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# Determination of thymol in human plasma by automated headspace solid-phase microextraction–gas chromatographic analysis

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**Abstract**

A reliable and sensitive method was developed for determination of thymol in human plasma by automated headspace solid-phase microextraction (SPME). After enzymatic cleavage of thymol sulfate thymol was extracted by a 65 μm polydimethylsiloxane–divinylbenzene crimped fiber (Supelco) after addition of sodium chloride and phosphoric acid (85%). Desorption of the fiber was performed in the injection port of a gas chromatograph at 220°C (HP 5890; 50 m×0.2 mm I.D., 0.2 μm HP Innowax capillary column; flame ionization detection). Fibers were used repeatedly up to 40 analysis. The recovery was 5% after 35 min of extraction. The calibration curve was linear in the range of 8.1-203.5 ng $ml^{-1}$ with a limit of quantitation (LOQ) of 8.1 ng $ml^{-1}$. The within-day and between-day precision and accuracy were ≤20% at the LOQ and <15% at higher concentrations according to international guidelines for validation of bioanalytical methods. After administration of a thymol-containing herbal extract only thymol sulfate, no free thymol, could be detected in human plasma, thus analysis of thymol was after enzymatic cleavage of thymol sulfate. It is concluded that the newly developed automated method can be used in clinical trials on bioavailability and pharmacokinetics of thymol-containing herbal medicinal products.


*Keywords:* Thyme; Bioavailability; Thymol

## 1. Introduction

Herbal medicinal products (HMPs) containing natural volatiles are used in the treatment of gastrointestinal diseases, pain, colds and bronchitis. Pharmacological studies demonstrate a wide variety of in vitro effects, with antiinflammatory and antimicrobial activities investigated most frequently. In comparison, relatively few studies on the bioavailability and pharmacokinetics of putative active components have been carried out. However, such studies are discussed to demonstrate the relevance of the in vitro activity to the therapeutic effects found in clinical trials or stated in textbooks of phytotherapy. Thus, studies with essential oils and their single compounds providing supporting evidence of efficacy and demonstrating systemic availability are necessary. These data could also be important with regard to safety aspects [1].

To address these issues for thymol – one of the active compounds in HMPs containing thyme – a

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bioavailability study after oral administration of a single dose of a respective preparation (BronchipretTP) was carried out. The major prerequisite for this study was a reliable and sensitive method for determination of thymol in human plasma. Since only unconjugated thymol can be detected by headspace solid-phase microextraction (HS-SPME), enzymatic hydrolysis of its phase II metabolite thymol sulfate was carried out prior to analysis.

As thyme or thymol are frequently found in food and pharmaceuticals, different methods have been published for thymol assay from various matrices. A quantitative thin-layer chromatography (TLC) method using a scanning densitometer was used for analysis of thymol in the essential oil of *Nigella sativa* [2]. For the determination of thymol in thyme dry herb spectrophotometric methods [3] and gas chromatographic (GC) analysis after microdistillation of plant material were applied [4]. High-performance liquid chromatography (HPLC)–UV methods were used for analysis of thymol in halothane anaesthetic preparations where it is used as a stabilizing agent, and for quantification of thymol in cough mixtures [5,6].

As plasma concentrations of thymol after oral intake of therapeutic doses of a thyme preparation were in the lower ng $ml^{-1}$ range in preliminary investigations, a very sensitive bioanalytical method was required to obtain reliable results at this concentration level. Spectrophotometric detection, TLC and HPLC–photodiode array detection (PDA) methods would not provide sufficient sensitivity and selectivity, respectively, to analyze thymol at lower ng $ml^{-1}$ concentrations in human plasma. Therefore, GC combined with flame ionization detection, which is a very sensitive method for quantification of volatile compounds, was chosen for the analysis of thymol in human plasma in our bioavailability study. Regarding sample preparation prior to TLC, HPLC and GC analysis, common concentration and extraction steps cannot be applied easily to volatile compounds. Thus, HS-SPME was chosen as sample preparation technique, which uses coated fused-silica fibers to selectively extract volatile analytes from aqueous samples. This method integrates extraction, concentration and sampling in one single step and helps saving time and effort on sample preparation. Moreover, organic solvents which are often expensive, toxic or carcinogenic are not necessary using SPME [7]. Therefore, SPME has already been used for the determination of compounds in food, natural products and pharmaceuticals. It is also regarded as an established method in different fields like toxicology, environmental analysis and forensics for the analysis of volatiles from biological matrices such as plasma, urine, saliva, and hair [7–9].

