considerable attention due to its high efficiency, high enantioselectivity, mild reaction condition, and low environmental pollution (Ni and Xu 2012; Sheldon 2012). Literature also report that *A. alternata* is a good biocatalyst for bioreduction reaction of ketones to alcohols (Kurbanoglu et al. 2007; Ogórek and Jarosz 2013). Our study confirmed these reports. Nevertheless, we noticed that the biotransformation process was dependent on the structure of the substrate. Generally, in case of acetophenone (**2**), 2-acetylthiophene (**3**) and α,α,α-trifluoroacetophenone (**4**), both product enantiomers have been detected, whereas biotransformation of acetylcyclohexane (**1**) afforded only one enantiomer. However, the number of products was dependent mainly on enzyme selectivity and probably the effect of the type of extraction and SPME fibre was negligible. Therefore, the ratio of both enantiomers obtained from a specific substrate was similar for all extraction methods tested because the enantiomers behave in a similar way when extracted on a non-chiral phase. The apparent conversion of the substrates amounted from 71.2 to 98.8%, and it was also dependent on enzyme selectivity (Table 3). However, literature report that the species of filamentous fungi as *A. alternata* may convert the substrates

examined by us at the same time with a higher yield than in our study (Ogórek and Jarosz 2013). This is probably related to the age of the fungal culture. Because very often microorganisms, especially microscopic fungi, lose their phenotypic properties as well as enzymatic abilities along with the number of passages and life time *in vitro* (Ogórek et al. 2016).

Stereoselective and enantiospecific reduction of ketones to form chiral alcohols in one of the most useful reactions in organic chemistry, and it is catalysed by carbonyl reductases. Those enzymes belong to the oxidoreductase family that often need cofactors such as NAD(H) or NADP(H) to be functionally active (Gao et al. 2013). However, whole cells of microorganisms contain all the necessary cofactors and metabolic pathways for their regeneration. Carbonyl reductases secreted by the fungus used in our research reduced simple aliphatic and aromatic ketones according to Prelog's rule, which means that the stereochemistry can be determined by looking at the size of the two groups. This rule states that the enzyme has a large and a small pocket that makes up the active site, in which the substrate binds, and controls the stereochemistry of the product based on the geometry of the substrate. Consequently, the product as (S)-alcohols is obtained from simple aliphatic and aromatic ketones (Prelog 1964). However, this should not always be generalized and caution should be exercised in particular, when Prelog's rule is applied to whole cells due to the presence of multiple enzymes inside the cells (Csuk and Glaenzer 1991). This is probably one of the reasons for obtaining, in some cases, a product mixture rather than a single product.

Conclusions

The proposed method based on SPME which is a fast and versatile technique, using no cosolvents and compatible with GC was successfully applied to evaluate the results of microbial biotransformation of ketones of varying volatility. The best way to extract all the components of the mixture (alcohols, aldehydes) in quantities similar to those obtained by extracting with methylene chloride was the use of fibres coated with a combination of a nonpolar and a polar material. However, the enantiospecificity was dependent mainly on enzyme selectivity and probably the effect of the used the type of extraction and the type of SPME fibre was negligible. Nevertheless, the commercial PDMS/DVB fibre was most suitable for the extraction of the product mixture obtained after biotransformation of acetylcyclohexane and acetophenone. On the other hand, the best results were obtained for 2-acetylthiophene, α,α,α-trifluoroacetophenone and their derivatives using DVB/CAR/PDMS fibre. In addition, our study showed that whole-cell of *A. alternata* is a good biocatalyst for bioreduction reaction of ketones to alcohols with a high enantiospecificity and substrate conversion. Moreover, carbonyl reductases secreted by the *A. alternata* reduced ketones used in this study in accordance with Prelog's rule and hence with high predominance of (S)-isomers. The exception was the reduction of α,α,α-trifluoroacetophenone, with predominance of (R)-isomers.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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