universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„A supercritical fluid workflow for the fast separation, isolation, and quantification of major constituents in *Liquidambar* (styrax) resins“

verfasst von / submitted by

Johanna Scheuba

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag.pharm.)

Wien, 2018 / Vienna, 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Univ.-Prof. Mag.pharm. Dr. Judith M. Rollinger
Mitbetreut von / Co-Supervisor:	Mag.pharm. Dr. Ulrike Grienke

Acknowledgements

First of all, I want to thank Univ.-Prof. Mag. Dr. Judith M. Rollinger for enabling this diploma thesis for me, offering me the possibility of working with and welcoming me into her team. I appreciate very much how she sets great value upon a well-functioning team, and her efforts to ensure that. It is rewarding that I was able to experience the good working atmosphere everyone around her enjoys so much. I am also thankful that she had time for consultations, reviewing my work and helping me with whatever needed, despite her busy schedule.

I want to thank my co-supervisor Mag. Dr. Ulrike Grienke, who guided me through the whole process of the diploma thesis with all the kindness and patience one could hope for, always ready to help and happy to answer questions of all kind. Her encouraging way of taking concerns of others to heart whilst keeping her professional manner, makes her the ideal supervisor. I am convinced, that being guided well through scientific work with everything that goes along with it, is the most crucial and important part of this process. I can call myself very lucky that I was supervised so well. I do not take it for granted, and I am incredibly grateful for it.

I also want to thank Valerie Wronski for being such a great working partner and her help, especially with the software Empower®.

Further, I want to thank ao. Univ.-Prof. Mag. Dr. Ernst Urban for recording NMR data for me, as well as Christina Sykora for her assistance with mass spectrometry.

I want to thank the team of Pharmacognosy for welcoming me at the department so openly and kindly. It made my time there so enjoyable and I was comfortable around so many nice people. I was happy to get to know them and I appreciated to get insights into their work. The exchange of views on ongoing work was very helpful, especially in the Journal Club that was held on a regular basis by Univ.-Prof. Mag. Dr. Judith M. Rollinger.

I want to thank my boyfriend Rainer, who was always there, listening to my thoughts and concerns, helping me to reflect and see things clearer, when necessary. He has been my rock, when I needed one, supporting and encouraging me with his endless patience. That was worth so much.

And last but not least, I want to thank my friends and family for being there for me, listening to my thoughts, which helped bringing forward new ideas.

To all of you: Thank you!

Table of Contents

List of Abbreviations

APT	attached proton test
$CDCl_3$	deuterated chloroform
CO_2	carbon dioxide
COSY	correlated spectroscopy
GC-MS	gas chromatography – mass spectrometry
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
LC-HRMS	liquid chromatography – high resolution mass spectrometry
LC-MS	liquid chromatography – mass spectrometry
MeOD	deuterated methanol
MeOH	methanol
NMR	nuclear magnetic resonance
PDA	photo diode array (detector)
Prep-15	name of the preparative scale SFC device
SF	supercritical fluids
SFE	supercrticial fluid extraction
SFC	supercritical fluid chromatography
UPC^2	ultra performance convergence chromatography

1 Abstract/ Zusammenfassung

This diploma thesis shows that the polar compounds, o-vanillin (**1**), styracin (**2**), vanillin (**3**), trans-cinnamic acid (**4**), vanillic acid (**5**), and shikimic acid (**6**), can be extracted, analysed, quantified and isolated with devices operating with CO_2 based supercritical fluids (SF). This includes the following SFx technologies: SFE (supercritical fluid extraction), analytical SFC (supercritical fluid chromatography), and preparative scale SFC. For each step, a protocol was established and the analytical method was validated following the ICH guidelines.

Compounds **2**-**6** can be found in the balsam, a resinous sap (styrax) of *Liquidambar* tree species which belong to the Hamamelidaceae family. As an application example for the developed protocol, eight styrax samples (R1-R8) were chosen for the quantification of the phenolic compounds **1**-**5**, and the phenolic precursor **6**. The extraction was performed on a SFE device with a mixture of ethanol and CO_2 (1:1). An ultra-performance convergence chromatography (UPC^2) SFC device was used for the analysis and quantification of the compounds. A baseline separation of **1**-**6** was achieved within 4 min using the Acquity UPC^2 BEH 2-EP (3.0 x 100 mm, 1.7 µm) as stationary phase and supercritical CO_2 and methanol with 0.1 % phosphoric acid as mobile phase. The addition of phosphoric acid reduced the tailing especially of the acids **4**-**6** effectively, ensuring their protonated, uncharged state. The predominant compounds **2**, **4**, and a third, unknown compound (compound **7**) were separated and isolated from R6 with preparative scale SFC (Prep-15). This was conducted within 6 min with a scaled up method from the UPC^2 method, using Viridis BEH 2-EP (10 x 250 mm; 5 µm) as column material. In addition to the manuscript which was published based on these results, compound **7** was structurally elucidated as 3-phenylpropyl-(E)-cinnamate by the interpretation of 1D and 2D NMR spectra. Furthermore, **5** and **6** were extracted and quantified from vanilla beans and star anise, as they were barely or not present in the resin samples. This was done with the same methods as for the styrax samples.

As another additional part of this diploma thesis, an extraction protocol for triterpenes from the medicinal mushroom *Ganoderma lucidum* was established by SFE.

Zusammenfassung

Diese Diplomarbeit zeigt, dass die polaren Verbindungen, o-Vanillin (**1**), Styracin (**2**), Vanillin (**3**), trans-Zimtsäure (**4**), Vanillinsäure (**5**) und Shikimisäure (**6**) mit Geräten, die mit überkritischen CO_2 arbeiten, extrahiert, analysiert, quantifiziert und isoliert werden können. Diese Technologien beinhalten die SFE (überkritische Flüssigextraktion), analytische SFC (überkritische Flüssigchromatographie) und SFC im präparativen Maßstab. Für jeden dieser Schritte wurde ein Protokoll erstellt. Die analytische Methode wurde gemäß den ICH-Leitlinien validiert. Die Verbindungen **2**-**6** kommen im Balsam, einem harzigen Saft (Styrax) von Liquidambar-Arten, die zur Hamamelidaceae-Familie gehören, vor. Als Anwendungsbeispiel für das entwickelte Protokoll wurden acht Styraxproben (R1-R8) für die Quantifizierung der phenolischen Verbindungen, **1**-**5**, und **6**, einer phenolischen Vorstufe, ausgewählt. Die Extraktion wurde mit einem SFE-Gerät mit einer 1:1 Mischung aus Ethanol und CO_2 durchgeführt. Ein Ultra-Performance-Convergency-Chromatographie (UPC^2) SFC-Gerät wurde für die Analyse und Quantifizierung der Verbindungen verwendet. Mit der stationären Phase Acquity UPC^2 BEH 2-EP (3.0 x 100 mm, 1.7 µm) wurde eine Grundlinientrennung der Verbindungen **1**-**6** innerhalb von 4 min erreicht. Die mobile Phase bestand aus überkritischem CO_2 und Methanol mit 0,1 % Phosphorsäure. Die Zugabe von Phosphorsäure reduzierte die Peakschultern, insbesondere jene der Säuren **4** und **6** effektiv, indem ihr protonierter, ungeladener Zustand sichergestellt wurde. Die vorherrschenden Verbindungen **2**, **4** und eine dritte, unbekannte Verbindung (Verbindung **7**) wurden aus R6 mittels präparativer SFC (Prep-15) isoliert und rein dargestellt. Die präparative Methode wurde innerhalb von 6 min mit einem Maßstabsverfahren der UPC^2-Methode unter Verwendung der Viridis BEH 2-EP (10 x 250 mm; 5 µm) als Säulenmaterial durchgeführt. Zusätzlich zu dem auf diesen Ergebnissen basierenden Manuskript wurde Verbindung **7** durch Interpretation von 1D- und 2D-NMR-Spektren als 3-Phenylpropyl-(E)-cinnamat strukturell aufgeklärt. Weiters wurden **5** und **6** aus Vanilleschoten und Sternanis extrahiert und quantifiziert, da sie in den Styrax-Proben kaum beziehungsweise nicht vorhanden waren. Dies wurde mit den gleichen Methoden wie für die Styraxproben durchgeführt.

Als weiterer Teil dieser Diplomarbeit wurde mit SFE ein Extraktionsprotokoll für Triterpene aus dem Medizinalpilz *Ganoderma lucidum* erstellt.

2 Aim of the Work

The goal of this work was to explore the suitability of supercritical fluid technologies (SFx) for the extraction, quantification and isolation of polar natural compounds. We decided to concentrate on polar constituents, as almost no application examples have been published on polar analytes investigated by supercritical fluid devices. Hence, we decided to explore the polarity limit for SFx technologies.

Styrax, the balsam obtained from the bark of *Liquidambar* tree species, is known for its polar and volatile constituents. It was the ideal natural product to probe the polarity limit of supercritical fluid applications and an application example for the so called SFx workflow. This workflow included the extraction, quantification, and isolation of six polar constituents, each with devices that operate with supercritical CO_2. Samples of American, Asian (native in Minor Asia), and Chinese styrax, were acquired. Different *Liquidambar* tree species occur in certain areas of these continents of origin. Accordingly, the composition of styrax constituents varies and an analytical comparison was made.

Furthermore, the validation of the method for the quantification of the polar constituent was envisaged.

3 Results and Discussion

3.1 Part I - Manuscript

In the course of this diploma thesis the vast majority of the work resulted in the publication of a paper. It was published in the journal Planta Medica in August 2017 (Scheuba et al., 2017).

My contribution was, first of all, the practical laboratory work, with the introductory support of my co-supervisor. This included the acquisition and preparation of the samples for the extraction, as well as the extraction, analysis, quantification, evaluation of obtained data, isolation, and structure elucidation. Final decisions were made by my co-supervisor, Mag. Dr. Ulrike Grienke, who was open for my opinion and contribution concerning strategical questions. I did that by introducing and discussing my ideas for the further approach. I contributed the most time and effort into the writing of the paper and therefore, I was named as first author.

Original Papers

Thieme

Fast and Green – CO_2 Based Extraction, Isolation, and Quantification of Phenolic Styrax Constituents*

Authors
Johanna Scheuba, Valerie-Katharina Wronski, Judith M. Rollinger, Ulrike Grienke

Affiliation
Department of Pharmacognosy, University of Vienna, Vienna, Austria

Key words
Liquidambar orientalis, Hamamelidaceae, supercritical fluid extraction, supercritical fluid chromatography, ultra-performance convergence chromatography, styrax

received January 25, 2017
revised February 23, 2017
accepted March 4, 2017

Bibliography
DOI http://dx.doi.org/10.1055/s-0043-105499
Published online March 16, 2017 | Planta Med 2017; 83: 1068–1075 © Georg Thieme Verlag KG Stuttgart · New York | ISSN 0032-0943

Correspondence
Univ.-Ass. Mag. pharm. Dr. Ulrike Grienke
Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna
Althanstrasse 14, 1090 Vienna, Austria
Phone: + 43 1 4 27 75 52 62, Fax: + 43 1 4 27 78 55 2 62
Ulrike.Grienke@univie.ac.at

Supporting information available online at http://www.thieme-connect.de/products

ABSTRACT

In this study the first supercritical fluid based protocol for the extraction, analysis, and isolation of six polar compounds, i.e., o-vanillin (**1**), styracin (**2**), vanillin (**3**), trans-cinnamic acid (**4**), vanillic acid (**5**), and shikimic acid (**6**), was developed. First, eight styrax resin products (R1–R8) obtained from various *Liquidambar* tree species, which are known to contain compounds **2**–**6**, were extracted with a 1 : 1 mixture of supercritical CO_2 and EtOH. Within 4 minutes, the compounds were successfully baseline separated on an Acquity UPC² BEH 2-EP (3.0 × 100 mm, 1.7 µm) column using a mobile phase of supercritical CO_2 and MeOH with 0.1% phosphoric acid. The compounds were quantified and the method was validated according to current ICH guidelines. Scaling up to preparative supercritical fluid chromatography using a Viridis BEH 2-EP (10 × 250 mm, 5 µm) column allowed for a fast separation and isolation of the selected constituents **2** and **4** from R6 within 7 minutes. This supercritical fluid protocol is easily adaptable to compounds of similar polarity. The increase in speed and its environmental friendliness underline its superiority over conventional set-ups.

ABBREVIATIONS

SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SFx	supercritical fluid
UPC²	ultra-performance convergence chromatography

Introduction

In the recent past, supercritical fluid chromatography (SFC) has undergone a real boom, which is not simply a revival of an old technology from the early 1960s [1, 2]. SFC has re-emerged with various new facets, e.g., as ultra-performance convergence chromatography (UPC²), combining SFC with revolutionary stationary phases with sub-2 µm particles [3]. Besides instrumental improvements in the field of analytics, supercritical fluid (SFx) technology has found entrance into sample preparation processes, e.g., supercritical fluid extraction (SFE) [4], and preparative scale applications [5, 6].

In principle, SFC is like HPLC, however, instead of an aqueous solution SFC runs with supercritical CO_2 as the primary mobile phase. The inert and environmentally neutral CO_2 is converted to a supercritical fluid above 31 °C and 74 bar revealing a polarity

* Dedicated to Professor Dr. Max Wichtl in recognition of his outstanding contribution to pharmacognosy research.

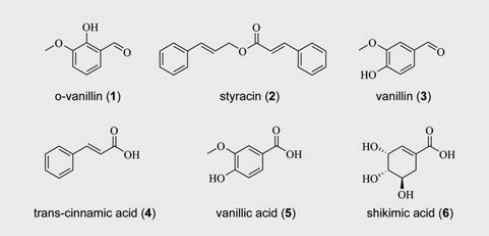
similar to heptane. This is why SFC is especially suitable for non-polar compounds. However, solvent strength can be adjusted by adding miscible organic co-solvents up to 50% which makes an analysis of more polar compounds possible. Moreover, to improve peak shapes (e.g., for compounds with ionisable features) and to promote selectivity, a third component, i.e., an acidic or basic additive, can be included in low concentrations in the mobile phase [7]. The increasing popularity of SFC is also based on high efficiency separations with short analysis times which are reached due to the high diffusivity and low viscosity of the mobile phase [8].