In the course of method development, various factors have previously been identified to influence method performance in the analysis of phenols which are structurally similar to thymol, such as propofol [10–12]. An indication that thymol was suitable for HS-SPME was given by Kroll and Borchert using thymol as an internal standard [13]. Recently, the influence of fiber coatings in HS-SPME–GC analysis of thyme and other aromatic plants, among them thyme was investigated [14]. For the determination of phenolic compounds such as thymol and carvacrol the 65 μm polydimethylsiloxane–divinylbenzene fiber showed the highest concentration factors determined from the ratios of the peak areas after HS-SPME and headspace analysis [14].

Decreasing the solubility of phenolics in the aqueous phase in order to force them into the headspace was achieved by saturation with salt and acidification to below pH 1. Magnetic stirring or sonication was used for the SPME extractions to ensure proper mixing of the samples and to reduce equilibration time, respectively [10,11]. Additionally, heating of the samples reduced the equilibrium time [15].

Automated HS-SPME devices available so far did not allow sufficient adjustment of these parameters and thus adequate sensitivity could not be achieved. Therefore, an additional device was developed to allow optimization of automated HS-SPME parameters. The automated, but not the previously developed manual method then fulfilled the acceptance criteria required for bioavailability studies according to international guidelines for pharmaceutical bioavailability, pharmacokinetic and bioequivalence studies [16,17]. Furthermore, the analysis of a large number of samples of bioavailability studies which is still not generally suitable for routine GC–MS analysis was facilitated by automated HS-SPME combined with flame ionization detection. This method was used for the analysis of thymol in

human plasma samples in the course of a bioavailability and pharmacokinetic study of a thyme preparation in humans.

## 2. Experimental

### 2.1. Chemicals

Thymol as working standard was purchased from Fluka (Thymol purum >99.9% GC; 89330), Deisenhofen, Germany. It was intercalibrated with a primary reference standard of thymol obtained from Zentralinstitut Arzneimittelforschung, Sinzig, Germany, lot 377452/1, certificated by quantitative $^{1}$H-nuclear magnetic resonance (NMR) spectroscopy. β-Glucuronidase (Typ HPII crude solution from *Helix pomatia*; G7017) was obtained from Sigma, Deisenhofen, Germany. NaCl Suprapur, phosphoric acid (85%), acetic acid (100%), *o*-cresol and methanol Lichrosolv were purchased from Merck, Darmstadt, Germany. Water was obtained from a Milli-Q system from Millipore (Eschborn, Germany). Analyte-free plasma was obtained from healthy volunteers.

### 2.2. Sample preparation

To 1.0 ml of human plasma 80 μl acetic acid 0.58 *M* (adjustment to pH 5, which is the pH optimum of the enzyme) and 100 μl of the β-glucuronidase solution were added and incubated for 30 min at 37°C. After incubation 50 μl water (50 μl thymol stock solution for validation) and 50 μl *o*-cresol internal standard solution were added to the plasma. A 1.0-g amount of sodium chloride, 100 μl phosphorous acid 85% and a stirring bar were put in a 10-ml vial (equipped with PTFE-silicon septa and magnetic caps, Achroma, Müllheim, Germany) and afterwards the plasma was added to the 10-ml vial and it was closed airtight.

The following automated procedure was carried out by a Combi-PAL autosampler (CTC Analytics, Bern, Switzerland) equipped with a magnetic mixer/heater (Chromtech, Idstein, Germany) and a bake out device (CTC Analytics). One vial at a time was taken automatically from the tray and inserted in the magnetic mixer, heated at 80°C for 35 min and stirred at 750 rpm simultaneously. During that time a 65 μm polydimethylsiloxane–divinylbenzene crimped fiber (Supelco, Belfonte, PA, USA) was exposed to the headspace of the vial for headspace equilibration. Afterwards the fiber was inserted immediately into the GC injection port for desorption of the compounds (220°C for 5 min). To avoid carry over of analytes in following samples the fiber was desorbed prior to subsequent runs in the bake out device for 10 min at 270°C.

