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„SFC based analysis, identification and quantification of hop ingredients"

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Mag. Carmen Schretter, MBA

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Betreut von / Supervisor:	Univ.-Prof. Mag. pharm. Dr. Judith M. Rollinger
Mitbetreut von / Co-Supervisor:	Mag. pharm. Dr. Ulrike Grienke

Acknowledgement

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List of Abbreviations

6-PN	6-Prenylnaringenin
8-PN	8-Prenylnaringenin
AA	Acetic Acid
BEH 2-EP	Bridged-Ethylene-Hybrid 2-Ethylpyridine
BSM	Binary Solvent Manager
DCM	Dichloromethane
ELSD	Evaporative light scattering detector
FA	Formic acid
Hex	*n*-hexane
HLD	*Humulus lupulus* dichloromethane extract
HPLC	High Performance Liquid Chromatography
Hulu	Hulupinic acid
IPA	Isopropanol
ISM	Isocratic Solvent Manager
IXN	Isoxanthohumol
MeOH	Methanol
PDA	Photodiode Array
PZN	Pharmazentralnummer (Pharmaceutical Central Number)
QDa	QDa mass detector
SFC	Supercritical Fluid Chromatography
TFA	Trifluoroacetic acid
TLC	Thin Layer Chromatography
UPC^2	UltraPerformance Convergence Chromatography^{TM}
UV-Vis	Ultraviolet visible spectroscopy
VF	Volumetric flask
XN	Xanthohumol

1 ABSTRACT

In this study the first supercritical fluid based protocol for the **identification and quantification** of five characteristic polar reference substances in **hops products** was developed. One chalcone (xanthohumol), three flavanones (isoxanthohumol, 8-prenylnaringenin, 6-prenylnaringenin) and hulupinic acid, an oxidation product of hops bitter acids, were used as reference compounds.

Six mono hops dietary products were obtained from online pharmacies and extracted with EtOH 96% according to a published extraction protocol. A **UPC²** (Acquity UltraPerformance Convergence Chromatography™) method was developed for the five reference compounds using a mobile phase consisting of CO_2 and isopropanol (IPA). Within 5 minutes all 5 reference substances could be baseline separated using a Viridis BEH 2-EP column (3.0 x 100 mm; 1.7 µm particle size).

All compounds were quantified in the six commercial products and the **method was validated according to ICH guidelines**.[1] The optimized protocol and validation on UPC² achieved good results - also compared to other chromatographic methods like HPLC.

In addition, a second UPC² method was successfully established to separate and detect **all compounds** (from non-polar to polar) within a complex hops crude extract.

The last step was a scaling up of this method to **SFC Prep-15** (preparative supercritical fluid chromatography) carried out with a Viridis BEH 2-EP column (10 x 250 mm; 5 µm particle size). However, separation and isolation of pure compounds were not satisfying on the SFC Prep-15 instrument due to the inherent instability of hops constituents.

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[1] http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000043.jsp&mid=WC0b01ac05800240cb

2 ZUSAMMENFASSUNG

In der vorliegenden Studie wurde die erstmalige Erstellung eines SFC Protokolls (supercritical fluid chromatography) zur **Identifizierung und Quantifizierung** von fünf charakteristischen, polaren Referenzsubstanzen in **Hopfen Produkten** erarbeitet. Als Referenzsubstanzen dienten ein Chalkon (Xanthohumol), drei Flavanone (Isoxanthohumol, 8-Prenylnaringenin, 6-Prenylnaringenin) und Hulupinsäure - ein Oxidationsprodukt der Hopfenbittersäuren.

Sechs Mono-Hopfen-Produkte wurden von Online Apotheken bezogen und gemäß einer optimierten Extraktionsmethode mit 96%-igem Ethanol extrahiert. Für die fünf Referenzsubstanzen wurde eine **UPC²** (Acquity UltraPerformance Convergence Chromatography^{TM}) Methode erstellt. Als mobile Phase diente eine Mischung aus CO_2 and Isopropanol (IPA). Innerhalb von 5 Minuten konnten mittels einer Viridis BEH 2-EP Säule (3.0 x 100 mm; 1.7 μm Partikelgröße) alle 5 Referenzsubstanzen Basislinien-getrennt werden.

Alle Komponenten wurden in den sechs kommerziellen Produkten quantifiziert und in einem weiteren Schritt einer **Methodenvalidierung gemäß ICH Guidelines[2]** unterzogen. Das optimierte Protokoll sowie die Validierungsmethode für UPC² zeigten gute Ergebnisse – auch im Vergleich zu anderen chromatographischen Techniken wie beispielsweise HPLC.

Zusätzlich wurde erfolgreich ein weiteres Protokoll erarbeitet, um aus dem komplex aufgebauten Gesamtextrakt **alle Komponenten** (apolar bis polar) zu trennen und zu detektieren.

Als letzter Schritt erfolgte ein Upscaling auf ein **SFC Prep-15** Instrument (präparative supercritical fluid chromatography) unter Verwendung einer Viridis BEH 2-EP Säule (10 x 250 mm; 5 μm Partikelgröße). Die Ergebnisse hinsichtlich Basislinientrennung und Isolierung von reinen Substanzpeaks waren aber aufgrund der inhärenten Instabilität der Hopfenkomponenten nicht zufriedenstellend.

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[2] http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000043.jsp&mid=WC0b01ac05800240cb

3 AIM OF WORK

The diversity and complexity of components within hops (*Humulus lupulus L.*) makes analysis and quality control for hops products very challenging. Hop cones provide a complex pool of secondary metabolites. Especially **prenylchalcones** (xanthohumol, desmethylxanthohumol), **prenylflavanones** (isoxanthohumol, 6-prenylnaringenin, 8-prenylnaringenin) and **prenylphloroglucinols** – the so called bitter acids or hop acids – are of particular importance regarding health benefits. (Prencipe et al., 2014).
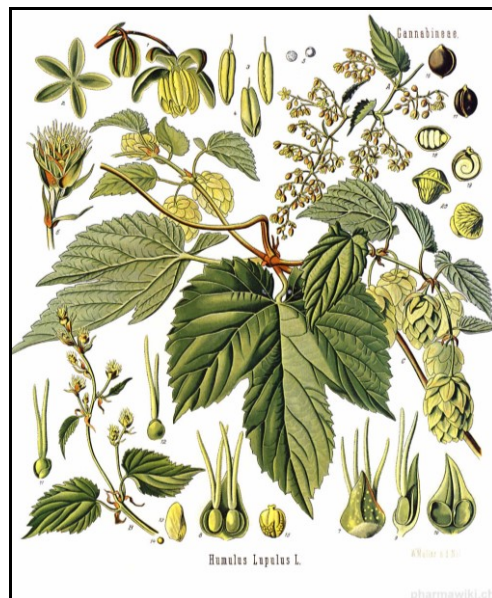
Figure 1 - Humulus lupulus L. [3]

The first aim of the present study was to establish a supercritical fluid based protocol for the **identification and quantification** of five characteristic reference substances i.e xanthohumol (XN), isoxanthohumol (IXN), 6-prenylnaringenin (6-PN), 8-prenylnaringenin (8-PN) and hulupinic acid (Hulu) in different commercial **mono hops products** including **method validation.**

Traditionally, non-polar or volatile hop compounds are analyzed with gas chromatography (GC) while polar substances like the marker substance xanthohumol are investigated with high performance liquid chromatography (HPLC) (Sticher et al., 2015). Over the last few years SFC (supercritical fluid chromatography) has become an interesting and powerful tool in natural product analysis as it is applicable for both polar and nonpolar constituents (Eisath et al., 2017). For SFC analysis supercritical CO_2 is used as mobile phase and according to low viscosity and high solute diffusivity separation results can be improved (Gao et al., 2016).

Therefore, a **second aim** of the study was to evaluate the applicability of UPC² for the detection of non-polar and polar substances in hops extracts in one single analytical workflow.

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[3] https://www.pharmawiki.ch/wiki/index.php?wiki=Hopfen

4 INTRODUCTION

4.1 *HUMULUS LUPULUS* L.

Hops (*Humulus lupulus* L.) is a species of the Cannabaceae family (Auster et al., 1958). In crop growing, only female plants (Figure 2) are cultivated because they contain all valuable components (Sticher et al., 2015). Hops is a climbing plant reaching up to 10 meters height and can be characterized as perennial, dioecious, flexuous and herbaceous (van Wyk et al.,2004).

Figure 2 – (A) Female hop flowers, (B) hop flower resin covering flower bracts (Liu et al. 2015), (C) hops plant[4]

Hops is not only used in the beer brewing industry but is also a source of many biologically active molecules. Therefore, it is interesting not only for the food industry but also in the fields of pharmaceuticals, nutraceuticals and cosmetics (Prencipe et al., 2014). Nevertheless it has not been approved as an herbal medicine so far because of missing evidence and lack of clinical studies (Štulíková et al., 2018).

According to the **Ph. Eur.** (European Pharmacopoeia, Vol. 9) no content is defined for hop cones. Only a content of extractable components (25%) carried out with ethanol 70% (V/V) is mentioned. Nevertheless hop cones for pharmaceutical purposes – were the dried extract is used - should at least contain 0.82% α-acids, 0.43% β-acids and 0.28% xanthohumol. In stored hops (more than 1 year), hops extracts and drugs (containing hops extracts) usually no genuine bitter acids are present anymore (due to thermal decomposition); but 2-methyl-3-buten-2-ol,

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[4] http://www.tipdisease.com

xanthohumol and flavonoids can be detected. Traditionally 2-methyl-3-buten-2-ol, an auto-oxidative product from bitter acids, can be analyzed with GC (gas chromatography). Hops acids and the analytical marker xanthohumol are analyzed separately with HPLC (high performance liquid chromatography) (Sticher et al., 2015).

4.1.1 Application and use

Hops products can be used **internally** (the drug itself as tee infusion or tincture; the extracts as capsule or coated tablet) and **externally** (hop pillow, lipid extracts for aroma therapy). Specially for internal use, the preferred preparations are fixed combinations of hops (*Humulus lupulus* L.), valerian (*Valeriana officinalis* L.), melissa (*Melissa officinalis* L.*)*, passion flower (*Passiflora incarnata* L.) or St. John`s wort (*Hypericum perforatum* L.). They provide additive, sedative effects and stability of hops constituents can be increased. (Wichtl, 2009; http://www.theplantlist.org; Sticher et al., 2015).

In general mono-hops products are very rare. According to the Austrian "Arzneispezialitätenregister"[5] were all pharmaceutical products are listed – only one mono-hops product – a homeopathic one - is mentioned: Hopfen Bioxera Kapseln"; *Humulus lupulus* D2. Other mono-hops products are dietary products.

In general, hops products are used in phytotherapy, as a sedative or for the treatment of restlessness, anxiety, nervousness, sleeping disorders or menopausal symptoms (Wichtl, 2009). In a randomized placebo-controlled trial with 120 women the effects of hop extracts (in tablet form) on menopausal symptoms and hot flashes was tested. Hops effectively reduced early menopausal symptoms (Aghamiri V., 2016).

Further interesting effects of hops are digestive, anti-inflammatory and antimicrobial effects as well as strong estrogenic activity. Hops products can also be used in the treatment of skin diseases (atopic eczema, contact dermatitis, pigment disorders, skin infections, skin aging, skin cancer, photo protection) and cure of neurodegenerative diseases (Chen et al., 2017; Štulíková et al., 2018; Liu et al., 2015). Hops can also be interesting for the cure of thyroid diseases since XN stimulates the iodide uptake (proven in rat model) and could therefore be used in radio iodide therapy.[6]

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[5] https://aspregister.basg.gv.at

[6] https://onlinelibrary.wiley.com/doi/pdf/10.1002/mnfr.200500053
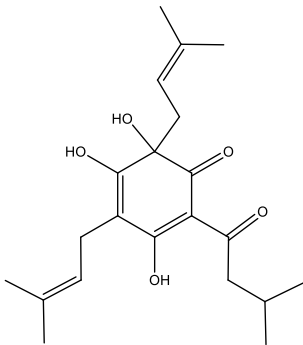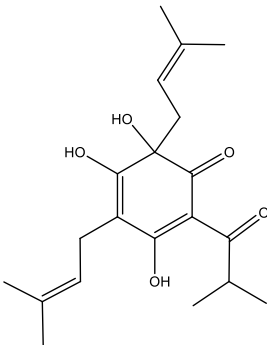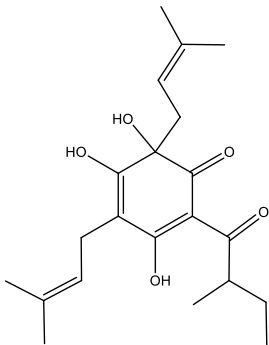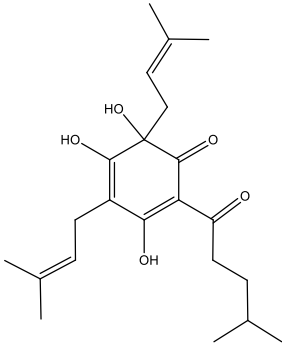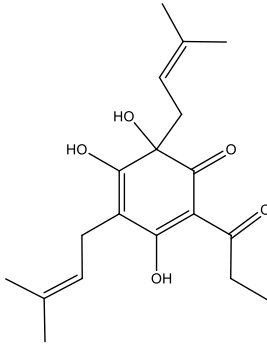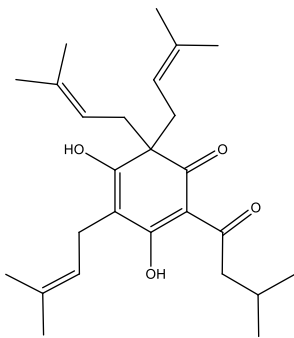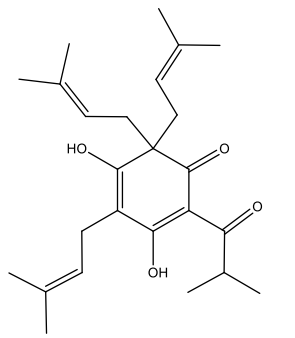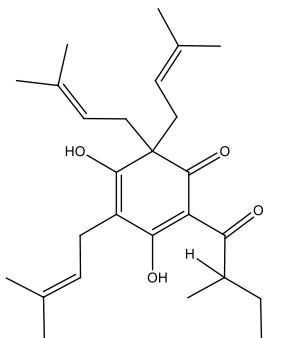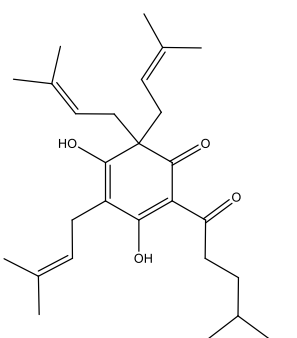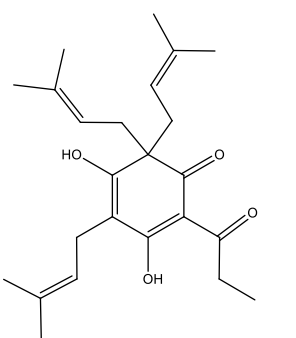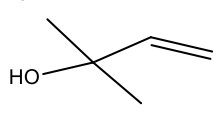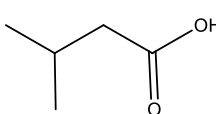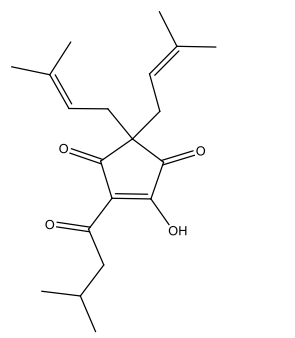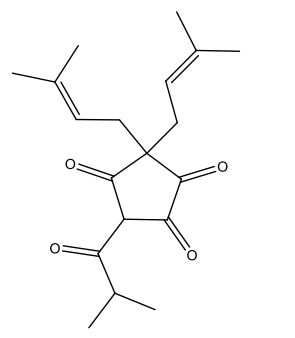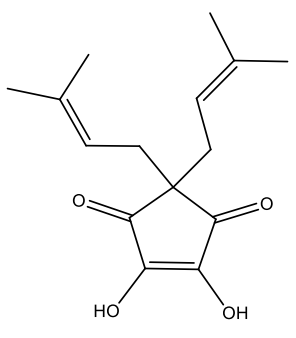
4.1.2 Bioactive compounds

Because of the **complex profile of compounds** - so far not fully understood - hops and its active constituents including prenylated flavonoids and hop bitter acids have become an interesting field of studies since the late 1990s (Gerhäuser et al., 2005).

Main compounds (Figure 3) are a resin fraction (mostly in hops glands) containing approximately 50% **bitter acids** or so called acylphloroglucinoles, **flavonoids** (0.5-1.5 %) and **essential oils** (0.3-1 % mono- and sesquiterpenes mainly without oxygen). Further substances like tanning agents (2-4% proanthocyanidines), phenolic carboxylic acids, proteins, lipids and polysaccharides are contained (Teuscher et al., 2004; van Wyk et al., 2004). Several hundred different constituents can be biosynthesized in female hops plants which shows the complexity of this natural product (Bland et al., 2015). Figure 3 gives an overview of the most important and bioactive constituents of hops.

Alpha Bitter Acids		*3-7%*

Humulone $C_{21}H_{30}O_5$

Cohumulone $C_{20}H_{28}O_5$

Adhumulone $C_{21}H_{30}O_5$

MW: 362.466 g/mol

MW: 348.439 g/mol

MW: 362.466 g/mol

Prehumulone $C_{22}H_{32}O_5$

Posthumulone $C_{19}H_{26}O_5$

MW: 376.463 g/mol

MW: 334.412 g/mol

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Beta Bitter Acids		2.5-4%

Lupulone / $C_{26}H_{38}O_4$

MW: 414.586 g/mol

Colupulone / C25H36O4

MW: 400.559 g/mol

Adlupulone / $C_{26}H_{38}O_4$

MW: 414.586 g/mol

Prelupulone / $C_{27}H_{40}O_4$

MW: 428.613 g/mol

Postlupulone / $C_{24}H_{34}O_4$

MW: 386.532 g/mol

Oxidation products	

2-Methyl-3-buten-2-ol / $C_5H_{10}O$

MW: 86.134 g/mol

Isovalerianic acid / $C_5H_{10}O_2$

MW: 102.133 g/mol

Hulupone / $C_{20}H_{28}O_4$

MW: 332.55 g/mol

Co-hulupone / $C_{19}H_{26}O_4$

MW: 318.413 g/mol

Hulupinic acid / $C_{15}H_{20}O_4$

MW: 264.321 g/mol
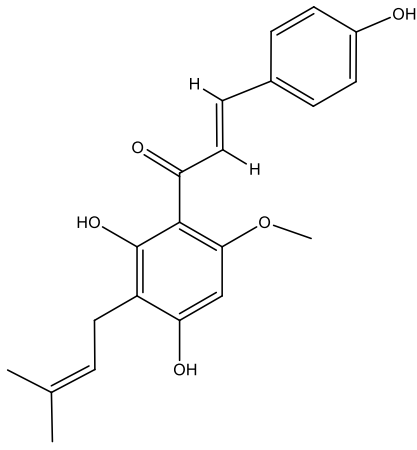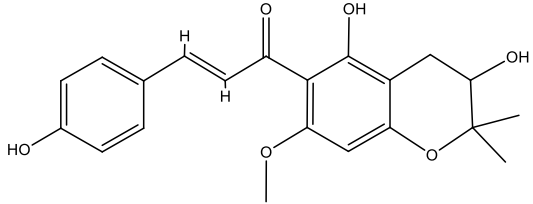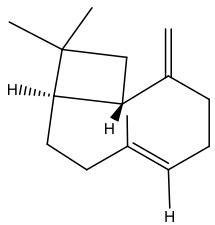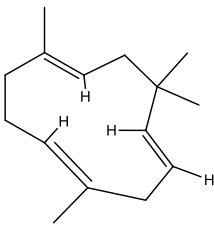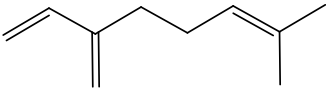
Flavonoids	**0.5-1.5%**	
(further compounds see 4.1.4)		

Xanthohumol / $C_{21}H_{22}O_5$	Xanthohumol B / $C_{21}H_{22}O_6$
MW: 354.40 g/mol	MW: 370.401 g/mol

Essential Oil	**0.3-1%**	

β-Caryophyllene / $C_{15}H_{24}$	Humulene / $C_{15}H_{24}$	β-Myrcene / $C_{10}H_{16}$
MW: 204.357 g/mol	MW: 204.357 g/mol	MW: 136.238 g/mol

Figure 3 - Overview of characteristic hop constituents[7]

Bitter acids and flavonoids are the most important specific compounds. Some of them can only be found in hops and they are responsible for bioactivities.

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[7] https://www.ncbi.nlm.nih.gov/pccompound ; Sticher et al. 2015

4.1.3 <u>Acylphloroglucinoles / hop acids</u>

Acylphloroglucinoles (bitter acids or hop acids) with α- and β-bitter acids and their auto-oxidative products are prenylated derivatives of 1-acylphloroglucide (Table 1). They form the hops resin that is contained in hops cones (15-30%) and hops glands (50-80%). Bitter acids are responsible for the characteristic bitter taste in fresh hops in which α-bitter acids are strongly bitter and β-bitter acids are barely bitter (Sticher et al., 2015).

Table 1 - Overview of hop acids

α-bitter acids are also called **humulones**. Basic structure: 3 x C_2 plus 3 x C_5	They are **solvable** in water which explains their strong bitterness. 5 similar structures can be distinguished (Figure 3): humulone, cohumulone, adhumulone, prehumulone, posthumulone
β-bitter acids are also called **lupulones**. Basic structure: 3 x C_2 plus 4 x C_5	They are **not solvable** in water which explains the low bitterness and appear in 2 tautomeric forms. 5 similar structures can be distinguished (Figure 3): lupulone, colupulone, adlupulone, prelupulone, postlupulone

Bitter acids are very instable and during drying processes, storage or manufacturing a lot of derivatives result through oxidation, isomerization or polymerization. One example is 2-methyl-3-buten-2-ol - a volatile compound that is formed through auto-oxidation and can reach up to 0.15% in stored (> 2 years) hops (Figure 3 and Figure 4). Many of these substances are shown to have an impact on health issues.

2-methyl-3-buten-2-ol, the degradation product of bitter acids, is important regarding the sedative effects of hops (as well as xanthohumol). Applied to mice at concentrations of 0.8 g/kg 2-methyl-3-buten-2-ol was able to produce narcosis that lasted for 8 hours. But so far it could not be clarified if 2-methyl-3-buten-2-ol also results out of hop acids *in vivo* after oral administration (Franco et al., 2012).
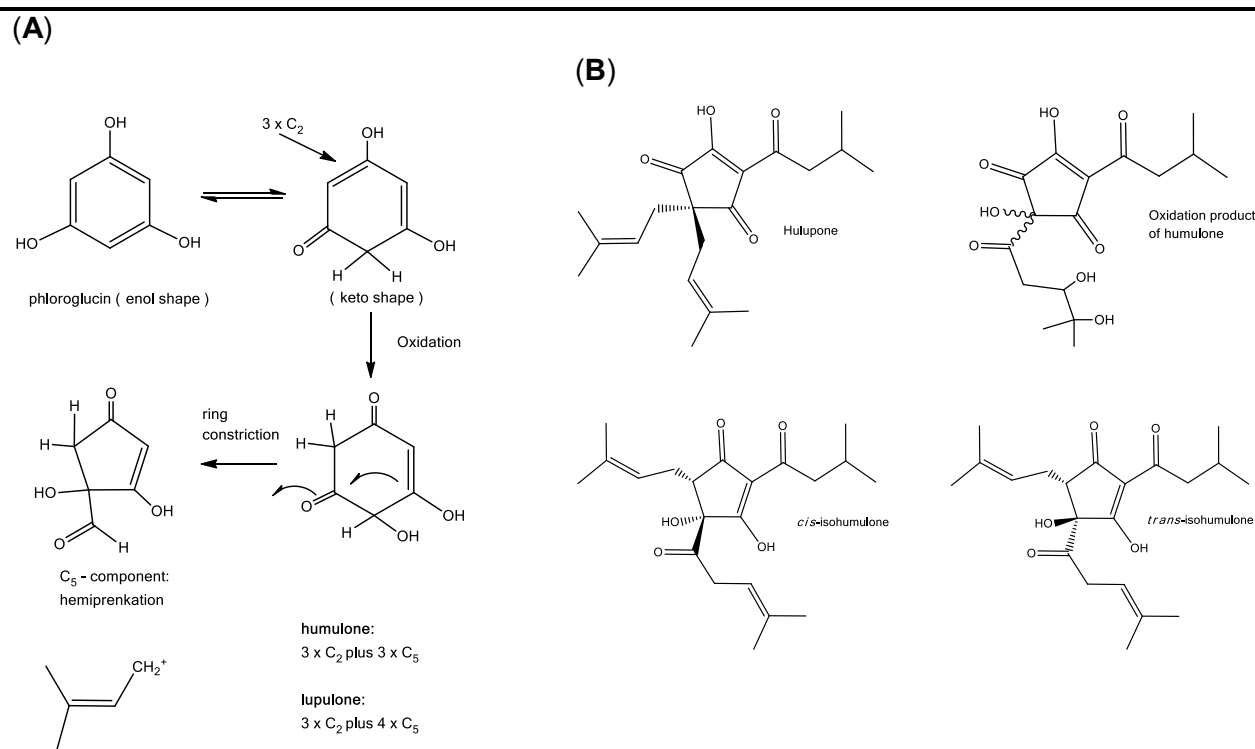
Figure 4 - (A) Genuine bitter acids (appearing in fresh hops): humulone and lupolone (B) isomerisation and oxidation products of bitter acids; hulupon (ring constriction), cis- and trans-isohumulone (Sticher et al., 2015)

Isohumulones have a very broad range of physiological effects: they can reduce metabolic inflammation and insulin resistance. Furthermore they have a positive impact on dyslipidemia and obesity. So they might be interesting bioactive agents in the treatment of chronic diseases associated with metabolic inflammation and medical nutrition therapy (Bland et al., 2015).

In a mouse model **colupulone** was showing to be a potent inducer of hepatic CYP enzymes (P4503A) (Mannering et al., 1996).

4.1.4 Flavonoids

Flavonoids (0.5-1.5%) especially **prenylated flavonoids** - with a big amount of chalcones like xanthohumol (up to 1% in dried hop cones; 80-90% of total flavonoid amount) and xanthohumol B - **prenylated flavanones** like isoxanthohumol, 6-prenylnaringenin, 8-prenylnaringenin and glycosides of quercetin and kaempferol can be found in hops.

The basic core structure of prenylated flavonoids is a flavane with 2 benzene rings (A and B) linked together by a pyrane ring (C) and a prenyl group (C_5H_9, 3-methyl-2-butenyl-group) attached to the flavane nucleus. XN and desmethylxanthohumol (DMX; desmethyl analogue of XN) are prenylated chalcones, IXN, 8-PN and 6-PN are prenylated flavanones (Štulíková et al., 2018). These structures are very instable and as Figure 5 demonstrates isomerization is responsible for changes in the chemical structure.
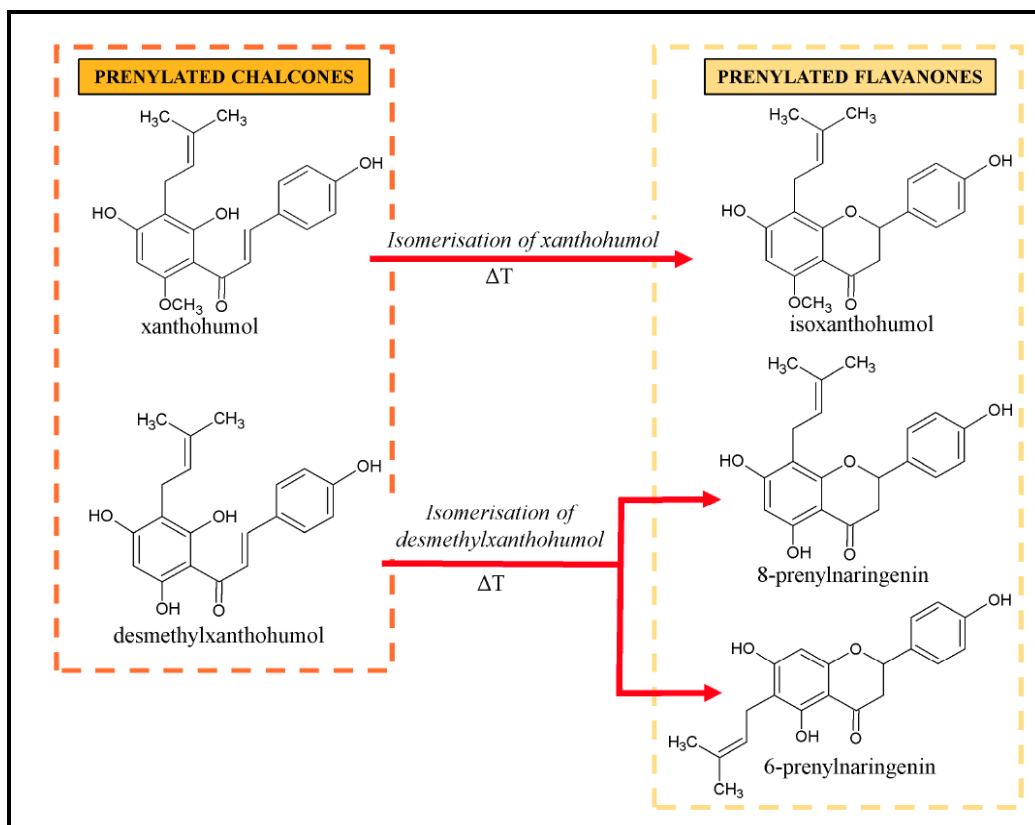
Figure 5 - Prenylated flavonoids in hops (Štulíková et al., 2018)

The natural compound **xanthohumol** can only be found in hop plants (*Humulus lupulus* L.) (Ortega et al., 2016) and can reach up to a content of 1% of dry weight in the female florescence. It is secreted mainly as part of the hop resin – but it can also appear in trichomes underneath young leaves (Liu et al. 2015). In Greek "xantho" means blond or bright – responsible for the color of the compound. Due to thermal conversion xanthohumol turns into the isoflavanone isoxanthohumol which explains that in beer the xanthohumol concentration is only around 0.1 mg/l (Gerhäuser et al., 2005).

XN has antioxidative, antimicrobial, chemopreventive and anti-inflammatory properties. Furthermore, effects on lipid metabolism could be shown. In western-type diet-fed apolipoprotein-E-deficient mice it could be proven, that XN supplements reduced plasma cholesterol and hepatic triglyceride concentrations significantly. So atherosclerotic lesion areas and plasma concentrations of the proinflammatory cytokine MCP-1 could be reduced (Doddapattar et al., 2013). MCP-1 (monocyte chemoattractant protein-1) is a key chemokine and potent chemotactic factor that regulates migration and infiltration of monocytes and macrophages. It is involved in many clinical pictures (Deshmane et al., 2009). The combination of reduced plasma cholesterol and MCP-1 concentrations resulted in atheroprotective effects of XN. Also positive effects on liver and cardiovascular diseases, diabetes, arthrosis, neurodegenerative diseases (Alzheimer`s, Parkinson`s, demential) and malaria infection have been reported. All these effects could be proven in animal models (Biendl M., 2018).