Its versatility makes SFx technology attractive for extraction, separation, and purification of compounds over a broad range of polarities. However, the polarity limit for compounds that can be extracted, analysed, or purified with SFE and SFC is not yet defined and the application of modern SFx technology in the field of natural product research is still at an early stage [9]. Whilst analytical questions regarding the quantitation of various pharmaceutically relevant natural product structure classes have been increasingly addressed with UPC^2 (with or without hyphenation to MS) [10–16], reports about the scale-up from analytical to preparative SFC or complete SFx workflows starting from extraction to analytics and preparative scale purification are still scarce [4, 17, 18].

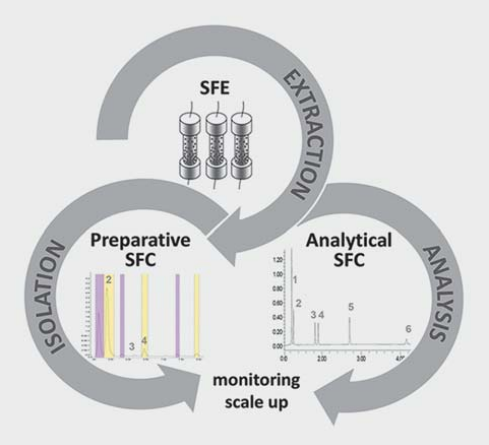
Hence, this study was set up to evaluate the suitability of SFx technologies for polar natural compounds regarding extraction, analysis, and isolation. For this endeavour, six small molecules including o-vanillin (**1**), styracin (**2**), vanillin (**3**), trans-cinnamic acid (**4**), vanillic acid (**5**), and shikimic acid (**6**) were selected (▶ **Fig. 1**). Except for compound **6**, which is a metabolic phenolic precursor, these compounds can be classified as phenolics. Besides compound **1**, which is a position isomer of **3**, all of the selected compounds are known as polar constituents of styrax [19–23].

Styrax (synonyma: Liquidambaris resin, oriental sweetgum, storax) is a resinous sap collected as exudate from the damaged bark of four tree species including *Liquidambar orientalis* Mill. (growing in the Southwest of Turkey), *Liquidambar formosana* Hance, *Liquidambar acalycina* H. T. Chang (both native to Eastern Asia), and *Liquidambar styraciflua* L. (growing in Eastern North America and Central America). All species belong to the Hamamelidaceae family [24, 25].

In their countries of origin, the resins are traditionally used for the treatment of peptic ulcer symptoms, skin problems, and coughs [25, 26]. Moreover, antihypertensive [25], wound healing [26], anticonvulsant [27], and antibacterial effects [28] have been reported. Well-studied constituents of styrax are volatile components present in the essential oil and triterpenoids [29–32]. Phenolic styrax constituents, however, have been studied to a lesser extent, although being of relevance as bioactive plant constituents, food additives, and synthetic building blocks [33–38]. Hence, as an application example for the developed protocol, the goal was to quantitatively analyse **1**–**6** in eight authentic styrax products (R1–R8) originating from Turkey, Honduras, and China. In previous years, different gas- and liquid-chromatography approaches, e.g., HPLC-PDA-fluorimetry [38], GC and/or GC-MS [38–42], have been developed to characterise volatile and non-volatile styrax components with or without chromophore groups.



▶ **Fig. 1** Chemical structures of standards.



▶ **Fig. 2** Schematic overview on the applied supercritical fluid workflow.

All these methods are characterised by often laborious sample preparation and long analysis times.

Finally, preparative scale SFC was used to isolate selected phenolic compounds (**2** and **4**) based on optimised conditions. To the best of our knowledge, this study is the first report on the evaluation of the suitability of SFx technologies for polar natural compounds with the aim to rapidly extract, analyse, and isolate them in one workflow.

Results and Discussion

As a starting point for this CO_2 based workflow (▶ **Fig. 2**), a UPC^2 method was generated using a standard mix of polar compounds previously reported as ingredients of styrax (**2**–**6**) and o-vanillin (**1**), which we included as position isomer of vanillin (**3**).

Method development was accomplished in three steps: (i) rapid column and co-solvent screening with a generic gradient,

▶ **Table 1** Optimised parameters for SFE, UPC², and preparative SFC.

SFx technique	SFC						SFE (MV-10)		
scale	analytical (UPC²)			preparative (Prep-15)					
injection volume [µL]	1			250			–		
flow rate [mL/min]	1.5			15			10		
wavelength [nm]	220			220			–		
mobile phase A	CO_2			CO_2			CO_2		
mobile phase B	MeOH + 0.1 % phosphoric acid			MeOH			EtOH		
gradient	**time [min]**	**% A**	**% B**	**time [min]**	**% A**	**% B**	**time [min]**	**% A**	**% B**
	0	100	0	0	95	5	0	50	50
	2.5	75	25	4	75	25	10	50	50
	4.5	75	25	8	75	25			
	4.6	100	0	10	95	5			
	5.0	100	0	11	95	5			
back pressure	2000 psi (~ 138 bar)			100 bar			300 bar		
temperature [°C]	50			50			40		
stationary phase	Acquity BEH 2-EP (3.0 × 100 mm, 1.7 µm)			Viridis BEH 2-EP (10 × 250 mm, 5 µm)			–		

(ii) defined gradient optimisation with a specified stationary phase chemistry and co-solvent, and (iii) detailed optimisation regarding additive, flow rate, column temperature, and back pressure.

For the first step, four different co-solvents [MeOH, EtOH, isopropanol (IPA), and CH_3CN] were probed with a generic gradient (from 0% to 25% co-solvent) on four different stationary phases (same dimensions of 3.0 × 100 mm). These achiral column chemistries included 2-ethyl pyridine bound to ethylene bridged hybrid particles (BEH 2-EP, 1.7 µm), plain ethylene bridged hybrid particles (BEH, 1.7 µm), fluoro-phenyl based on charged surface hybrid technology (CSH FP, 1.7 µm), and a C18 material based on high-strength silica technology (HSS C18 SB, 1.8 µm). As a result of this screening, MeOH evolved as best suitable co-solvent and BEH 2-EP was the stationary phase giving the best separation (**Fig. S1**, Supporting Information).

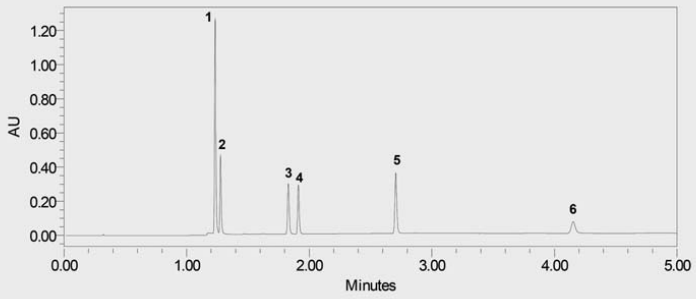
Hence, in step two, variations of gradients with and without isocratic steps were evaluated on BEH 2-EP using CO_2 and MeOH as co-solvent (data not shown). In a third step, the effects of different acidic additives to the co-solvent were evaluated to reduce the tailing of these moderately acidic compounds. Out of the tested additives (**Fig. S2**, Supporting Information), 0.1% phosphoric acid showed the best result on peak shape. The impact of different flow rates on the retention time was evaluated in particular with respect to the challenging separation of **1** and **2**. While considering a fast separation time of all analytes, the best resolution was achieved with a flow rate of 1.5 mL/min. Elevating the back pressure slightly increased the retention time especially for later eluting compounds but had only little influence on peak resolution (data not shown). For ideal conditions depicted in ▶ **Table 1**, a temperature of 50 °C was selected and the automatic back pressure regulator (ABPR) was set to 2000 psi. A chromatogram showing the separation of the six compounds with the finally optimised method is depicted in ▶ **Fig. 3**.

As an application example for the established UPC² method, eight authentic styrax resin products (R1 to R8) originating from Turkey (R1, R2, R3), Honduras (R4, R5, R6) and China (R7, R8) were selected. For R3, R5, and R6 a direct solution of the viscous crude material was possible in EtOH followed by sonication and centrifugation prior to the analysis. The five other products (R1, R2, R4, R7, and R8) were not directly soluble in EtOH and needed to be extracted from their matrices.

To generate extracts of these natural materials (R1, R2, R4, R7, and R8), an SFE method was established whereby the optimisation followed a similar protocol as described before for UPC², except that the parameter of column chemistry was not applicable. For SFE method development, the impact of three co-solvents (in different percentages) was evaluated in isocratic mode. Extractions with 10%, 20%, and 30% of MeOH, EtOH, or IPA were conducted. The generated extracts were further analysed by UPC² revealing that both MeOH and EtOH served as best suitable co-solvents to extract the polar compounds (**1**–**6**) of interest. For further optimisation, EtOH was selected as preferred co-solvent since it is less toxic and also better to use from a food-grade perspective. In the final SFE method, 50% of EtOH was used as co-solvent to obtain the highest yields of the focused polar constituents (▶ **Table 1**).

Following SFE method development, extraction efficiency was determined with sample R4. Therefore, the plant material was extracted four times for 9 min, respectively. Since over 95% of compound **4**, which was used as a reference, were extracted within the first 9 min (data not shown), the final extraction time was set to 10 min to ensure exhaustive extraction.

To confirm that the generated protocols are reliable and suitable for the quantification of compounds **1**–**6** in styrax samples



▶ **Fig. 3** UPC² chromatogram of standard mixture containing compounds **1** to **6**. Conditions as described in the Materials and Methods section and compound numbering according to ▶ **Fig. 1**.

▶ **Table 2** Summarised UPC² method validation data.

parameter	compound					
	1	**2**	**3**	**4**	**5**	**6**
regression equation (y =)	3270x – 264	2350x – 373	2720x – 448	3460x – 3540	4410x – 5250	1740x – 9780
correlation coefficient (R^2 =)	0.9999	0.9998	0.9996	0.9998	0.9994	0.9973
linearity range [µg/mL]	0.167–122	0.160–117	0.167–122	0.150–109	0.145–106	0.139–101
LOD [µg/mL]	0.1	0.1	0.1	0.2	0.1	3.7
LOQ [µg/mL]	0.5	1.4	1.5	2.7	1.3	11.2
precision						
▪ intra-day[a]	n. d.[b]	0.1	0.2	0.1	n. d.	n. d.
▪ inter-day[c]	n. d.	0.7	3.0	6.3	n. d.	n. d.
accuracy[d]						
▪ high spike	n. d.	75.0	72.6	75.3	n. d.	n. d.
▪ medium spike	n. d.	94.8	82.4	87.4	n. d.	n. d.
▪ low spike	n. d.	85.7	76.8	81.8	n. d.	n. d.

[a] standard deviation within one day based on peak area in percent (n = 5), [b] n. d. = not determined since compounds were not detected in sample R4, [c] standard deviation over three days based on peak area in percent (n = 3), [d] expressed as recovery rate in percent

(R1–R8), the procedure was validated according to ICH guidelines [43]. Results of this validation including the evaluation of linearity, limit of detection (LOD), limit of quantification (LOQ), as well as precision and accuracy are presented in ▶ **Table 2**.

The calibration curves were obtained by injecting a series of the six compound solutions at increasing concentrations. The calculated linearity ranged from 0.139 to 122 µg/mL. Linear regression analysis showed a good linearity for compounds **1** to **5** ($R^2 \geq 0.9994$), with the limits of detection (LOD) at 0.1 µg/mL (**1**–**3**, and **5**) or 0.2 µg/mL (**4**) and the limits of quantification (LOQ) between 0.5 µg/mL (**1**) and 2.7 µg/mL (**4**) (▶ **Table 2**).

The validation values obtained for compound **6** differed significantly from the other compounds which can be explained by the missing phenolic character of this compound and therefore a generally lower response with UV detection.

The precision of the developed method was assessed by performing intra-day and inter-day variation experiments. The RSD of intra-day and inter-day were in the range of 0.1% to 0.2% and 0.7% to 6.3%, respectively, suggesting a good precision of this method.

All eight resin samples (R1 to R8) were extracted and quantified according to the developed SFx protocol. The UPC² chromatograms of the eight samples are shown in **Fig. S3**, Supporting Information. The compounds were assigned to standards by matching retention times and UV spectra. The quantitative composition of the six focused polar compounds in the eight commercial styrax samples varied significantly (▶ **Table 3**). For instance,

▶ **Table 3** Percentage of polar styrax compounds (**1**–**6**) in investigated resin samples (R1–R8; n = 3).

cpd	samples (origin)							
	R1 (Turkey)	R2 (Turkey)	R3 (Turkey)	R4 (Honduras)	R5 (Honduras)	R6 (Honduras)	R7 (China)	R8 (China)
1	n. d.[a]	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
2	0.31 ± 0.00	1.98 ± 0.01	22.2 ± 0.06	0.06 ± 0.00	24.6 ± 0.02	26.4 ± 0.11	0.03 ± 0.00	0.61 ± 0.00
3	0.70 ± 0.01	0.34 ± 0.00	0.22 ± 0.00	0.50 ± 0.00	0.44 ± 0.00	0.41 ± 0.00	n. d.	0.16 ± 0.00
4	2.70 ± 0.05	1.76 ± 0.01	0.41 ± 0.01	0.80 ± 0.00	2.36 ± 0.01	2.42 ± 0.01	n. d.	0.15 ± 0.00
5	0.10 ± 0.00	0.05 ± 0.00	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
6	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.