### 2.3. Apparatus

Completely automated HS-SPME was carried out by a Combi-PAL autosampler (CTC Analytics), providing room for 32 10-ml vials at the tray. For all analysis so-called ''crimped'' fibers were used. The septum of a ''crimped'' fiber was fixed by means of a metal lid instead of glue and hence, provided a longer lifetime for the fibers. The autosampler is equipped with a single magnet mixer/heater, which is basically an incubation device with magnetic stirring feature (Chromtech). The magnet system for single vial incubation is controlled by a stepper motor with a programmable choice of rpm. Heating is provided by a heating foil in 1°C steps up to 150°C. One vial can be inserted in the magnetic mixer at once. In addition a bake out device was mounted to provide desorption of the fibers at high temperature. GC was performed on an Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a split–splitless injection port with a narrow bore insert (0.75 mm I.D.; Supelco) and flame ionization detection. Column (HP Innowax, 50 m×0.2 mm I.D., 0.2 μm film thickness, Agilent Technologies) temperature was set at 80°C for 5 min and then programmed from 80°C to 240°C at 4°C min$^{-1}$ and held constant for 10 min. Injector and detector temperatures were 220°C and 250°C, respectively, helium flow-rate 1 ml min$^{-1}$. Hydrogen flow-rate was 33 ml min$^{-1}$, air 370 ml min$^{-1}$ and nitrogen as make up gas 31 ml min$^{-1}$. The samples were injected in the splitless mode. The chromatograms were recorded using software of Hewlett-Packard, HP Chemstation.

## 3. Validation results

Validation of the method was performed with human plasma obtained from healthy volunteers (written informed consent was given), blank and spiked with thymol stock solutions. Thymol stock solutions were prepared with methanol and diluted further with water; methanol concentration in spiked samples was maximal $0.7 \cdot 10^{-3}$% (v/v). As internal standard *o*-cresol was chosen. Due to its structural and physicochemical similarity it met the criteria required from an internal standard. Solutions of *o*-cresol were prepared in the same way as described for thymol.

### *3.1. Selection and conditioning of the fiber*

For thymol for the 65 μm polydimethylsiloxane–divinylbenzene fiber a high partition coefficient (HS phase/polymeric fiber coating) and concentration factor (peak area obtained by an SPME fiber/peak area obtained by headspace) was found compared to other coatings available [14]. This coating turned out to be the most sensitive and robust one for our method as well. The 85 μm polyacrylate fiber provided sufficient sensitivity but did not resist the low pH level. Prior to the first use of every crimped fiber it was cleaned from glue residues and interfering peaks by desorption in the bakeout device at 270°C for 1 h, exposition to methanol for 1 min and heating for 1 h at 220°C again.

### *3.2. Lifetime of the fiber coating*

The phosphoric acid (85%) and acetic acid in the samples caused reduced lifetime of the fiber coating compared to other headspace SPME methods. After about 40 replicate assays the fiber coating turned black and chromatography was not satisfactory any more. Also a decrease in peak areas was observed, but ratios remained relatively constant. Repeated use of fibers was therefore restricted to 40 replicate samples.

### *3.3. Calibration of exposure time*

The first step in the method development procedure was to determine when headspace fiber equilibrium had reached its plateau phase. Equilibrium time for the analytes in the fiber coating and the sample corresponds to the optimum sampling time. Stock solutions of thymol and *o*-cresol were used to spike plasma samples in order to determine the absorption time profile. Appropriate exposure time was determined to be 35 min (Fig. 1). Equilibrium of *o*-cresol was reached within the exposure time, which is a major prerequisite for an internal standard in SPME.

### *3.4. Selectivity*

Selectivity of the method was examined with plasma samples of six different volunteers. All blank plasmas showed an interfering peak at the retention time of thymol, which was identified as 2′-tetradecene. There was no interference observed between *o*-cresol and plasma compounds. Different types of columns and different changes concerning chromatography did neither allow shorter GC analysis time nor achieve separation of the interfering peak from the thymol peak. However, peak areas were consistent in the range of 1927 to 2602 area counts. The determination of limit of detection (LOD) was not possible within this method because of the interfering peak. The peak area of the interfering peak was constant within the precision of the method and thus the meaning of the data presented is not falsified.