The Medical University of Vienna (Institute for Cancer Research; project group TA-XAN) was the first institution that carried out studies on healthy humans. It could be shown that **a daily dose of 12 mg of XN** had significant protective effects against alterations in genetic material (DNA mutations). This DNA protective effect was investigated in collaboration with the Linus Pauling Institute at the Oregon State University in Corvallis (USA) who carried out a clinical trial with a larger number of healthy probands. As the clinical trial was supported by the FDA a health claim (like "Xanthohumol can protect DNA") is very likely to be approved by the American Food and Drug Administration (FDA) and European Food Safety Authority (EFSA), respectively – so it could be considered a nutritional supplement. However it has to be stated that further and more complex studies have to be carried out to get an approval for XN as a licensed herbal medicinal product (Biendl M., 2018).

8-PN is considered the most potent phytoestrogen **discovered so far**. Its effects could be observed in several *in vitro* and animal studies (Štulíková et al., 2018).

Desmethylxanthohumol may have chemopreventive effects but its expression in hops is only 1/5 of XN. DMX might nevertheless be interesting because it isomerizes into 8-PN and 6-PN (Nikolić et al., 2013).

IXN is a 5-O-methyl-analogon of 8-PN. It has much weaker estrogenic activity but studies (*in vitro* and *in vivo*) have shown that IXN can be converted into 8-PN enzymatically which is why IXN can be considered a pro-estrogen (Nikolić et al., 2013).

4.2 ANALYSIS OF *HUMULUS LUPULUS*

The analysis of hops compounds is very challenging. A recent study published the effects of different ethanol concentrations (10%, 30%, 50%, 70% and 95%) on the stability of active constituents of hops. 95% *Humulus lupulus* ethanolic extracts were determined to give best results and highest levels of constituents (Gagnon et al., 2014). These conclusions were included in this study during extraction optimization.

According to chromatographic analysis, GC (gas chromatography) and HPLC (high performance liquid chromatography) are frequent methods. For the analysis of volatile compounds and essential oil, GC is commonly used, whereas HPLC is used for the analysis and quantification of prenylated flavonoids and bitter acids. For example Taniguchi et al. (2015) developed a preparative method for the bitter acid oxide fractions from hops using HPLC-PDA-ESI/HRMS and MS². Moreover when using LC-MS and LC-MS-MS methods, LOD (limit of detection) and LOQ (limit of quantification) values between 1-30 ng/ml for flavonoids in hop extracts and beer could be concluded in another study (Nikolić et al., 2013).

In the last few years, **SFC** (supercritical fluid chromatography) has been used successfully for natural product analysis and became a powerful instrument not only for non-polar compounds (like fatty acids, essential oils or fat-soluble vitamins) but also for polar ones. It could be demonstrated in several studies that a broad range of compounds like flavonoids, carotenoids, water-unstable ginkgolides and highly polar triterpene saponins with several sugar residues can be analyzed (Eisath et al., 2017).

SFC uses environmentally neutral CO_2 as primary mobile phase. It is highly lipophilic and when converted into a supercritical fluid (above 31°C and 74 bar) its polarity is similar to heptane. Supercritical CO_2 has lower viscosity and higher diffusivity and therefore results in higher efficiency and faster separations than normal phase HPLC. Also consumption of organic solvents can be reduced (Zhao et al., 2013). The addition of co-solvents to the mobile phase to a maximum of 50% influences the solvent strength and makes the analysis of polar compounds possible. Furthermore acidic or basic solvents can be added to the mobile phase in low concentrations to achieve better peak shapes and selectivity. SFC is a very efficient and high selective separation method providing fast analysis with short run times (Scheuba et al., 2017). It furthermore protects thermolabile compounds (Wang et al., 2017).

The general principle of SFC is to use supercritical conditions (above critical temperature and pressure) but it has to be taken into account that changes in the mobile phase influence these supercritical conditions. In general there is an ongoing transition between the supercritical and subcritical (liquid) state. So it has to be concluded that SFC is often carried out in subritical state with temperature or pressure below the critical value (Hartmann et al., 2015).

SFx technology can be divided into SFE (supercritical fluid extraction), analytical SFC and preparative SFC (prep-SFC). SFE was first applied industrially for the preparation of extracts from hops for breweries. It was concluded that there is a linear relation between XN solubility and CO_2 density (Kostrzewa et al., 2013).

Using UPC² (Acquity UltraPerformance Convergence Chromatography[TM]) as an analytical instrument has a lot of advantages. As a stationary phase, columns packed with sub-2µm particles are used. They have big influence on efficiency, repeatability, stability and reliability of results (Yang et al., 2016). An aspect that has to be kept in mind is that these small particles affect pressure drops dramatically (Wang et al., 2017). A prep-SFC instrument is used for the isolation and purification of compounds. Advantages are faster separations than with HPLC, convenient use as the final evaporation of solvent is reduced and the use of a broad number of stationary phases that can be used for separation (Hartmann et al, 2015).

The complexity of hops with its valuable components (reaching from non-polar to very polar molecules) makes SFC technology an interesting instrument. To the best of our knowledge this study is the first report that presents the analysis and quantification of polar compounds in hops extracts and mono hops dietary products using UPC². Moreover an evaluation of suitability of this technology for the successful analysis and separation of non-polar and polar compounds in hops extracts in one single workflow was carried out successfully.

5 RESULTS AND DISCUSSION

5.1 SAMPLE PREPARATION AND EXTRACTION

Conclusion: The best and most efficient extraction for dried plant material and dietary supplement products was achieved with **EtOH 96%** hence it was used for preparation of all samples.

The evaluation of extraction efficiency was carried out with Prod5. The extraction was carried out with accurately weighted dried plant material that was pulverized in a mortar and extracted with 6 ml of extraction solvent. After sonification for 10 min at ambient temperature the sample was filtered into a 20 ml volumetric flask (VF 1). This procedure was repeated two more times and the flask VF 1 (= concentrated sample) was filled up to the volume with the extraction solvent. After a fourth extraction under same conditions the sample was filtered into a 20 ml volumetric flask (VF 2 = diluted sample) and filled up to the mark. 1 ml of each solution (VF 1 and VF 2) was transferred into a vial and dried with the sample concentrator. Then the dried extracts were each resolved in 500 µl of Hex/IPA (70:30) before running each sample on UPC² under optimized conditions (n = 3).

Apart from hulupinic acid, all reference compounds were used to estimate the extraction efficiency of different extraction procedures. It could be proved that EtOH at a concentration of 96% had the best impact on stability and extraction of target compounds - as suggested by Gagnon who already tested the impact of different EtOH concentrations (Gagnon et. al., 2014).

While testing DCM and Hex/IPA could not reach the **required limit of 95%** (calculated as AUC VF 2 / (AUC VF 1 + VF2) *100) for all reference substances.

DCM resulted in an extraction efficiency of 93.70% for IXN and also for 6-PN it was only 95.49%. Hex/IPA was resulting in 94.09% for IXN and only 92.45 % for XN.

With EtOH 96%, the extraction efficiency was 96.32% for 8-PN, 98.96% for 6-PN, 99.32% for IXN and 97.68% for XN (Table 9).

5.2 THIN LAYER CHROMATOGRAPHY (TLC)

The **best separation** results on TLC plates were achieved with n-hexane - isopropanol - formic acid (80:15:5) as mobile phase as described in trial 7, Table 11. The optimum detection reagent was a mixture of vanillin 1% (in MeOH) and sulfuric acid 5% (in MeOH).

Wagner (Wagner et al., 1983) suggested a mixture of n-heptane – isopropanol – formic acid (90:15:0.5) as a mobile phase for an optimal TLC system for hops extracts. This mobile phase was slightly modified and adjusted to get an impression of comprised components within all investigated samples.

As a more non polar mobile phase was used, polar components had a low retention factor (R_f) whilst non polar substances traveled a longer distance on the polar TLC plate. To identify all reference substances in the purchased products a TLC plate comprising all samples (each at a concentration of 25 mg/ml) was prepared applying 30 µl of each sample. Figure 6 to Figure 11 present the results (non derivatised and derivatised at different wavelengths).
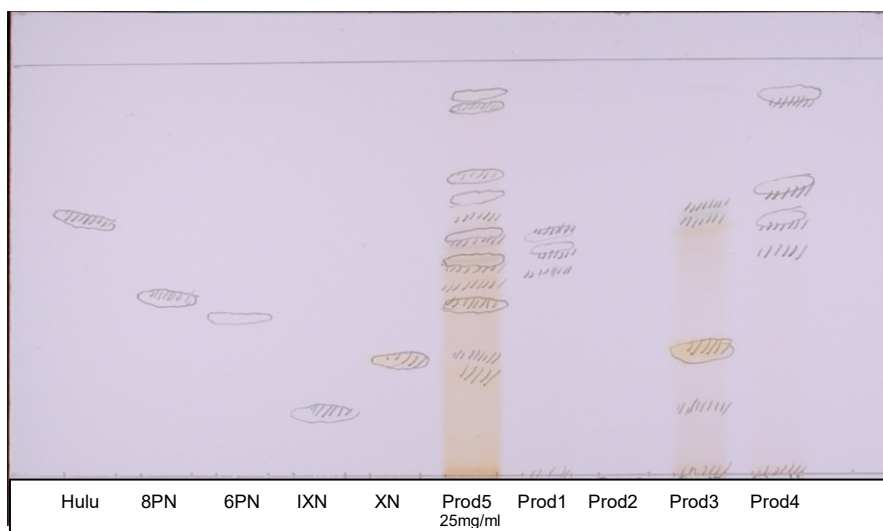
Figure 6 - all components - UV Vis non-derivatised
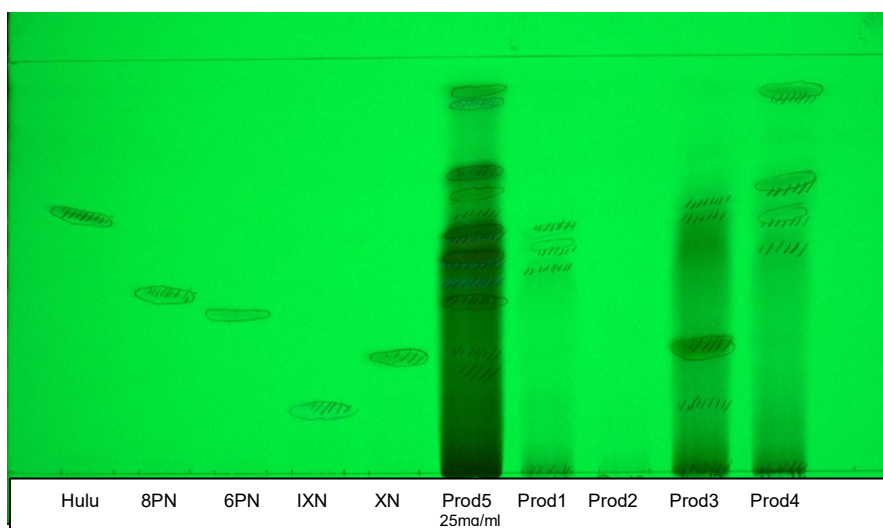
Figure 7 - all components – 254 nm non-derivatised
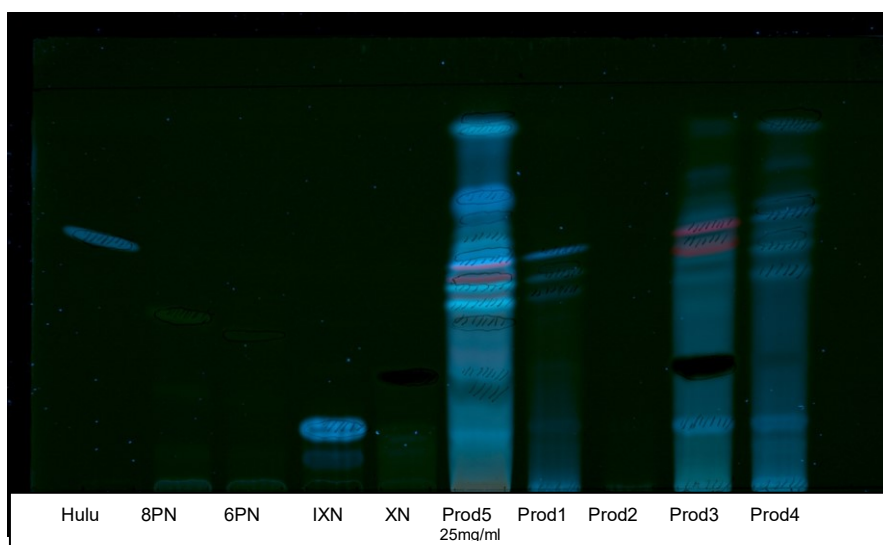
Figure 8 - all components – 366 nm non-derivatised
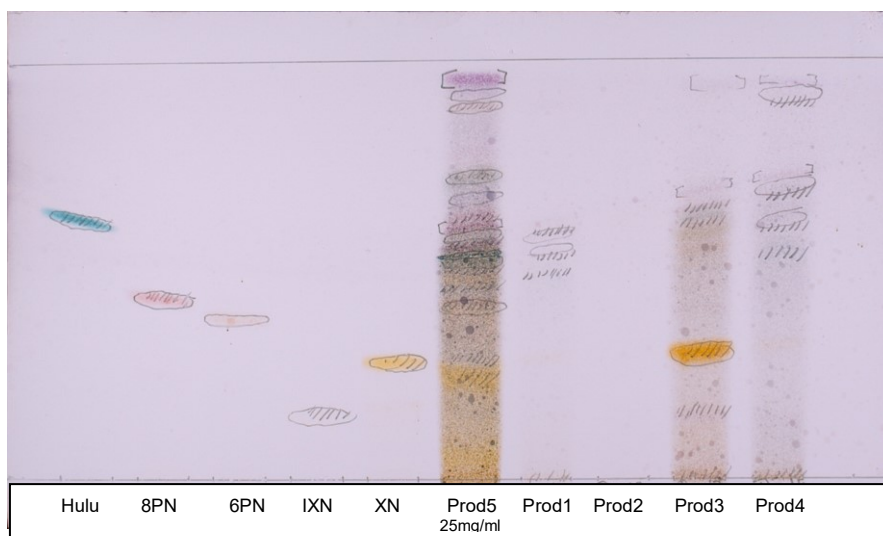
Figure 9 - all components - UV Vis derivatised
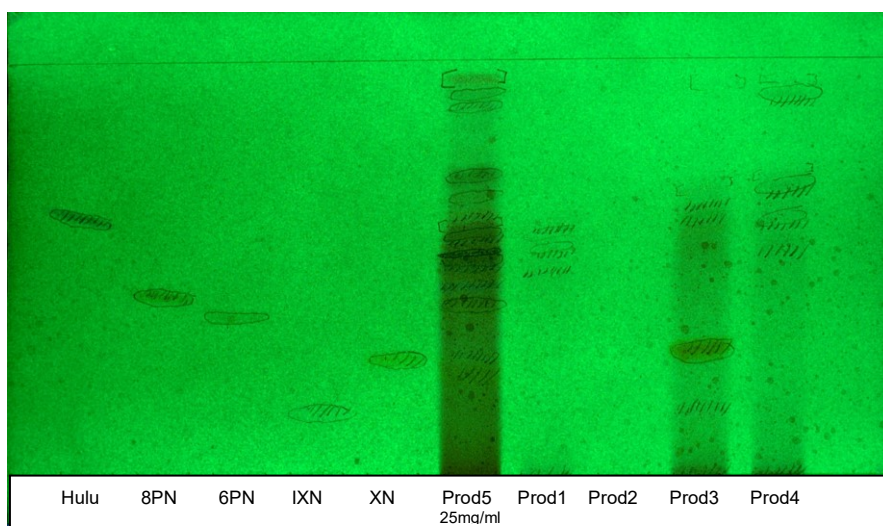
Figure 10 - all components – 254 nm derivatised
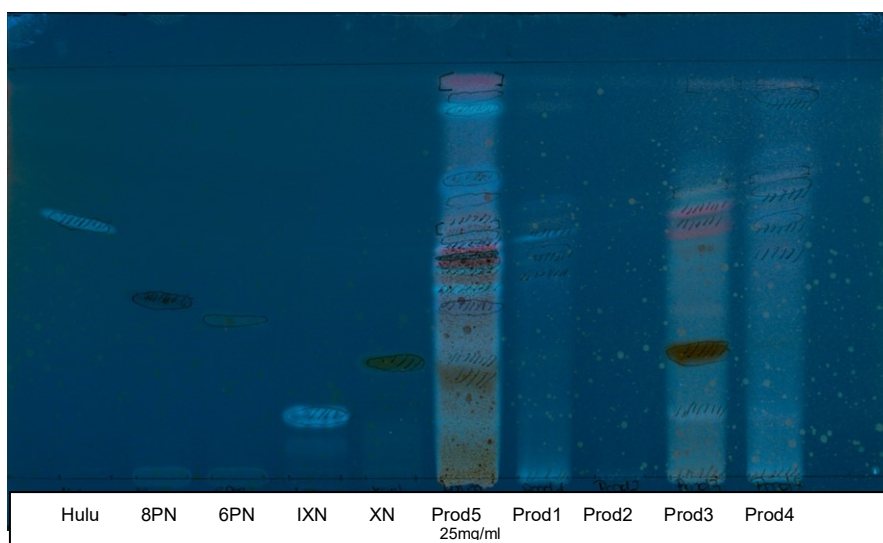
Figure 11 - all components – 366 nm derivatised

In general, interpretation in UV 366 nm gave good results as well as the derivatised TLC plate in visible light. All reference substances resulted in good and intense bands (Hulu: turquoise, 8-PN: light pink, 6-PN: light yellow, IXN: whitish-yellow, XN: intense yellow). Hulu as the most non polar substance had the highest hR_f value (hR_f = 62.35). IXN resulted in highest retention with hR_f = 14.15. Reference bands were compared within all products. The reference substances could not be detected in all products (applied concentrations: 25 mg/ml) and also the intensity of bands was varying a lot.

Table 2 - Interpretation of bands on TLC for purchased products

	Interpretation of bands
Prod1	only hulupinic acid could be found before and after spraying (UV Vis, UV 254 nm, UV 366 nm)
	intensity of the band was nevertheless very low
Prod2	no bands at all
Prod3	very intense band for XN
	less intense band for IXN before and after spraying specially at UV 366 nm
	very light band for 8-PN
Prod4	very light bands for XN, IXN and Hulu especially when non derivatised UV 366 nm conditions
	very light band for 8-PN
Prod5	all bands for the reference substances could be detected
	especially XN and Hulu resulted in intense bands

It can be concluded that the concentration of target substances in **Prod1** is very low, in **Prod2** it was below detection limit. For **Prod3** the intense band for XN was expected, as the product was enriched with Xanthohumol (see Table 8). **Prod4** clearly contained XN, IXN and Hulu. For the unambiguous identification of 8-PN within Prod3 and Prod4 the concentration was too little. **Prod5** was showing all reference bands just like **Prod6** (see Figure 29). However, the intensity of bands was higher in Prod6.

5.3 CHROMATOGRAPHIC CONDITIONS UPC²

For all experiments an Acquity UltraPerformance Convergence Chromatography™ (UPC²) instrument from Waters (Milford, MA, USA) was used. UPC² was equipped with binary solvent-, column-, convergence- and sample manager as well as with PDA-, QDa- and ELS detector. The operating software was Empower 3, release 2 from Waters.

As presented in Figure 12 and Figure 13 optimum separation as well as retention time was achieved by using a **Viridis BEH 2-EP column** (1.7 µm particle size, dimension: 3.0 x 100 mm).
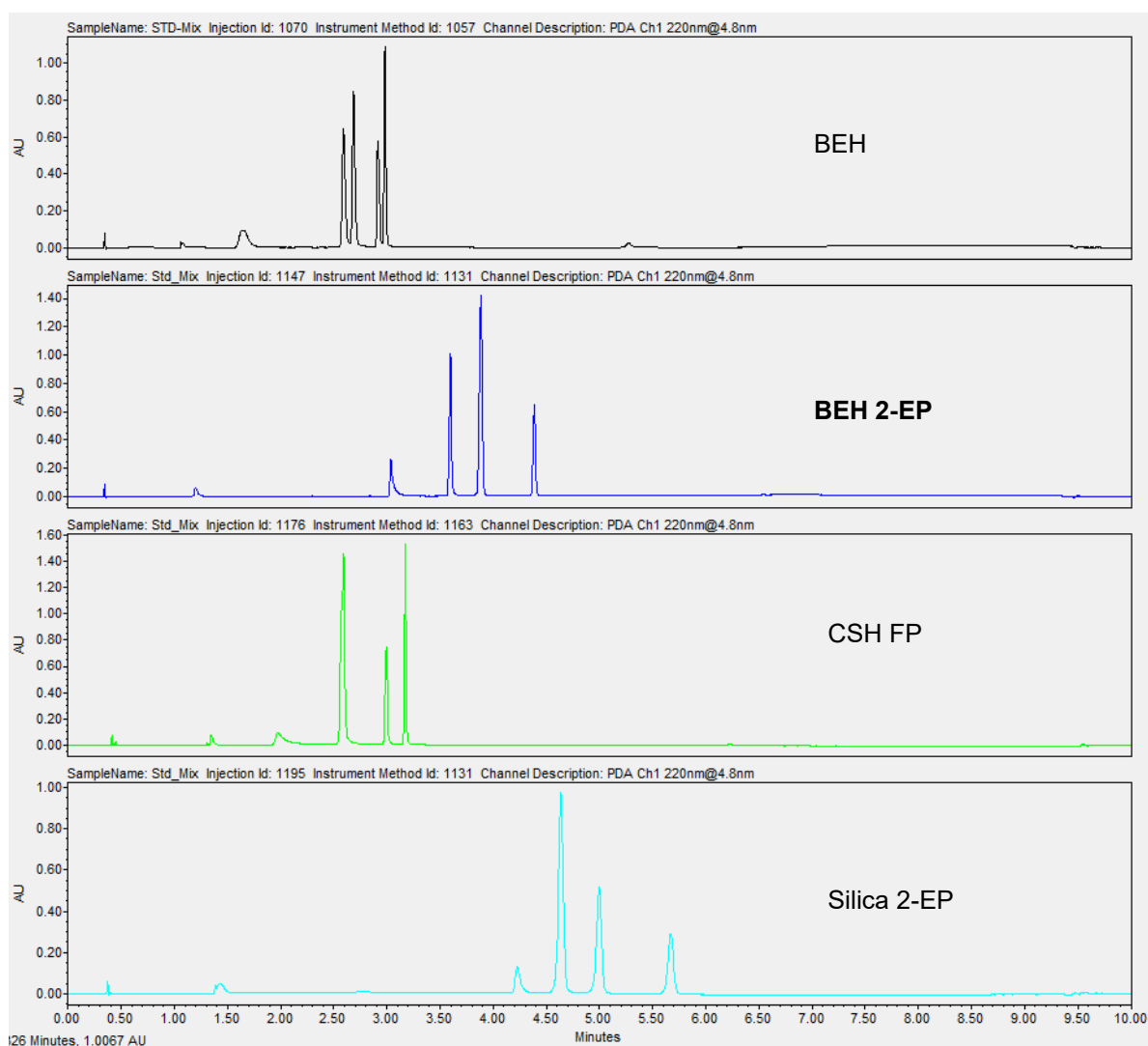
Figure 12 - Column Screening 1
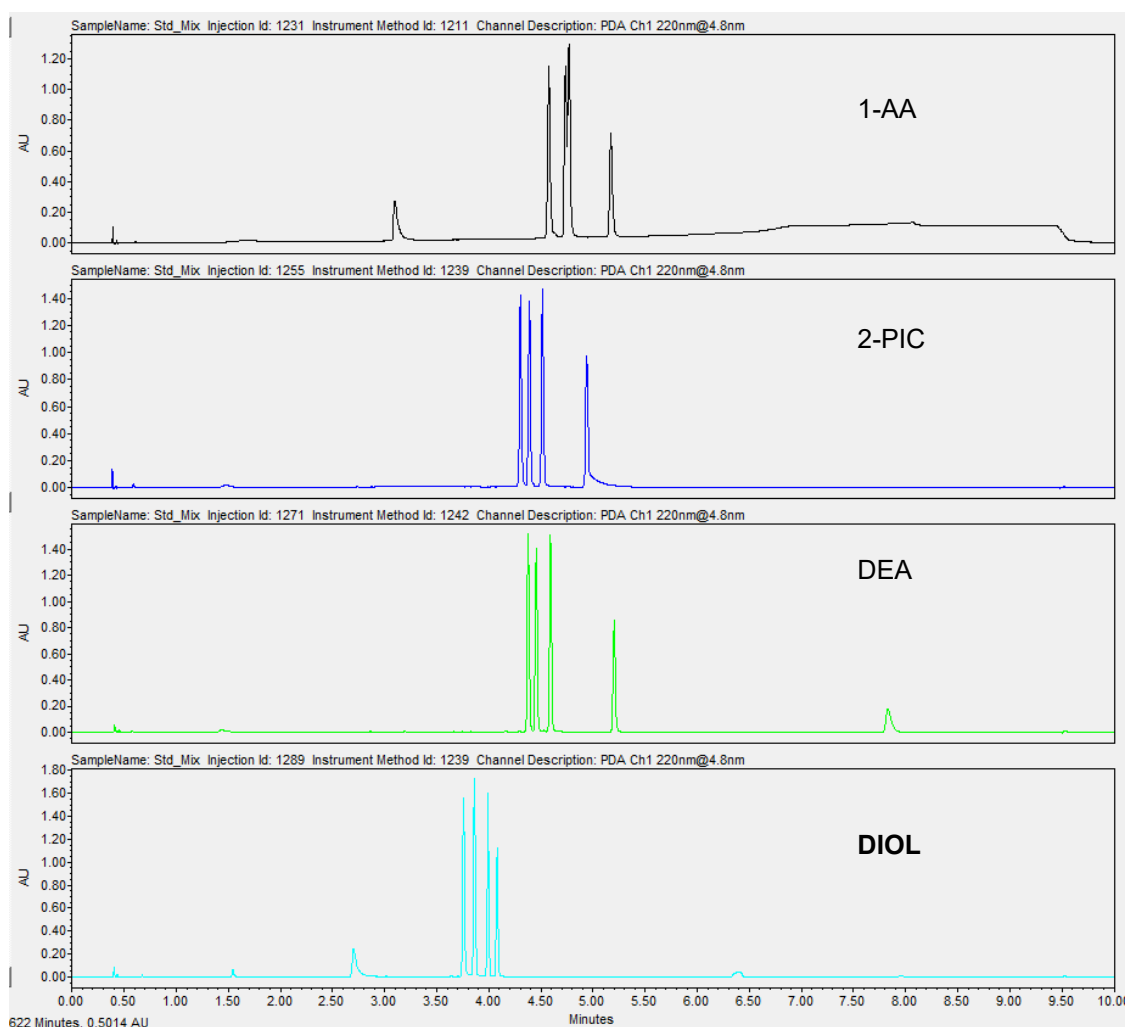
Figure 13 - Column Screening 2

Other column materials like BEH or DEA did not separate the peaks very well. With 1-AA and 2-PIC just 4 out of 5 substances could be detected. Using CSH FP, the peak resolution was very low. Results for Silica 2-EP were good but the column was excluded because it was not suitable for the hops crude extract (6.4.2.1).

Only the DIOL column was resulting in good peak shape and was tested along with BEH 2-EP within the co-solvent screening.

Co-solvents have a big influence on separation and peak shape. Four different solvents were tested: methanol (B1), acetonitrile (B2), ethanol (B3), isopropanol (B4). Figure 14 presents the results for the DIOL column (high-density diol). For this column MeOH was the best co-solvent. Figure 15 gives an overview of results on BEH 2-EP. Here IPA was giving best results.

Comparing those two chromatograms (DIOL plus MeOH and BEH-2EP with IPA) regarding peak shape, separation and retention time the BEH 2-EP (Bridged-Ethylene-Hybrid 2-Ethylpyridine) column was considered to be better than the DIOL column.
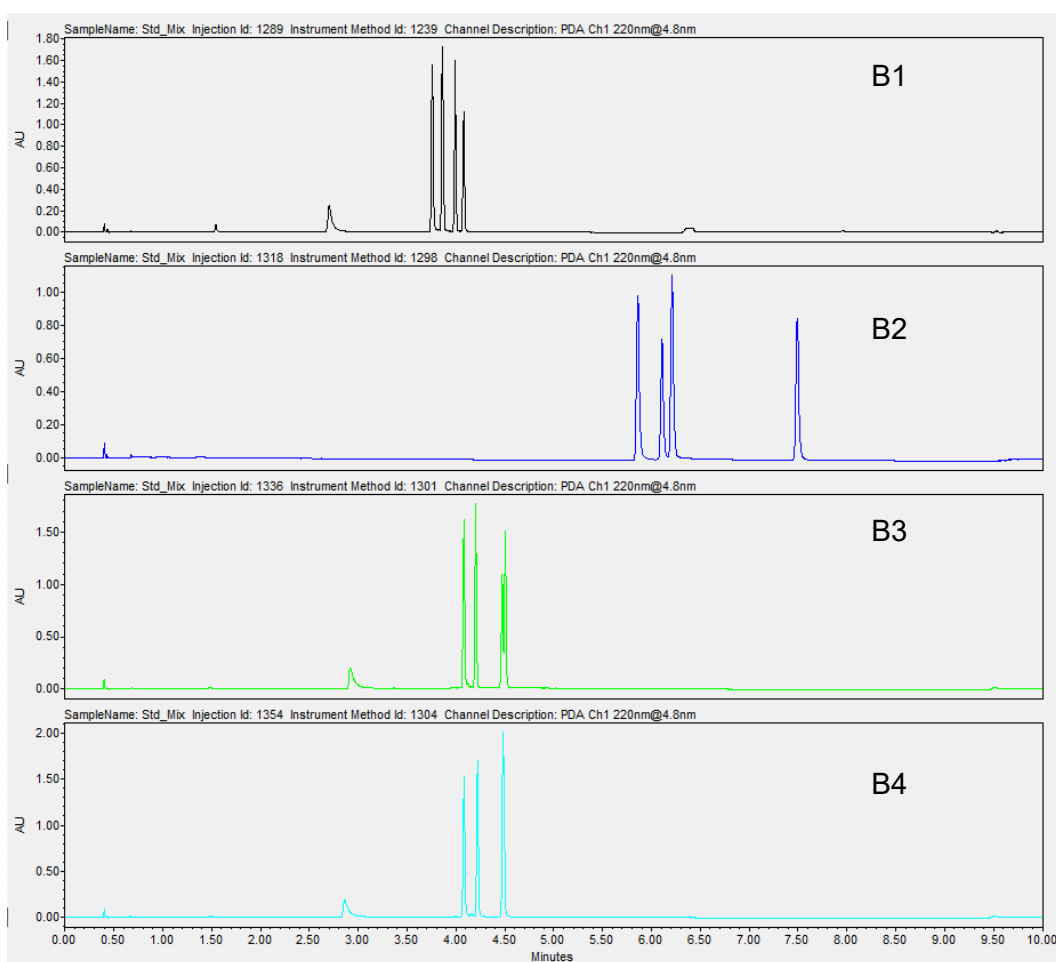
Figure 14 - Co-solvent screening on DIOL column with MeOH (B1) giving best results.
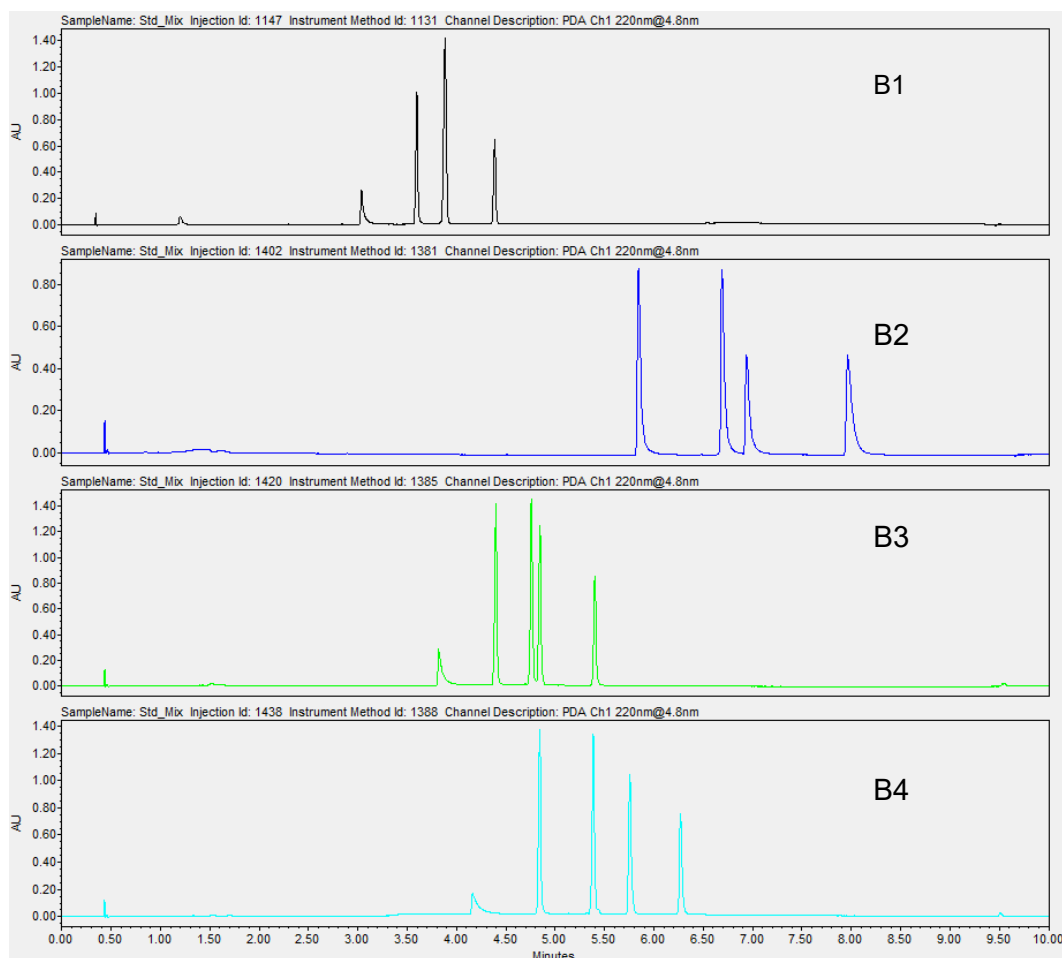
Figure 15 - Co-solvent screening on BEH 2-EP column with IPA (B4) giving best results

According to the Waters Handbook[8] no additive to the mobile phase was suggested when using the Viridis BEH 2-EP column. This could be confirmed as **pure isopropanol** without any additives was showing best results in peak shape as well as in peak separation.