[a] n. d. = not detectable

the highest percentages of compound **2** were found in R3, R5, and R6 (between 22.2% and 26.4%), whereas almost none of the six focused compounds could be detected in R7. Moreover, compounds **1** and **6** were not found in any of the investigated samples. For compound **1**, this is not surprising, since it has not been reported as a constituent of styrax resins before, and was included in this study to investigate, if it is possible to successfully separate it from its position isomer compound **3**. Interestingly, compound **6** was not detected in any of the resin products, although the seeds and bark of *L. styraciflua* were previously mentioned as source for **6** to be used for the semi-synthesis of oseltamivir, a well-established anti-influenza drug [44, 45].

As mentioned before, the investigated resin products originate from different countries and therefore different plant species. According to the literature, resins from Turkey (R1, R2, and R3) derived from *L. orientalis*, are known to contain around 2% of compound **3** and 4–30% of compound **4** as well as around 20% of compound **2** [22, 46]. Taking into account the results of the present study, much lower amounts of these compounds have been found except for compound **2** which was contained in R3 in a similar amount as described previously. Regarding resin samples from Honduras (R4, R5, and R6) derived from *L. styraciflua*, 5 to 10% of compounds **2** and **4** as well as trace amounts of compound **3** are known [46]. In this study, the determined percentages of compounds were outside of these ranges. Concerning the Chinese resin products (R7 and R8) derived from *L. formosana* or *L. acalycina*, no values for the investigated compounds were given in the literature. Moreover, based on the content of the polar compounds analysed, a clear discrimination of the resin samples from different origins was not visible.

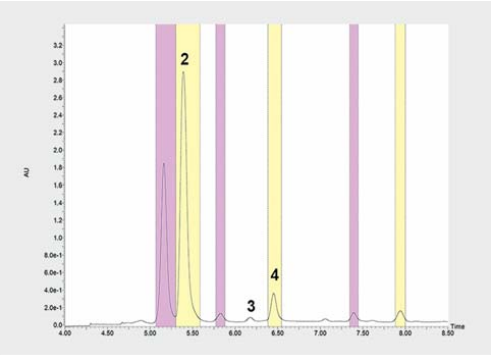
For the final validation of the SFE and SFC method, a spike recovery test was performed to evaluate the accuracy of the method. Therefore, standard compounds **2, 3**, and **4** were prepared in different amounts at three levels (low, medium, and high) and subsequently spiked into styrax sample R4, where the content of the focused compounds had already been determined before. Compounds **1, 5** and **6** were not used for accuracy experiments since they were not detected in the originally analysed sample R4. The spiked samples were extracted and analysed according to the established protocol. As presented in ▶ **Table 2**, good recovery percentages were found for all three standard compounds, indicating that the presented method is suitable for the analysis of polar phenolic compounds in styrax samples.

As a last step in this SFx workflow, we performed a scale up of the UPC^2 method to the preparative SFC (Prep-15) for validating its suitability to purify and isolate the respective polar compounds. Since none of the investigated resin products contained all six compounds, R6 and the isolation of its two main analytes **2** and **4** were selected as an application example. The optimised UPC^2 conditions were transferred to the preparative instrument with only little adaption (▶ **Table 1**). This is due to a couple of differences between analytical and preparative SFC. For instance, modifier-stream injection used with the preparative device introduces the sample into the co-solvent stream before it is mixed with the CO_2. Accordingly, an initial concentration of 5% co-solvent is mandatory. Moreover, non-volatile acidic additives, such as phosphoric acid, should be avoided for preparative SFC applications for reasons of concentrating the acid during the isolation step. Therefore, the original UPC^2 gradient was slightly adapted and no additive was used resulting in a decrease of peak shape. The parameters for the final scale-up to the preparative SFC are depicted in ▶ **Table 1**.

Using this protocol, the two selected phenolic compounds, **2** and **4**, could be successfully isolated from R6 (▶ **Fig. 4**; **2** at 5.42 min, **4** at 6.45 min). For the proof of purity and identity, the respective fractions/pure compounds were analysed by HPLC-PDA, HPLC-ESI-MS, and comparison of MS fractionation patterns of standard compounds **2** and **4** with the respective isolates (**Figs. S4** and **S5**, Supporting Information). They both showed a purity of > 95% determined by HPLC-ESI-MS.

Isolating these two compounds from the complex extract of R6 was a final proof of concept, offering the possibility of an overall workflow, going from extraction and quantification to isolation, all with liquid CO_2 based mobile phases.

Comparing this protocol with conventional workflows [38–42], the SFx based approach is a cost-saving environmental friendly alternative characterised by high efficiency and selectivity. Moreover, time-consuming sample preparation protocols (e.g., distillation and silylation for GC) and typical analysis times of more than 50 min are considerable disadvantages of standard methods.



▶ **Fig. 4** Preparative scale SFC separation of constituents present in R6 (c = 5 mg/mL). The shaded areas correspond to collected fractions (fractions containing compounds **2** and **4** are indicated).

The developed workflow is easily adaptable and has already been successfully applied in our lab to vanilla pods and star anise for the extraction and analysis of **3** and **6** (data not shown). Especially, in respect to the need for highly pure reference standards for quality control of herbal drugs or the demand for food-grade extraction, SFx workflows offer many new possibilities which will definitely be in the focus of future natural product research.

Materials and Methods

Standards, samples, and solvents

The standard compounds o-vanillin (**1**; purity 99%), styracin (**2**; purity ≥ 95%), vanillin (**3**; ≥ 97%), trans-cinnamic acid (**4**; purity ≥ 99%), and vanillic acid (**5**; purity ≥ 97%) were purchased from Sigma Aldrich. Shikimic acid (**6**; purity ≥ 95%) was obtained from the US National Cancer Institute. Standard stock solutions were prepared at a concentration of 0.1 mg/mL in *n*-hexane/IPA (7/3) and kept in 10 mL volumetric flasks at 8 °C prior to analysis.

The eight investigated commercial resin products (named R1 to R8) were purchased from different online providers (batch/lot numbers if given: R5: RB002; R7: SC092013). They were all declared as "Styrax" or "Liquidambaris resin". Their origins are Turkey (R1, R2 and R3), Honduras (R4, R5, and R6), and China (R7, R8). Voucher specimens of all samples are stored at the Department of Pharmacognosy, University of Vienna, Austria; voucher specimen numbers: JR-20121107-A1 (R1), JR-20121107-A2 (R2), JR-20160920-A2 (R3), JR-20121107-A3 (R4), JR-20160916-A1 (R5), JR-20160920-A1 (R6), JR-20140115-A1 (R7), JR-20130703-A1 (R8).

Solvents were of HPLC grade and purchased from VWR Chemicals. Compressed 4.5 grade CO_2 with a purity ≥ 99.995% (purchased from Messer) was used.

Supercritical fluid extraction

Extractions were performed on a Waters MV-10 SFE system comprising a fluid delivery module (connected to an Accel 500 LC chiller by Thermo Scientific), a Waters column oven holding the extraction cells, a back pressure regulator, a heat exchanger, a make-up pump, and an extract collector. The operating software was Waters ChromScope 1.6. The system was used with 5 mL extraction vessels. 500 mg of ground resin product (R1, R2, R4, R7, and R8) was placed in the extraction vessel and filled up with inert diatomaceous earth (Thermo Fisher Scientific). The optimum extraction method is presented in ▶ **Table 1**. The obtained extracts were transferred to volumetric flasks and filled up to 100 mL with EtOH. Samples were stored at 8 °C until analysis.

Analytical SFC device and separation conditions

For analytical experiments a Waters Acquity UPC^2 instrument consisting of a convergence-, sample-, binary solvent-, and column manager as well as a PDA detector were used. The operating software was Empower 3. The best separations were reached with parameters given in ▶ **Table 1**. An equilibration of 1.6 min with initial conditions was done before each run.

Method validation

For method validation, a dilution series for each of the six standards was prepared. The initial standard stock solution had a concentration of 0.1 mg/mL in a mixture of *n*-hexane/IPA (7/3). By serial dilution using a 1 : 3 ratio, further calibration levels were obtained. Each level was injected in triplicates and integration of the peaks was performed on Empower 3 software. Limit of detection (LOD) and limit of quantification (LOQ) values were evaluated visually as the concentration of the signal-to-noise ratio of at least 3 and 10, respectively. For selectivity confirmation, PDA data including the peak purity tool of the Empower 3 software were used. Precision was assessed by performing intra-day (evaluation within one day) and inter-day (evaluation over three days) experiments with sample R4. For accuracy, recovery rates were determined for R4 samples spiked with high (125%), medium (100%), or low (75%) amounts of respective standard compound. The summarised validation data can be found in ▶ **Table 2**.

Preparative scale SFC device, separation, and fractionation conditions

A preparative SFC system from Waters Corporation, Prep-15 SFC, was used for the purification of the two major phenolic compounds **2** and **4**. The system comprises a fluid delivery module (connected to an Accel 500 LC chiller by Thermo Scientific), a Waters 2767 sample manager, a ten-port column oven, a back pressure regulator, a heat exchanger, a make-up pump, a Waters 2998 PDA, and a Waters 2424 ELSD. The system was operated by the software MassLynx V4.1. The optimised preparative SFC method parameters are presented in ▶ **Table 1**. MeOH was used as a make-up solvent (flow rate: 5 mL/min) and fractionation was triggered by a minimum peak intensity threshold of 70 000.

Evaluation of purity and identity via HPLC-MS

The collected SFC fractions were analysed on a HPLC-MS system: UltiMate 3000 RSLC-series system (Dionex/Thermo Fisher Sci-

entific) hyphenated to an HCT 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (Bruker Daltonics). As a stationary phase a Phenomenex HyperClone DS (C18) (4.6 × 150 mm, 5 µm) column was used. The mobile phase contained water with 0.1% acetic acid and 0.9% formic acid (A) and acetonitrile (B). The gradient started at 10% B rising to 98% B in 20 min, keeping that percentage for 10 min. Flow rate, temperature, and detection wavelength were set to 1 mL/min, 35 °C, and 270 nm, respectively. The ESI ion source was operated as follows: capillary voltage: + 3.5/-3.7 kV, nebulizer: 26 psi (N_2), dry gas flow: 9 L/min (N_2), and dry temperature: 340 °C.

Supporting information

Chromatograms (UPC²) showing the method development and the eight resin products (R1–R8) with optimised parameters, as well as chromatograms (HPLC) of isolated pure compounds **2** and **4** including relevant mass spectra are available as Supporting Information.

Acknowledgements

The authors thank the Waters Corporation for providing extended instrument access to the UPC² and the MV-10 device and in particular, I. François, M. Gingras, L. Montis, and T. Polly for technical support and discussions.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Saito M. History of supercritical fluid chromatography: Instrumental development. J Biosci Bioeng 2013; 115: 590–599

[2] Klesper E, Corwin AH, Turner DA. High pressure gas chromatography above critical temperatures. J Org Chem 1962; 27: 700–701

[3] Novakova L, Perrenoud AGG, Francois I, West C, Lesellier E, Guillarme D. Modern analytical supercritical fluid chromatography using columns packed with sub-2 µm particles: A tutorial. Anal Chim Acta 2014; 824: 18–35

[4] Sánchez-Camargo AD, Parada-Alfonso F, Ibáñez E, Cifuentes A. On-line coupling of supercritical fluid extraction and chromatographic techniques. J Sep Sci 2017; 40: 213–227

[5] Rajendran A. Design of preparative-supercritical fluid chromatography. J Chromatogr A 2012; 1250: 227–249

[6] Ebinger K, Weller HN, Kiplinger J, Lefebvre P. Evaluation of a new preparative supercritical fluid chromatography system for compound library purification: The TharSFC SFC-MS Prep-100 system. J Lab Autom 2011; 16: 241–249

[7] Lemasson E, Bertin S, West C. Use and practice of achiral and chiral supercritical fluid chromatography in pharmaceutical analysis and purification. J Sep Sci 2016; 39: 212–233

[8] Desfontaine V, Guillarme D, Francotte E, Novakova L. Supercritical fluid chromatography in pharmaceutical analysis. J Pharmaceut Biomed 2015; 113: 56–71

[9] Hartmann A, Ganzera M. Supercritical fluid chromatography – Theoretical background and applications on natural products. Planta Med 2015; 81: 1570–1581

[10] Winderl B, Schwaiger S, Ganzera M. Fast and improved separation of major coumarins in *Ammi visnaga* (L.) Lam. by supercritical fluid chromatography. J Sep Sci 2016; 39: 4042–4048

[11] Pfeifer I, Murauer A, Ganzera M. Determination of coumarins in the roots of *Angelica dahurica* by supercritical fluid chromatography. J Pharm Biomed Anal 2016; 129: 246–251

[12] Ganzera M. Supercritical fluid chromatography for the separation of isoflavones. J Pharm Biomed Anal 2015; 107: 364–369

[13] Aichner D, Ganzera M. Analysis of anthraquinones in rhubarb (*Rheum palmatum* and *Rheum officinale*) by supercritical fluid chromatography. Talanta 2015; 144: 1239–1244

[14] Qi NL, Gong X, Feng CP, Wang XX, Xu YW, Lin LJ. Simultaneous analysis of eight vitamin E isomers in *Moringa oleifera* Lam. leaves by ultra performance convergence chromatography. Food Chem 2016; 207: 157–161

[15] Zhao TJ, Qi HY, Chen J, Shi YP. Quantitative analysis of five toxic alkaloids in *Aconitum pendulum* using ultra-performance convergence chromatography (UPC²) coupled with mass spectrometry. Rsc Adv 2015; 5: 103869–103875

[16] Murauer A, Ganzera M. Quantitative determination of lactones in *Piper methysticum* (Kava-Kava) by supercritical fluid chromatography. Planta Med 2017; DOI: 10.1055/s-0043–100632