The limit of quantitation (LOQ) was defined at 8.14 ng $ml^{-1}$ which corresponds to an average of 5284 area counts (including interfering peak), and thus is considerably higher than the interfering peak by itself. Fig. 2 shows a detail of a chromatogram of

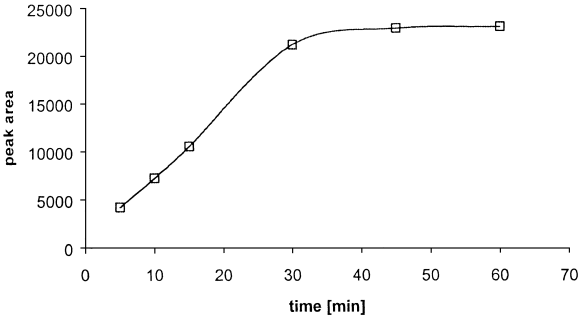


Fig. 1. Mean adsorption–time profile for thymol in human plasma at 80°C; concentration: 61.5 ng $ml^{-1}$; $n=2$.

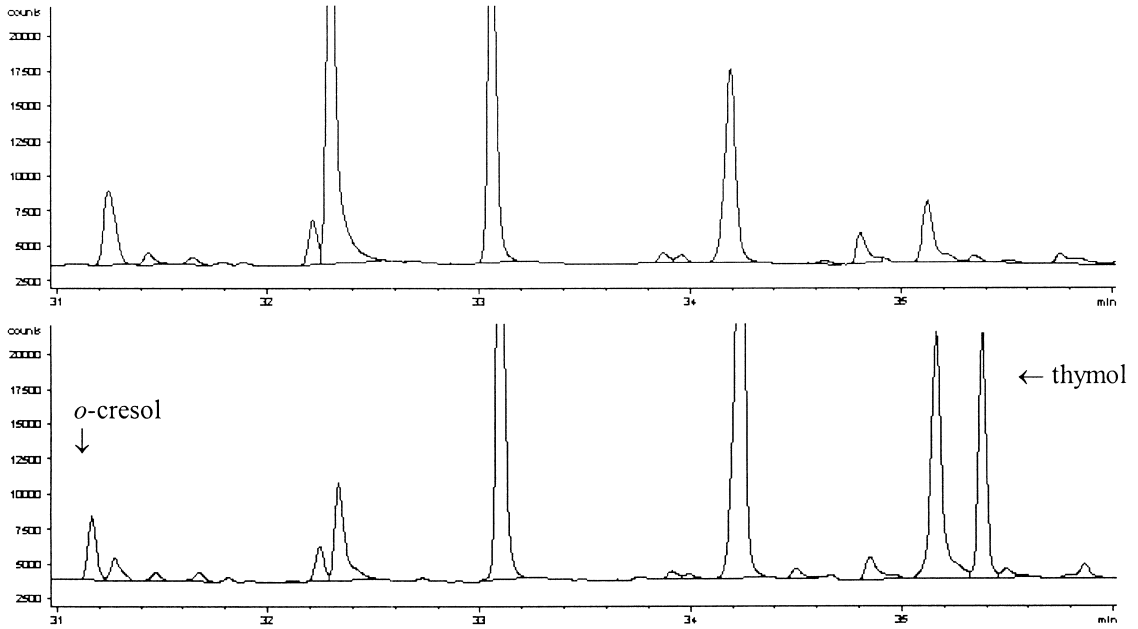


Fig. 2. Details of chromatograms of blank plasma (above) and after application of one BronchipretTP tablet (cp=122 ng $ml^{-1}$) and addition of internal standard *o*-cresol (below).

a blank plasma sample in comparison to an exemplary sample obtained in the bioavailability study at $t=1.5$ h after application of one BrochipretTP tablet (equivalent to 1.08 mg thymol) [18]. The identity of the concerning peak was confirmed by GC–MS.

### 3.5. Linearity

For linearity studies different standard thymol solutions were prepared and added to plasma samples. All calibration curves were always obtained from plasma matrices of the same source. Concentrations were set at 8.14, 10.18, 20.36, 30.54, 40.07, 50.9, 101.8, 152.7 and 203.5 ng $ml^{-1}$. Since calculation of samples with known concentrations by one single calibration curve did not achieve values in the range required (relative standard deviation, RSD, and accuracy, RE, ≤15% and ≤20% at LOQ) especially at low concentrations, calculations were carried out by two separate calibration curves. Each calibration curve was repeatedly performed with crimped fibers from different batches. The standard curves were linear in the range from 8.1 to 40.07 ng $ml^{-1}$ and from 30.54 to 203.5 ng $ml^{-1}$ with correlation coefficients in the range of $R^2=0.97138$–0.99854 at the lower concentrations and $R^2=$ 0.99036–0.99730 at higher concentrations, respectively ($n=3$) (Table 1).