The optimized **mobile phase consisted of CO_2 (A) and isopropanol (B) as co-solvent**.

Further additives to IPA (0.1% FA, 0.1% AA and 0.1% TFA) did not have any influence on peak shape – just on retention time. In general the influence of acids did not show any better results than using IPA alone (Figure 31). It was furthermore observed that ammonium acetate and ammonium formate did not have an impact on peak shape or peak separation but on the ionization of analytes detected by the QDa (see 6.4.2.2). Therefore, further testing with these buffers as an ISM solvent for the QDa was carried out.

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[8] http://www.waters.com

To identify peaks in the QDa, a make-up solvent or so called ISM solvent (isocratic solvent manger solvent) for the ionization of analytes had to be used. As ammonium formate resulted in good effects in co-solvent screening regarding mass detection, it was included in the testing. The impact of three different make-up solvents on QDa detection was tested (6.4.2.3.).

Best ionization and therefore selected make-up solvent was **10 mM ammonium formate in 99% MeOH and 1% H$_2$O** (HPLC). Especially the **negative mode** was giving good results regarding mass detection on QDa.

As the addition of a make-up solvent for the QDa also correlates with flow rates, the flow rates for BSM and ISM had to be adjusted and a stabile ABPR (active backpressure regulator) had to be secured too. According to the Waters Handbook[9] an ISM flow rate between 0.3-0.8 ml/min was recommended for QDa detection. At a flow rate of 0.3 ml/min (ISM) not enough substance reached the QDa to derive significant conclusions. BSM flow rates higher than 1.0 ml/min resulted in overpressure errors.

The optimized conditions and flow rates were **1.0 ml/min** (for BSM) and **0.6 ml/min** (for ISM) with ABPR of 2000 psi (equals about 130 bar).

When testing the influence of column temperature, it could be demonstrated, that at a temperature of 60°C early peaks (non-polar) could be separated very well but overall baseline separation was bad and also later peaks did not appear which might be due to a thermal decomposition. Also at a temperature of 55°C thermal decomposition was noticed.
Best results could be seen at 50°C: there was a good baseline separation and also peaks were more intense compared to other temperature programs. When testing different injection volumes (see 6.4.2.4) an injection volume of 1 µL was giving best results regarding peak shape and baseline separation.

Ideal column temperature, injection volume and detection wavelength for QDa were as follows: **50°C**, **1 µL** and **220 nm**. At these conditions the intensity of peaks was good and also all relevant components were stable. With higher temperature programs a degradation of thermo-labile components could be observed (see Figure 38). Especially for Hulu the decomposition was significant.

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[9] http://www.waters.com

During the experiment, the influence of ELSD on the chromatograms and mass detection was tested. In general, the Acquity ELS Detector has the ability to analyze a broad variety of compounds in a single analytical run. It can be used to monitor compounds with poor or no response to UV Vis (PDA detector) or for compounds that do not ionize very well in mass spectrometry.[10] That was supposed to be interesting for the complexity of compounds in the hops extract. However, by trying to use this triple-detection (PDA – ELSD – QDa), there were too many problems in finding the right equilibrium of BSM and ISM solvent flow. Also the backpressure was very variable and instable and higher flow rates in BSM and ISM would have been necessary to keep it stable. However, this would have resulted in an overpressure during gradient elution. Therefore, the triple-detection might only work at isocratic conditions.

Due to disturbances and dissatisfying results all experiments were carried out **without ELSD**.

Gradients were optimized for the standard mix and the extract of the toto drug (Prod5).

The optimized gradient for the **standard mix** was set as follows: at 0 min - 80A/20B, at 4 minutes 60A/40B, held at this composition for 1 minute (total run time: 5 minutes) and then equilibrated at initial conditions for 1 minute. It resulted in good peak shape and baseline separation as well as intensity of peaks (Figure 16).
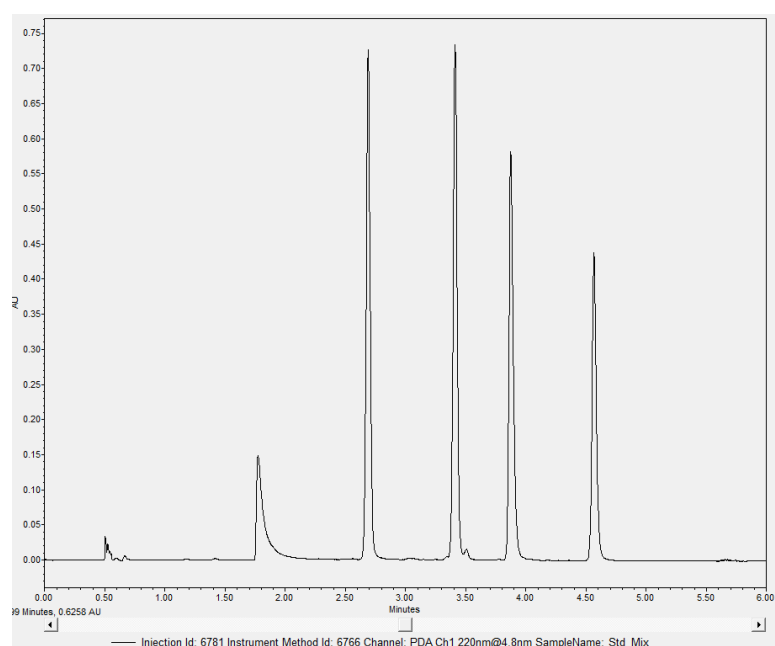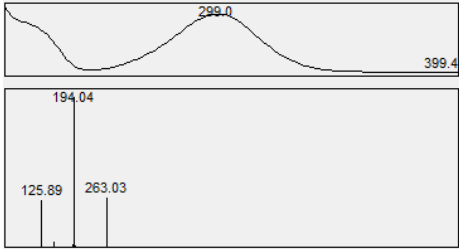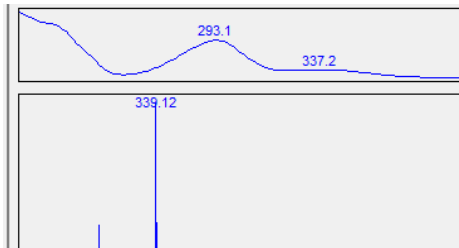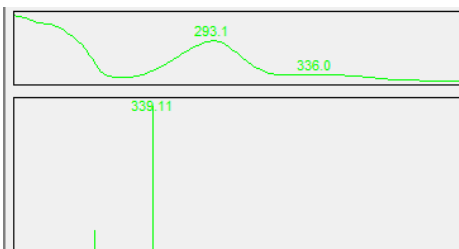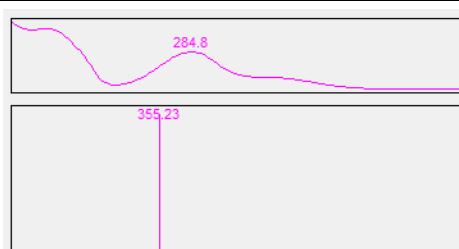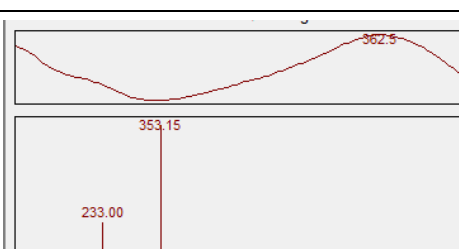
Figure 16 - Results with optimized gradient for standard mix

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[10] http://www.waters.com

To identify all reference substances also in the complex extract, the QDa spectra of the standard mix were evaluated showing the following results presented in Table 3:

Table 3 – Positive or negative mode QDa and UV spectra of reference substances

Hulupinic Acid		
UV$_{max}$	299.0	
m/z	194.04 // 263.03 [M-H]$^-$ // 125.89	
Molecular weight	264 g/mol	

8-Prenylnaringenin		
UV$_{max}$	293.1 // 337.2	
m/z	339.12 [M-H]$^-$	
Molecular weight	340 g/mol	

6-Prenylnaringenin		
UV$_{max}$	293.1 // 336.0	
m/z	339.11 [M-H]$^-$	
Molecular weight	340 g/mol	

Isoxanthohumol		
UV$_{max}$	284.8	
m/z	355.23 [M+H]$^+$	
Molecular weight	354 g/mol	

Xanthohumol		
UV$_{max}$	362.5	
m/z	353.15 [M-H]$^-$ // 233.00	
Molecular weight	354 g/mol	

Hulu, 8-PN, 6-PN and XN were detected in **negative scan mode** on QDa, which means that 1H had to be added to the mass of the detected ion to derive the molecular weight of the substance. **IXN** was detected in **positive scan mode**, which means that 1H had to be subtracted from the mass of the detected ion to derive the molecular weight.

For 8-PN, 6-PN, XN and IXN the derived molecular weight correlates with the de facto molecular weight of the substance. For Hulu the conditions for mass detection and separation were not ideal (throughout the whole experiment) and so the highest peak of m/z did not correspond with the de facto molecular weight - the second highest (263.03 m/z) did.

To unambiguously identify the reference peaks, the chromatogram of the standard mix was compared with the chromatograms of each single compound (carried out under the same optimized conditions) and also with the appropriate QDa spectra.

Std_Mix_Inj. 6781
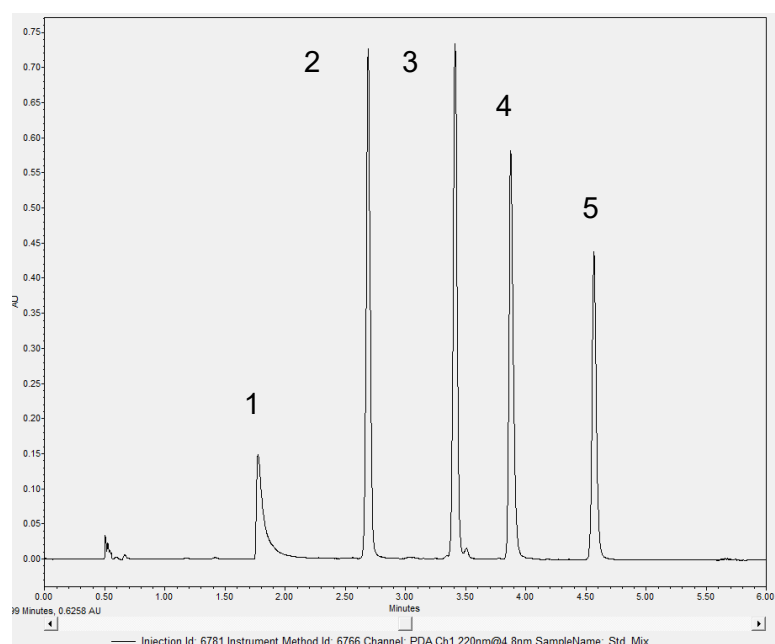
Figure 17 - Optimized conditions for standard mix (220 nm)

Peaks in the standard mix (Figure 17) could be identified as follows: (1) Hulu, (2) 8-PN, (3) 6-PN, (4) IXN, (5) XN.

Extracts of all purchased products were subjected to UPC² analysis using the optimized extraction method (with EtOH 96%) and the optimized UPC² method. The results can be found in Figure 39 to Figure 44. All chromatograms were compared (overlay) with the standard mix and also with UV spectra and QDa spectra of the single reference components.

Summarized optimum conditions for the standard mix are the following:

DATA OVERVIEW	**Standard Mix – Run time: 5 minutes**
UPC² conditions	**Optimized gradient**

time (min)	A (CO_2 %)	B (IPA %)
0	80	20
4	60	40
5	60	40
5.1	80	20
6	80	20

UPC² conditions

System:	Acquity UPC2
Detector:	PDA, QDa
Software:	Empower 3
Column:	Viridis BEH 2-EP
	3.0 x 100 mm
	1.7 µm particle size

SOLVENTS:

Mobile phase **A**:	CO_2
Mobile phase **B**:	Isopropanol (IPA)
Make up:	10 mM Ammonium Formate in 99% MeOH and 1% H_2O
Strong wash:	Hex/IPA (70:30)
Weak wash:	MeOH
Flow rate:	1,0 ml/min (BSM)
	0,6 ml/min (ISM)
ABPR:	2000 psi
Column temp.:	50°C
Sample temp.:	8°C
Injection volume:	1 µL

Chromatogram

Figure 18 - Optimized UPC² method for standard mix (220 nm)

Table 4 - Interpretation of UPC² chromatograms of extracts of purchased products (6.4.2.7)

	Interpretation of peaks
Prod1	very low peaks for 6-PN, IXN and XN
	no peaks for Hulu and 8-PN
Prod2	only very low peaks for IXN and XN
	no other peaks identified
Prod3	intense peaks for IXN and XN
	small peak for 6-PN
	very small peak for 8-PN
	no identification of Hulu
Prod4	intense peaks for IXN and XN
	small peak for 6-PN
	very small peak for 8-PN
	Hulu could not be identified explicitly
Prod5	all peaks could be identified
	the explicit authentication for Hulu was problematic (also with QDa spectra)
Prod6	all peaks could be identified
	intensity of peaks was higher compared to Prod5 (also toto drug); especially for Hulu

Compared with TLC the following conclusion can be drawn:

Prod1 resulted in very small peaks on UPC² - on TLC these peaks could not be identified.

Prod2 resulted in very small peaks on UPC² - on TLC these peaks could not be identified.

Prod3 resulted in intense peaks for IXN and XN – also on TLC these bands were intense. A small peak for 6-PN and 8-PN was detected on UPC² - on TLC only a light band for 8-PN was detected.

Prod4 resulted in intense peaks for IXN and XN – also on TLC these bands were identified. 8-PN could be detected on both chromatographic systems too.

Prod5 and Prod6: all substances could be identified on both chromatographic systems. Intensity of bands on TLC and intensity of peaks on UPC² were both higher in Prod6.

For the optimization of the gradient for the total **hops extract** (carried out with Prod5) the gradient had to be adjusted so that especially the non-polar compounds that elute first were optimally separated. Therefore the starting conditions for the mobile phase had to be 97% CO_2 and 3% IPA. Also the gradient had to be flatter. Best results regarding peak and baseline separation as well as peak shape could be achieved with the following gradient: starting with 97A/3B at 0 min, at 5.5 min 90A/10B, at 14 min 60A/40B, held at this composition for 1 minute (total run time: 15 minutes) and then equilibrated at initial conditions for 1 minute.
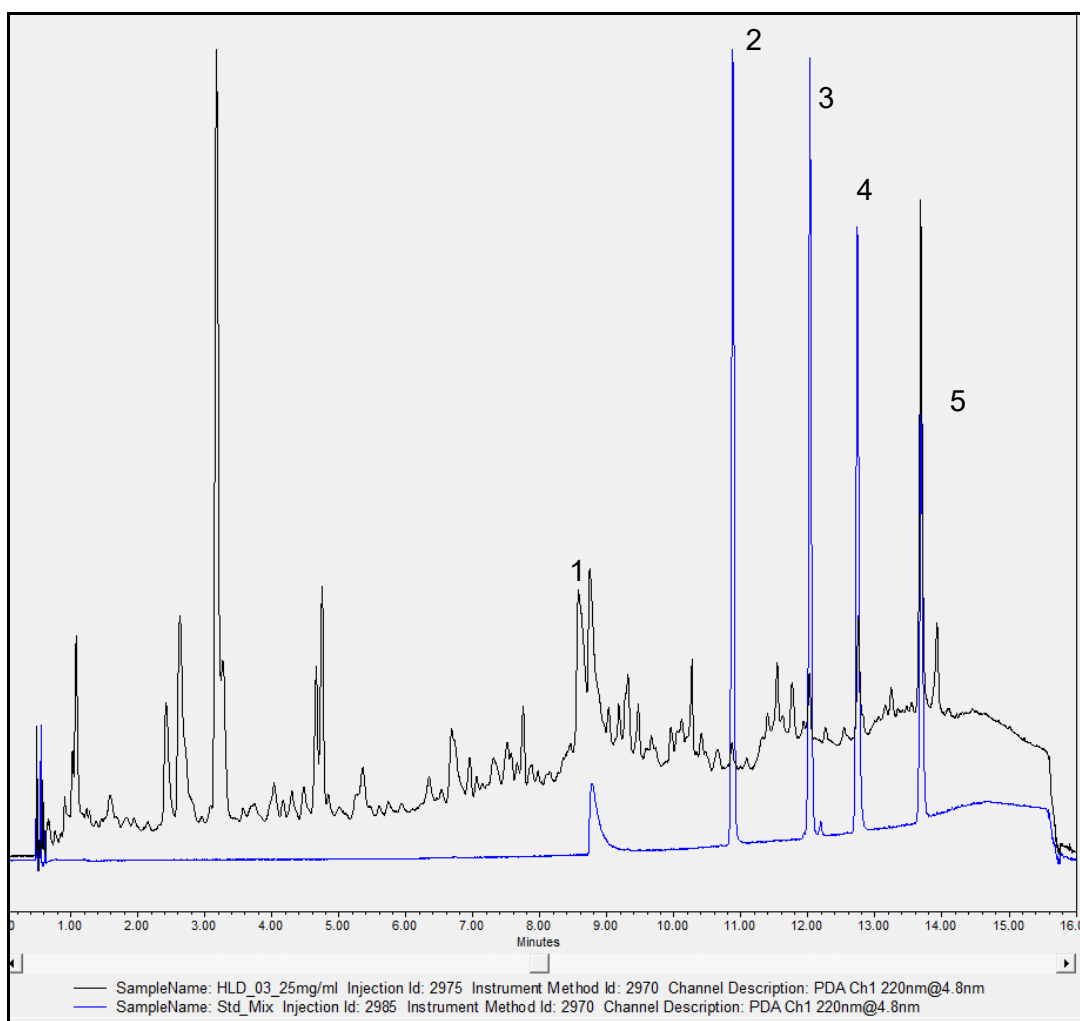
Figure 19 - Overlay of standard mix and hops extract (220 nm)

All relevant reference substances could be identified in the extract: hulupinic acid (1), 8-prenylnaringenin (2), 6-prenylnaringenin (3), isoxanthohumol (4), xanthohumol (5). The identification of peaks was carried out by overlay with the standard mix and comparison of UV spectra. Also mass spectra of the compounds and extract were compared.

During the optimization for the extract not only all reference substances could be identified but it could also be proven that on UPC² the **elution of non-polar and polar compounds is possible in a single procedure**. Also good separation results could be achieved. Only the accurate detection of hulupinic acid was not clearly possible due to overlaps in peaks and m/z.

Summarized optimum conditions for the hops extract (Prod5) are the folloowing:

DATA OVERVIEW

UPC^2 conditions

System:	Acquity UPC2
Detector:	PDA, QDa
Software:	Empower 3
Column:	Viridis BEH 2-EP
	3.0 x 100 mm
	1.7 µm particle size

SOLVENTS:

Mobile phase **A**: CO_2

Mobile phase **B**: Isopropanol (IPA)

Make up:	10mM Ammonium Formate in 99% MeOH and 1% H_2O
Strong wash:	Hex/IPA (70:30)
Weak wash:	MeOH

| Flow rate: | 1,0 ml/min (BSM) |
| | 0,6 ml/min (ISM) |

ABPR:	2000 psi
Column temp.:	50°C
Sample temp.:	8°C
Injection volume:	1 µL
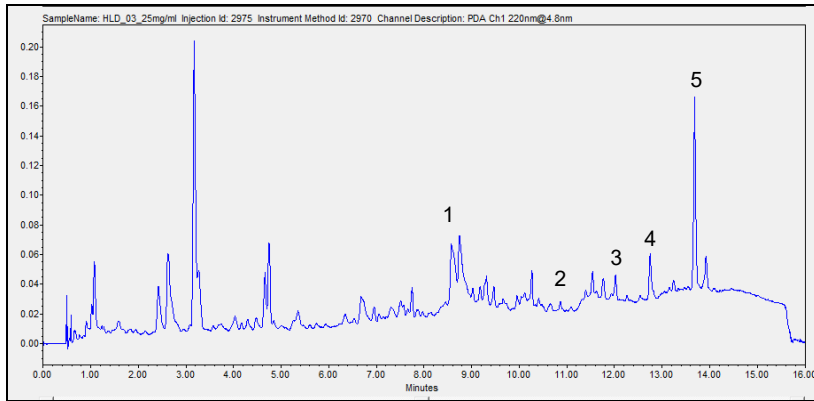
Hops extract (Prod5) – **Run time: 15 minutes**

Optimized gradient

time (min)	A (CO_2 %)	B (IPA %)
0	97	3
5.5	90	10
14	60	40
15	60	40
15.1	97	3
16	97	3

Chromatogram

Figure 20 - Optimized UPC² method for hops extract (220 nm)

5.4 OVERVIEW OF FURTHER POTENTIAL SUBSTANCES DETECTED IN THE EXTRACT

An overview of possible constituents within the whole extract (Figure 21) was carried out during the studies. UV spectra and mass peaks of hops specific large and intense peaks were compared with literature.

Total amount of integrated peaks (Inj. ID 2018; Method ID 2013):　　　　108
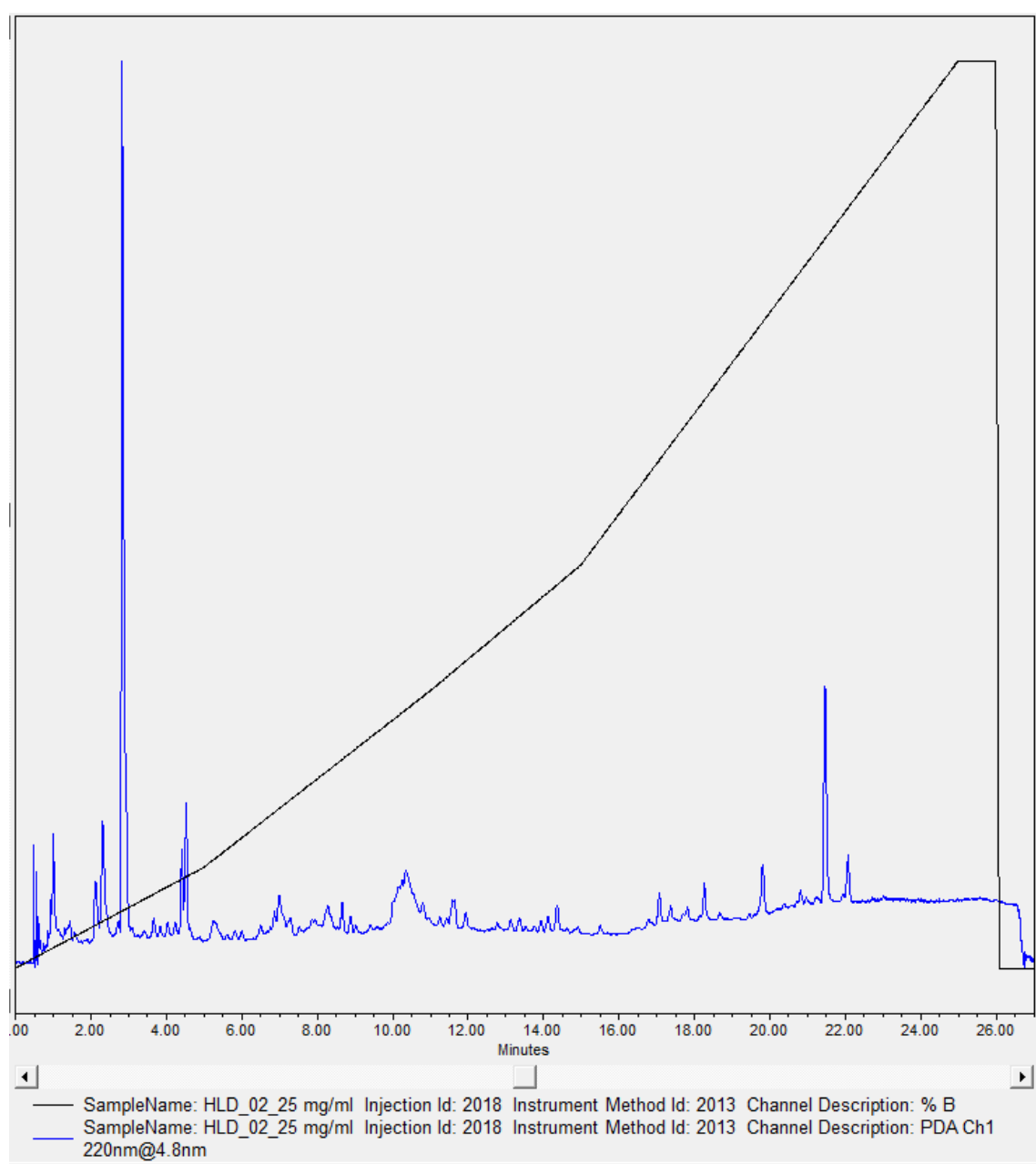
Figure 21 - Extract for identification of possible compounds (Inj. ID 2018) with gradient

The following table (Table 5) summarizes interesting peaks (out of 108 integrated peaks) within the hops extract and possible substances containing the CAS number (chemical abstract service number); peaks are listed by their retention time. The extensive list gives a good impression of the complexity of hops extracts.

Some of these substances are an interesting field of research as most of them are exclusively found in hop. Some examples:

- Garcinielliptone HC (MW was determined 416 g/mol) was identified as a major component of hop flower extracts to inhibit Aβ production in a cell model. In mice models, the administration of hop extracts also resulted in a decrease of Aβ. This small peptide (derived from amyloid precursor protein APP) can be found in pathologic deposition/accumulation in the brain of Alzheimer disease patients (Sasaoka et al., 2014).
- Desmethyl-xanthohumol (MW 340.375 g/mol) can be metabolized into a mixture of 8-PN and 6-PN. Especially 8-PN has been shown to be a potent phyto-oestrogen which means that desmethyl-xanthohumol serves as a pro-oestrogenic compound (EMA/HMPC Assessment Report).
- In a mouse model colupulones (MW 400.559 g/mol) were showing to be a potent inducer of hepatic CYP enzymes (P4503A) (Mannering et al., 1996).