[17] Song W, Qiao X, Liang WF, Ji S, Yang L, Wang Y, Xu YW, Yang Y, Guo DA, Ye M. Efficient separation of curcumin, demethoxycurcumin, and bisdemethoxycurcumin from turmeric using supercritical fluid chromatography: From analytical to preparative scale. J Sep Sci 2015; 38: 3450–3453

[18] Zhao Y, McCauley J, Pang X, Kang LP, Yu HS, Zhang J, Xiong CQ, Chen R, Ma BP. Analytical and semipreparative separation of 25 (R/S)-spirostanolsaponin diastereomers using supercritical fluid chromatography. J Sep Sci 2013; 36: 3270–3276

[19] Pastorova I, Weeding T, Boon JJ. 3-phenylpropanylcinnamate, a copolymer unit in Siegburgite fossil resin: a proposed marker for the Hammamelidaceae. Org Geochem 1998; 29: 1381–1393

[20] Chalchat JC, Garry RP, Mathieu JP. Composition of the volatile fraction from *Honduras styrax*, *Liquidambar styraciflua* L. J Essent Oil Res 1994; 6: 73–75

[21] Burger P, Casale A, Kerdudo A, Michel T, Laville R, Chagnaud F, Fernandez X. New insights in the chemical composition of benzoin balsams. Food Chem 2016; 210: 613–622

[22] Wang F, Cui HH, Wang SM. Studies on chemical constituents of Styrax. Zhongguo Shiyan Fangjixue Zazhi 2011; 17: 89–91

[23] Hafizoglu H. Analytical studies on the balsam of *Liquidambar orientalis* Mill by gas-chromatography and mass-spectrometry. Holzforschung 1982; 36: 311–313

[24] Hoey MT, Parks CR. Isozyme divergence between Eastern Asian, North-American, and Turkish Species of *Liquidambar* (Hamamelidaceae). Am J Bot 1991; 78: 938–947

[25] Lingbeck JM, O'Bryan CA, Martin EM, Adams JP, Crandall PG. Sweetgum: An ancient source of beneficial compounds with modern benefits. Pharmacogn Rev 2015; 9: 1–11

[26] Ocsel H, Teke Z, Sacar M, Kabay B, Duzcan SE, Kara IG. Effects of oriental sweet gum storax on porcine wound healing. J Invest Surg 2012; 25: 262–270

[27] Guo J, Duan JA, Tang Y, Li Y. Sedative and anticonvulsant activities of styrax after oral and intranasal administration in mice. Pharm Biol 2011; 49: 1034–1038

[28] Sağdic O, Ozkan G, Ozcan M, Ozcelik S. A study on inhibitory effects of Sigla tree (*Liquidambar orientalis* Mill. var. *orientalis*) storax against several bacteria. Phytother Res 2005; 19: 549–551

[29] Lee YS, Kim J, Lee SG, Oh E, Shin SC, Park IK. Effects of plant essential oils and components from Oriental sweetgum (*Liquidambar orientalis*) on growth and morphogenesis of three phytopathogenic fungi. Pestic Biochem Phys 2009; 93: 138–143

[30] Yao F, Qiu Q, Cui Z, Su D. Chemical components of essential oils from *Liquidambar orientalis* Mill. Yaowu Fenxi Zazhi 2005; 25: 859–862

[31] Fukuda Y, Yamada T, Wada SI, Sakai K, Matsunaga S, Tanaka R. Lupane and oleanane triterpenoids from the cones of *Liquidamber styraciflua*. J Nat Prod 2006; 69: 142–144

[32] Dat NT, Lee IS, Cai XF, Shen G, Kim YH. Oleanane triterpenoids with inhibitory activity against NFAT transcription factor from *Liquidambar formosana*. Biol Pharm Bull 2004; 27: 426–428

[33] Chien SC, Xiao JH, Tseng YH, Kuo YH, Wang SY. Composition and antifungal activity of balsam from *Liquidambar formosana* Hance. Holzforschung 2013; 67: 345–351

[34] Kalogeropoulos N, Konteles SJ, Troullidou E, Mourtzinos I, Karathanos VT. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. Food Chem 2009; 116: 452–461

[35] Fache M, Boutevin B, Caillol S. Vanillin production from lignin and its use as a renewable chemical. Acs Sustain Chem Eng 2016; 4: 35–46

[36] Su D, Yao F, Shi Z. Supercritical carbon dioxide extraction and analysis of chemical components in *Liquidambar orientalis* Mill. Huaxi Yaoxue Zazhi 2005; 20: 409–411

[37] Prince PSM, Dhanasekar K, Rajakumar S. Preventive effects of vanillic acid on lipids, bax, bcl-2 and myocardial infarct size on isoproterenol-induced myocardial infarcted rats: A biochemical and *in vitro* study. Cardiovasc Toxicol 2011; 11: 58–66

[38] Hovaneissian M, Archier P, Mathe C, Culioli G, Vieillescazes C. Analytical investigation of styrax and benzoin balsams by HPLC-PAD-fluorimetry and GC-MS. Phytochem Analysis 2008; 19: 301–310

[39] Gurbuz I, Yesilada E, Demirci B, Sezik E, Demirci F, Baser KH. Characterization of volatiles and anti-ulcerogenic effect of Turkish sweetgum balsam (*Styrax liquidus*). J Ethnopharmacol 2013; 148: 332–336

[40] Ma L, Li WL, Qin LP, Yao S. Identification of volatile components in grafted *Liquidambar orientalis* Mill. Adv Mat Res 2013; 641–642: 773–776

[41] Modugno F, Ribechini E, Colombini MP. Aromatic resin characterisation by gas chromatography-mass spectrometry. Raw and archaeological materials. J Chromatogr A 2006; 1134: 298–304

[42] Fernandez X, Lizzani-Cuvelier L, Loiseau AM, Perichet C, Delbecque C, Arnaudo JF. Chemical composition of the essential oils from Turkish and Honduras Styrax. Flavour Frag J 2005; 20: 70–73

[43] ICH (The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use). Guideline: Validation of analytical Procedures: Text and Methodology, Q2(R1). Geneva: ICH: 2005: 1–13

[44] Enrich LB, Scheuermann ML, Mohadjer A, Matthias KR, Eller CF, Newman MS, Fujinaka M, Poon T. *Liquidambar styraciflua*: a renewable source of shikimic acid. Tetrahedron Lett 2008; 49: 2503–2505

[45] Martin E, Duke J, Pelkki M, Clausen EC, Carrier DJ. Sweetgum (*Liquidambar styraciflua* L.): extraction of shikimic acid coupled to dilute acid pretreatment. Appl Biochem Biotechnol 2010; 162: 1660–1668

[46] Hager H, Greiner S, Heubl G, Stahl-Biskup E, Hänsel R, Keller K, Rimpler H, Schneider G. Hagers Handbuch der Pharmazeutischen Praxis: Drogen P–Z Folgeband 2. Berlin: Springer; 2013: 836–843

Supporting Information

Fast and Green - CO_2 Based Extraction, Isolation, and Quantification of Phenolic Styrax Constituents*

Johanna Scheuba, Valerie-Katharina Wronski, Judith M. Rollinger, Ulrike Grienke

Affiliation

Department of Pharmacognosy, University of Vienna, Vienna, Austria

Correspondence

Univ.-Ass. Mag. pharm. Dr. Ulrike Grienke

Department of Pharmacognosy

Faculty of Life Sciences

University of Vienna

Althanstrasse 14

1090 Vienna

Austria

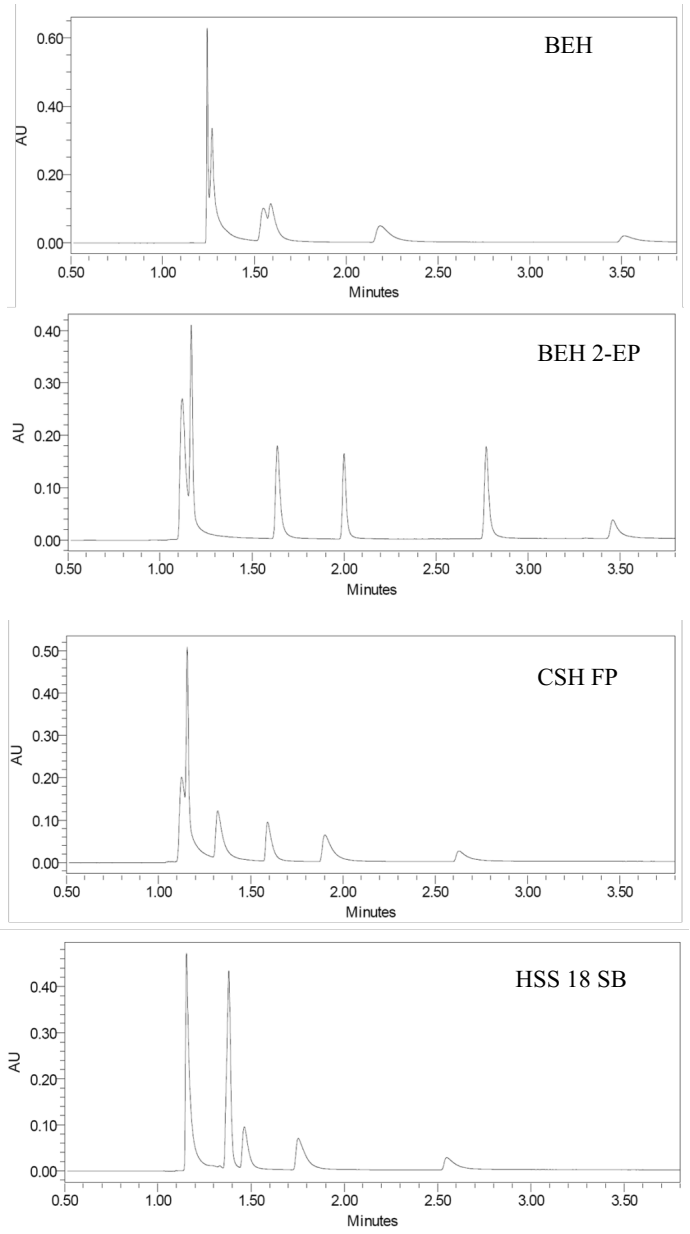
Phone: +43 1 4277 55262

Fax: +43 1 4277 855262

Ulrike.Grienke@univie.ac.at

*Dedicated to Professor Dr. Max Wichtl in recognition of his outstanding contribution to pharmacognosy research.

Fig. S1 Column screening with MeOH as co-solvent: Acquity UPC^2 BEH (1.7 µm), Acquity UPC^2 BEH 2-EP (1.7 µm), Acquity UPC^2 CSH FP (1.7 µm), and Acquity UPC^2 HSS C18 SB (1.8 µm). Column dimensions: 100 mm × 3 mm.



2

Fig. S2 Screening of different additives in MeOH.