### 3.6. Precision and accuracy

The precision was determined by the RSD of the peak area ratios at three different concentrations of

Table 1
Calibration curves for thymol in human plasma, $n=3$

| | Slope (mean±SD) | Intercept (mean±SD) | $R^2$ (mean) |
|---|---|---|---|
| | Concentrations: 8.14; 10.18; 20.36; 30.54; 40.07; 50.9; 101.8; 152.7; 203.5 ng $ml^{-1}$ | | |
| 8.14–40.07 ng·$ml^{-1}$ | 0.025±0.008 | 0.322±0.154 | 0.982 |
| 30.54–203.5 ng·$ml^{-1}$ | 0.027±0.005 | 0.220±0.285 | 0.993 |

Table 2
Within-day precision and accuracy (for experimental see text)

| Concentration (ng ml$^{-1}$) | Day | Mean (ng ml$^{-1}$) | RSD (%) | RE (%) | *n* |
|---|---|---|---|---|---|
| 8.14 | 1 | 9.26 | 18.26 | 13.72 | 5 |
| | 2 | 6.66 | 12.23 | −18.15 | 5 |
| | 3 | 9.66 | 9.72 | 18.7 | 5 |
| 61.5 | 1 | 59.69 | 3.87 | −2.94 | 5 |
| | 2 | 55.55 | 9.3 | −9.68 | 5 |
| | 3 | 61.78 | 11.98 | 0.45 | 5 |
| 203.5 | 1 | 174.57 | 8.01 | −14.22 | 5 |
| | 2 | 192.97 | 6.32 | −5.17 | 5 |
| | 3 | 192.49 | 13.48 | −5.41 | 5 |

thymol. Five measurements were made for within-day precision. Between-day precision was calculated from the average of each day's measurement. Accuracy (RE) was determined by running five replicate samples at three different concentrations.

The within-day precision and accuracy were $<$20% at the LOQ and $<$15% at higher concentrations and thus met the criteria required for bioanalytical methods validation [16,17]. The between-day precision of 19.1% indicated that 8.14 ng ml$^{-1}$ was the LOQ (see Tables 2 and 3).

### 3.7. Recovery

Recovery of thymol after 35 min of extraction was calculated by comparing the peak areas obtained from the spiked plasma samples with known concentrations with those obtained from solutions injected directly. Recovery was determined at three concentrations (8.1, 61.5, 203.5 ng on column) running five replicate samples (Table 4).

Table 3
Between-day precision and accuracy (for experimental see text)

| Concentration (ng ml$^{-1}$) | Mean (ng ml$^{-1}$) | RSD (%) | RE (%) |
|---|---|---|---|
| 8.14 | 8.53 | 19.10 | 4.76 |
| 61.5 | 59.01 | 5.37 | −4.06 |
| 203.5 | 186.68 | 5.62 | −8.27 |

Table 4
Recoveries and their relative standard deviations (RSDs) at different concentrations of thymol from human plasma by headspace SPME ($n$=5)

| | Amount on column or added to 1 ml plasma (ng) | | |
|---|---|---|---|
| | 8.1 | 61.5 | 203.5 |
| Recovery (%) | 4.9±0.7 | 5.3±0.7 | 4.5±0.3 |
| RSD (%) | 14.1 | 12.5 | 5.9 |

## 4. Discussion

### 4.1. Apparatus

Concentrations of single essential oil compounds in human plasma after administration of therapeutic doses were in the ng ml$^{-1}$ level [19–21]. Therefore single dose bioavailability studies with HMPs containing essential oils as active principle require very sensitive bioanalytical methods.

HS-SPME prior to GC combined with flame ionization detection is a very sensitive tool for the determination of volatiles from biological matrices. Most of the applications described previously used manual HS-SPME methods [14,22–26]. However, our manual HS-SPME method developed for determination of thymol from human plasma did not meet the validation criteria required from international guidelines for validation of bioanalytical methods concerning precision and accuracy [17].