Table 5 - Overview of potential substances in hops extract by retention time (https://pubchem.ncbi.nlm.nih.gov)

Peak nr.	Retention time [min.]	Proposed molecular weight [g/mol]	Supposed structure	CAS-nr:
4	0.811	306	Leukoanthocyanidol	93527-39-0
			Epigallocatechol	970-74-1
			Gallocatechol	970-73-0
5	0.997	398	Lupulone B	842121-74-8
6	1.041	398	Lupulone B	842121-74-8
8	1.446	477	Isoxantholupone	1691256-96-8
12	2.118	416	2''-Hydroxyxanthohumol M	1691257-01-8
			6-Hydroxycolupulone	43161-61-1
			Lupulone E	29366-64-1
			Colupox a	18944-21-3
			Garcinielliptone HC	1008376-89-3
13	2.305	416	2''-Hydroxyxanthohumol M	1691257-01-8
			6-Hydroxycolupulone	43161-61-1
			Lupulone E	29366-64-1
			Colupox a	18944-21-3
			Garcinielliptone HC	1008376-89-3
14	2.724	430	Lupulone P	n.a.
			Lupulone C	613683-50-4
15	2.828	400	Isoxanthohumol P	1691256-98-0
			Xanthohumol P	1691256-97-9
			Colupulone	43160-56-1
			Colupulone I	21621-94-3
			n-Butyrolupuphenone	35049-56-0
23	4.408	430	Lupulone D	613683-51-5
			Lupulone C	613683-50-4
24	4.518	416	2''-Hydroxyxanthohumol M	1691257-01-8
			6-Hydroxycolupulone	43161-61-1
			Lupulone E	29366-64-1
			Colupox a	18944-21-3
			Garcinielliptone HC	1008376-89-3
31	6.856	416	2''-Hydroxyxanthohumol M	1691257-01-8
			6-Hydroxycolupulone	43161-61-1
			Lupulone E	29366-64-1
			Colupox a	18944-21-3
			Garcinielliptone HC	1008376-89-3
32	6.990	430	Lupulone D	613683-51-5
			Lupulone C	613683-50-4

Peak nr.	Retention time [min.]	Proposed molecular weight [g/mol]	Supposed structure	CAS-nr:
33	7.291	416	2''-Hydroxyxanthohumol M	1691257-01-8
			6-Hydroxycolupulone	43161-61-1
			Lupulone E	29366-64-1
			Colupox a	18944-21-3
			Garcinielliptone HC	1008376-89-3
49	11.637	362	5-Deprenyllupulone c	1383844-41-4
			cis-Alloisoadhumulone	1270005-82-7
			trans-Alloisoadhumulone	1270005-80-5
			Isotricycloadhumene	1270005-76-9
			Isotricyclohumene	1194013-50-7
			Tricyclohumene	1194013-48-3
			n-(+)-Adhumulone	1000974-81-1
			cis-Iso-n-humulone	860020-69-5
			cis-Isoadhumulone	96614-01-6
			trans-Isoadhumulone	68107-76-6
			cis-Alloisohumulone	65914-00-3
			Racemic isohumulone	38574-02-6
			Alloisohumulone A	1533-83-1
			(-)-(R)-Humulone	26472-41-3
			Isohumulone (6CI)	25522-96-7
			Isoadhumulone (6CI)	25422-83-7
			Isohumulone B (6CI)	1534-03-8
			Isohumulone A (6CI)	467-72-1
			α-Lupulic acid = Humulone	23510-81-8
			(-)-(R)-Humulone	26472-41-3
			n-Humulone	16520-91-5
			Lupuloxinic Acid	464-80-2
			Humulon	26472-41-3
			Adhumulone	31769-65-0
53	12.513	378	Tricyclooxyisoadhumulone B	1821029-94-0
			Tricyclooxyisoadhumulone A	1821029-92-8
			Tricyclooxyisohumulone B	1611526-15-8
			Tricyclooxyisohumulone A	1611526-14-7
			trans-Alloisoadhumulone hydroxide	1233663-83-6
			trans-Alloisohumulone hydroxide	1233663-81-4
			cis-Alloisoadhumulone hydroxide	1233663-77-8
			cis-Alloisohumulone hydroxide	1233663-75-6
			Adhumulinone	1185198-13-3
			Humulinone (6CI)	981-03-3

Peak nr.	Retention time [min.]	Proposed molecular weight [g/mol]	Supposed structure	CAS-nr:
54	12.783	306	Leukoanthocyanidol	93527-39-0
			Epigallocatechol	970-74-1
			Gallocatechol	970-73-0
58	13.759	377	Adprehumulone	212394-62-2
			Prehumulone	59122-94-0
60	14.121	422	1'',2''-Dihydroxanthohumol F	1691256-99-1
			5'-Prenylxanthohumol	189299-04-5
61	14.364	379	Tricyclooxyisoadhumulone B	1821029-94-0
			Tricyclooxyisoadhumulone A	1821029-92-8
			Tricyclooxyisohumulone B	1611526-15-8
			Tricyclooxyisohumulone A	1611526-14-7
			trans-Alloisoadhumulone hydroxide	1233663-83-6
			trans-Alloisohumulone hydroxide	1233663-81-4
			cis-Alloisoadhumulone hydroxide	1233663-77-8
			cis-Alloisohumulone hydroxide	1233663-75-6
			Adhumulinone	1185198-13-3
			Humulinone (6CI	981-03-3
62	14.613	379	Tricyclooxyisoadhumulone B	1821029-94-0
			Tricyclooxyisoadhumulone A	1821029-92-8
			Tricyclooxyisohumulone B	1611526-15-8
			Tricyclooxyisohumulone A	1611526-14-7
			trans-Alloisoadhumulone hydroxide	1233663-83-6
			trans-Alloisohumulone hydroxide	1233663-81-4
			cis-Alloisoadhumulone hydroxide	1233663-77-8
			cis-Alloisohumulone hydroxide	1233663-75-6
			Adhumulinone	1185198-13-3
			Humulinone (6CI)	981-03-3
65	15.506	340	(±)-8-Prenylnaringenin	53846-50-7
			(±)-6-Prenylnaringenin	68236-13-5
			Desmethylxanthohumol	115063-39-3
69	17.07	332	Ad-hulupone	1185198-15-5
			n-Hulupone	38574-31-1
78	19.457	332	Ad-hulupone	1185198-15-5
			n-Hulupone	38574-31-1

Peak nr.	Retention time [min.]	Proposed molecular weight [g/mol]	Supposed structure	CAS-nr:
79	19.812	354	Xanthohumol N	1691257-00-7
			1",2"-Dihydroxanthohumol K	688360-14-7
			Xanthogalenol	265659-35-6
			Xanthoangelol H	265652-89-9
			7-O-Methyl-6-prenylnaringenin	261776-61-8
			1",2"-Dihydroxanthohumol C	250603-94-2
			7-O-Methyl-8-prenylnaringenin	201805-81-4
			3'-Hydroxy-4'-O-methylglabridin	175554-11-7
			1",2"-Dihydroisoxanthohumol C	115063-44-0
			(-)-Isoxanthohumol	72247-79-1
			Xanthohumol (6CI,7CI)	6754-58-1
83	20.822	370	Xanthohumol I	688360-06-7
			Xanthohumol B	189308-10-9
			Xanthohumol D	274675-25-1
86	21.469	354	Xanthohumol N	1691257-00-7
			1",2"-Dihydroxanthohumol K	688360-14-7
			Xanthogalenol	265659-35-6
			Xanthoangelol H	265652-89-9
			7-O-Methyl-6-prenylnaringenin	261776-61-8
			1",2"-Dihydroxanthohumol C	250603-94-2
			7-O-Methyl-8-prenylnaringenin	201805-81-4
			3'-Hydroxy-4'-O-methylglabridin	175554-11-7
			1",2"-Dihydroisoxanthohumol C	115063-44-0
			(-)-Isoxanthohumol	72247-79-1
			Xanthohumol (6CI,7CI)	6754-58-1
88	22.075	370	Xanthohumol I	688360-06-7
			Xanthohumol B (Dehydroxanthoh.)	189308-10-9
			Xanthohumol D	274675-25-1

5.5 METHOD VALIDATION

To confirm method reliability and suitability for quantification of reference substances in the purchased products (Prod1 – Prod6), the UPC² method was validated according to ICH guidelines. The results of the validation including evaluation of linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy are summarized in Table 6.

Table 6 - Results of method validation

	Hulu	8-PN	6-PN	IXN	XN
Regression equation (y =)	$(1.48 \cdot 10^6)$ x - $4.33 \cdot 10^4$	$(4.12 \cdot 10^6)$ x - $9.80 \cdot 10^3$	$(4.13 \cdot 10^6)$ x - $1,24 \cdot 10^3$	$(4.19 \cdot 10^6)$ x - $2.93 \cdot 10^4$	$(3.58 \cdot 10^6)$ x - $3.82 \cdot 10^4$
Levels used	5	8	8	7	6
R²	0,9952	0,9995	0,9996	0,9983	0,9995
Linear range[a]	46-990	1.296-1020	1.296-1050	3.7-1010	11.7-1040
LOD[a]	3.8	1.3	1.3	1.2	1.3
LOQ[a]	35	3.9	3.9	3.7	3.9
Precision					
intra-day[b]	0,22	0,14	0,10	0,14	0,41
inter-day[c]	0,04	0,04	0,01	0,01	0,09
Accuracy[d]					
high spike	113,1	99,2	100,7	105,1	92,5
medium spike	95,2	92,3	96,7	93,7	91,3
low spike	92,8	92,3	91,4	88,9	77,0

a) µg/ml
b) standard deviation within one day based on peak area in percent (n = 5)
c) standard deviation over three days based on peak area in percent (n = 3)
d) expressed as recovery rate in percent

Calibration curves were derived by serial injection of the 5 reference compounds at increasing concentrations. The calculated linearity ranged from 1.296 to 1050 µg/ml. That means that a very high concentration of approximately 1 mg/ml was necessary for each reference substance to meet the demands of ICH guidelines where at least 5 data points are requested for a good calibration.

Linear regression analysis was showing **very good linearity** for 8-PN, 6-PN and XN (R² ≥ 0.9995) and **good linearity** for IXN (R² ≥ 0.9983). Hulu resulted in a linearity of R² ≥ 0.9952.

Limits of detection (**LOD**) were 1.2 µg/ml for IXN, 1.3 µg/ml for 8-PN, 6-PN, XN and 3.8 µg/ml for Hulu. Limits of quantification (**LOQ**) were between 3.7 µg/ml and 3.9 µg/ml for 8-PN, 6-PN, IXN, XN and 35 µg/ml for Hulu.

Validation values for Hulu differed significantly from the other components which can be explained because of overlaps in the peak area and also because still higher concentrations of the extract would have been necessary for accurate detection of Hulu. It was not possible to detect m/z and UV spectra precisely in the extract.

Compared to other chromatographic methods like HPLC linearity was good. A recent study (Prencipe et al., 2014) published linearity ranges for bioactive hops components between 1.3 – 177.0 µg/ml and R^2 values between 0.9984 (8-PN) and 0.9997 (XN) using HPLC-UV/DAD. LOD and LOQ values varied a lot compared to other studies. Moreover, hops extracts and beer which were analysed with HPLC-MS (LC-MS and LC-MS-MS) resulted in much lower LOQ values, for example for 8-PN, 6-PN, IXN, XN between 10 and 30 ng/ml (Nikolic, 2013).

For validation of **precision** an intra-day and inter-day experiment was carried out.

Standard deviation of intra-day and inter-day ranged from 0.10% – 0.41% and 0.01% – 0.09% respectively. **Good precision** of the method could be achieved.

The final validation was a spike recovery test to evaluate the **accuracy** of the presented method. Therefore, the 5 standard compounds were prepared in different amounts at three different levels reaching from low (75%), to medium (100%) to high (125%) spike. They were subsequently added to all prepared product samples and extracted with the optimized extraction method. Then all samples were analysed on UPC² in triplicates according to the established protocol. Good accuracy could be found for all 5 reference compounds indicating a good and suitable method for the detection of these compounds in hops samples.

Recovery rates were not lower than 88.89% (low spike) except for Xanthohumol with only 76.97% and not higher than 113.08% (high spike).

Another study using HPLC-DAD for validation of 8-PN, 6-PN, XN and IXN in hops resulted in mean recovery rates between 98.37 % (XN) and 100.1 % (8-PN) found in the crude extract. Recovery rates for the all 4 compounds in capsules were reported between 96.13 % and 97.22% (Dhooghe et al., 2010).

The **quantitative composition** of the 5 reference substances in the 6 commercial products could be derived after quantification (Table 26).

Hulu could only be quantified in Prod5 and Prod6 (toto drugs) in the range of 0.115% and 0.207 %, respectively. 8-PN could be quantified in Prod3-Prod6 with a percentage of 0.014 %, 0,005%, 0,004% and 0,005%. 6-PN could be quantified in Prod1 (0.001%) as well as Prod3-Prod6 (0.026%, 0.003%, 0.005%, 0.006%). IXN and XN were quantified in all purchased products.

In general, the percentage of each component except for Hulu was at its highest in Prod3. This is due to the fact that this product was enriched with Xanthohumol. The highest percentage for Hulu could be found in Prod6 with 0.207 %.

When percentages for each reference substance and recommended daily intakes of the purchased products (Prod1 – Prod4) are calculated, the following results could be concluded:

Figure 22 - Calculated daily intake of reference substances per purchased product

Product	Recommended max. daily intake	Total sample weight [mg][a]	Calculated amount of compounds [µg]				
			Hulu	8-PN	6-PN	IXN	XN
Prod1	2 capsules	839.80	n.d.[b]	n.d.	8.40	33.60	58.80
Prod2	3 capsules	1380.33	n.d.	n.d.	n.d.	110.42	165.64
Prod3	3 capsules	1035.54	n.d.	144.98	269.24	1739.71	**10.53** [c]
Prod4	3 coated tablets	911.58	n.d.	45.58	27.35	255.24	255.24

a) Prod1 – Prod3: sample weight is without capsule

b) n.d. = not detectable

c) component expressed in mg

According to the manufacturer[11] Prod3 was enriched with 16 mg Xanthohumol per daily dose (3 capsules). According to the calculation the daily dose contains 10.53 mg of Xanthohumol. So Prod3 can be considered as a valuable nutritional supplement. Biendl (2018) reported that in the first clinical studies carried out on humans, a daily dose of 12 mg of XN had significant protective effects against alterations in genetic material (DNA-mustations).

The Advisory Committee for Plant Preparation limits the daily intake for *H. lupulus* with 400 µg of 8-PN (Dhooghe et al., 2010). All 4 products meet these demands.

For the toto drugs (Prod5 and Prod6) no recommended dose was given by the supplier.

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[11] http://www.alcura.de

5.6 CHROMATOGRAPHIC CONDITIONS SFC PREP-15

As a last step of the study the optimized conditions of UPC² for the **hops extract** were transferred to a preparative SFC Prep-15 as it is a very interesting field of research to separate, isolate and identify especially non polar compounds of the total extract.

A Supercritical Fluid Chromatography (SFC), SFC Prep-15, Purification System from Waters (Milford, MA, USA) was used for all experiments. The instrument was equipped with a 515 HPLC pump, backpressure regulator, column oven, ELS and PDA detector, flow splitter, fluid delivery module, heat exchanger, pump control module II and sample manager. The operating software was MassLynx®.

In a first step the optimized UPC² method was transferred to an SFC Prep-15.

Best results on the **analytical column** were achieved with a **Viridis BEH 2-EP column** (5.0 µm particle size, dimension: 4.6 x 250 mm) and a **mobile phase comprising CO_2 (A) and isopropanol (B)**. These two parameters could be transferred directly from UPC² to SFC Prep-15.

With BEH-2 EP the intensity of peaks, peak shape and peak amount were better than with the other columns like for example CSH Fluoro-Phenyl were all substances eluted very early and at the same time (Figure 23 and Figure 24).
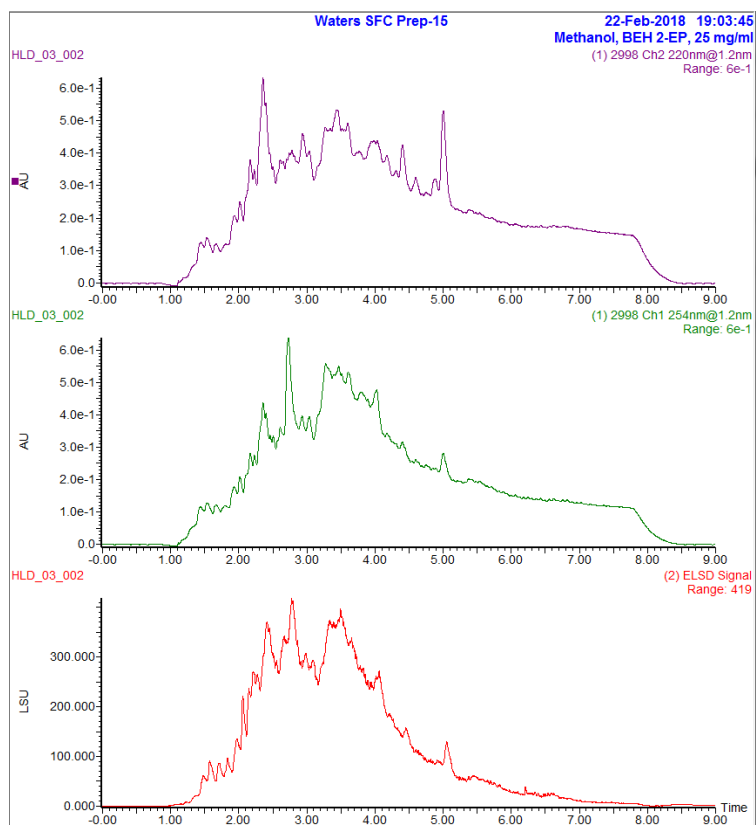
Figure 23 - Column screening on analytical BEH-2 EP (220 nm, 254 nm, ELSD Signal)
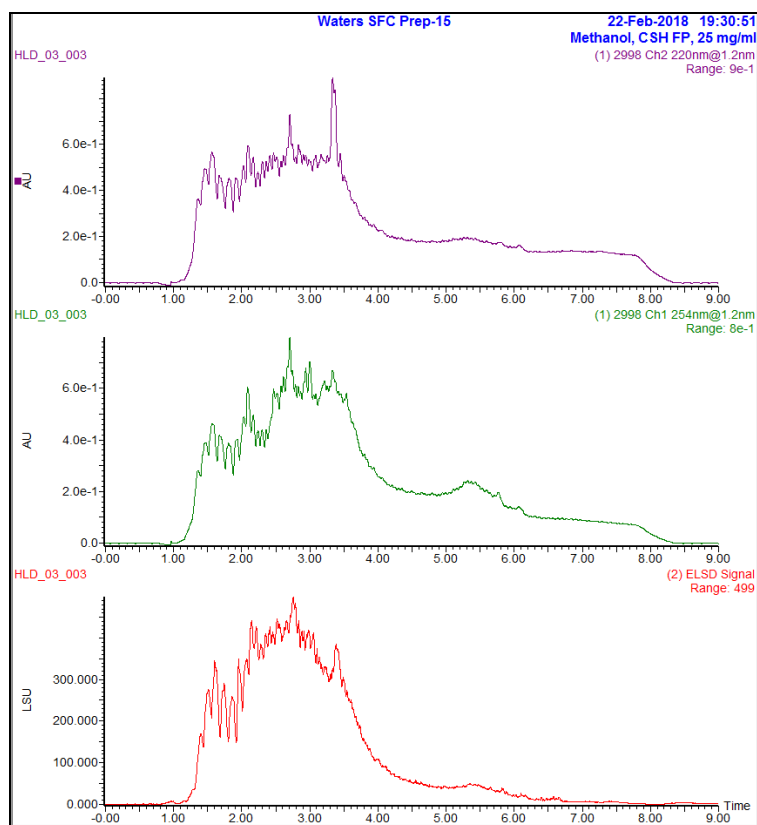
Figure 24 - Column screening on analytical CSH Fluoro-Phenyl (220 nm, 254 nm, ELSD Signal)

But the UPC² method could not be transferred directly as columns and particle sizes vary. Furthermore, the biggest challenge was that non polar compounds were not eluted and separated as good as on UPC² because the composition of the mobile phase on SFC demands at least 95% CO_2 and 5% co-solvent. On UPC² a composition of 97% CO_2 and 3% co-solvent was used – and this was absolutely necessary to separate early non-polar peaks in the extract.

For good results on the analytical column, flow rates, concentration of extracts and injection volumes had to be adjusted.

Best results regarding separation and peak shape could be achieved with a concentration of **50 mg/ml** and an injection volume of **40 µl** (Figure 45, Figure 46). An injection volume of 30 µl was tested under the same conditions but resulted in bad peak shape.

Also flow rates, temperature and injection volume had to be adjusted.

Ideal flow rates for binary solvent manager and isocratic solvent manager, column temperature and detection wavelength were as follows: 5.0 ml/min, 3.0 ml/min, 40°C and 220nm. Backpressure ABPR was 120bar.

The optimized gradient was: from 95A/5B in 13 minutes, to 50A/50B within another 3.5 minutes, held at this composition for 1.5 minutes (total run time: 18 minutes) and then equilibrated at initial conditions for 1 minute.

Figure 25 is presenting the summarized optimum conditions for the hops extract (Prod5):

DATA OVERVIEW	Hops Extract (Prod5)
SFC Prep-15 analytical conditions	**Optimized gradient**

SFC Prep-15

analytical conditions

System:	Acquity SFC Prep-15
Detector:	PDA
Software:	MassLynx®
Column:	BEH 2-EP
	4.6 x 250 mm
	5.0 µm particle size

SOLVENTS:

Mobile phase **A**: CO_2

Mobile phase **B**: Isopropanol (IPA)

Make-up: MeOH

Wash solvent: MeOH

Flow rate: 5 ml/min (BSM)

Make-up flow: 3 ml/min (ISM)

APBR: 120 bar

Column temp.:   40°C

Injection volume: 40 µl

Hops Extract (Prod5)

Optimized gradient

time (min)	A (CO_2 %)	B (IPA %)
0	95	5
7	95	5
13	50	50
16.5	50	50
18	95	5
19	95	5

Chromatogram

Figure 25 - Optimized method for analytical SFC Prep-15

As a last step in the SFx workflow a scale up of the established method to preparative SFC (Prep-15) was carried out with the target to validate its suitability to purify and isolate **non polar** compounds. The optimized conditions of the analytical SFC Prep-15 were slightly modified regarding flow rates (15 ml/min for BSM and 5 ml/min for make-up) and injection volume (200 µl).
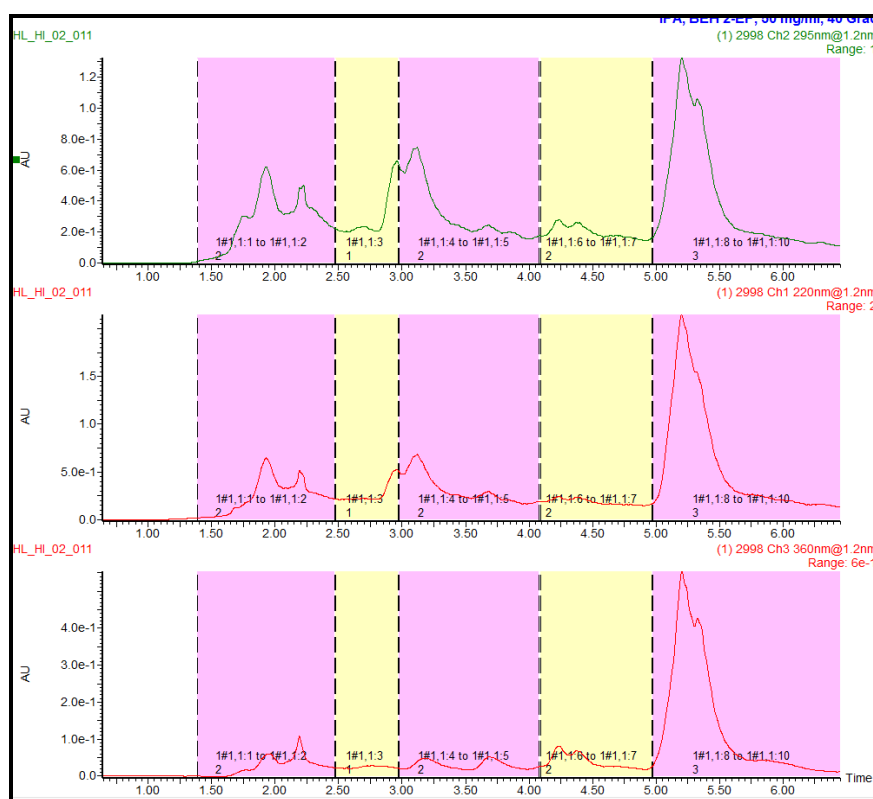
Figure 26 - Fractioning and peak collection on SFC Prep-15

After fractionation and peak collection (Figure 26) results were monitored on the UPC[2] instrument. Further preparative studies and optimization of parameters for SFC Prep-15 will be carried out in another diploma study.

6 MATERIAL AND METHODS
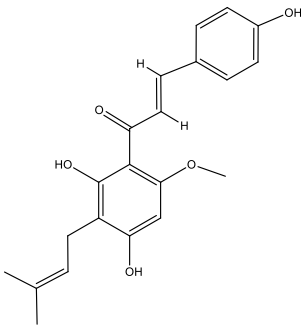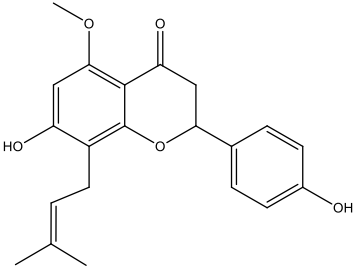
This chapter describes all used materials as well as extraction and chromatographic methods. Regarding chromatographic methods Thin Layer Chromatography (**TLC**), UltraPerformance Convergence Chromatography (**UPC²**) and **SFC Prep-15** (Supercritical Fluid Chromatography) were used.

6.1 MATERIALS

6.1.1 <u>Reference substances</u>

Five reference substances (Table 7) with a purity of more than 97% (method: HPLC) were purchased from PhytoLab, Vestenbergsgreuth, Germany. (http://www.phytolab.com). A standard stock solution for each reference substance was prepared at a concentration of 0.1 mg/ml in hexane/IPA (70:30) and kept in 10 ml volumetric flasks at 8°C prior to analysis. The standard stock solutions were referred to as **HL_XN, HL_IXN, HL_6-PN, HL_8-PN** and **HL_Hulu**. A standard mix solution containing all 5 standards was prepared too. The standard mix was referred to as **Std_mix**.

Table 7 - Reference Substances included in the study (https://pubchem.ncbi.nlm.nih.gov)

Xanthohumol	**XN**	
Article Number:	89292	
CAS Number:	6754-58-1	
Charge:	12192	
Certificate-Number	3191366.7	
Quantity:	10.30 mg	
Purity:	99.87%	

MG = 354.402 g/mol
Molecular Formula: $C_{21}H_{22}O_5$

Isoxanthohumol	**IXN**	
Article Number:	89234	
CAS Number:	70872-29-6	
Charge:	5922	
Certificate-Number	3191363.2	
Quantity:	10.21 mg	
Purity:	99.57%	

MG = 354.402 g/mol
Molecular Formula: $C_{21}H_{22}O_5$

6-Prenylnaringenin **6-PN**

Article Number: 80520
CAS Number: 68236-13-5
Charge: 9946
Certificate-Number   3191364.0
Quantity: 10,71 mg
Danger Symbol: GHS07
Purity: 97.31%

MG = 340.375 g/mol
Molecular Formula: $C_{20}H_{20}O_5$
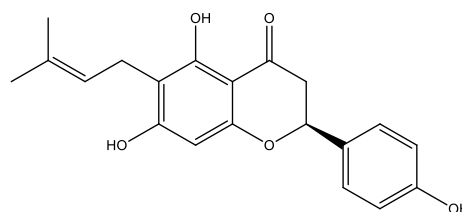
8-Prenylnaringenin **8-PN**

Article Number: 89886
CAS Number: 53846-50-7
Charge: 7425
Certificate-Number   3191365.9
Quantity: 10,43 mg
Danger Symbol: GHS07
Purity: 99.72%

MG = 340.375 g/mol
Molecular Formula: $C_{20}H_{20}O_5$
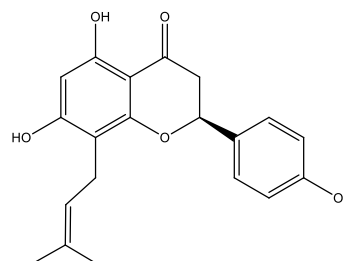
Hulupinic acid   **Hulu**

Article Number: 89899
CAS Number: 1891-42-5
Charge: 7110
Certificate-Number   3191362.4
Quantity: 10,50 mg
Purity: 100%

MG = 264.321 g/mol
Molecular Formula: $C_{15}H_{20}O_4$
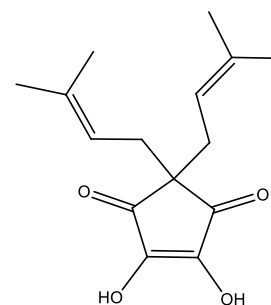
6.1.2 Product samples

Six commercial mono-hops dietary supplements (Prod1-Prod6) purchased from online pharmacies[12] were included in this diploma thesis. They contain 4 dietary supplements and 2 toto drugs. HL_Prod6 was purchased from Dr. Kottas (Eitnergasse 8, Vienna) in 2013 and since then stored in a freezer. Voucher specimens of all samples are kept at the Department of Pharmacognosy, University of Vienna, Austria.

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[12] http://www.shop-apotheke.at; http://www.apotheke.at

Table 8 - Purchased products included in the study

	Prod1
Product Name:	**Hopfen Kapseln**
	(=Hops capsules)
Company Name:	Arnimont-Pharma GmbH // Vienna, Austria
Package size:	60 capsules à 29 g
Active component:	1 capsule contains 40 mg of hops extract
Dosage:	2 capsules per day
Charge Number:	193446
Date of expiry:	04/2020
PZN:	PZN-06971041 for 60 capsules

	Prod2
Product Name:	**Hopfen 125 mg GPH Kapseln**
	(=Hops 125 mg GPH capsules)
Company Name:	Gall Pharma GmbH // Judenburg, Austria
Package size:	60 capsules à 32 g
Active component:	1 capsule contains 125 mg hops extract
Dosage:	1-3 x 1 capsule per day
Charge Number	246.44
Date of expiry:	02/2020
PZN:	PZN-9645307

	Prod3
Product Name:	**Hopfenextrakt Kapseln mit Xanthohumol aus Hopfen**
	(= hops extract capsules with xanthohumol from hops)
Company Name:	Allcura Naturheilmittel GmbH // Wertheim, Germany
Package size:	90 capsules à 35 g
Active component:	1 capsule contains 77% hops extract (equals approx. 27 g)
Dosage:	3 x 1 capsule per day
Charge Number:	L-A1707072
Date of expiry:	07/2019
PZN:	PZN-04632010

	Prod4
Product Name:	**Klosterfrau Nervenruh Hopfen Beruhigungs-Dragees**
	(= Klosterfrau Nervenruh tranquillization-coated tablet)
Company Name:	DIVAPHARMA GmbH // D-12277 Berlin
Package size:	120 coated tablets
Active component:	1 coated tablet contains 125 mg dry-extract from hops
	(4-5:1) with MeOH 50% (V/V)
Dosage:	2-3 x 1 capsule per day
Charge Number:	004067
Date of expiry:	05/2019
PZN:	PZN-09287575

	Prod5
Product Name:	**Hopfenzapfen ganz,** *Humuli Lupuli strobuli tot.*
	(= Hops flowers whole)
Company Name:	ApoFit Arzneimittelvertrieb GmbH // Bamberg, Germany
Package size:	100 g
Charge Number:	91687
Date of expiry:	06/2020
PZN:	PZN-10023769

	Prod6
Product Name:	**Flos Lupuli Freinschnitt nach PhE**
	(= Hops flowers fine cut)
Company Name:	Kottas Pharma // Eitnergasse 8 // A-1230 Vienna
Package size:	1 kg
Charge Number:	W12203440
Date of expiry:	09/2015

6.1.3 Used instruments

- ACQUITY UPC^2 ^{TM}, Waters with the following modules: binary solvent manager, column manager, convergence manager, ELS detector, isocratic solvent manager, PDA detector, QDa detector, sample manager
  Software: Empower 3, release 2
- Analytical Balance, Sartorius BP210D
- Centrifuge, Labofuge 400, Function Line, Heraeus Instruments
- Centrifuge, mini spin, Eppendorf
- Chiller (for SFC Prep-15), Accel 500LC, Thermo Scientific Polar Series
- Sample concentrator FSC400D, Techne
- Heatgun, HG 2000 E, Steinel
- Nitrogen Generator (for SFC Prep-15), Genius NM32LA, Peak Scientific Instruments Ltd.
- Rotavapor R-210 and Heating Bath B-491, Büchi Laboratorium Technologie AG
- SFC Prep-15, purification system, Waters with the following modules: 515 HPLC pump, backpressure regulator, column oven, ELS detector, flow splitter, fluid delivery module, heat exchanger, PDA detector, pump control module II, sample manager
  Software: MassLynx®
- TLC Spray Cabinet II, CAMAG®
- TLC Visualizer, CAMAG®
- Ultrasonic bath, Transsonic T460, Elma
- UV-Lamp, CAMAG®
- Vacuum Pump, V-710, Büchi

6.1.4 Solvents and reagents

Sample preparation and extraction
- dichloromethane, ÖAB, distilled, Rectapur
- ethanol, 96%
- methanol, ÖAB, distilled
- isopropanol (2-Propanol) for HPLC, HiPerSolv Chromanorm
- hexane for HPLC, HiPerSolv Chromanorm

Chromatographic methods

- 1% vanillin in MeOH; spraying solvent
- 5% methanol. H_2SO_4; spraying solvent
- acetic acid
- acetonitrile for HPLC, HiPerSolv Chromanorm
- ammonium formate (>99%), HiPerSolv Chromanorm
- ethanol for HPLC (>99,7%), HiPerSolv Chromanorm
- formic acid 99%
- isopropanol (2-Propanol) for HPLC, HiPerSolv Chromanorm
- isopropanol (2-Propanol), AnalaR Normapur
- methanol for HPLC, HiPerSolv Chromanorm
- n-hexane, Normapur
- natural spraying reagent
- hexane for HPLC, HiPerSolv Chromanorm
- H_2O for HPLC purified by ion exchanger

Others

- DMSO, Emsure, Merck

6.2 SAMPLE PREPARATION AND EXTRACTION

6.2.1 Sample preparation of standard mix

All weighted samples (Hulu: 1.22 mg, 8-PN: 1.01 mg, 6-PN: 1.18 mg, IXN: 0.99 mg; XN: 1.04 mg) were together solved in a mixture of hexane/isopropanol (70:30) in a 10 ml graduated flask to get a stock solution of reference substances (Std_mix).