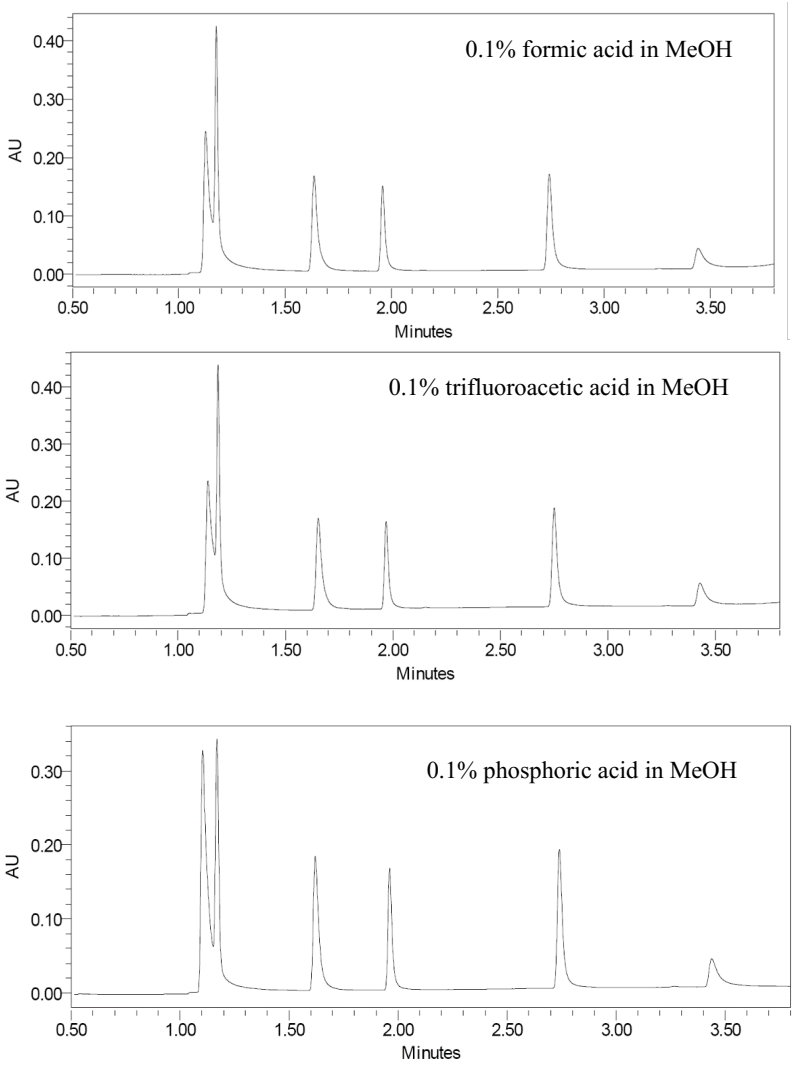
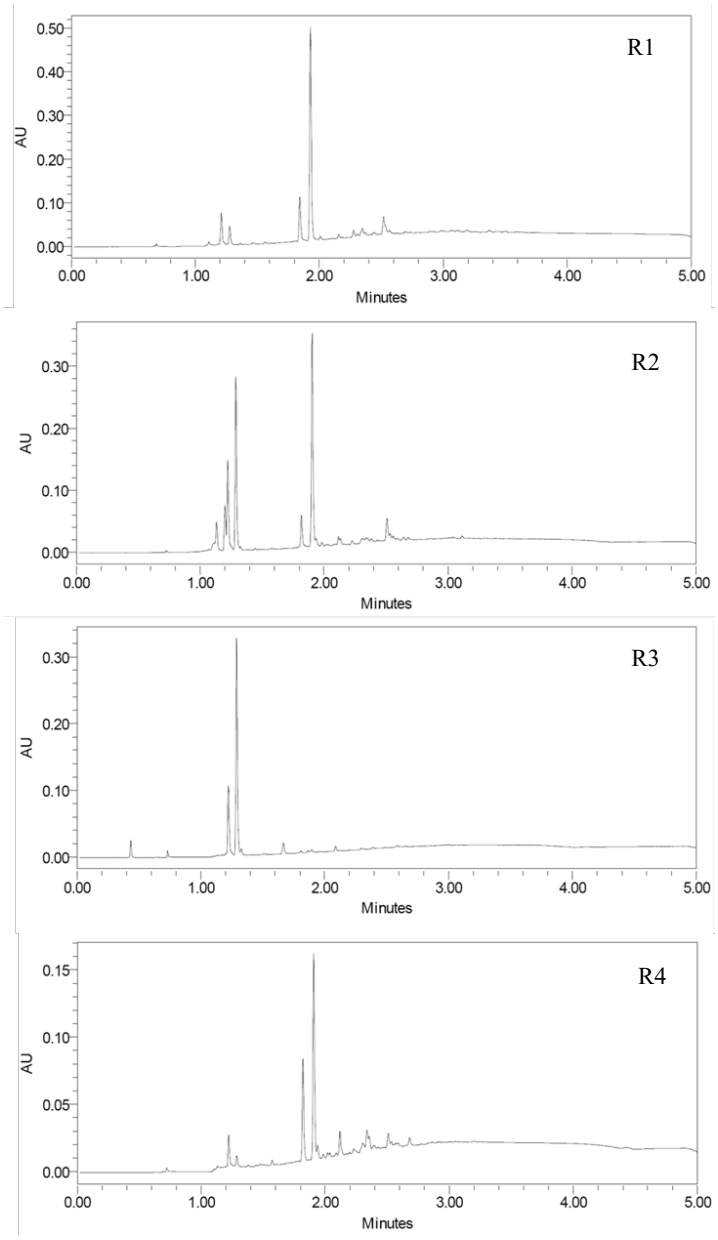
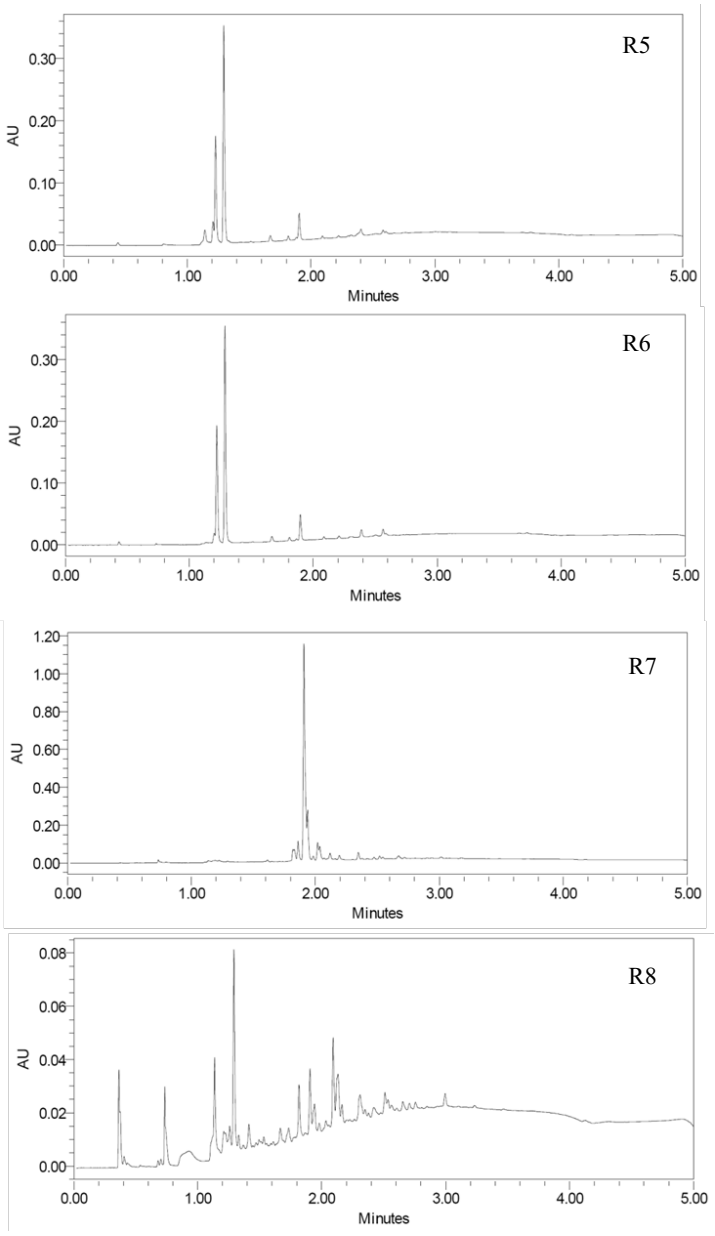


Fig. S3 UPC^2 chromatograms of the eight resin products (R1-R8) with optimised parameters.





5

Fig. S4 Analysis of isolated **2** and **4** by HPLC (> 95% pure).

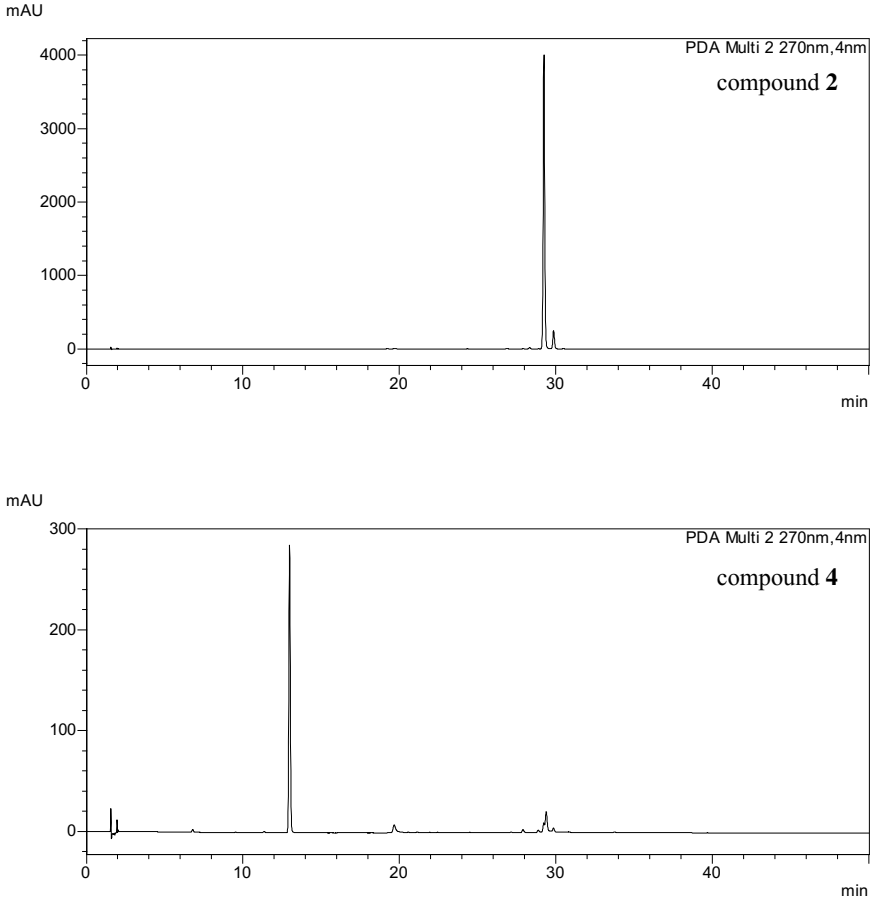
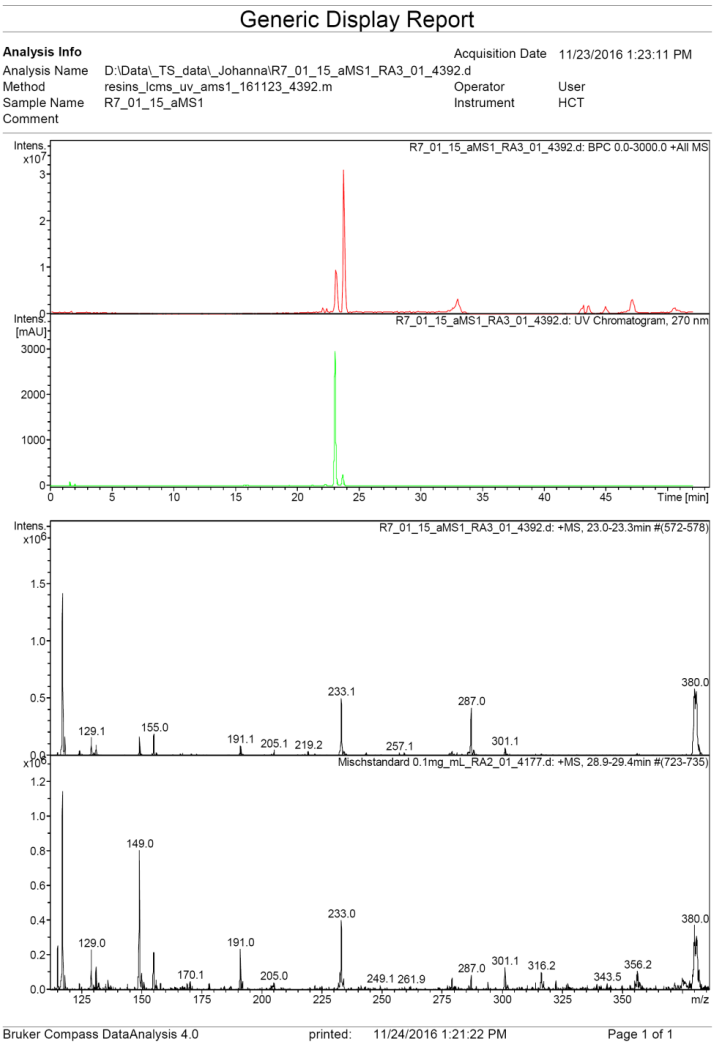
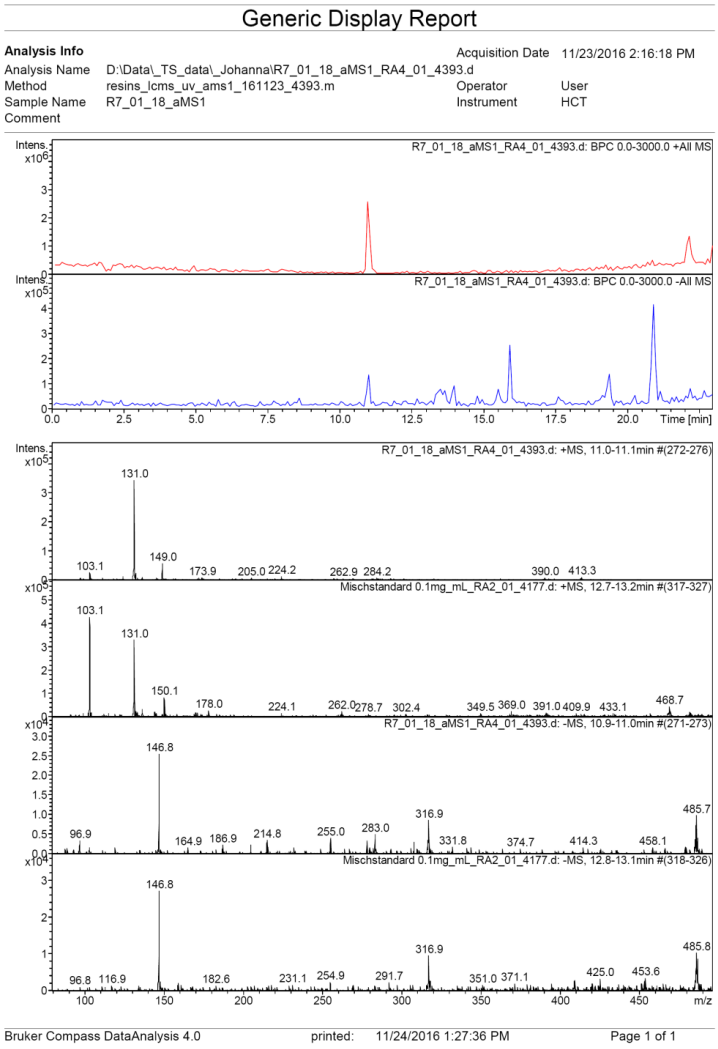


Fig. S5 LC-MS data for determination of purity and identity of isolated **2** and **4**, compared to the fragmentation pattern of the respective standard compounds, respectively.

compound **2**:



compound **4**:



3.2 Part II – Additional Results

3.2.1 Styrax – isolation and structure elucidation of compound 7

As part of the third SFx workflow step, the two main compounds, styracin (**2**) and trans-cinnamic acid (**4**), were isolated from the styrax sample R6 (as described in 3.1 Part I - Manuscript). In the course of that, a third unknown compound (compound **7**) was isolated from R6 as another main compound, besides **2** and **4**. No new method had to be established, because the method applied for the preparative fractionation of R6 was a scale-up method from the UPC^2 analytical method. It is described in 3.1 Part I - Manuscript, Table 1. This demonstrates a huge, time saving advantage of the SFx workflow. Compound **7** eluted slightly before, and baseline seperated from styracin and was therefore collected separately (see Figure 1). The purity of compound **7** was monitored with HPLC-PDA (see Figure 2), and found to be > 95 %. Again, it eluted at a similar retention time compared to styracin (29.86 min for compound **7** and 29.24 min for styracin). (The figure of the HPLC-PDA chromatogram of styracin can be found in 3.1 Part I - Manuscript, Supporting information.) Furthermore, LC-MS data confirmed similar fractionation patterns for compound **7** and styracin. All this suggested a structural similarity of compound **7** and styracin (data not shown).