In order to accurately match the method parameters such as equilibration time, temperature and stirring, as well as to provide a high sample throughput in the course of the pharmacokinetic study, automation of the method was planned by means of a Combi-PAL autosampler. The commercially available device, however, did not provide magnetic stirring but shaking of the samples. Shaking of the samples with high frequency was only possible prior to fiber exposition whereas shaking with low frequency was carried out during fiber exposition. Thus, an enormous increase in equilibration time was observed. Additionally, due to shaking of the samples plasma splashed to the fiber which decreased recovery and lifetime of the fibers. Therefore, an magnetic mixer/heater was developed in co-opera-

tion with Chromtech, which provided heating and magnetic stirring of the sample simultaneously and thus lead to considerably shorter exposition time. With this newly developed device optimization of the HS-SPME parameters was performed.

### 4.2. Extraction conditions and matrix effects

Decreasing the solubility of thymol in the sample in order to force it into the headspace was achieved by saturation with sodium chloride and acidification to below pH 1 with phosphorous acid. Additionally, acidification ensured maximum protonation of the phenol. Magnetic stirring was used for the SPME extractions to ensure proper mixing of the samples and to reduce equilibration time [10,11]. As seen from several methods applied for extraction of volatiles or semi volatiles from human body fluids pH adjustment, addition of salt and heating are the basic conditions [9,22–26]. In combination with sample heating and addition of salt the whole treatment procedure is likely to liberate analytes from protein binding in plasma. High protein binding of the analytes (antidepressants and clozapine) appeared to be the main limiting factor concerning a faster extraction in plasma and recovery [24,27]. However, these investigations were carried out using direct immersion SPME and did not use any salt, heating or sufficient acidification in order to break down the proteins. Recently it has been shown that the plasma protein binding of lidocain (74% at pH 9.5) could be reduced enormously by acidification [28].

Thymol could not be separated from an interfering plasma peak. However, peak areas did not vary significantly from subject to subject. Regarding the interfering plasma peak it might have been reasonable to choose a more selective detection system such as nitrogen–phosphorus detection (NPD) (only available for compounds containing nitrogen) or even MS. With respect to any further method development concerning bioavailability studies with HMPs as complex active compounds emphasis should be put on selecting a detection system which provides sufficient selectivity. Thus, detection of possible metabolites or other compounds than the main compound of HMPs could be provided. However, in the course of a pharmacokinetic study there are too many samples to be routinely analysed by such a cost-intensive device like GC–MS. Moreover, by the development of a method using flame ionization detection it is available for a broad range of users, which do not have access to GC–MS analysis. However, at least the identification of the compounds concerned is required to be performed by GC–MS.

### 4.3. Validation criteria

The method applied is regarded as being suitable for performing bioavailability, bioequivalence and pharmacokinetic studies, because all validation criteria are in the ranges required by international guidelines for validation of bioanalytical methods [16,17]. Appropriate equilibration time was 35 min, which is reasonable considering that the GC analysis took 55 min. Linearity of the method was given in the range from 8.14 to 203.5 $ng \cdot ml^{-1}$ as shown in Table 1. This calibration range was suitable as the concentrations in plasma after oral application of one tablet of BronchipretTP (equivalent to 1.08 mg thymol) ranged from 8.14 ng $ml^{-1}$ 15 min after application to 125 ng $ml^{-1}$ at maximum plasma concentrations 2 h after application. The plasma concentrations came down to 8.14 ng $ml^{-1}$ again after 38 h.

For determination of accuracy and precision different fibers were used, i.e., precision may be described as *inter*-fiber precision and still the between-day precision is below 20% at the lowest concentration level. Sometimes absolute peak areas varied considerably between different fibers even from the same batch. Therefore calibration was carried out whenever using a new fiber.

The recovery of thymol from human plasma was determined to be 5%. Several SPME methods have been published for the analysis of compounds from human plasma or whole blood and recoveries ranged from 0.03 to 12.9% [25]. Addition of enzyme which is necessary for cleavage of the phase II conjugate resulted in a 33% decrease of recovery compared to samples without enzyme addition (data not shown). However, data on precision and accuracy demonstrate the suitability of the applied method.

## 5. Conclusion

The method presented here will be used as an analytical tool for the determination of thymol plasma levels in a clinical bioavailability study after oral application of therapeutic doses of an HMP containing thyme. Taken together, results presented here demonstrate appropriateness of automated headspace SPME assays for bioanalytical determination of volatile compounds in routine analysis.

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