6.2.2 Extraction of purchased products

For the testing of exhaustive extraction and extraction efficiency three different solvents were tested under the same conditions: DCM, Hex/IPA (70:30) and EtOH 96%. Testing was carried out with 1.08 mg of Prod5.

Extraction method: The dried plant material was accurately weighted, pulverized in a mortar and extracted with 6 ml of the extraction solvent by sonification for 10 min at ambient temperature. Then the sample was filtered into a 20 ml volumetric flask (VF 1). This procedure was repeated two more times and the flask (VF 1 = concentrated sample) was filled up to the volume with the extraction solvent. The drug was extracted a fourth time under the same conditions: 6 ml of extraction solvent, sonification for 10 min at ambient temperature, filtered into a 20 ml volumetric flask (VF 2 = diluted sample) and filled up to the mark.

1 ml of each solution (VF 1 and VF 2) was transferred into a vial and dried with the sample concentrator. Then the dried extracts were each resolved in 500 µl of Hex/IPA (70:30) before running each sample on UPC² under optimized conditions (n = 3).

For the calculation of the extraction efficiency, all results of the three extractions were compared. To calculate the most efficient extraction solvent, the AUC (area under the curve) for each reference substance in the diluted sample VF 2 should be less than 5% in relation to the AUC of VF1+VF2 (European Medicines Agency, 2014). Results are summarized in Table 9.

Table 9 - Extraction efficiency of different extraction solvents

	Extraction solvent and calculated extraction efficiency in %		
	DCM	Hex/IPA (70:30)	EtOH 96%
Hulu	98.12	99.997	n.d.
8-PN	n.d.	95.26	96.32
6-PN	95.04	97.86	98.96
IXN	**93.70**	**94.09**	99.32
XN	95.49	**92.45**	97.68

Efficient extraction was carried out for all purchased products afterwards. Sample weights are summarized in Table 10 (same concentration for each sample).

Table 10 - Weighted product samples for optimized extraction method

Product	Number of samples used	Hops extract per sample [mg]	Sample weight per unit [mg][a]	Total sample weight
Prod1	4 capsules	40	419.90	1679.60
Prod2	2 capsules	125	460.11	920.22
Prod3	1 capsules	300	345.18	345.18
Prod4	2 coated tablets	125	303.86	607.72
Prod5	Toto drug		-	1084.85
Prod6	Toto drug		-	1011.34

a) Prod1 – Prod3: sample weight is without capsule

6.3 PRELIMINARY TESTING AND THIN LAYER CHROMATOGRAPHY (TLC)

6.3.1 TLC plates

TLC plates: aluminum sheets, TLC silica gel 60 F_{254}, Merck 20x20, 1.05715.0001

Reagents for Detection 1	vanillin, 1% in MeOH
	sulfuric acid, 5% in MeOH
Reagents for Detection 2	natural product reagent A

6.3.2 Extraction of toto drug

The first extraction was carried out with 1.01 mg of Prod5 (*Humuli lupuli strobuli tot.*) and 20 ml DCM for 10 minutes on the ultrasonic bath (extract named **HLD**). The extract was centrifuged for 5 minutes at 3.500 U/min. As the drug was too big and bulky, centrifugation did not show any better separation of sediment and supernatant. So further processing was carried out without centrifugation. After reducing the volume of the extract to approximately 4 ml (40°C, rotary evaporator) it was filtered through cotton wool.

The same drug material was reused after drying and extracted with 20 ml MeOH (extract named **HLM**). After 10 minutes on the ultrasonic bath the extract was reduced to approximately 4 ml (40°C, Rotavapor) and filtered through cotton wool.

Both extracts were analyzed on TLC.

6.3.3 TLC solvent systems

For *Humulus lupulus* the recommend system consists of the mobile phase n-heptane : i-propanol : formic acid (90:15:0.5) and a TLC plate of 2 x 15 cm (Wagner et al., 1983). This system was modified, adjusted to the needs of the present study and optimized. Instead of n-heptane, n-hexane was used. The following systems (8 cm) were tested:

Table 11 - Optimization of TLC using different eluents

Trial Nr.	Solvent System		Reagents for Detection
1	8,31 ml	n-Hex	vanillin, 1% in MeOH
	1,64 ml	IPA	sulfuric acid, 5% in MeOH
	0,05 ml	FA	
2	8,50 ml	n-Hex	vanillin, 1% in MeOH
	1,00 ml	IPA	sulfuric acid, 5% in MeOH
	0,50 ml	FA	
3	9,00 ml	n-Hex	vanillin, 1% in MeOH
	0,50 ml	IPA	sulfuric acid, 5% in MeOH
	0,30 ml	FA	
	0,20 ml	AA	
4	7,50 ml	n-Hex	vanillin, 1% in MeOH
	2,00 ml	IPA	sulfuric acid, 5% in MeOH
	0,50 ml	FA	
5	7,50 ml	n-Hex	vanillin, 1% in MeOH
	1,80 ml	IPA	sulfuric acid, 5% in MeOH
	0,50 ml	FA	
	0,20 ml	AA	
6	7,50 ml	n-Hex	natural product reagent A
	1,80 ml	IPA	
	0,70 ml	FA	
7	**8,00 ml**	**n-Hex**	**vanillin, 1% in MeOH**
	1,50 ml	**IPA**	**sulfuric acid, 5% in MeOH**
	0,50 ml	**FA**	

Run time was approximately 19 minutes.

Systems 1 and 2 were tested for both **HLD_01** and **HLM_01**. The bands were equal but of course **HLM_01** had less intense bands because the drug material from **HLD_01** was reused for **HLM_01**. So systems 3-7 were only carried out for **HLD_01**.

Detection (= derivatization) with natural product reagent A did not show all relevant bands and was less intense.

6.3.4 TLC of reference substances

The weighted samples of reference substances (Hulu: 1.14mg, 8-PN: 1.01mg, 6-PN: 1.02mg, IXN: 1.03mg; XN: 1.13mg) were separately solved in MeOH (HPLC quality) in a 10 ml graduated flask. A representative sample of 500 µl was taken out of each flask for TLC analysis. It could be demonstrated that with 100 µl of each sample very intense bands could be identified. The extract **HLD_01** (dichloromethane extract of Prod5) was applied in the middle to identify the reference substances.

Figure 27 shows UV-Vis **derivatised** TLC:

- **Hulu**: intense turquoise band
- **8-PN**: light pink band
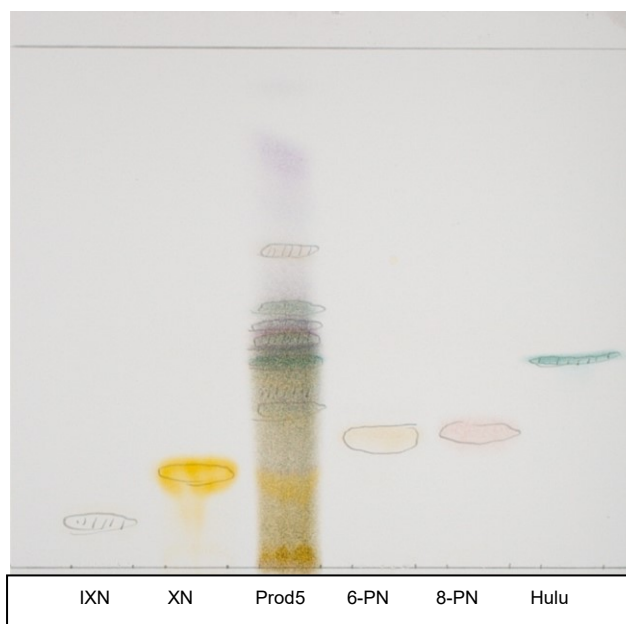- **6-PN**: light yellow
- **IXN**: whitish-yellow band
- **XN**: intense yellow band

Figure 28 shows UV 366 nm **derivatised** TLC:

- **Hulu**: intense light blue fluorescence
- **8-PN**: light pink fluorescence
- **6-PN**: light yellow fluorescence
- **IXN**: intense blue fluorescence
- **XN**: dark orange fluorescence

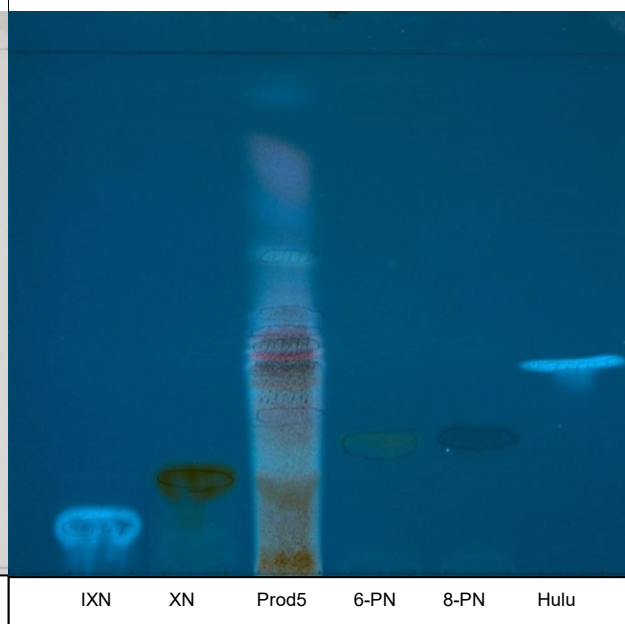Figure 27 - UV-Vis derivatised

Figure 28 - UV 366 nm derivatised

6.3.5 TLC of products and reference substances

To achieve a concentration of 25 mg/ml for the purchased products (capsules and coated tablet) they had to be prepared separately (Table 12). Solvent was hexane : isopropanol (Hex: IPA; 70:30).

Table 12 - Product preparation

Product	Active compound	Weighted sample	Hex : IPA (70:30)
Prod1	40 mg per capsule	2 capsules (80mg)	3,2 ml
Prod2	125 mg per capsule	1 capsule (125 mg)	5,0 ml
Prod3	300 mg per capsule	1 capsule (300 mg)	12,0 ml
Prod4	125 mg per coated tablet	1 coated tablet (125 mg)	5,0 ml

Each sample was extracted on the ultrasonic-bath for 2 minutes and filtered through cotton wool.

A TLC plate applying 30 µl of each component was carried out using the following samples:
- 25,7 mg of dried Extract of **Prod5** solved in 700 µl Hex and 300 µl IPA
- **Hulu, 8-PN, 6-PN, IXN** and **XN** from prepared stock solution
- **Prod1**, **Prod2, Prod3, Prod4** at a concentration of 25 mg/ml according to Table 12

Results of TLC plates (UV Vis, 254 nm, 366 nm) non-derivatised and derivatised can be found in Figure 6 to Figure 11.

For comparison of the two extracts of the toto drugs (Prod5 and Prod6) a concentration of 25 mg / ml was prepared for each sample. Both samples were run on a TLC plate.

Table 13 – Weighted samples for comparison of Prod5 and Prod6 on TLC

Product	Weighted sample	Solvent	Weighted sample dried extract (solved in 700 µL hexane and 300 µL IPA)
Prod5	3.0146g	DCM	25.46mg
Prod6	1.0113g	DCM	25.56mg

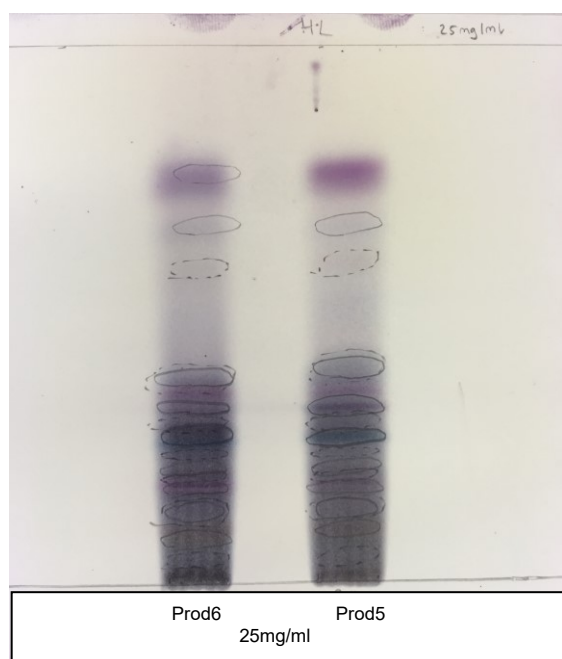Both toto drugs were giving the same bands with Prod6 being more intense (Figure 29).

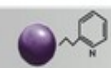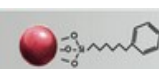Figure 29 - Comparison of Prod5 and Prod6 on TLC
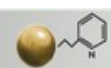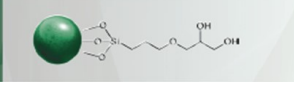
6.4 CHROMATOGRAPHIC OPTIMIZATION
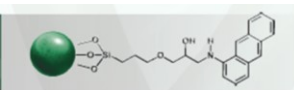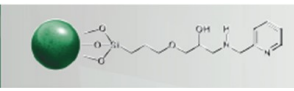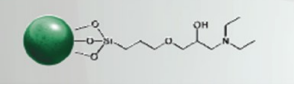
For the experiments an Acquity UPC² instrument and a SFC Prep-15 instrument – both Waters (Milford, MA, USA) - were used.

6.4.1 Column material

The columns described in Table 14 were used for column screening on UPC² and SFC Prep-15. Further details regarding dimensions for each instrument are described below.

Table 14 - Columns used for screening on UPC² or SFC Prep-15 (http://www.waters.com)

Series	Name	Chemistry	Ligand	Separation properties
Viridis	Bridged-Ethylene-Hybrid	BEH		strong interaction with polar functional groups
	Bridged-Ethylene-Hybrid 2-Ethylpyridine	BEH 2-EP		less polar surface than BEH, no use of mobile-phase additives necessary, also for well-retained basic achiral compounds
	Charged-Surface-Hybrid Fluoro-Phenyl	CSH FP		good retention for weak bases; alternate elution order for acid and neutral compounds
	Silica 2-Ethylpyridine	Silica 2-EP		good for achiral separation both with or without additives
Torus	1-Amino-anthracen	1-AA		wide range of compounds like hydrophobic, free fatty acids, natural products liposoluble vitamins, steroids; very robust
	2-Picolylamine	2-PIC		acid and basic compounds, excellent peak shape
	Diethylamine	DEA		good peak shape for strong bases; additional selectivity to 2-PIC
	High-density Diol	DIOL		more robust when pH value is acid or basic according to high surface density

6.4.2 UPC² UltraPerformance Convergence Chromatography™

The optimization for UPC² was carried out step by step. The optimized parameters were used for further testing.

6.4.2.1 Column screening

Column screening was carried out with 8 different columns (Table 15) under the same conditions (generic method: starting with 100A/0B, reaching 50A/50B within 7 minutes, holding at this composition for 2 minutes and then equilibrating for 1 minute at initial conditions). Total run time was 9 minutes. A standard mix containing approximately 1 mg of each of the 5 reference compounds, solved in 10 ml Hex/IPA (70:30) was tested.

Table 15 – Column dimensions used for column screening on UPC²

Series	Name	Chemistry	Dimension Particle Size
Viridis	Bridged-Ethylene-Hybrid	BEH	3.0 x 100 mm
			1.7 µm
	Bridged-Ethylene-Hybrid 2-Ethylpyridine	BEH 2-EP	3.0 x 100 mm
			1.7 µm
	Charged-Surface-Hybrid Fluoro-Phenyl	CSH Fluoro-Phenyl	3.0 x 100 mm
			1.7 µm
	Silica 2-Ethylpyridine	Silica 2-EP	3.0 x 100 mm
			5.0 µm
Torus	1-Aminoanthracene	1-AA	3.0 x 100 mm
			1.7 µm
	2-Picolylamine	2-PIC	3.0 x 100 mm
			1.7 µm
	Diethylamine	DEA	3.0 x 100 mm
			1.7 µm
	High-density Diol	DIOL	3.0 x 100 mm
			1.7 µm

In Figure 12 and Figure 13 the results of column screening are summarized.

6.4.2.2 Effects of co-solvent

2 columns (BEH 2-EP and DIOL) were used for further co-solvent screening. The following co-solvents were tested in a first run: B1: Methanol, B2: Acetonitrile, B3: Ethanol, B4: Isopropanol

B1 (**Methanol**) solvent gave the **best result on the DIOL column.** B4 (**IPA**) solvent gave the **best result on the BEH 2-EP column.** <u>Further testing</u> was carried out with **BEH 2-EP** since there were better peak shapes observed.

The following co-solvents were tested: methanol, acetonitrile, ethanol, isopropanol (Figure 15) and MeOH : IPA (1:1), IPA + 5% H_2O, 10 mM ammonium acetate in IPA, 10 mM ammonium formate in IPA (Figure 30).
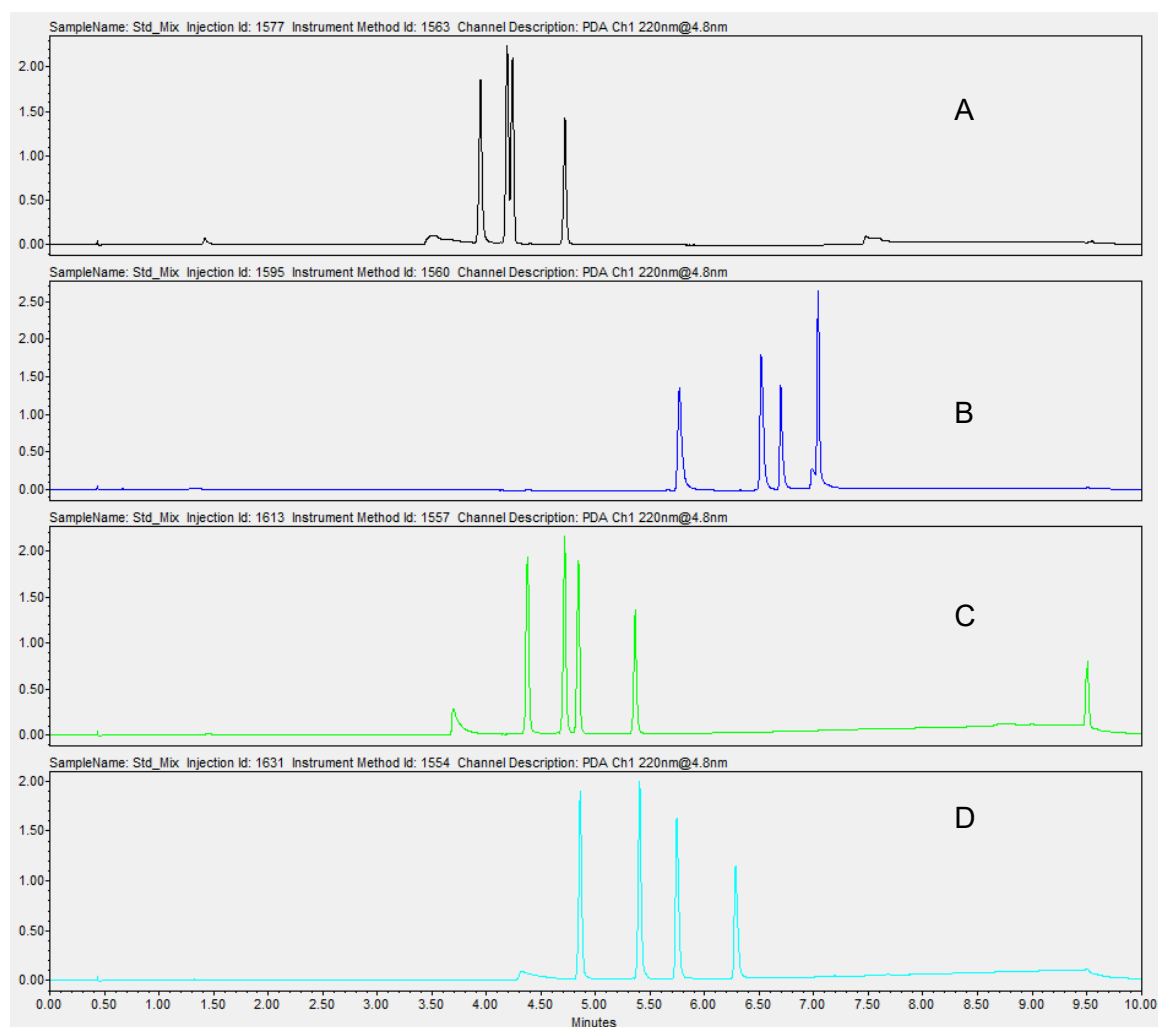
Figure 30 - Influence of different co-solvents **A**: MeOH : IPA (1:1), **B**: IPA + 5% H_2O, **C**: 10mM ammonium acetate in IPA, **D**: 10mM ammonium formate in IPA

Furthermore additives to IPA (0.1% FA, 0.1% AA, 0.1% TFA) were tested (Figure 31).
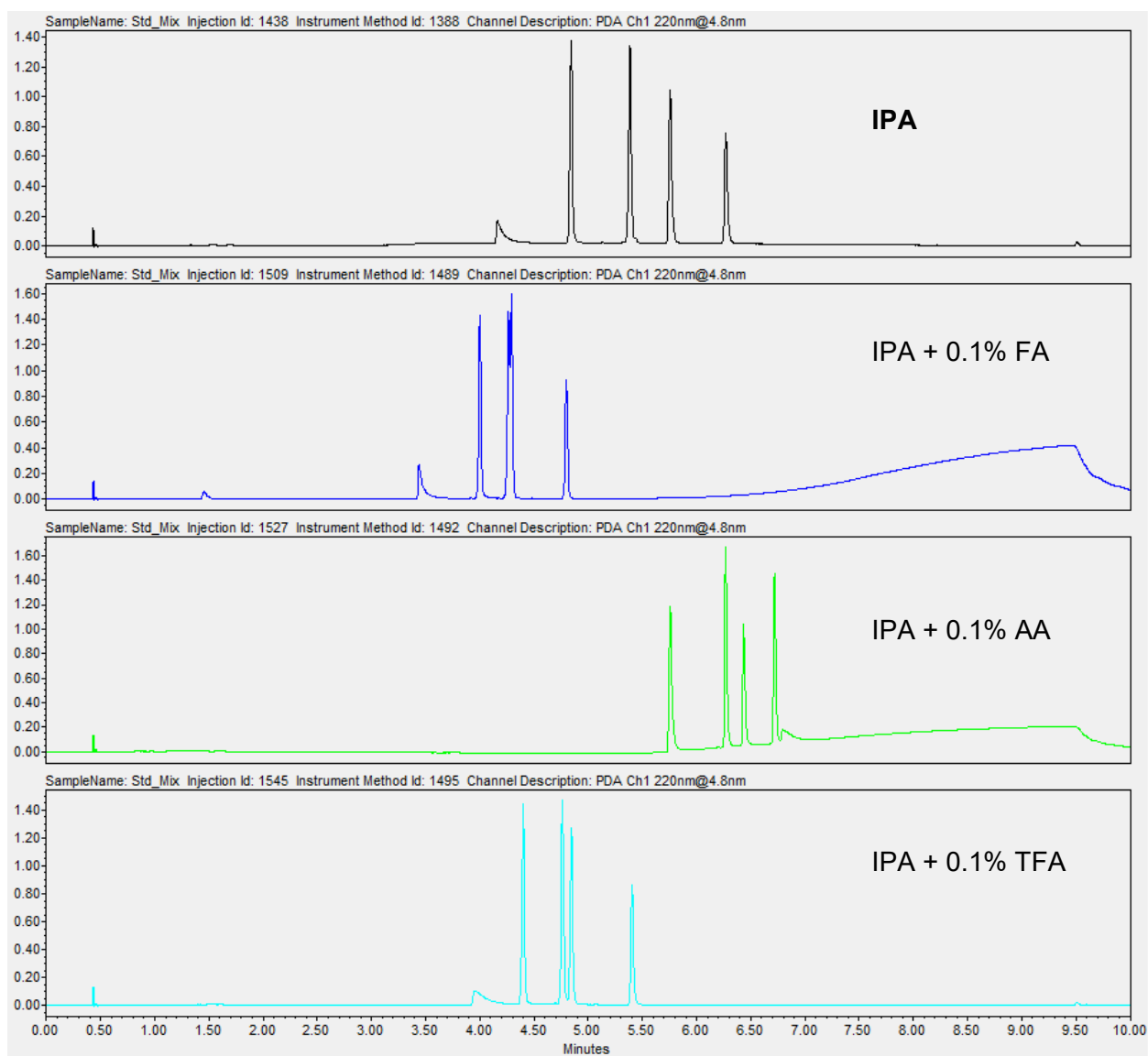
Figure 31 - Co-solvent IPA and IPA with different additives

During this screening a QDa detection using different co-solvents was carried out too (Figure 32 to Figure 34)
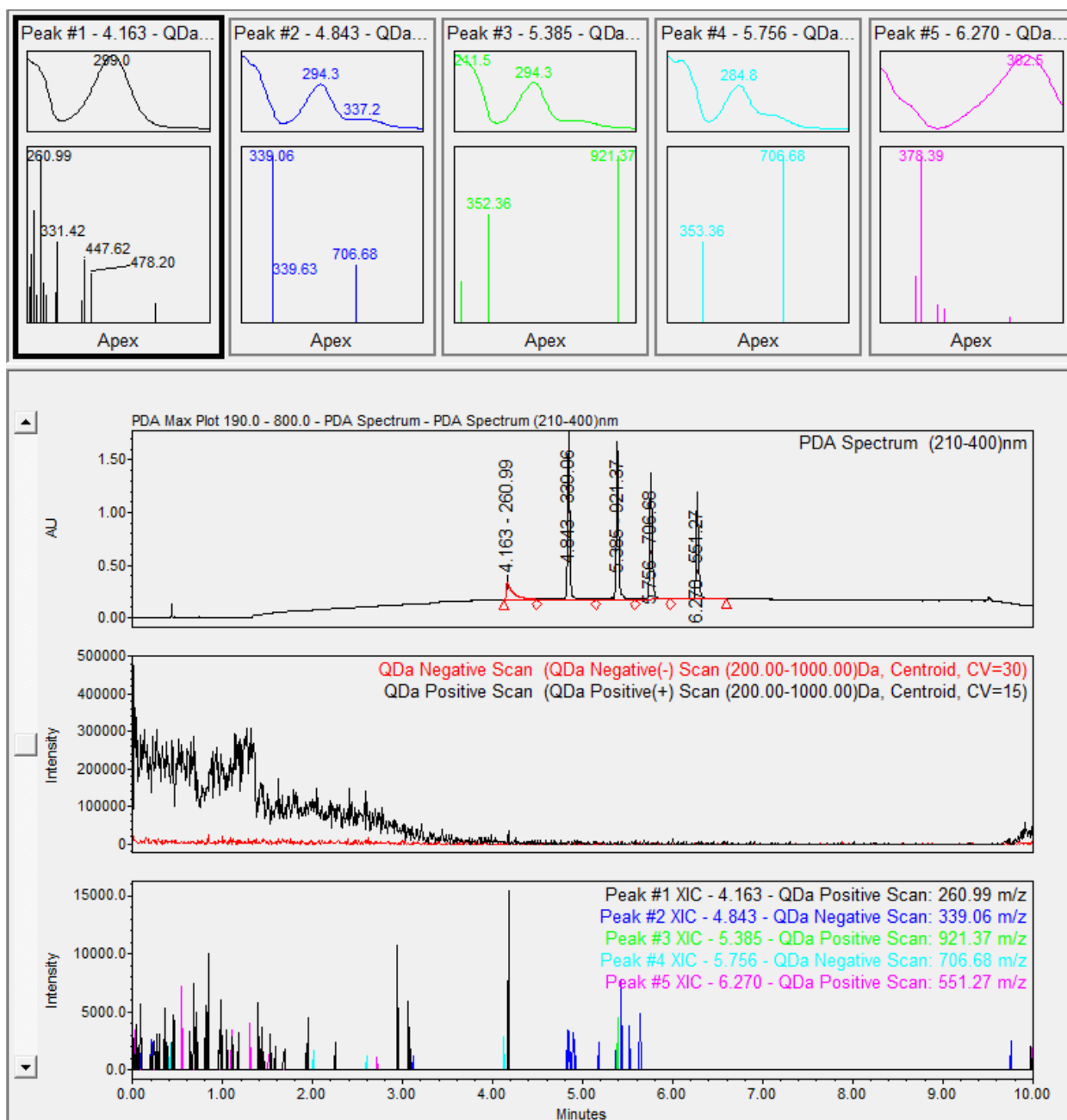
Figure 32 – Poor ionization with IPA alone (Inj. ID 1438)
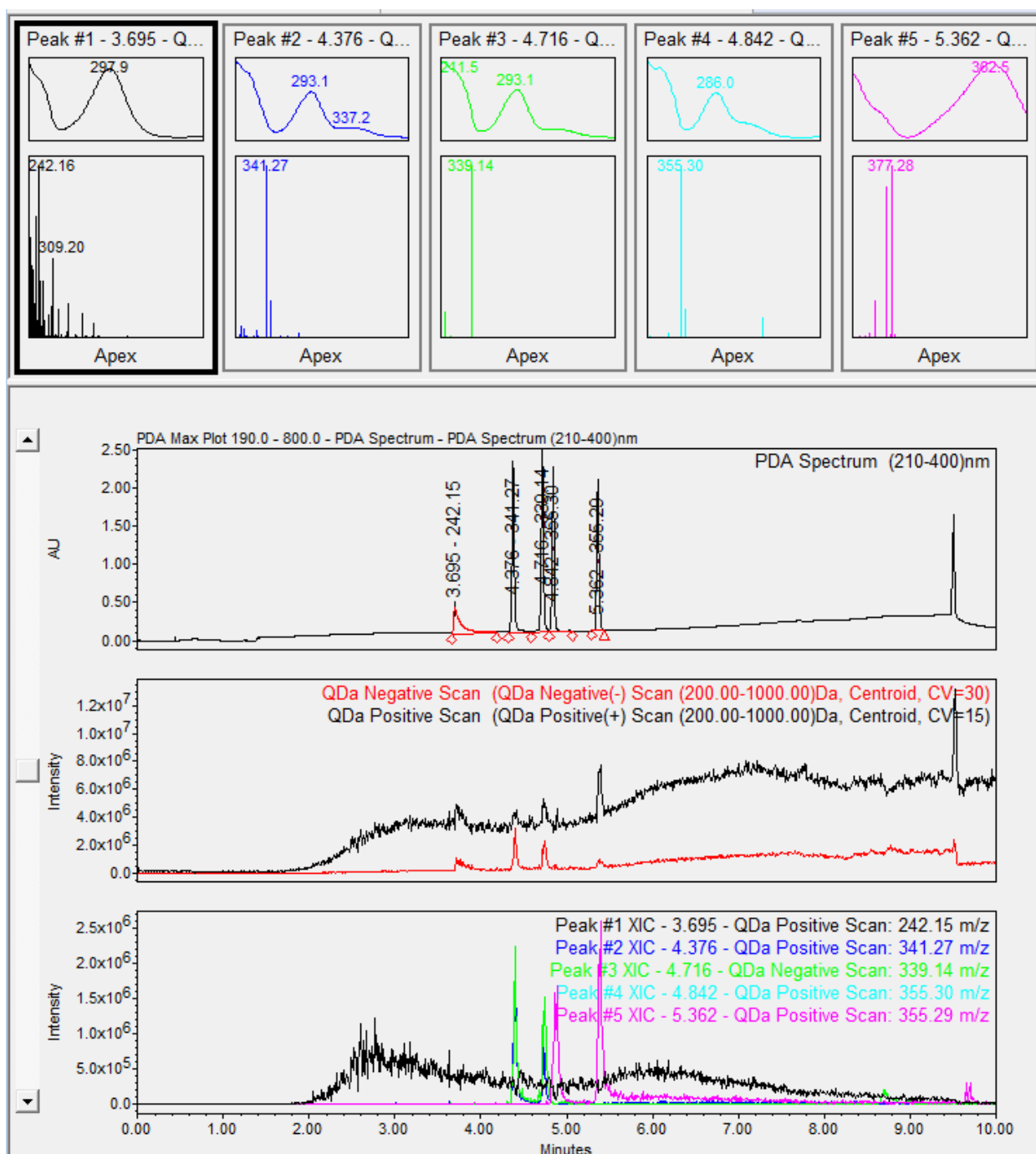
Figure 33 – Good ionization with IPA and 10mM ammonium acetate (Inj. ID 1613)
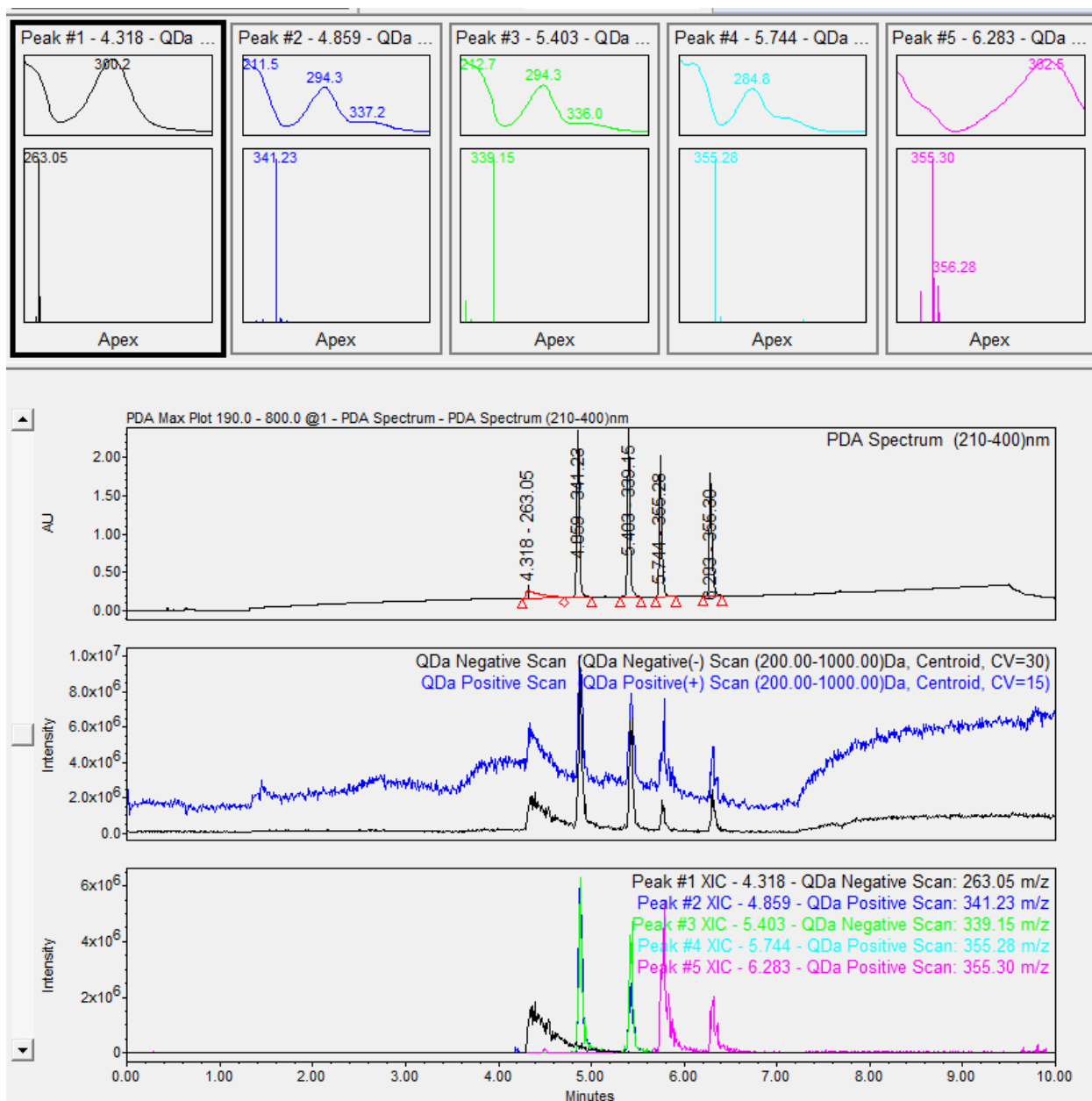
Figure 34 - Best ionization results with IPA and 10mM ammonium formate (Inj. ID 1631)