Figure 1: Preparative scale SFC fractionation of R6 styrax constituents (collected fractions are shaded).

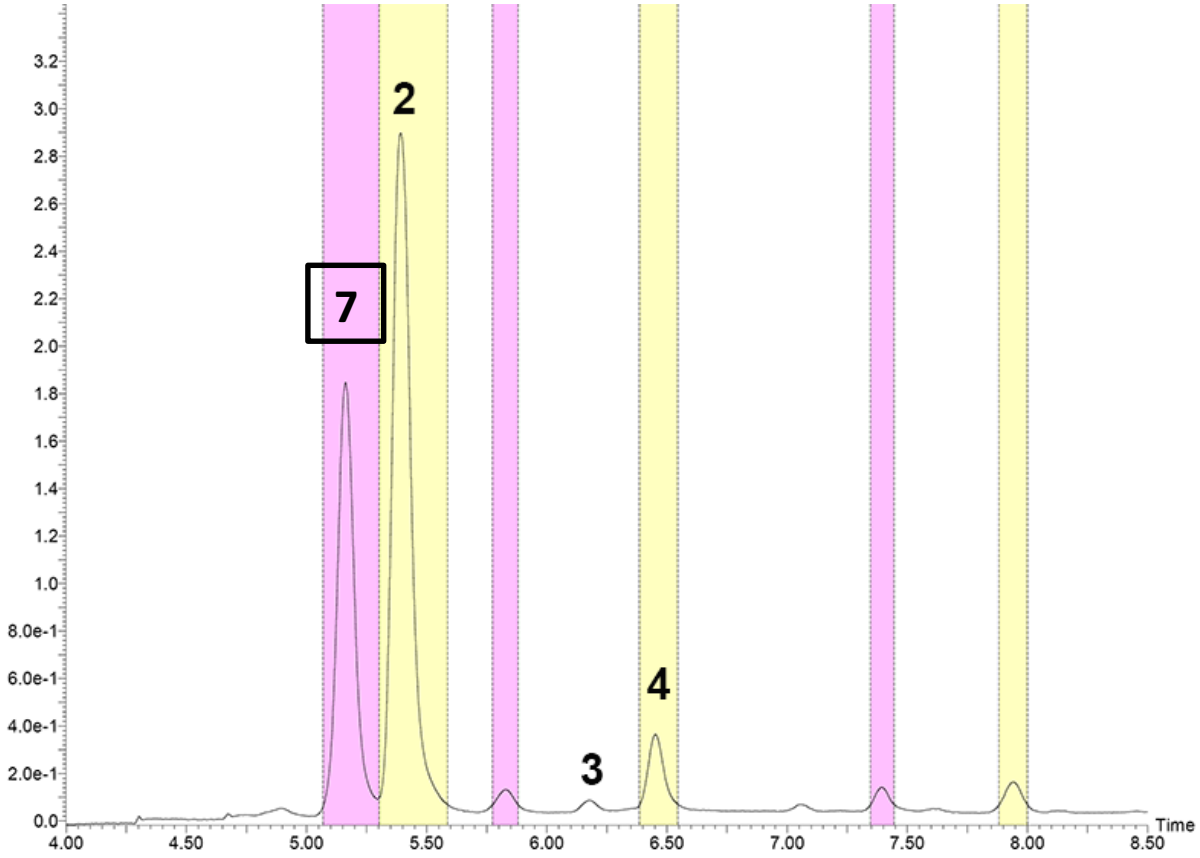
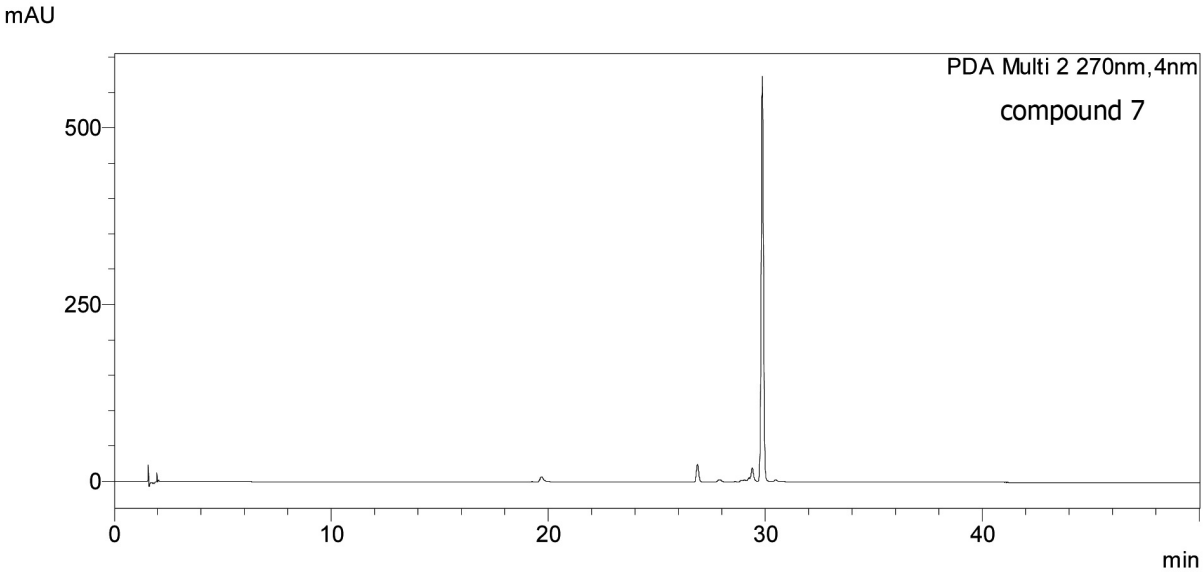


Figure 2: Analysis of compound 7 by HPLC-DAD at λ 270 nm (>95 % pure).



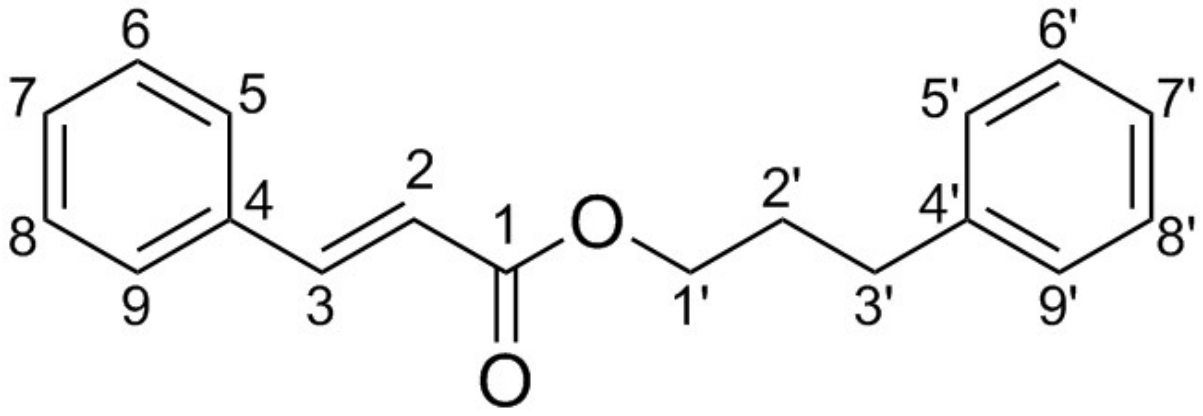
The unknown compound **7** displayed UV maxima at 205, 275, 342, and 359 nm (measured in 98 % acetonitril (A) and 2 % water with 0.1 % acetic acid and 0.9 %

formic acid (B)). Subsequently, the collected Prep-15 fraction of compound **7** was evaporated and yielded a yellow, oily liquid. LC-HRMS confirmed the purity of compound **7**, and revealed a molecular weight of 266 g/mol and a molecular formula of $C_{18}H_{18}O_2$ (data not shown).

Similar structures, like styracin and other cinnamic acid derivatives, are commonly structurally identified by NMR (Lotti, 2012; Rao, 2014; Villano, 2014). Thus, 1D and 2D NMR spectra were recorded for structural elucidation.

The ^{13}C NMR, ^{1}H NMR, HSQC, HMBC, and COSY spectroscopic data of compound **7** are listed in Table 1, as well as the corresponding HMBC and COSY correlations.

Figure 3: Chemical structure of compound 7 with a molecular weight of 266 g/mol.



The spectroscopic data revealed two phenolic ring systems, an ester function in conjugation with a double bond, and an aliphatic chain of three carbon molecules.

Quarternary C's were not visible as cross peaks in HSQC, but were determined via HMBC correlations. There were three of them: C1 (at δ 168.4) correlating with H1' and H3; C4 (at δ 135.4) correlating with H2, H6, and H8; and C4' (at δ 142.3) correlating with H2', H3', H6', and H8'.

Table 1: ^{13}C and ^{1}H NMR, HMBC and COSY data of 3-phenylpropyl-(E)-cinnamate in MeOD.

position[a]		δ H [ppm] (multiplicity)	δ C [ppm]	δ C (HMBC) [ppm]	δ H (COSY) [ppm]	δ H (Lit.)[b] [ppm]	δ C (Lit.)[c] [ppm]
1	C		168.4				166.9
2	CH	6.54 (d)	118.9	135.4	7.67	6.45 (d)	118.1
3	CH	7.67 (d)	146.3	129.3, 168.4	6.54	7.68 (d)	144.7
4			135.4				134.4
5, 9	C6H5-	7.62	129.3		7.42	7.52-7.53 (m)	128.0
6, 8		7.41	131.5	135.4		7.34-7.42 (m)	130.2
7		7.42	130.0		7.62		128.8
1'	CH2	4.20	65.1	168.4		4.23 (t)	66.8
2'	CH2	2.03	31.5	33.2, 65.1, (142.3)	2.75, 4.20, 7.23	2.00-2.07 (m)	30.2
3'	CH2	2.75	33.2	31.5, 65.1, 129.3, 142.3		2.74 (t)	32.2
4'			142.3				141.2
5', 9'	C6H5-	7.23 (d)	129.5	129.3		7.20-7.23 (m)	128.38
6', 8'		7.27 (t)	129.5	129.3, 142.3	2.03	7.27-7.31 (m)	128.40
7'		7.17 (t)	127.0			7.20-7.23 (m)	126.0

[a] positions of C's
[b,c] corresponding shifts found in literature (Zhang, 2012), recorded in $CDCl_3$

The first phenolic ring system (meaning the left one in Figure 3) had resonating ^{13}C NMR signals at δ 129.3, δ 130.0, δ 131.5 (C5-C9). Through HMBC correlations C4 (δ 135.4) was located as the quarternary C of this ring system, as mentioned above. ^{13}C NMR signals of the second phenolic ring (the right one in Figure 3) resonated at δ 127.0 and δ 129.5 (C5'-C9') and the HMBC correlations identifying C4' (δ 142.3) as the quarternary C, as already mentioned. The ten phenolic ^{1}H NMR signals correlated very well with these findings, the values being δ 7.41, δ 7.42, and δ 7.62 (H5-H9 of the first phenolic ring system) and at δ 7.17, δ 7.23, and δ 7.27 (H5'-H9' of the second phenolic ring system). The exact position of each C in the ring was identified by HMBC correlations (see Table 1).

Three aliphatic ^{13}C NMR signals at δ 31.5, δ 33.2, and δ 65.1 (C1'-C3'), and six aliphatic ^{1}H NMR signals at δ 2.03, δ 2.75, and δ 4.20 (H1'a and H1'b – H3'a and H3'b) proof their alignment along with their HMBC and COSY correlations. They also reveal the aliphatic chain, linking the second phenol ring to the ester function.

(C1' showed a typical shift of an oxygen bond, an HMBC correlation to C1, C2 and C3. C2' and C3' both correlated with C4' in HMBC spectra, but only C3' correlated with the phenolic C's, C5' and C9'.)

The downfield resonance δ 168.4 (C1) of a quarternary C and the two oxygen atoms that added up to the molecular weight of compound **7**, led to the elucidation of an ester function at the position C1. HMBC correlations of H1' with C1 (H1' and C1'

showed a shift of an oxygen bond) and H3 with C1 proof the position of the ester group.

The two remaining C's (C2 and C3) appeared at ^{13}C NMR shifts of δ 118.9 and δ 146.3 with the corresponding ^{1}H NMR signals at δ 6.54 and δ 7.67 (H2 and H3). The values of the shift signals show that there is a double bond at that position. H2 correlated with C4 in HMBC spectra and COSY data show the spatial proximity of H2 and H3. H3 itself correlated with C1, C5 and C9, proofing its position.

The conjugation of the double bond with the ester function is clarified by the especially downfield shift of C3 (δ 146.3). It can be explained by the mesomeric effect of an ester function. It deshields C3, meaning C3 is more exposed to the magnetic field of the NMR device. The resulting stronger interactions with it lead to a higher chemical shift value of C3. HMBC correlations of H3 with C1 proof its position, as mentioned before.

Subsequently, compound **7**, a yellow oil, was identified as 3-phenylpropyl-(E)-cinnamate. In literature, it is reported to be both, a yellow oil (Lotti, 2012) as well as a colourless one (Zhang, 2012). Published NMR data of 3-phenylpropyl-(E)-cinnamate correspond to compound **7**'s spectroscopic data (Zhang, 2012; Sinha, 2010; Lotti, 2012). Exemplarily, NMR data of Zhang, 2012 are listed in Table 1.