6.4.2.3 Effect of make-up solvent and flow rates

The following make up solvents were tested regarding their potential for ionization:

- H_2O : MeOH (1:99) + 0.1% FA

  no mass could be detected (Figure 35)

- 10 mM ammonium formate in 95% IPA and 5% HPLC H_2O

  even a reduced ISM flow rate (0,3 ml/min) and a BSM flow rate of 1.2 ml/min resulted in overpressure in the BSM

- 10 mM ammonium formate in 99% MeOH : 1% H2O

  specially the negative mode (QDa Negative Scan) was showing very good results regarding mass detection (Figure 36)
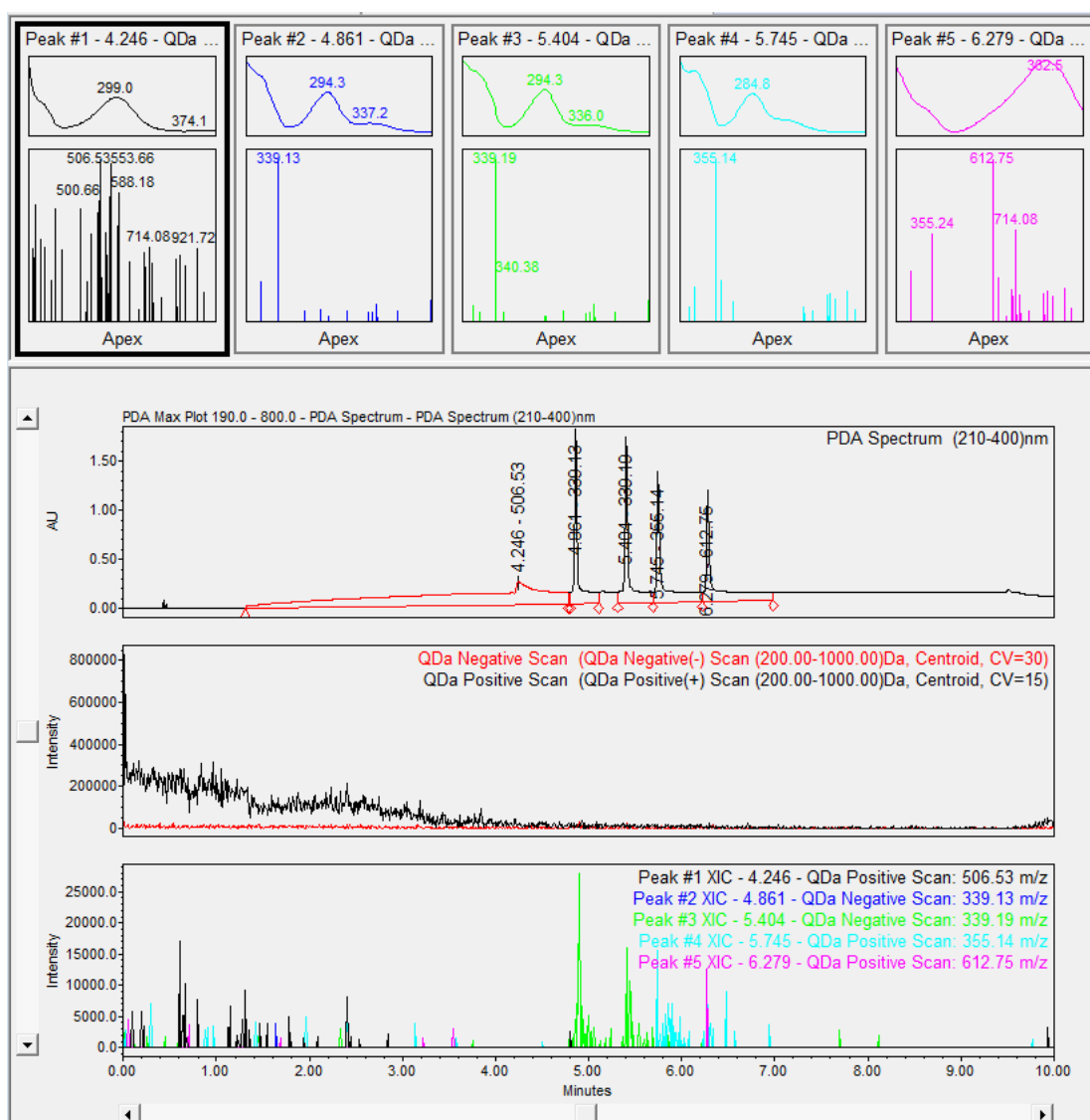
Figure 35 - Mass detection with H2O : MeOH (1:99) + 0.1% FA as make up solvent (Inj. ID 1749)
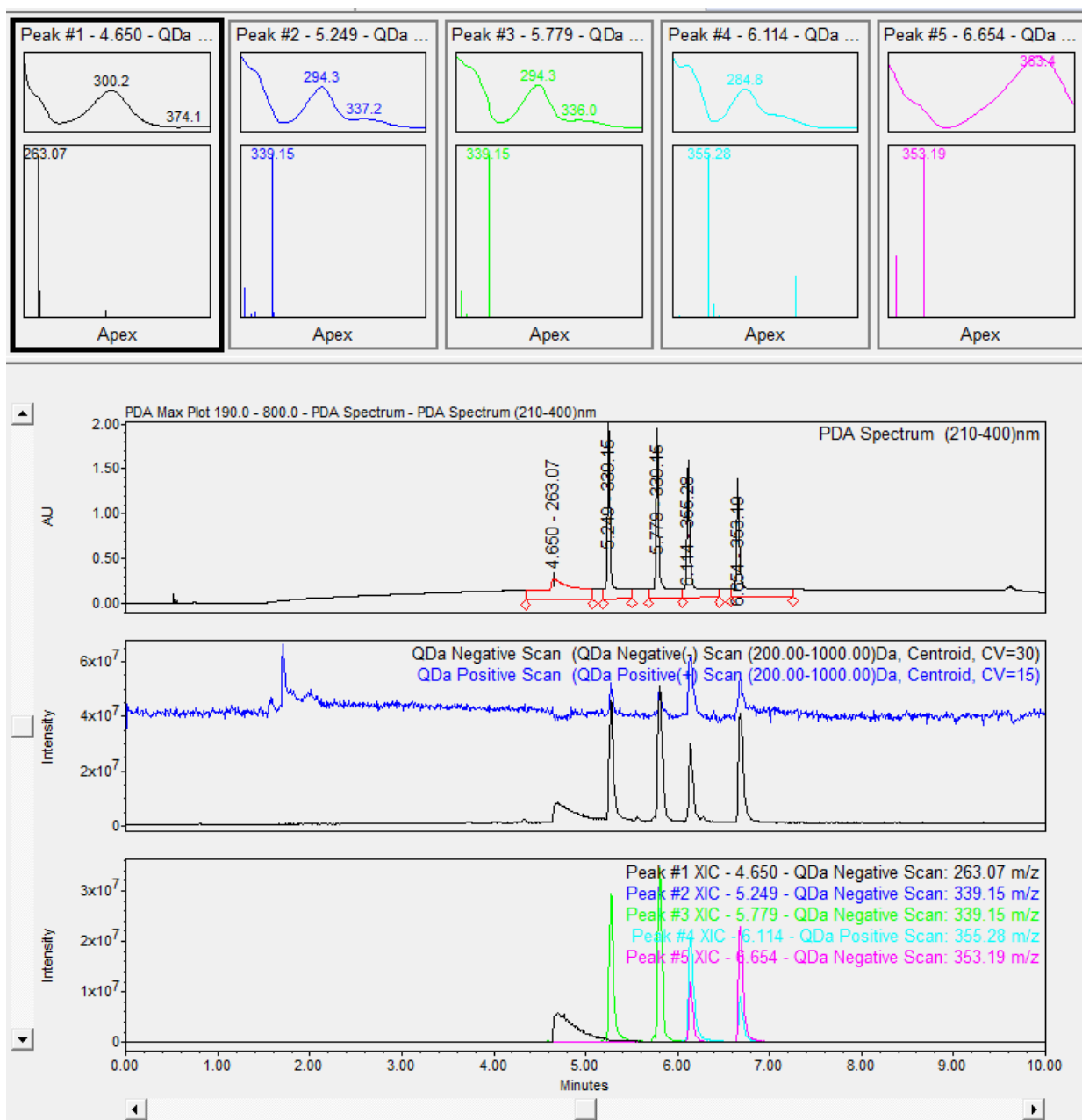
Figure 36 – Mass detection with optimized flow rates and 10 mM ammonium formate in 99% IPA and 1% H2O as make up solvent (Inj. ID 1770)

6.4.2.4 Effect of injection volume

Working with the so far optimized parameters, the next step was to find the optimum injection volume. 1, 2 and 3 µL of samples (extract of Prod5) were injected.
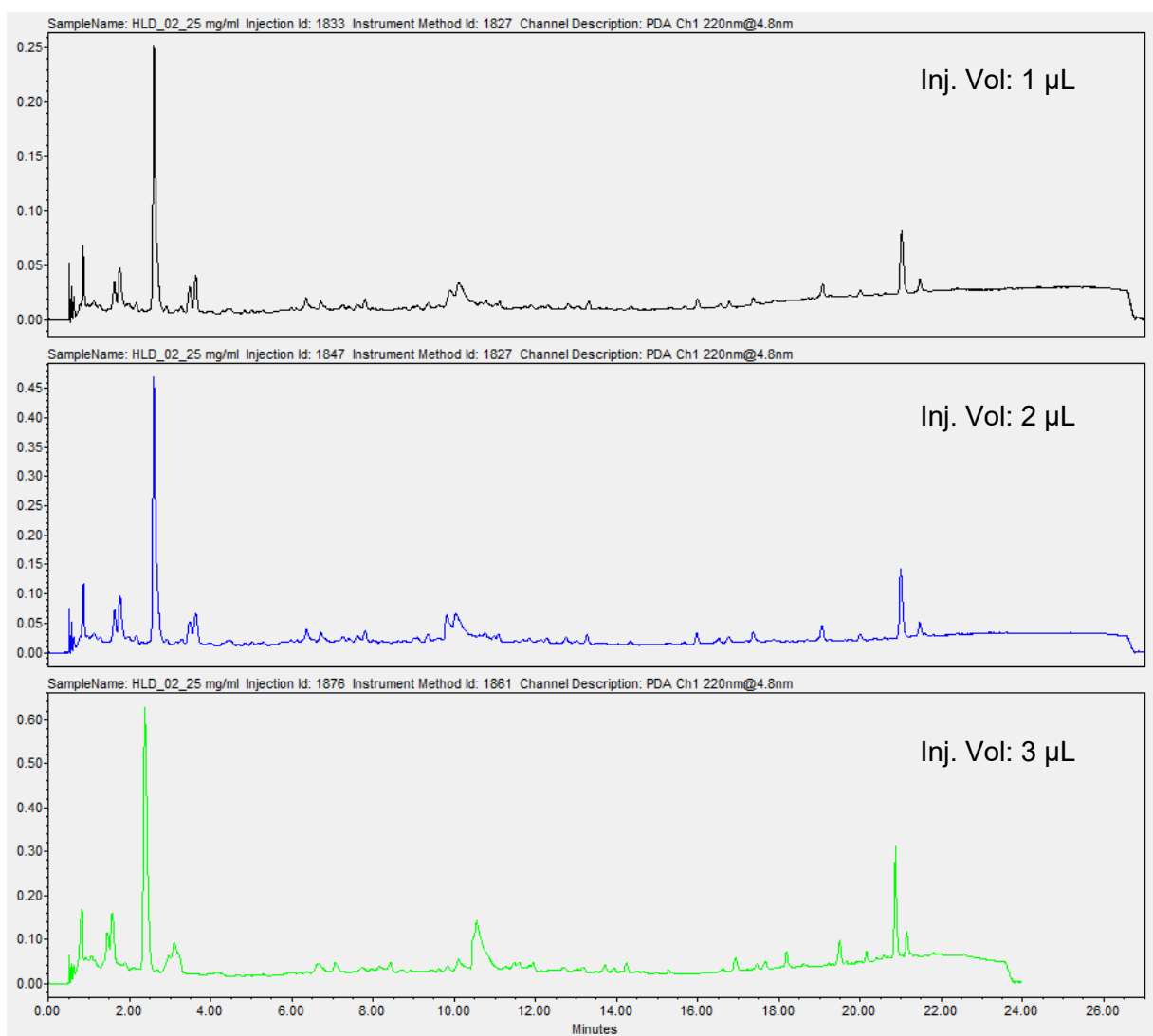
Figure 37 - Influence of different injection volumes on peak shape and retention tested with the extract of Product 5

6.4.2.5 Effect of temperature

To assess the influence on peaks and retention time different column temperatures were tested comprising 40°C, 50°C, 55°C, 60°C.
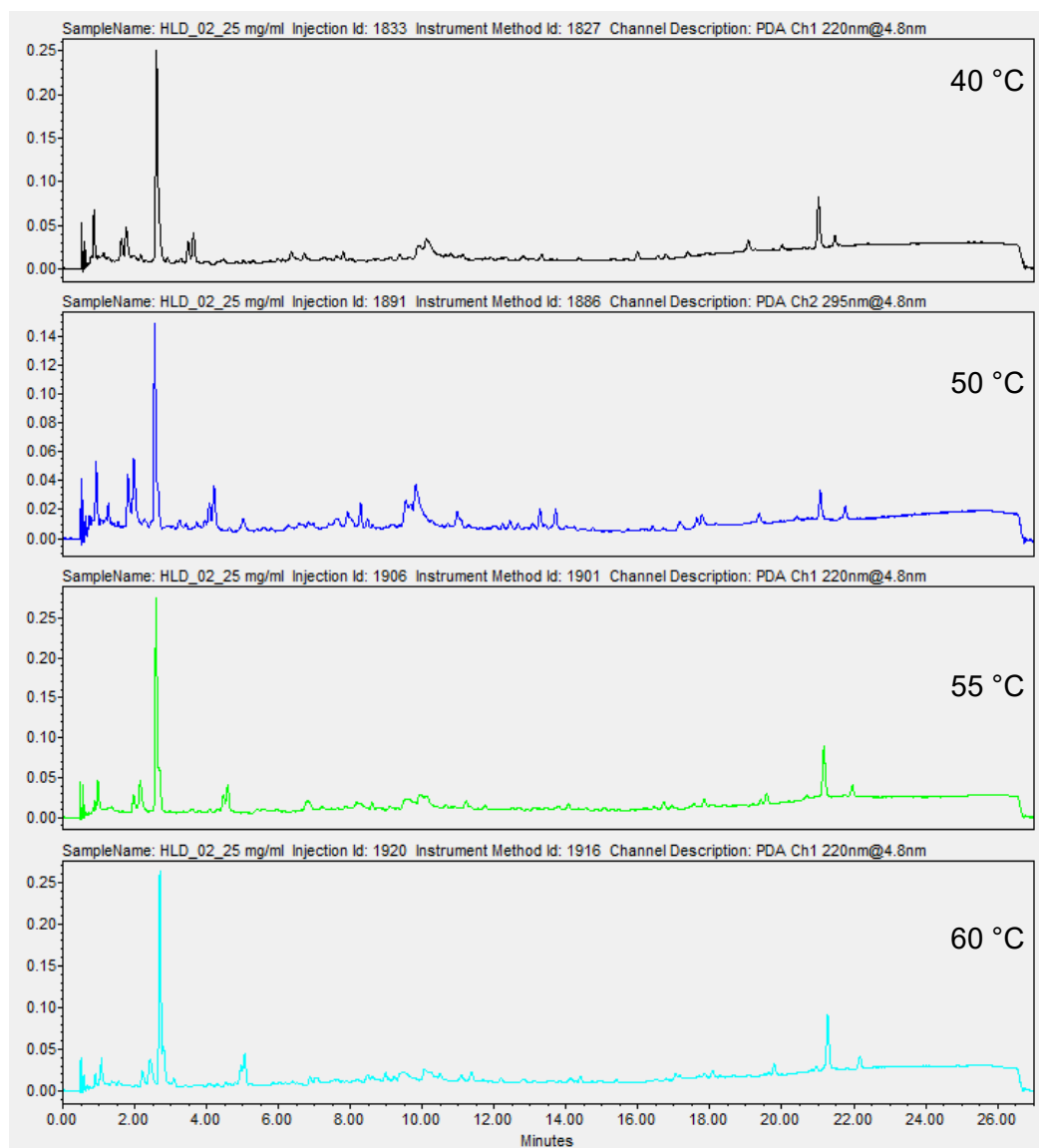
Figure 38 - Comparison of different column temperature on the extract of Product 5

6.4.2.6 Effect of gradient

Different gradients were tested for both the standard mix and the extract of *Humulus lupulus* (Product 5).

Optimized gradient for standard mix:

For the standard mix, gradient optimization was quite easy to achieve as the different compounds were already quite well separated (previously optimized parameters) and also peak shape was ideal. The gradient optimization for the Standard Mix resulted in a short run time (6 minutes). This method was carried out for all purchased products (6.4.2.7).

Table 16 - Optimized gradient for standard mix

Time (min)	A = CO_2 (%)	B = Co-Solvent (%)
0	80	20
4	60	40
5	60	40
5.1	80	20
6	80	20

Optimized gradient for the extract:

The challenging part of gradient optimization for the extract was to get a good baseline separation and peak shapes for volatile/non-polar as well as polar compounds of the extract. Therefore the run time had to be extended.

Table 17 - Optimized gradient for the extract carried out with Prod5

Time (min)	A = CO_2 (%)	B = Co-Solvent (%)
0	97	3
5.5	90	10
14	60	40
15	60	40
15.1	97	3
16	97	3

6.4.2.7 Optimized method for standard mix carried out with all purchased products

HL_E_Prod1_in 0,5ml Hex/IPA
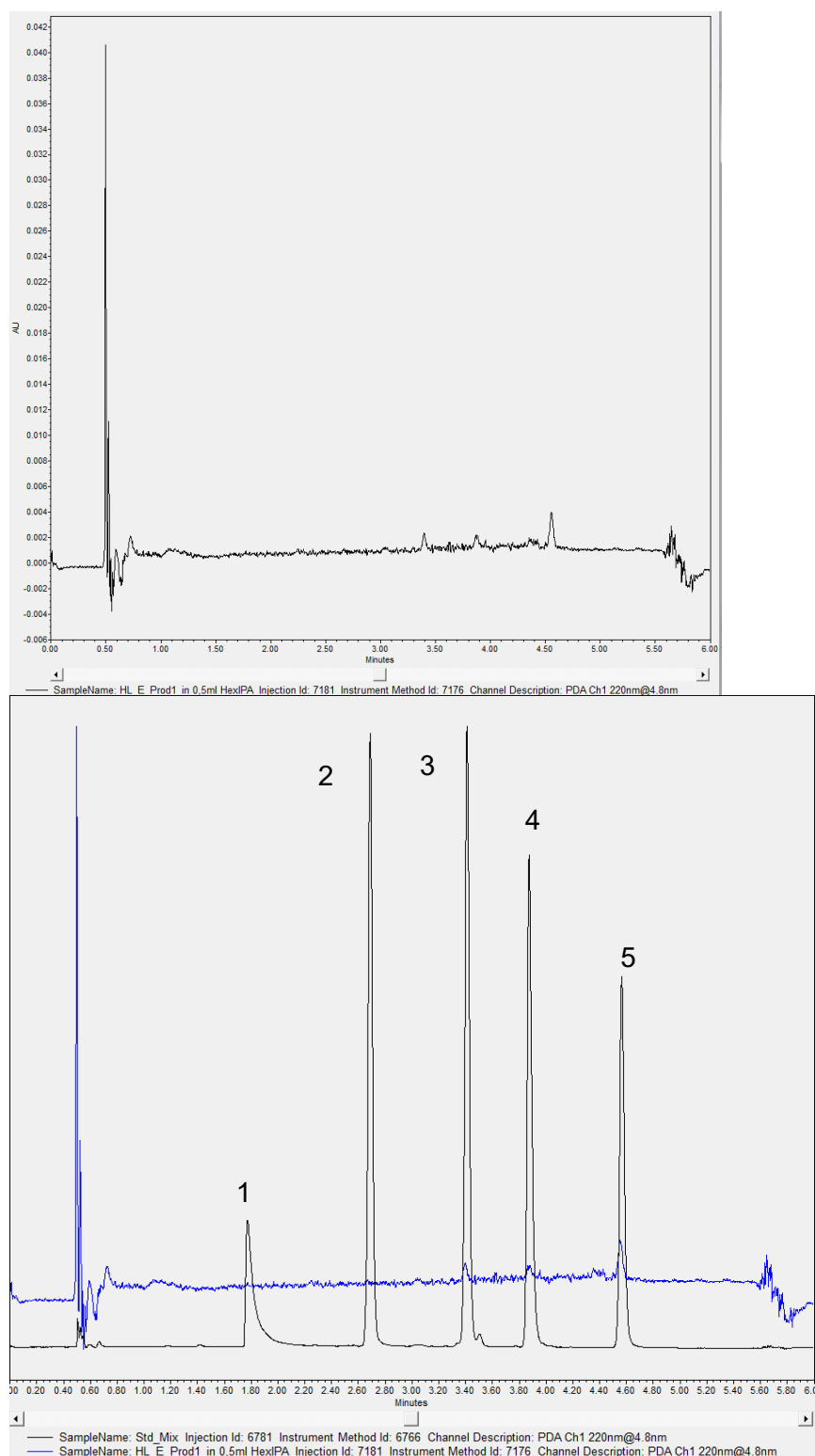
Figure 39 - Optimized conditions carried out for Prod1 alone and overlay with standard mix (220 nm)

HL_E_Prod2_in 0,5ml Hex/IPA
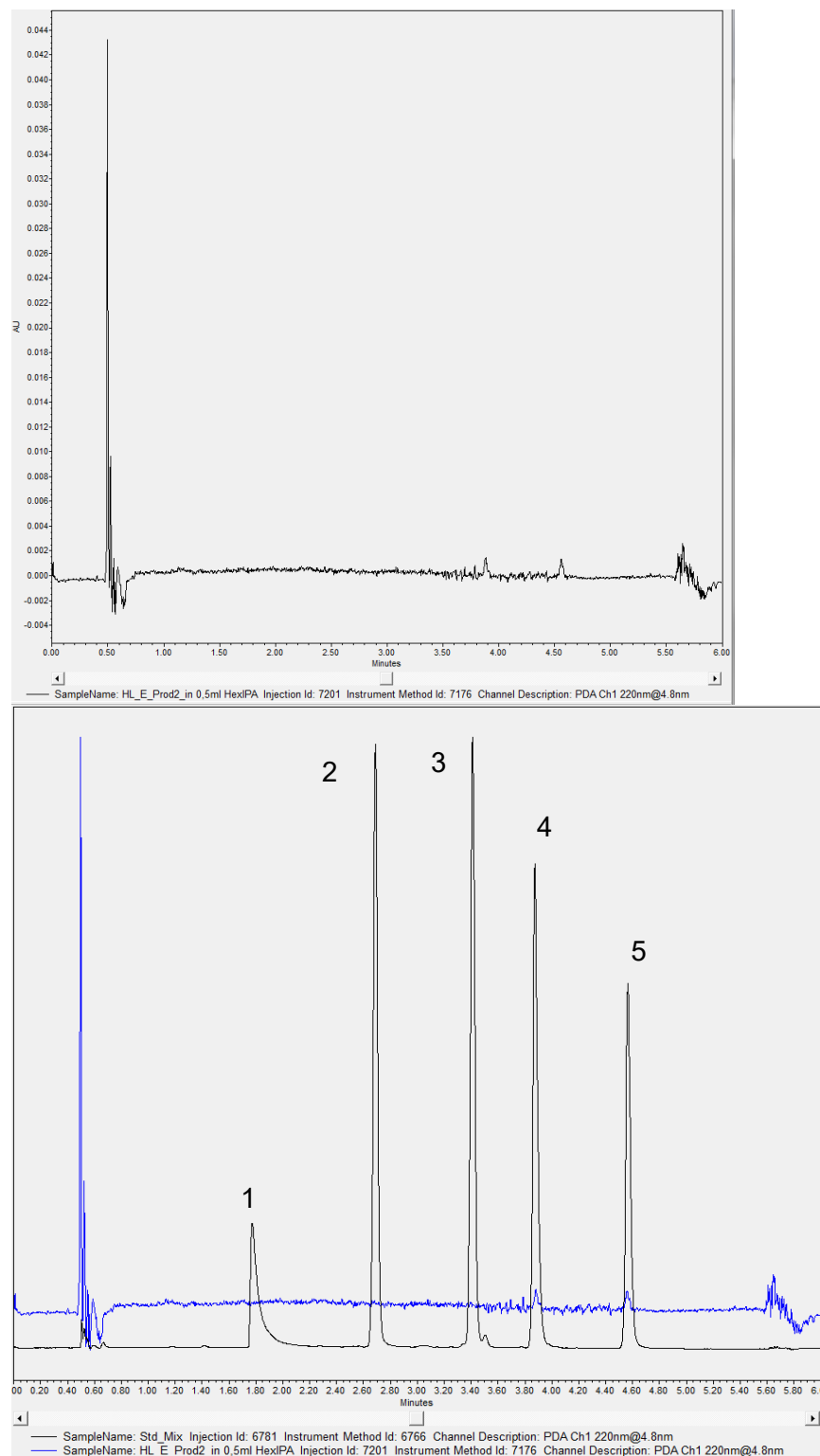
Figure 40 - Optimized conditions carried out for Prod2 alone and overlay with standard mix (220 nm)