Honduras styrax, as the styrax sample R6 that was investigated, is known to contain 3-phenylpropyl-(E)-cinnamate and styracin as main constituents (Chalchat, 1994). Besides, this so called American styrax gum, Asian styrax gum is also a documented source of 3-phenylpropyl-(E)-cinnamate (Fernandez, 2005).

Styrax in general, is known to contain various cinnamic acid derivatives. Especially cinnamic acid, a hydrolysis product of 3-phenylpropyl-(E)-cinnamate, is well known for its antioxidant and antibacterial effects (Lingbeck, 2015). Furthermore, cinnamic esters are organic compounds of immense importance due to their wide spread application in flavour, perfum and pharmaceutical industries (Sinha, 2010; Steffen, 1994).

This shows that cinnamic esters, like 3-phenylpropyl-(E)-cinnamate, are valuable natural compounds, perfectly suitable for the extraction, quantification, and isolation with supercritical fluid devices.

3.2.2 Vanilla pod and star anise

The analysis of the eight acquired resin samples revealed that shikimic acid (**6**) was not present in any of them, and vanillic acid (**5**) in only two out of the eight samples with merely up to 0.10 % (see 3.1 Part I - Manuscript, Table 3).

Since this affected the two most polar compounds **5** and **6**, the aim was to verify that they were indeed quantitatively extractable with supercritical fluid devices.

Therefore, two well-known natural sources of **5** and **6** were acquired from local stores. Vanilla beans are well known to contain **5** (Pérez-Silva, 2006; Ramachandra Rao, 2000); whereas compound **6** is reported as major ingredient of Chinese star anise (Ohira, 2009; Karpf, 2009). In parallel to SFE, extracts by classical ultrasound extraction were prepared in order to compare the results of the different extraction procedures.

Three samples of vanilla pods and Chinese star anise, respectively, were extracted for 10 min by SFE with a co-solvent mixture consisting of equal amounts of ethanol and water. The same amount of samples were extracted by sonication with pure ethanol for 10 min.

The analysis of the extracts was conducted on the UPC^2 SFC device with the optimized method, as established in 3.1 Part I - Manuscript, Table 1. Quantified results for **5** were exactly the same for both extraction methods (0.06%). 3,13 µg/ml **5** were present in 5,00 mg/ml extract obtained with SFE, and 7,40 µg/ml **5** were present in 12,98 mg/ml extract obtained with ultrasound extraction. Concerning **6**, sonication seems to be superior with 2,19% **6** (1,16 mg/ml in 52,82 mg/ml extract), compared to 0.97% **6** (49,23 µg/ml in 5,05 mg/ml extract) with SFE. This shows that up to 4,92 mg **6** are extractable from 505,15 mg star anise seeds (0.97%) within 10 min with the chosen parameters on the MV-10 SFE device. It might be the polarity limit of this extraction method.

It can be concluded that SFE, a common way of extracting lipophilic compounds from natural products, is also a valuable extraction method for polar compounds, including polar phenolics.

3.2.3 Ganoderma lucidum

Ganoderma lucidum of the Ganodermataceae family is a traditionally used medicinal mushroom, especially in China. It is popular for the treatment of various diseases (Xu, 2017) and has been subject to previous studies by the Department of Pharmacognosy, University Vienna. Especially the accumulation and isolation of lipophilic triterpenes was in the focus of interest. Besides those, the mushroom is known to contain other lipophilic compounds like sterols or fatty acids (Li, 2006; Shiao, 2003; Akihisa, 2007). Since SFE is well-known for the extraction of lipophilic compounds, the MV-10 SFE was regarded as promising extraction device. Furthermore, also more polar compounds, like polysaccharides, have been previously extracted by SFE from *Ganoderma lucidum* (Fu, 2009).

While the liquid extracts are collected with the MV-10 SFE, the high system pressure of the device is elevated to atmospheric pressure. At that point, CO_2 converts back to the gas state, leaving the co-solvent as the only remaining liquid mobile phase. In case there is less than 3 ml/min of total mobile phase, it could easlily happen that the extract precipitates in the tubes of the extract collector. In order to keep the extracted compounds solved the addition of a make-up fluid was necessary, especially for extracts which were prepared with pure carbon dioxide and only 10 % or 20 % of methanol co-solvent. Therefore, the make-up pump was set to a certain flow rate making sure that enough pure solvent was added to deliver the finished extract through the tubing when pressure was released.

The mushroom samples were extracted at different lipophilicity levels, ranging from pure CO_2 up to 1:1 CO_2 and methanol. In total, six different mobile phase compositions were applied, with decreasing CO_2 amounts in steps of 10 %.

Due to technical problems with the instrument, the extractions were interrupted. An instrument alarm required a system restart, which meant extraction parameters were reset as well. In further consequence, it took some time for the system to reach the

extraction parameters again. During this initial phase of set up, the mobile phase was led through the extraction vessels. Hence the extraction process proceeded even though the set parameters for the extraction were not reached yet. Since the alarms occurred randomly and the set-up time differed, depending among others on the mobile phase composition, resulting extraction times and thus extracted amounts of compounds are not comparable. The extracts may also contain lipophilic compounds that are only present because of the initial set up phase, when pure or a higher percentage of carbon dioxide was led through the extraction vessels. Nonetheless, the extracts obtained are comparable in respect of polar compounds, as the preset amounts of methanol in the mobile phase were never exceeded. They are therefore adequate for further qualitative analysis.

3.3 Part II - Materials and Methods

3.3.1 Styrax – isolation and structure elucidation of compound 7

3.3.1.1 Sample, solvents and preparation

An unknown compound (compound **7**) was isolated from the styrax resin sample R6 by preparative scale SFC (Prep-15) as the third step of the SFx workflow. The method used for the purification was scaled up from the UPC^2 method (For details see 3.1 Part I - Manuscript, Table 1).

The gathered fractions were monitored for their purity on the HPLC device Shimadzu UFLC XR. As stationary phase, a Phenomenex® HyperClone ODS (C18) with 150 mm column length and an internal diameter of 4.60 mm was used. It consisted of 5 µm silica particles of 120 Å pore size. The mobile phase was set up as a gradient, starting with 90 % of solvent A (water). As additives 0.1 % acetic acid and 0.9 % formic acid were added to A. Solvent B was acetonitrile. The gradient started at 10 % going up to 98 % of acetonitrile within 40 min, holding that solvent composition for 10 min. Solvents used were of HPLC grade. The overall run time of 50 min was performed at a flow rate of 1.0 ml/min at 35 °C. 10 µl was the amount of sample injected, the PDA detector was set to 270 nm, and a 10 min post run time was added after each run.

The Prep-15 fraction of compound **7** was evaporated, revealing a yellow oil.

Confirmation of purity and molecular weight were obtained with the same HPLC-MS system as used for the analysis of compound **2** and **4** (see 3.1 Part I - Manuscript, Materials and Methods – Evaluation of purity and identity via HPLC-MS).

3.3.1.2 NMR – structural elucidation

For structural elucidation, a BRUKER Avance 500 MHz NMR spectrometer (UltraShield) was used in combination with a switchable cryo probe. ^{13}C and ^{1}H NMR, HSQC, COSY, and HMBC spectra were recorded in MeOD (deuterated methanol). Collected NMR spectra were evaluated with MestReNova (Mestrelab Research). Chemical shift values are in reference to the non-deuterated solvent signal of MeOH.

3.3.2 Vanilla pod and star anise

3.3.2.1 Samples, standards and solvents

The vanilla bean and the star anise samples were purchased from a food store.

Standard compounds vanillic acid (**5**) and shikimic acid (**6**), as well as the standard stock solutions, solvents and compressed CO_2 were the same ones as used for the method development and validation of compounds **1**-**6**. For details see 3.1 Part I - Manuscript, Materials and Methods.

3.3.2.2 Sample preparation

For comparison of the extraction methods, vanilla bean pulp of *Vanilla planifolia* Jacks. ex Andrews (Orchidaceae) and the ground seeds of star anise of *Illicium verum* Hook.f. (Schisandraceae) were both extracted with supercritical fluid extraction (SFE) and by means of sonication in ethanol.

SFE was performed on a Waters MV-10 SFE system comprising of different modules, as listed in 3.1 Part I - Manuscript. From both samples, three 500 mg weighed portions were extracted. They were placed in a 5 ml extraction vessel, respectively, and filled up with inert diatomaceous earth (Thermo Fisher Scientific). Optimized extraction conditions, as established for styrax (see 3.1 Part I - Manuscript, Table 1), consisted of a 1:1 mobile phase mixture of ethanol and CO_2 at

a flow rate of 10 ml/min, and an extraction time of 10 min. System pressure and vessel oven temperature were set to 300 bar and 40 °C, respectively. Obtained extracts were filled up to 100 ml in volumetric flasks with ethanol.

For sonication (Elma® Transsonic 460, Germany) 50 mg ground sample were extracted with 10 ml ethanol for 10 min. This was repeated three times. After centrifugation (Labofuge 400R, Heraeus Instruments Ges.m.b.H.) for 5 min at 2500 rpm, the samples were filtrated through a 0.45 µm pore size membrane filter (Satorius, Germany). Collected extracts were evaporated, weighed and diluted to 3 mg/ml with ethanol.

3.3.2.3 Analytical conditions

For analytical experiments each extract was analyzed threefold with the Waters® Acquity UPC^2 supercritical fluid chromatography (SFC) instrument. For the parameters of the best separation method see 3.1 Part I - Manuscript, Table1).

3.3.3 Ganoderma lucidum

3.3.3.1 Samples and solvents

The fruit bodies of *Ganoderma lucidum* (Curtis) P. Karst. were purchased from Plantasia (Oberndorf, Salzburg) and a voucher is stored at the Department of Pharmacognosy, University of Vienna, Austria, under the voucher specimen number: JR-20080605-A1.

Methanol, the solvent used, was of analytical grade and purchased from VWR Chemicals. CO_2, compressed and of grade 4.5, had a purity of 99.995 % and was purchased from Messer.

3.3.3.2 Supercritical fluid extraction

Extracts were conducted on a Waters MV-10 SFE system with the same system, software and modules used for the extractions of styrax resins (see 3.1 Part I - Manuscript, Table 1).

The dry sample was ground in an electrical coffee bean mixer. For each run, a 5 ml extraction vessel was filled with 700 mg ground sample. Six weighed out sample

portions were extracted with the generic method parameters 300 bar, 40°C, and 10 ml/min mobile phase flow rate. The six samples were extracted for 15 min, respectively, each with a different mobile phase composition. The mobile phase comprised, besides 100 % CO_2, of rising methanol portions in steps of ten percent (10, 20, 30, 40, and 50 %). Accordingly, a polarity range from pure carbon dioxide to a 1:1 rate of methanol and CO_2 was covered with these six different extracts. With pure CO_2, a make-up flow rate of 3 ml/min of methanol was necessary, as well as 2 ml/min and 1 ml/min make-up flow for a 9:1 and 8:2 mobile phase composition of CO_2 and methanol.

3.3.3.3 Extract storage

Obtained extracts were concentrated on a rotary evaporator. The remaining few ml were transferred into separate glass vials, respectively, and put under a fume hood to dry completely. When the remaining methanol had evaporated, the extracts were vacuum dried over silica gel in a desiccator. After a few days, they were covered with a lid and put for storage to the fridge at 8 °C.

4 Conclusion/ Outlook

The SFx workflow, as described in the published manuscript, shows a variety of advantages compared to conventional approaches, such as gas chromatography-mass spectrometry (GC-MS) (see 3.1 Part I - Manuscript). It is characterized by low operating costs and faster analysis times than with conventional HPLC. Further, sustainability aspect due to the usage of significantly less amounts of organic solvents are the main advantages SFx technologies offer when compared to established procedures. The only drawback is probably the high acquisition costs of these instruments (Hartmann, 2015).

The aim to show that supercritical fluid devices are fit for the analysis of polar natural compounds was met successfully. A method for six selected standard compounds (**1**-**6**) was developed and validated on the UPC^2 SFC. Standards **1**-**6** were quantified with SFC in eight different styrax samples (R1-R8), whereof five (R1, R2, R4, R7, R8) were extracted with SFE (MV-10) prior to the analysis with SFC. Three compounds (**2**, **4**, and the additional compound **7**) were isolated with preparative SFC (Prep-15) and **7** was structurally elucidated with NMR spectroscopy as 3-phenylpropyl-(E)-cinnamate.

Since **5** and **6** were merely or not present in the resin samples, they were additionally extracted from known natural sources. Extraction and analysis was performed with the same methods as with the resins. It proved that **5** is quantitatively extractable with SFE, and **6** almost up to 50 % from star anise. In detail, 0,97 % **6** was extracted with SFE compared to 2,19 % **6** with ultrasound sonication from star anise, respectively. This shows that polar compounds, like **1**-**6**, are fit for the application of the SFx workflow, including extraction, analysis, quantification and isolation by supercritical fluid based devices. Compound **6**, shikimic acid, seems to be at the polarity limit of the applied method, as it is only partially extractable with SFE. Overall, this outcome emphasises the opportunities, which SFx technologies offer not only for apolar, but also for polar analytes.

Conclusion/ Outlook

The SFE obtained *Ganoderma lucidum* extracts were evaporated, vacuum dried and stored at 8 °C. They are ready for further qualitative analysis, especially regarding the polarity of extractable compounds.

These results are valuable findings for follow-up studies for the Phytochemistry group of the Department of Pharmacognosy as well as for every scientist working with supercritical fluid devices.