HL_E_Prod3_in 0,5ml Hex/IPA
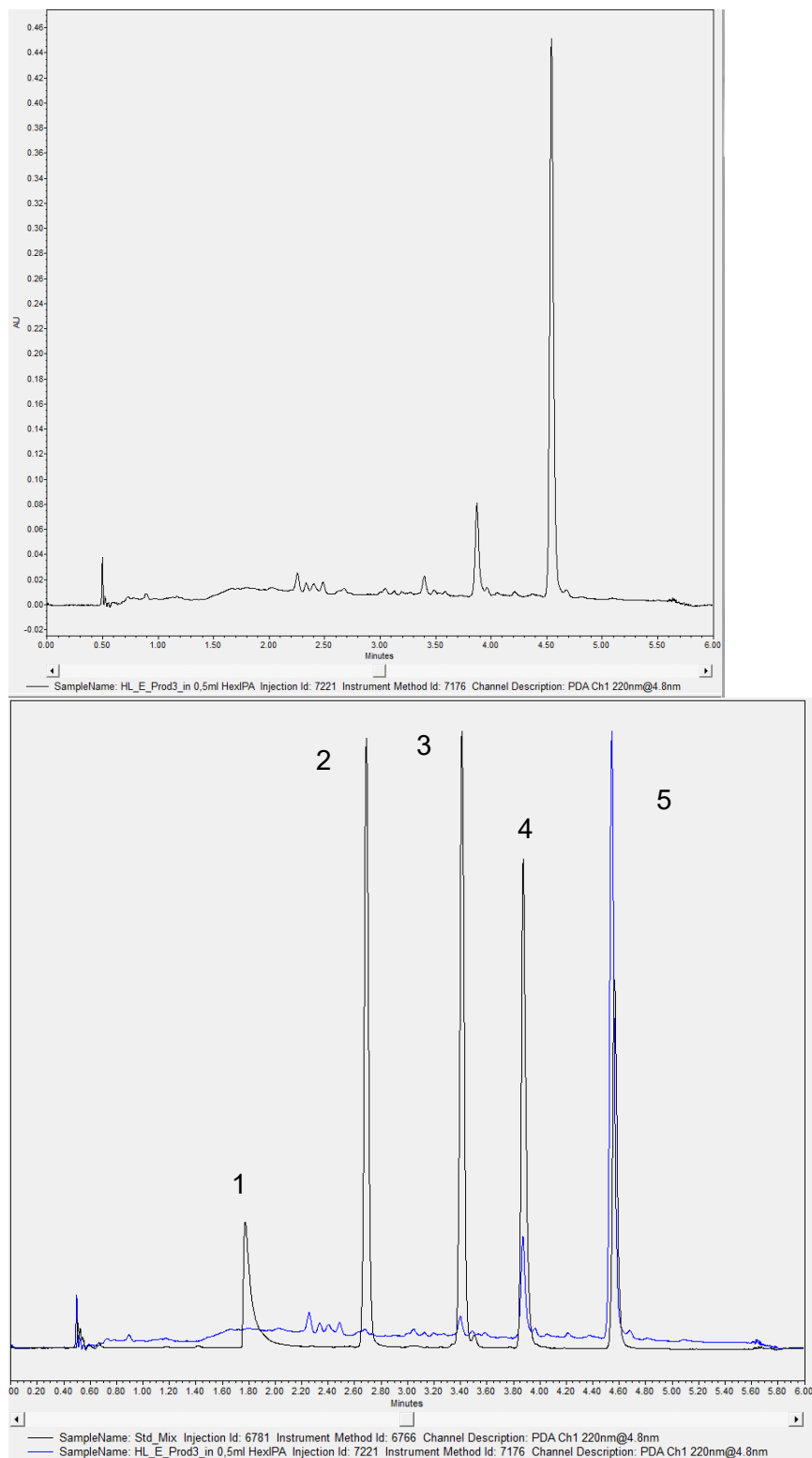
Figure 41 - Optimized conditions carried out for Prod3 alone and overlay with standard mix (220 nm)

HL_E_Prod4_in 0,5ml Hex/IPA
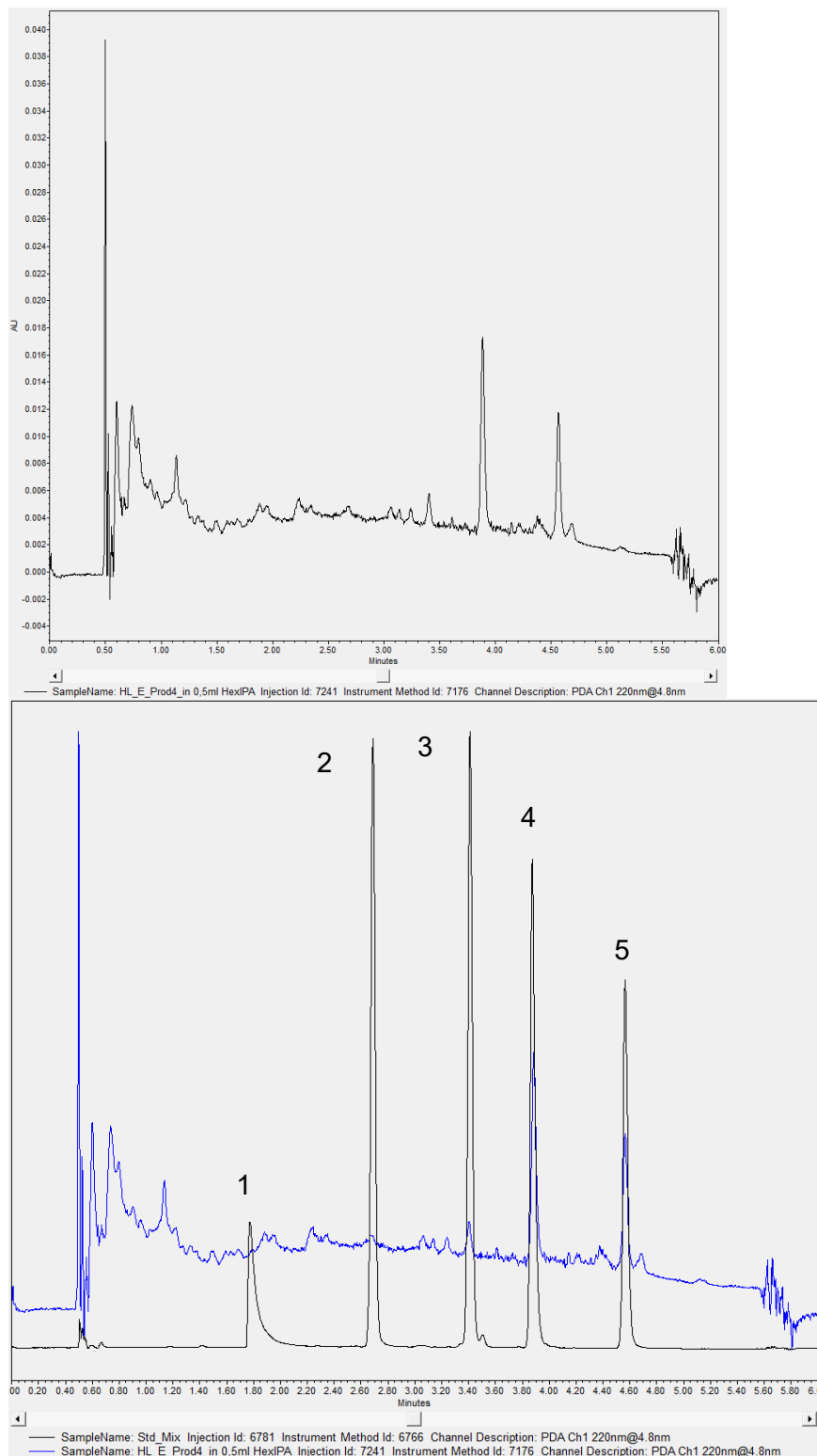
Figure 42 - Optimized conditions carried out for Prod4 alone and overlay with standard mix (220 nm)

HL_E_Prod5_in 0,5ml Hex/IPA
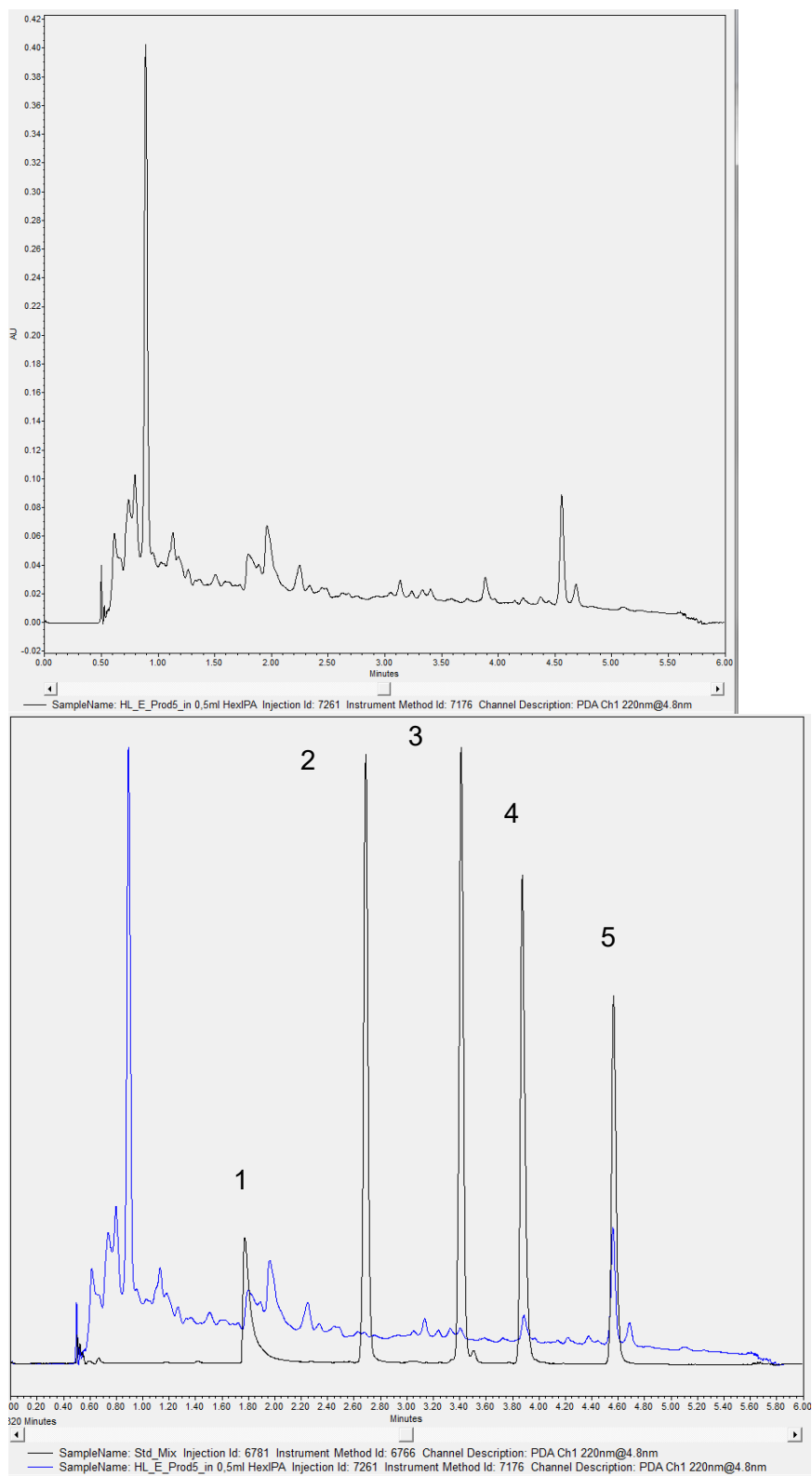
Figure 43 - Optimized conditions carried out for Prod5 alone and overlay with standard mix (220 nm)

HL_E_Prod6_in 0,5ml Hex/IPA
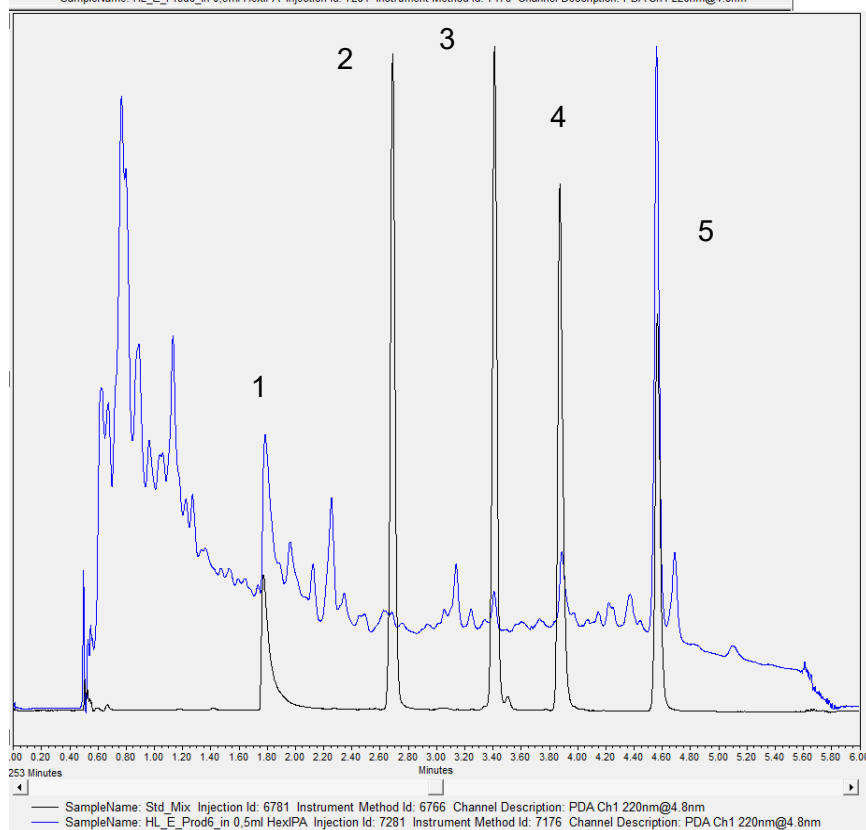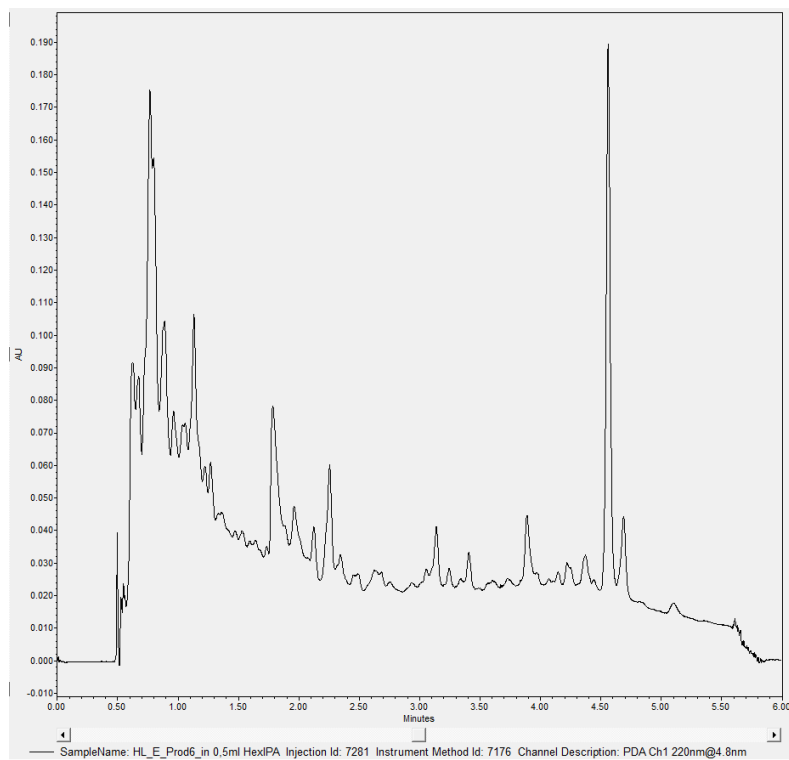
Figure 44 - Optimized conditions carried out for Prod6 alone and overlay with standard mix (220 nm)

6.4.3 SFC Prep-15

The optimized UPC² method for the hops extract was transferred to SFC Prep-15. Therefore, some pretesting and optimization of parameters (extract of Prod5) was carried out to separate early (non-polar) peaks on the analytical SFC.

6.4.3.1 Column Screening

The following columns were used for a first column screening of the extract.

Table 18 - Colum dimensions for column screening on Prep-15 SFC

Series	Name	Chemistry	Dimension Particle Size	
			analytical	preparative
Viridis	Bridged-Ethylene-Hybrid	BEH	4.6x250 mm	10x250 mm
			5.0 µm	5.0 µm
	Bridged-Ethylene-Hybrid 2-Ethylpyridine	**BEH 2-EP**	4.6x250 mm	10x250 mm
			5.0 µm	5.0 µm
	Charged-Surface-Hybrid Fluoro-Phenyl	CSH Fluoro-Phenyl	4.6x250 mm	10x250 mm
			5.0 µm	5.0 µm
	Silica 2-Ethylpyridine	Silica 2-EP	4.6x250 mm	10x250 mm
			5.0 µm	5.0 µm
Torus	1-Aminoanthracene	1-AA	4.6x250 mm	10x250 mm
			5.0 µm	5.0 µm

6.4.3.2 Method development for analytical column

With a run time of 9 minutes best results could be achieved with BEH 2-EP (Figure 23 and Figure 24) – the column that was previously used on UPC² too.

Injection volume, sample concentration, flow rates backpressure and column temperature during column screening were: 10 µl, 25 mg/ml, 5 m /min (BSM), 3 ml/min (ISM), 120 bar and 40°C. As co-solvent isopropanol gave better results than MeOH and was used for further processing.

6.4.3.3 Effect of other parameters on analytical column results

At a concentration of 25 mg/ml (as used for UPC² optimization) not enough substance was reaching the column. So it was necessary to adjust concentration as well as adapt injection volumes.

So a sample was prepared using 1.004 g of Prod5; the extraction was carried out with 25 ml hexane/isopropanol (70:30) on the ultrasonic bath for 10 minutes. The extract was filtered and solvents were removed by evaporation on the rotary evaporator. 50.48 mg of the dried extract were solved in 1 ml hex/IPA (70:30) and diluted (concentration: 50.48 mg/ml). The samples were run on SFC Prep-15 with different injection volumes as Figure 45 and Figure 46 demonstrate.
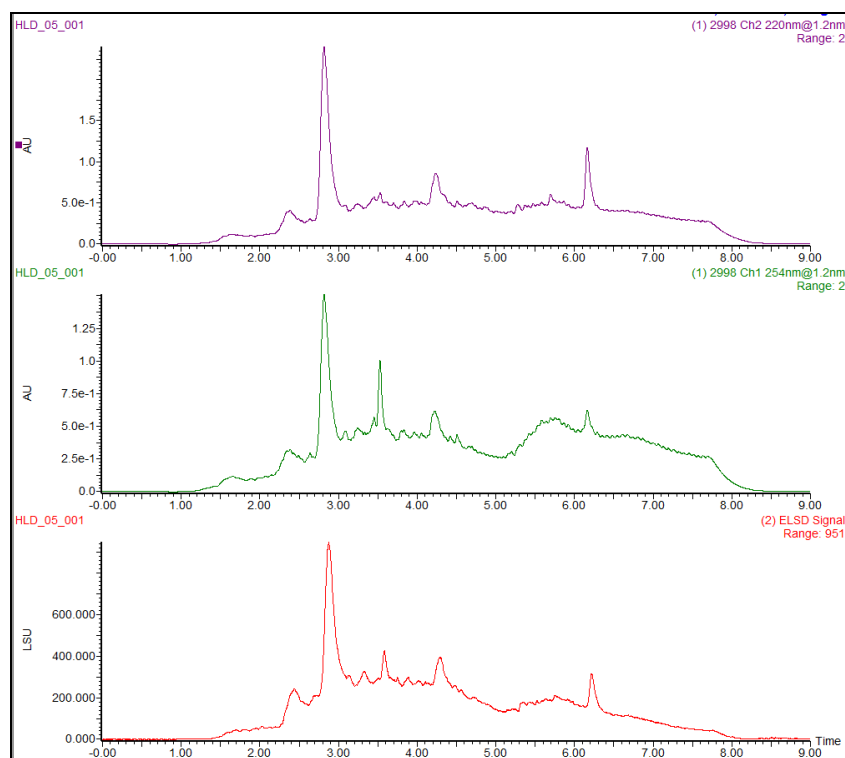
Figure 45 - Extract with an injection volume of 20 µl and a concentration of 50 mg/ml
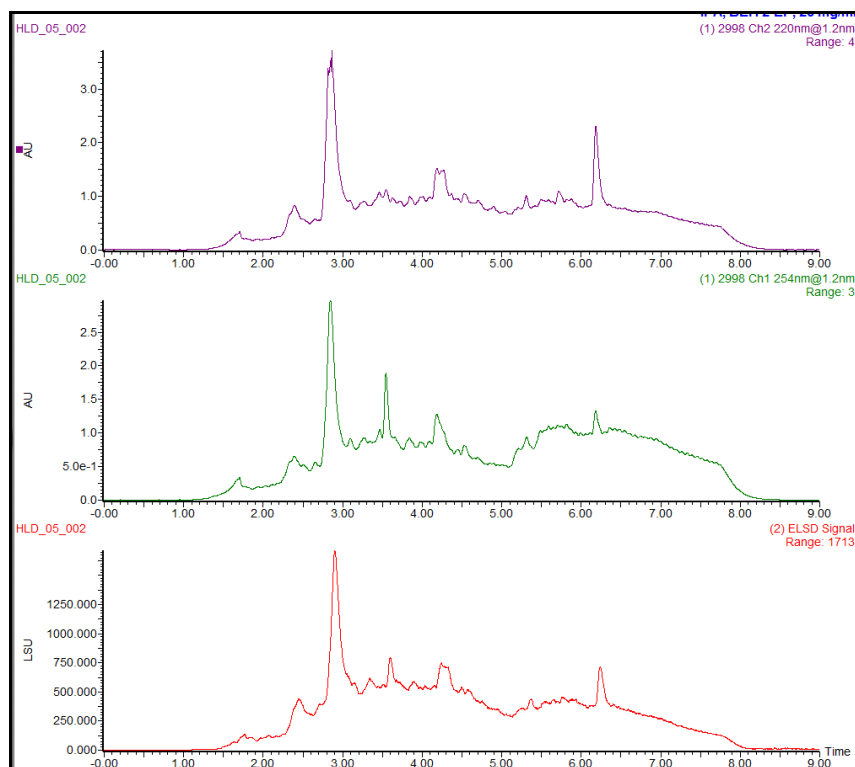
Figure 46 - Extract with an injection volume of 40 µl and a concentration of 50 mg/ml

Carrying out gradient optimization the run time for the extract had to be extended. Optimum conditions on the analytical column were:

Table 19 - Optimized gradient on analytical column

Time (min)	A = CO_2 (%)	B = Co-Solvent (%)
0	95	5
7	95	5
13	50	50
16.5	50	50
18	95	5
19	95	5

The optimized conditions were carried out with two different injection volumes: 40 µl and 20 µl as can be seen in Figure 47.
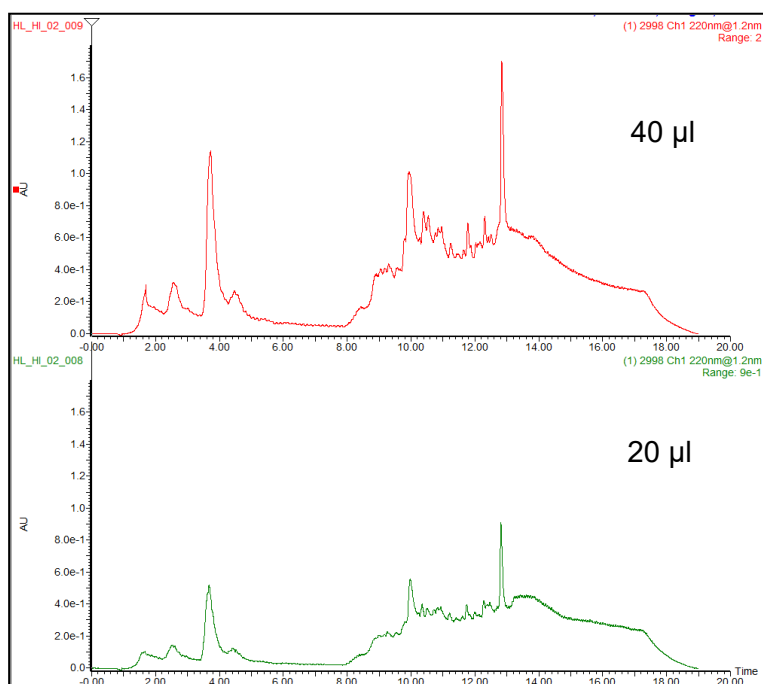
Figure 47 - Optimized gradient on analytical column with different injection volumes

6.4.3.4 Fractioning of nonpolar constituents on preparative column

According to other dimensions on the preparative column parameters like flow rate, make up flow rate and injection volumes had to be adjusted to the following conditions: 15 ml/min, 5 ml/min, 200 µl. Column temperature, detection wavelength, gradient and backpressure could be transferred directly.
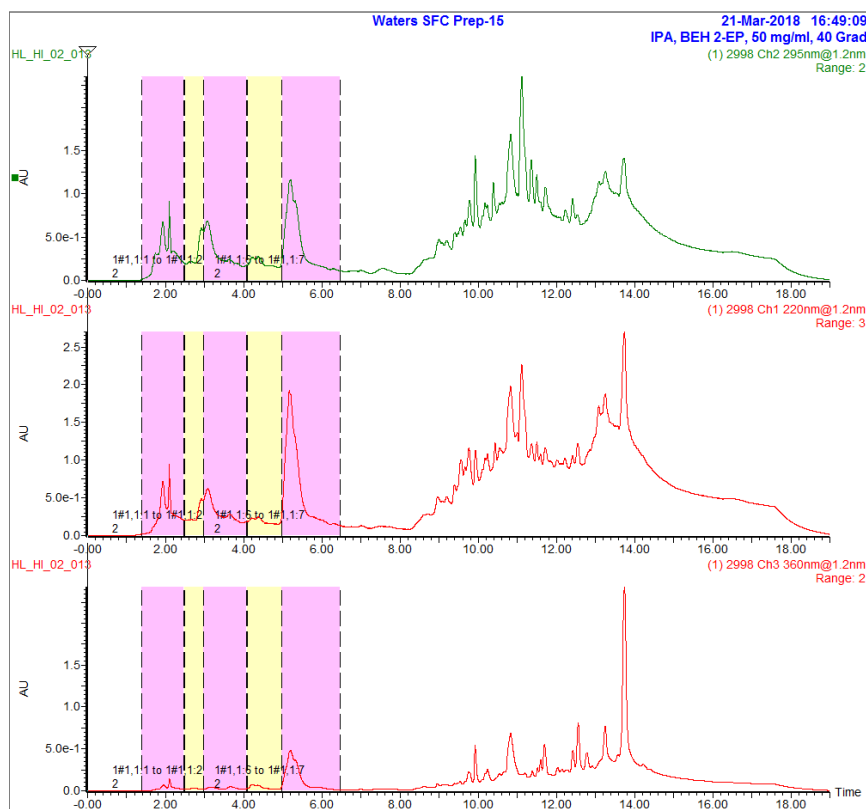
Figure 48 - Fractioning of non-polar constituents on preparative column

The following fractions were collected:

- Peak 1   Fractions 1:1 to 1:2
- Peak 2   Fractions 1:3
- Peak 3   Fractions 1:4 to 1:5
- Peak 4   Fractions 1:6 to 1:7
- Peak 5   Fraction 1:8 to 1:10

6.5 METHOD VALIDATION

For development of **calibration curves** on UPC² a series dilution of all 5 standard substances was prepared; serial dilution ratio was 1:3. At least 5 calibration levels were required to match the criteria for a good calibration curve stated in the ICH Guidelines. Each level was injected on UPC² in triplicates and integration of the peaks was performed with Empower 3 software.

Hulupinic acid:

5 levels used

Level	concentration (mg/ml)
0	0.99
1	0.33
2	0.11
3	0.105
4	0.035

Method	Hops_Hulu	Date/Time	4/19/2018 12:49:13 PM CEST	
System	UPC2_no ELSD	Channel	PDA Ch1 220nm@4.8nm	
Component	Hulupinic Acid	Time (min)	8.860	

Equation: Y = 1.48e+006 X - 4.33e+004

R^2	0.995251	R	0.997623	Standard Error	3.860984e+004
RSS	1.937936e+010	RSD	128.377172	Weighting	None

Codes

RSD(E) 9.180588

Norm Intercept/Slope -0.057196
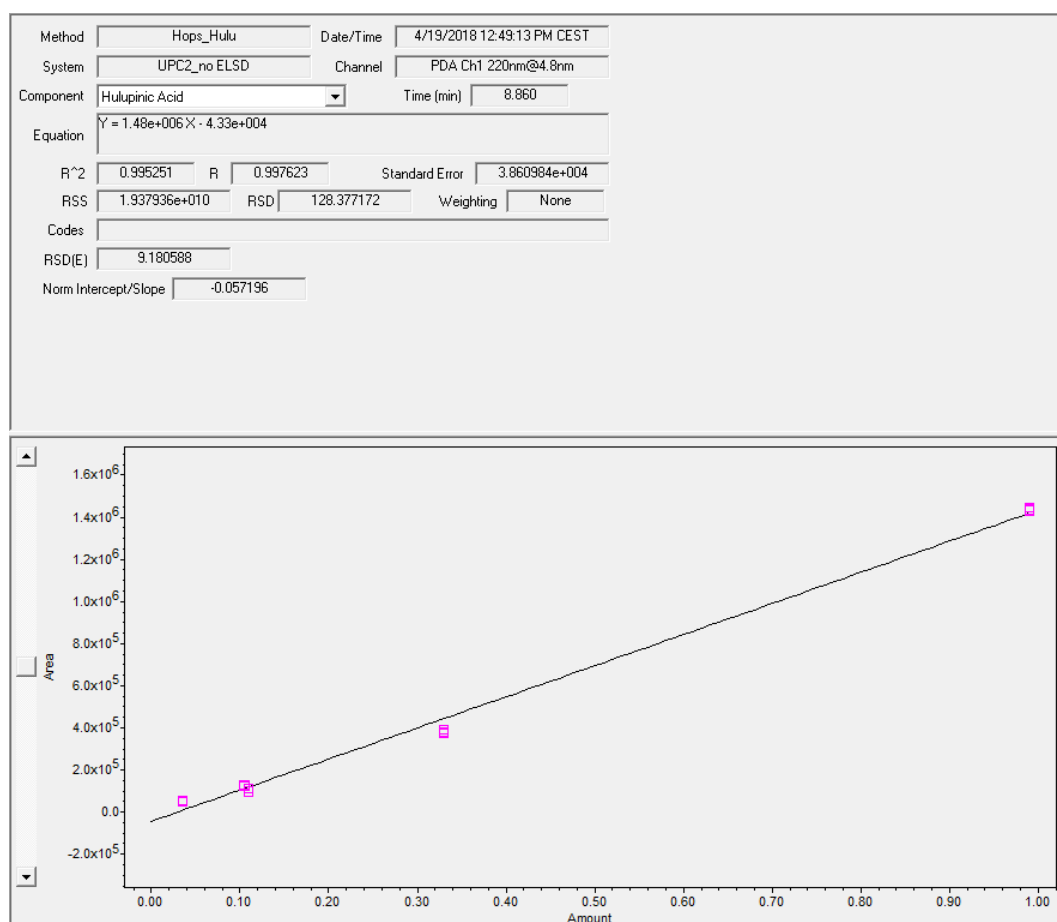
Figure 49 - Calibration curve for hulupinic acid (5 levels)

8-Prenylnaringenin:

8 levels used

Level	concentration (mg/ml)
0	1.02
1	0.34
2	0.1134
3	0.105
4	0.035
5	0.0117
6	0.00388
7	0.0013

Method	Hops_8Pre	Date/Time	4/19/2018 1:12:07 PM CEST	
System	UPC2_no ELSD	Channel	PDA Ch1 220nm@4.8nm	
Component	8-Prenylnaringenin	Time (min)	10.874	
Equation	Y = 4.12e+006 X - 9.80e+003			
R^2	0.999536	R	0.999768	Standard Error	3.018535e+004
RSS	2.004541e+010	RSD	165.280184	Weighting	None
Codes				
RSD(E)	3.638501			
Norm Intercept/Slope	-0.004660			
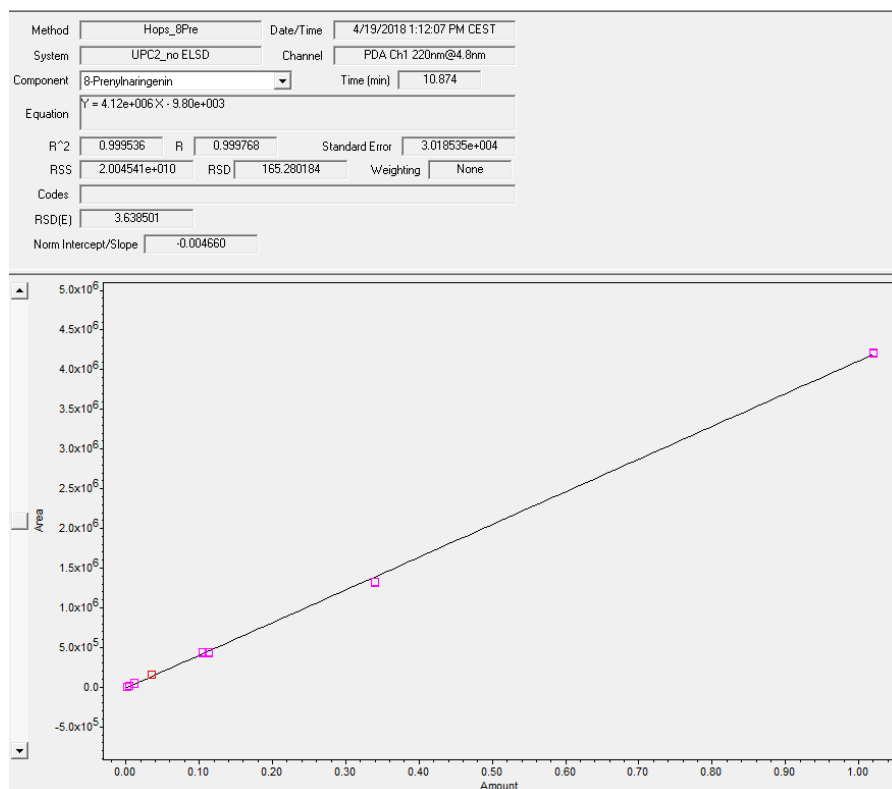
Figure 50 - Calibration curve for 8-Prenylnaringenin (8 levels)

6-Prenylnaringenin:

8 levels used

Level	concentration (mg/ml)
0	1.05
1	0.35
2	0.1167
3	0.105
4	0.035
5	0.0117
6	0.00388
7	0.0013
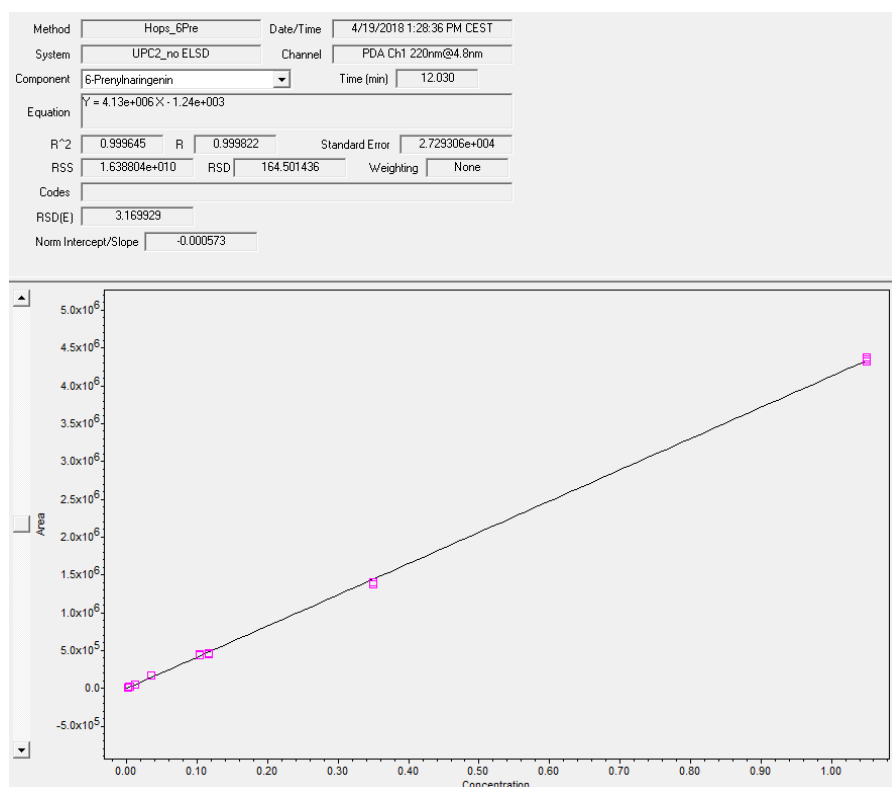
Figure 51 - Calibration curve for 6-Prenylnaringenin (8 levels)

Isoxanthohumol:

7 levels used

Level	concentration (mg/ml)
0	1.01
1	0.337
2	0.1123
3	0.10
4	0.033
5	0.011
6	0.0037
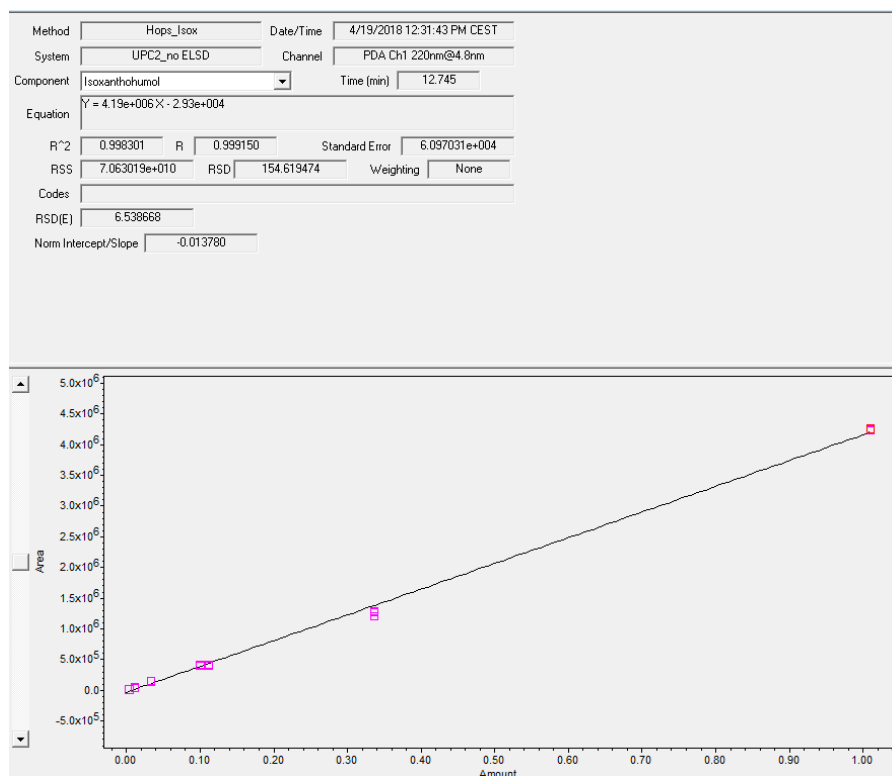
Figure 52 - Calibration curve for Isoxanthohumol (7 levels)

Xanthohumol:

8 levels used

Level	concentration (mg/ml)
0	1.04
1	0.347
2	0.1156
3	0.105
4	0.035
5	0.0117
6	0.0038
7	0.00129
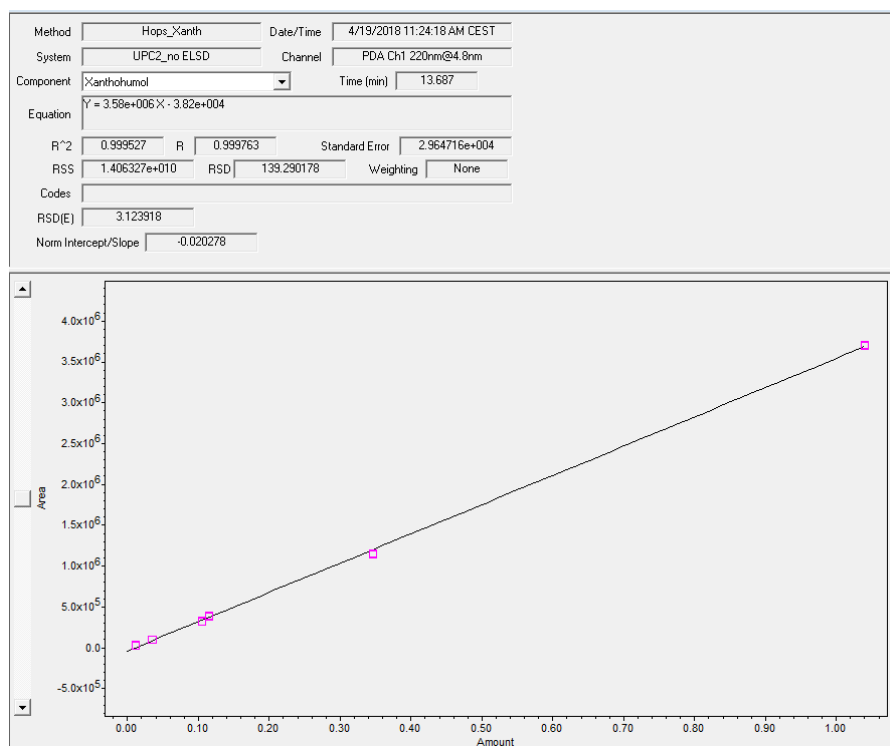
Figure 53 - Calibration curve for Xanthohumol (8 levels)

Linearity range was varying between reference substances. Especially for hulupinic acid a high concentration was necessary to identify the substance after integration with Empower 2. Limit of detection (**LOD**) and limit of quantification (**LOQ**) values were evaluated visually representing standard concentrations equivalent to signal-to noise-ratios of 3 and 10, respectively (Table 20). **Selectivity** was concluded by evaluation of PDA data including peak purity.

Table 20 - Linearity range, LOD and LOQ for compounds

	Linear range (µg/ml)	LOD (µg/ml)	LOD (µg/ml)
Hulu	46.0 – 990.0	3.800	35.00
8-PN	1.3 – 1050.0	1.296	3.88
6-PN	1.3 – 1050.0	1.296	3.88
IXN	3.7 – 1010.0	1.235	3.70
XN	11.7 – 1040.0	1.296	3.88

Intra-day (evaluation within one day; n = 5) and **inter-day** (evaluation over three days; n = 3) experiments were carried out with Prod5 to assess **precision** and showing the following results:

Table 21 - Reproducibility (intra-day)

	1	2	3	4	5	average	STADEV
Hulu	3.13	3.20	3.40	2.92	3.46	**3.22**	**0.217**
8-PN	0.85	0.72	0.51	0.78	0.83	**0.74**	**0.137**
6-PN	0.94	1.02	0.78	1.02	0.91	**0.93**	**0.099**
IXN	1.62	1.70	1.92	1.85	1.94	**1.81**	**0.140**
XN	3.44	3.92	3.96	2.99	3.85	**3.63**	**0.414**

The intra-day comparison was carried out with Prod5.
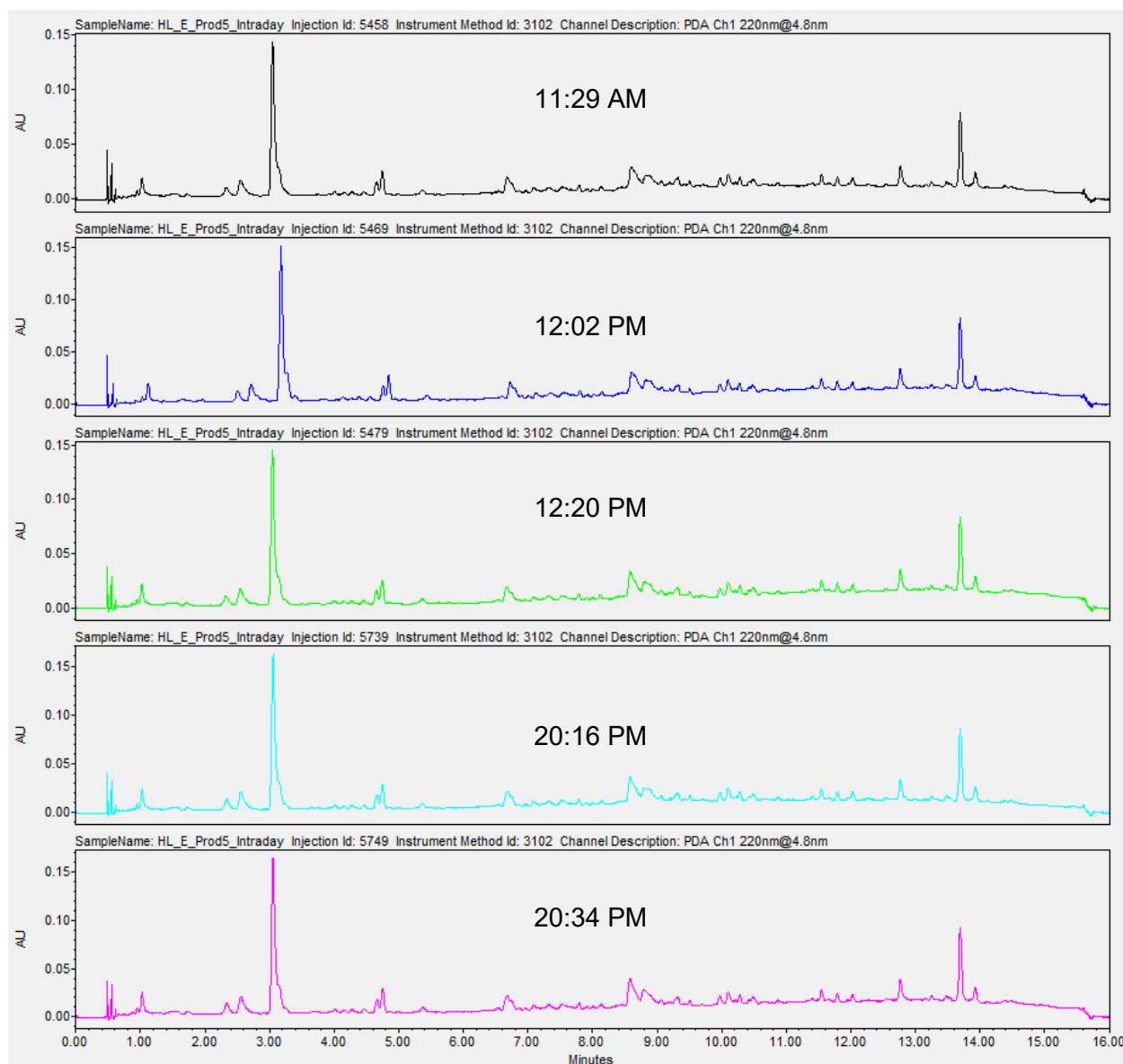
Figure 54 - intra-day comparison carried out with EtOH extract of Prod5

Table 22 - Reproducibility (inter-day)

	1	2	3	average	STADEV
Hulu	3.05	2.97	3.00	3.01	0.040
8-PN	0.71	0.66	0.72	0.70	0.036
6-PN	0.99	0.97	0.99	0.98	0.010
IXN	1.75	1.75	1.73	1.75	0.011
XN	3.91	3.99	4.09	4.00	0.090

For **accuracy**, recovery rates were determined for Prod5 spiked with 125% (high spike), 100% (medium spike) and 75% (low spike) amounts of respective standard compounds.

Weighted samples were pulverized in a porcelain mortar and prepared according to the optimized extraction method. Spiking volumes were calculated (Table 23), added to the pulverized drug material and dried with the sample concentrator before extraction. All samples were run on UPC² as triplicates and evaluated with Empower 3 software using the established calibration curves and processing methods for each reference substance.

Table 23 - Calculated volumes for spiking in µl

		Low spike (75%) 108.00 mg	Medium spike (100%) 108.10 mg	High spike (125%) 108.29 mg
Hulu		884	1180	1477
8-PN	volume in µl	31	41	51
6-PN		36	48	61
IXN		157	209	262
XN		465	620	776

Recovery rates for each reference substance were calculated and are summarized below (Table 24).

Table 24 - Accuracy of the assay

	Accuracy expressed as recovery rate in %		
	Low spike	Medium spike	High spike
Hulu	91.77	95.17	113.08
8-PN	92.31	92.31	99.15
6-PN	91.43	96.67	100.74
IXN	88.89	93.65	105.11
XN	76.97	91.33	92.52

For **quantificatio**n all purchased products (Prod1-Prod6) were prepared according to the optimized extraction method in a 20 ml volumetric flask (see 6.2.2) and run on UPC² with the optimized method. Sample preparation and the percentage of every compound are listed in Table 25 and Table 26.

Table 25 - Sample preparation for quantification

Sample name	content of dried extract	used amount		additional info
Prod1	40 mg/capsule	4	capsules	
Prod2	125 mg/capsule	2	capsules	
Prod3	300 mg/capsule	1	capsule	
Prod4	125 mg/coated tablet	2	coated tablets	pulverized in porcelain mortar
Prod5		1.08485 g		pulverized in porcelain mortar
Prod6		1.01134 g		pulverized in porcelain mortar

Table 26 - Percentage of reference compounds in investigated product samples (Prod1 - Prod6)

	sample name					
	Prod1	Prod2	Prod3	Prod4	Prod5	Prod6
Hulu	n.d.[a]	n.d.	n.d.	n.d.	0.115± 0.003	**0.207±** 0.004
8-PN	n.d.	n.d.	**0.014±** 0.000	0.005± 0.000	0.004± 0.001	0.005± 0.000
6-PN	0.001± 0.000	n.d.	**0.026±** 0.000	0.003± 0.000	0.005± 0.000	0.006± 0.000
IXN	0,004± 0.000	0.008± 0.000	**0.168±** 0.003	0.028± 0.000	0.019± 0.000	0.026± 0.001
XN	0.007± 0.000	0.012± 0.000	**1.017±** 0.010	0.028± 0.000	0.060± 0.001	0.137± 0.001

a) n.d. = not detectable

7 CONCLUSION

Complexity and diversity of hop component makes analysis very challenging. Isomerisation, polymerisation and oxidation processes lead to several hundreds of secondary metabolites.

The **first aim** of the study was to establish an SFx based protocol for **identification and quantification** of five polar, hop characteristic reference substances in six mono hop products.

Four flavonoid compounds – xanthohumol (XN), isoxanthohumol (IXN), 8-prenylnaringenin (8-PN) and 6-prenylnaringenin (6-PN) - and hulupinic acids (Hulu), the degradation product of bitter acids, were chosen as reference substances. The examined mono hop products (Prod1 – Prod6) were four dietary products (3 capsule and 1 coated tablet product) and two toto drugs.

Development of an optimized extraction method was basically easy to carry out. Results for extraction efficiency were over 96.32% for four out of five reference compounds (i.e. 8-PN, 6-PN, IXN, XN) when using EtOH 96% as extraction solvent. Only the vinylogous acid - hulupinic acid – could not be included in finding the optimum extraction solvent.

During method development a **thin layer chromatography (TLC) system** was carried out for all reference compounds and extracts of the purchased commercial products. The TLC gave a good first impression of the composition of Prod1 – Prod6. In general only **Prod3** – a capsule product enriched with XN, **Prod4** (coated tablet product) and the two toto drugs **Prod5** and **Prod6** were containing some respectively all bands of reference compounds.

Using **UPC²** as an analytical SFC instrument was showing good results for a prepared **standard mix**, comprising approx. 1 mg of each reference compound per 10 ml hexane:isopropanol (70:30). A short run time of only 5 minutes could be achieved. Peak separation and peak shape were optimized. Nevertheless it has to be noted that peak shape for hulupinic acid resulted in tailoring. So also on UPC² the peak for instable hulupinic acid was not perfectly sharp.

The addition of a make-up solvent made integrated mass detection (QDa detector) possible. But it also had strong impact on flow rates for BSM and ISM. Flow rates had to be adjusted to 1.0 ml/min and 0.6 ml/min, respectively as higher flow rates resulted in ABPR overpressure. With lower flow rates not enough substances reached the QDa. With QDa four reference compounds (XN, 8-PN, 6-PN and Hulu) were detected in negative scan mode. IXN was detected in positive

scan mode. Again for Hulu conditions for mass detection were not ideal and so the highest peak of m/z in the mass spectra did not correspond with the de facto molecular weight.

Reliability and suitability of the established 5 minute method for the quantification of reference substances in all purchased commercial products could be **proven in method validation**. It has to be considered that during preparation a very high concentration of approximately 1mg/ml of each reference substance was necessary to achieve the minimum required 5 data points for good calibration (demanded by ICH guidelines). This is very significant and again shows how instable compounds in hops are.

Good results regarding linearity, LOD and LOQ, precision and accuracy could be achieved; also compared to other chromatographic methods like HPLC. But also here hulupinic acid was showing the greatest variance and differed significantly from the other reference compounds.

The optimized UPC² method for the **standard mix** was carried out for all six commercial products. The results basically correlated with first results on TLC – but of course UPC² was more precise and sensitive.

When transferring the optimized UPC² method to **SFC Prep-15** the focus was lying on validation of its suitability to purify and isolate non-polar compounds. Gradient parameters could not be directly transferred from UPC² to SFC Prep-15. After fractioning and peak collection results were monitored on the UPC² instrument. As non-polar peak isolation in hops extracts is an interesting field of studies parameter optimization for the preparative instrument will be carried out in another diploma study.

In general the first SFx protocol for identification and quantification of polar substances in hops could successfully been carried out. Further studies on preparative instruments will be necessary to develop optimized methods that are suitable to also purify and isolate non-polar compounds.

Second aim of the current diploma study was testing the **applicability of UPC²** for detection of non-polar and polar compounds within the crude hops extracts within one single workflow. It was very **challenging** to establish a UPC² method for the whole crude hop extract (carried out with Prod5). During gradient optimization for the whole extract a very flat gradient starting with only 97% CO_2 and 3% co-solvent (isopropanol) was absolutely necessary to get good separation for non-polar compounds. So of course run time had to be elongated and resulted in 15 minutes.

> Nevertheless the applicability of UPC² for the detection of non-polar and polar substances within one workflow could definitely be proved.

By using results from the PDA detector (UV spectra) and the integrated QDa detector (mass detection) identification of reference compounds could be carried out. Furthermore QDa and PDA data were compared with literature and possible compounds within the complex hops extract could be listed by peak retention time.

The **complexity of hops** and the broad variety of secondary metabolites makes fast and efficient chromatographic methods like UPC² a very interesting field of studies. Only recently the first study on humans was carried out by the Medical University of Vienna and proved by the Linus Pauling Institute at the Oregon State University in Corvallis (USA). In the USA the study was supported by the FDA and it concluded that a **daily dose of 12 mg XN** was shown to have significant protective effects against alterations in genetic material. It seems quite likely that a **health claim for XN** like "Xanthohumol can protect your DNA" will be approved in future (Biendl, 2018). Within the commercial products reviewed in this diploma study only Prod3 (enriched XN capsule product) resulted in a high amount of XN after quantification. So it can be considered a valuable nutritional supplement. 10.53 mg of XN could be quantified in the recommended daily dose of 3 capsules. According to the manufactured the product was enriched with 16 mg XN per daily dose. Differences in the amounts could be the manufacturers processing, inhomogeneous capsule content or also within the extraction processing in this study. To validate the exact amounts a larger amount of capsules could be examined.

This example **underlines the importance** of a broader knowledge of the potent compounds in hops and also the necessity of short and fast analysis and quality control methods.

8 REFERENCES

8.1 BOOKS AND PAPERS

Aghamiri V., Mirghafourvand M., Mohammad-Alizadeh-Charandabi S., Nazemiyeh, H. (2016). *The effect of hop (Humulus lupulus L.) on early menopausal symptoms and hot flashes: A randomized placebo-controlled trial.* Complementary Therapies in Clinical Practice, 23, 130–135.

Auster F., Schäfer J., (1958). *Arzneipflanzen.* VEB Georg Thieme Leipzig

Biendl M. (2018). *A Milestone in Xanthohumol Research: Effects Confirmed in First Studies on Humans.* Hopfen - Rundschau International 2017/2018, 72 - 76

Bland J., Minich D., Lerman R., Darland G., Lamb J., Tripp M., Grayson N. (2015). *Isohumulones from hops (Humulus lupulus) and their potential role in medical nurtrition therapy.* PharmaNutrition, 3, 46-52

Chen S., Chen P., Hsu H., Wu M., Yen J. (2017). *Xanthohumol suppresses mylip/idol gene expression and modulates LDLR abundance and activity in HepG2 Cells.* Journal of Agricultural and Food Chemistry, 65, 7908-7918

Deshmane S., Kremlev S., Amini S., Sawaya B. (2009). *Monocyte Chemoattractant Protein-1 (MCP-1): An Overview.* Journal of Interferon & Cytokine Research, 313-326

Dhooghe L., Naessens T., Heyerick A., De Keukeleire D., Vlietinck A., Pieters L., Apers S. (2010). *Quantification of xanthohumol, isoxanthohumol, 8-prenylnaringenin, and 6-prenylnaringenin in hop extracts and derived capsules using secondary standards*. Talanta 83, 448-456

Doddapattar P., Radović B., Patankar J., Obrowsky S., Jandl K., Nusshold C., Kolb D., Vujić N., Doshi L., Chandak P., Goeritzer M., Ahammer H., Hoefler G., Sattler W., Kratky D. (2013). *Xanthohumol ameliorates atherosclerotic plaque formation, hypercholesterolemia, and hepatic steatosis in ApoE-deficient mice*. Molecular Nutrition & Food Research, 57, 10

Eisath N., Sturm S., Stuppner H. (2017). *Supercritical Fluid Chromatography in Natural Product Analysis – An Update.* Planta Med (published online)

Europäisches Arzneibuch (EuAB; European Pharmacopoeia Ph.Eur.), 9. Ausgabe, Grundwerk 2017, Band 2, Verlag Österreich, 2104-2105

European Medicines Agency (2014). *Assessment report on Humulus lupulus L., flos.* EMA/HMPC (Committee on Herbal Medicinal Products).

Franco L., Sánchez C., Bravo R., Rodríguez A., Barriga C., Romero E., Cubero J. (2012) *The sedative effect of non-alcoholic beer in healthy female nurses.* Plos ONE, 7, 7

Gagnon D., Wendakoon C., Smith R., Leker, J. (2014). *Stability of active constituents of hops (humulus lupulus) strobiles and their ethanolic extracts during storage.* European Journal of Medicinal Plants, 1302–1512.

Gao W., Dong X., Wang R., Liu X., Li P., Yuang H. (2016). *The use of ionic liquid as a mobile phase modifier in analytical supercritical fluid chromatography for the separation of flavonoids.* Royal Society of Chemistry. 61418 - 61422

Gerhäuser C., Frank R. (2005). *Xanthohumol, a new all-rounder?* Molecular Nutrition & Food Research, Volume 49, 821 - 823

Hartmann A., Ganzera M. (2015). *Supercritical Fluid Chromatography – Theoretical Background and Applications on Natural Products.* Planta Med (published online)

Kostrzewa D., Dobrzyńska-Inger A., Rój E. (2013) *Experimental data on xanthohumol solubility in supercritical carbon dioxide.* Fluid Phase Equilibria, 360. 445-450

Liu M., Hansen P., Wang G., Qiu L., Dong J., Yin H., Qian Z., Yang M., Miao J. (2015) *Pharmacological Profile of Xanthohumol, a Prenylated Flavonoid from Hops (Humulus lupulus).* Molecules, 20, 754-779

Mannering G., Shoeman J. (1996). *Murine cytochrome P4503A is induced by 2-methyl-3-buten-2-ol, 3-methyl-1-pentyn-3-ol (meparfynol), and tert-amyl alcohol.* Xenobiotica, 26, 5, 487-493

Nikolic D., Van Breemen R. (2013). *Analytical methods for quantitation of prenylated flavonoids from hops.* Current Analytical Chemistry, 71 - 85

Ortega A., Škerget M., Knez Ž. (2016). *Separation of xanthohumol from hop extracts by supercritical fluid chromatography.* Chemical Engineering Research and Design, 109, 335-345

Prencipe F., Brighenti V., Rodolfi M., Mongelli A., dall' Asta C., Ganino T., Bruni R., Pellati F. (2014). *Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in Humulus lupulus L.* Journal of Chromatography A, 1349, 50 - 59

Scheuba J., Wronski V., Rollinger J., Grienke U. (2017). *Fast and Green – CO_2 Based Extraction, Isolation, and Quantification of Phenolic Styrax Constituents.* Planta Med, 83, 1068-1075

Sticher O., Heilmann J., Zündorf I. (2015). *Hänsel / Sticher Pharmakognosie Phytopharmazie*. 10. Aufl., Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 682-685

Štulíková K., Karabín M., Nešpor J., Dostálek P. (2018). Therapeutic Perspectives of 8-Prenylnaringenin, a Potent Phytoestrogen from Hops. Molecules, 23, 660

Taniguchi Y., Matsukura Y., Taniguchi H., Koizumi H., Katayama M. (2015). *Development of preparative and analytical methods of the hop bitter acid oxide fraction and chemical properties of its components.* Bioscience, Biotechnology and Biochemistry, 79/10, 1684-1694

Teuscher E., Melzig M., Lindequist U. (2004). *Biogene Arzneimittel – ein Lehrbuch der Pharmazeutischen Biologie. 6. Aufl.,* Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 291-294

van Wyk B., Wink C., Wink M. (2004). *Handbuch der Arzneipflanzen*. 2. Aufl. Stuttgart: Wissenschaftliche Verlagsgesellschaft Stuttgart. 172

Wagner H, Bladt S., Zgainski E.M. (1983). *Drogenanalyse: dünnschichtchromatographische Analyse von Arzneidrogen.* Springer Verlag Berlin Heidelberg New York

Wang B., Liu X., Zhou W., Hong Y., Feng S. (2017). *Fast separation of flavonoids by supercritical fluid chromatography using a column packed with a sub-2 µm particle stationary phase.* Journal of Separation Science, 40, 1410-1420

Wichtl M. (2009). *Teedrogen und Phytopharmaka - Ein Handbuch für die Praxis auf wissenschaftlicher Grundlage*. 5. Aufl., Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 386-389

Yang W., Zhang Y., Pan H., Yao C., Hou J., Yoa S., Cai L., Feng R., Wu W., Guo D. (2016). *Supercritical fluid chromatography for separation and preparation of tautomeric 7-epimeric spiro oxindole alkaloids from Uncaria macrophylla.* Journal of Pharmaceutical and Biomedical Analysis, 134, 352-360

Zhao Y., McCauley J., Pang X., Kang L., Yu H., Zhang J., Xiong C., Chen R., Ma B. (2013). *Analytical and semipreparative separation of 25 (R/S)-spirostanol saponin diastereomers using supercritical fluid chromatography*. Journal of Separation Science, 36, 3270-3276

8.2 WEBSITES

http://www.shop-apotheke.at   (accessed October 2017)

http://www.apotheke.at   (accessed October 2017)

http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_0000
43.jsp&mid=WC0b01ac05800240cb   (accessed April 2018)

https://aspregister.basg.gv.at   (accessed May 2018)

http://www.phytolab.com   (accessed May 2018)

https://pubchem.ncbi.nlm.nih.gov   (accessed May 2018)

http://www.theplantlist.org   (accessed May 2018)

http://www.waters.com   (accessed May 2018)

http://www.alcura.de   (accessed May 2018)

https://onlinelibrary.wiley.com/doi/pdf/10.1002/mnfr.200500053   (accessed June 2018)

https://www.dkfz.de   (accessed June 2018)

https://www.pharmawiki.ch/wiki/index.php?wiki=Hopfen   (accessed June 2018)

http://www.tipdisease.com/2015/09/hops-humulus-lupulus-overview-health.html
                                                           (accessed June 2018)