References

Akihisa, T., Nakamura, Y., Tagata, M., Tokuda, H., Yasukawa, K., Uchiyama, E., ... & Kimura, Y. (2007). Anti-Inflammatory and Anti-Tumor-Promoting Effects of Triterpene Acids and Sterols from the Fungus Ganoderma lucidum. *Chemistry & biodiversity*, *4*(2), 224-231.

Chalchat, J. C., Garry, R. P., & Mathieu, J. P. (1994). Composition of the Volatile Fraction from Honduras Styrax, Liquidambar styraciflua L. *Journal of Essential Oil Research*, *6*(1), 73-75.

Fernandez, X., Lizzani-Cuvelier, L., Loiseau, A. M., Perichet, C., Delbecque, C., & Arnaudo, J. F. (2005). Chemical composition of the essential oils from Turkish and Honduras Styrax. *Flavour and Fragrance Journal*, *20*(1), 70-73.

Fu, Y. J., Liu, W., Zu, Y. G., Shi, X. G., Liu, Z. G., Schwarz, G., & Efferth, T. (2009). Breaking the spores of the fungus Ganoderma lucidum by supercritical CO2. *Food chemistry*, *112*(1), 71-76.

Hartmann, A., & Ganzera, M. (2015). Supercritical fluid chromatography–theoretical background and applications on natural products. *Planta medica*, *81*(17), 1570-1581.

Karpf, M., & Trussardi, R. (2009). Efficient Access to Oseltamivir Phosphate (Tamiflu) via the O-Trimesylate of Shikimic Acid Ethyl Ester. *Angewandte Chemie International Edition*, *48*(31), 5760-5762.

Li, C., Li, Y., & Sun, H. H. (2006). New ganoderic acids, bioactive triterpenoid metabolites from the mushroom Ganoderma lucidum. *Natural product research*, *20*(11), 985-991.

Lingbeck, J. M., O'Bryan, C. A., Martin, E. M., Adams, J. P., & Crandall, P. G. (2015). Sweetgum: An ancient source of beneficial compounds with modern benefits. *Pharmacognosy reviews*, *9*(17), 1.

Lotti, C., Piccinelli, A. L., Arevalo, C., Ruiz, I., Migliani De Castro, G. M., Figueira Reis De Sá, L., ... & Rastrelli, L. (2012). Constituents of Hondurian propolis with

inhibitory effects on Saccharomyces cerevisiae multidrug resistance protein Pdr5p. *Journal of agricultural and food chemistry*, *60*(42), 10540-10545.

Ohira, H., Torii, N., Aida, T. M., Watanabe, M., & Smith, R. L. (2009). Rapid separation of shikimic acid from Chinese star anise (Illicium verum Hook. f.) with hot water extraction. *Separation and Purification Technology*, *69*(1), 102-108.

Pérez-Silva, A., Odoux, E., Brat, P., Ribeyre, F., Rodriguez-Jimenes, G., Robles-Olvera, V., ... & Günata, Z. (2006). GC–MS and GC–olfactometry analysis of aroma compounds in a representative organic aroma extract from cured vanilla (Vanilla planifolia G. Jackson) beans. *Food chemistry*, *99*(4), 728-735.

Ramachandra Rao, S., & Ravishankar, G. A. (2000). Vanilla flavour: production by conventional and biotechnological routes. *Journal of the Science of Food and Agriculture*, *80*(3), 289-304.

Rao, G. K., Kumar, A., Singh, M. P., Kumar, A., Biradar, A. M., & Singh, A. K. (2014). Influence of pendent alkyl chains on Heck and Sonogashira C–C coupling catalyzed with palladium (II) complexes of selenated Schiff bases having liquid crystalline properties. *Journal of Organometallic Chemistry*, *753*, 42-47.

Scheuba, J., Wronski, V. K., Rollinger, J. M., & Grienke, U. (2017). Fast and green–CO2 based extraction, isolation, and quantification of phenolic styrax constituents. *Planta medica*, *83*(12/13), 1068-1075.

Shiao, M. S. (2003). Natural products of the medicinal fungus Ganoderma lucidum: occurrence, biological activities, and pharmacological functions. *The Chemical Record*, *3*(3), 172-180.

Sinha, A. K., Sharma, A., Swaroop, A., & Kumar, V. (2010).

Steffen, A. (1994). Perfume and flavor chemicals (aroma chemicals). *Vol. I & II, Allured Publishing Corporation, IL, USA*.

Villano, R., Acocella, M. R., & Scettri, A. (2014). Fe 3 O 4 nanoparticles/ethyl acetoacetate system for the efficient catalytic oxidation of aldehydes to carboxylic acids. *Tetrahedron Letters*, *55*(15), 2442-2445.

Xu, S., Dou, Y., Ye, B., Wu, Q., Wang, Y., Hu, M., ... & Guo, J. (2017). Ganoderma lucidum polysaccharides improve insulin sensitivity by regulating inflammatory cytokines and gut microbiota composition in mice. *Journal of Functional Foods*, *38*, 545-552.

Zhang, B., Feng, P., Cui, Y., & Jiao, N. (2012). NHC-catalyzed C–O or C–N bond formation: efficient approaches to α, β-unsaturated esters and amides. *Chemical Communications*, *48*(58), 7280-7282.

Appendix

Figure A.1: ^{1}H-NMR spectrum of 3-phenylpropyl-(E)-cinnamate (7); (CD_3OD, ref.: δ_H 3.31 ppm, 500 MHz NMR, ~1 mg).

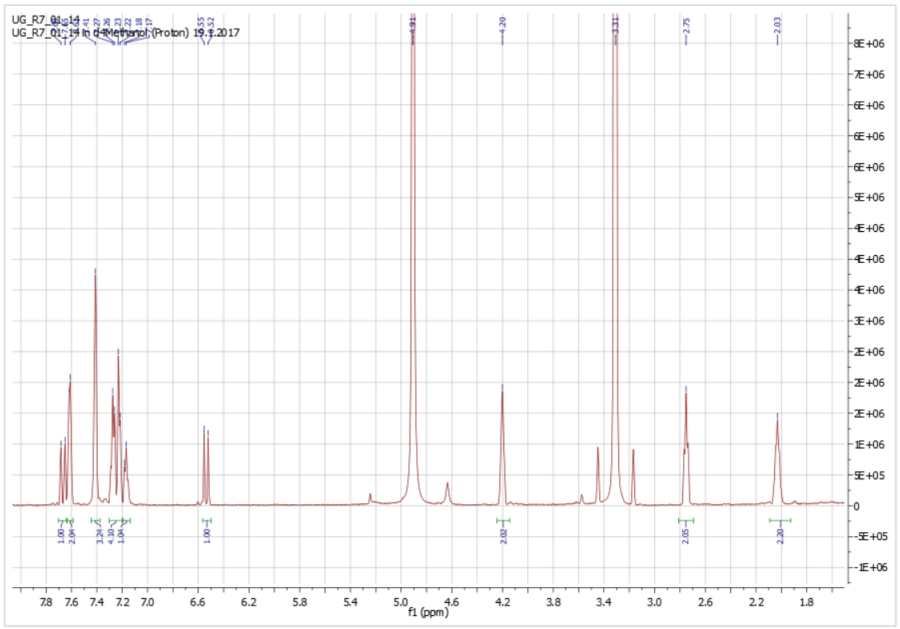


Figure A.2: ^{13}C-APT spectrum of 3-phenylpropyl-(E)-cinnamate (7); (CD_3OD, ref.: δ_C 49.00 ppm, 500 MHz NMR, ~1 mg).

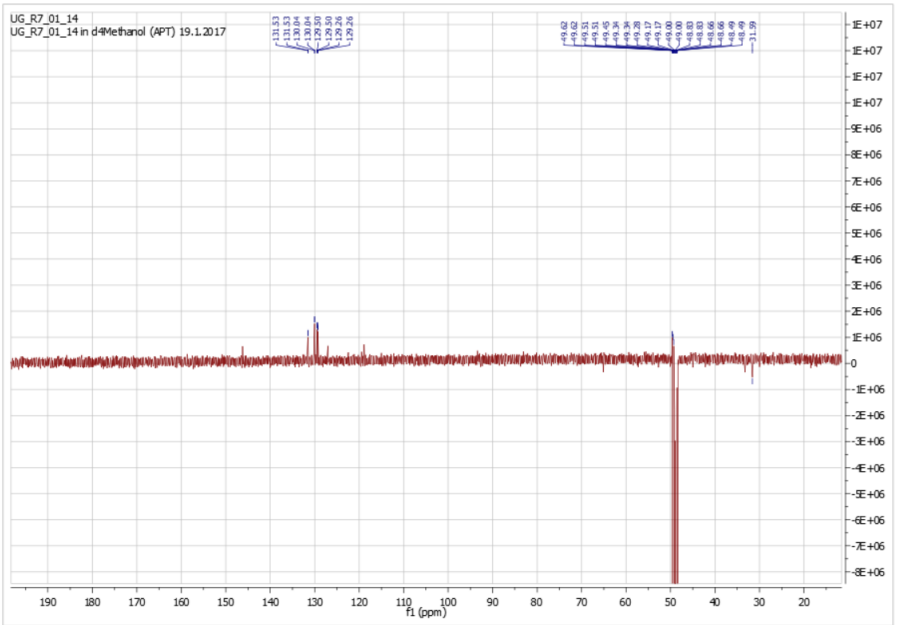


Figure A.3: HSQC spectrum (horizontal trace: ^{1}H-NMR spectrum, vertical trace: ^{13}C-APT) of 3-phenylpropyl-(E)-cinnamate (7); (CD_3OD, ref.: δ_H 3.31 ppm, δ_C 49.00 ppm, 500 MHz NMR, ~1 mg).

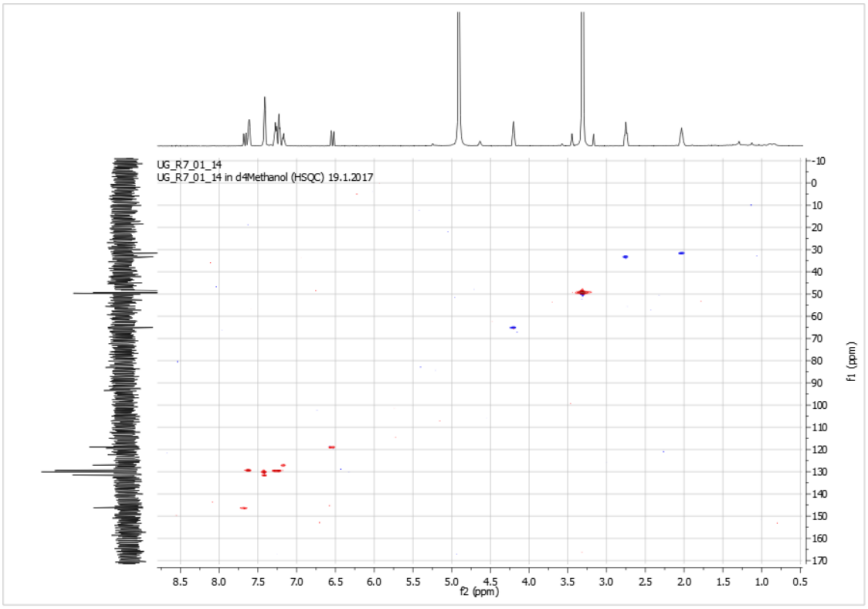


Figure A. 4: HMBC spectrum (horizontal trace: ^{1}H-NMR spectrum, vertical trace: ^{13}C-APT) of 3-phenylpropyl-(E)-cinnamate (7); (CD_3OD, ref.: δ_H 3.31 ppm, δ_C 49.00 ppm, 500 MHz NMR, ~1 mg).

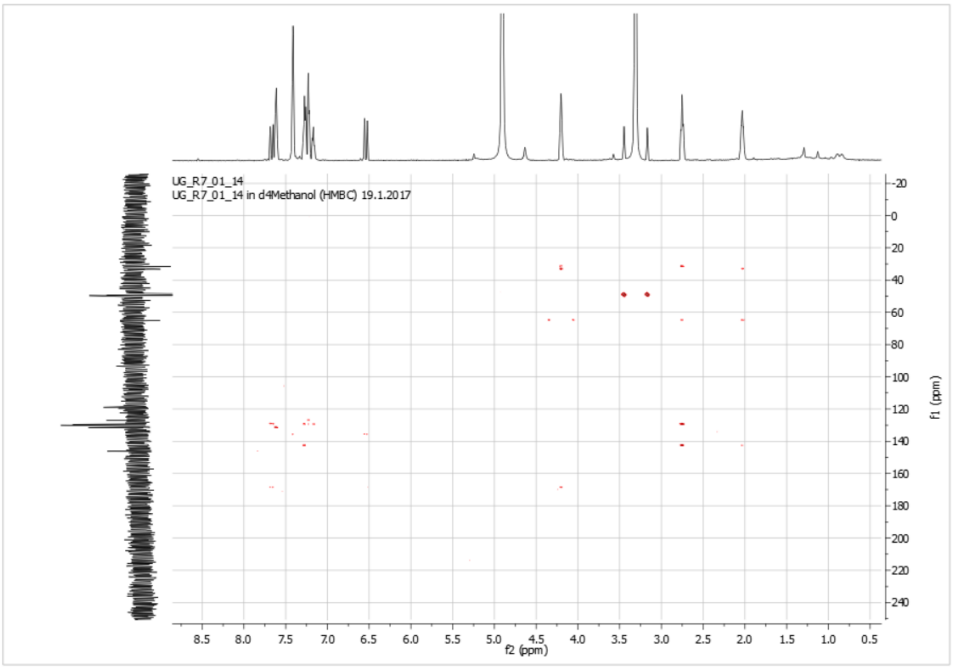


Figure A.5: HH-COSY spectrum (horizontal & vertical trace: ^{1}H-NMR spectrum) of 3-phenylpropyl-(E)-cinnamate (7); (CD_3OD, ref.: δ_H 3.31 ppm, 500 MHz NMR, ~1 mg).