Synthesis and Purification of Xanthohumol C from Xanthohumol Hop Extract

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Biological Engineering

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Abstract

The present work aims to investigate the use of liquid-liquid chromatography (LLC) in the separation of the major hop (*Humulus lupulus*) prenylfavonoid xanthohumol with the highest possible purity, and also to develop and evaluate a strategy for the production of hops minor chalcone xanthohumol C followed by purification through LLC. Unlike xanthohumol and other major hop compounds, the availability of xanthohumol C through isolation is limited. Therefore, three alternative methods were assessed for the production or concentration of xanthohumol C, chemical and microbial methods and centrifugal partition chromatography, respectively.

Countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC) were used for the purification of xanthohumol from a crude xanthohumol pre-concentrated extract from hop cones. A purification method was initially developed at the CCC apparatus and then linearly scaled up to the CPC apparatus. A solvent system consisting of n-hexane/ethyl acetate/methanol/water at a volume ratio of 6/4/6/4 (HEMWat-3), was found to be effective in the isolation of xanthohumol. The purity of xanthohumol was over 98% assayed by HPLC at 290 nm.

Xanthohumol C chemical synthesis, pre-concentration and purification were accomplished. The minor hop chalcone was obtained through an oxidative cyclization of xanthohumol by treatment with (diacetoxyiodo)benzene in the presence of 2,2,6,6-tetramethylpiperidinyloxyl. Countercurrent chromatography was used for the purification of xanthohumol C after synthesis. The solvent system HEMWat-4 at a volume ratio of 7/3/6/4 and a 1 mL min^{-1} flow rate were employed. The pre-concentration was performed using a centrifugal partition chromatograph, with solvent system HEMWat 0 at a volume ratio of 5/5/5/5.

Keywords**:** xanthohumol (XN), xanthohumol C (XN C), countercurrent chromatography (CCC), centrifugal partition chromatography (CPC), back-extrusion, breakthrough curve.

Resumo

O presente trabalho tem como objetivo investigar o uso de cromatografia líquido-líquido (LLC) na separação de xanthohumol, o mais abundante prenilflavonoide do lúpulo (*Humulus lupulus* L.), com a maior pureza possível, e ainda desenvolver e avaliar uma estratégia para a produção de xanthohumol C, uma calcona minoritária do lúpulo seguida de purificação por LLC. Contrariamente ao xanthohumol e outros compostos principais do lúpulo, a disponibilidade de xantohumol C através do isolamento é limitada. Portanto, três métodos alternativos foram avaliados quanto à produção ou concentração do mesmo: métodos químicos e microbianos e cromatografia centrifuga de partição, respetivamente.

Cromatografia contracorrente (CCC) e cromatografia centrífuga de partição (CPC) foram utilizadas para purificar xanthohumol a partir de um extrato bruto pré-concentrado de xanthohumol de cones de lúpulo. Inicialmente desenvolveu-se um método de purificação em CCC seguido de *scale up* linear para CPC. Verificou-se que o sistema constituído por n-hexano/acetato de etilo/metanol/água numa proporção volumétrica 6/4/6/4 (HEMWat -3) era eficaz no isolamento de xanthohumol. A pureza deste foi superior a 98%, quando analisado em HPLC a 290 nm.

A síntese química de xanthohumol C, a pré-concentração e a purificação foram realizadas. Este foi obtido através de uma ciclização oxidativa de xanthohumol por tratamento com (diacetoxiiodo)benzeno e 2,2,6,6-tetrametilpiperidiniloxilo. A cromatografia contracorrente foi utilizada na purificação de xanthoumol C após síntese. Utilizou-se o sistema de solventes HEMWat -4 em proporção volumétrica 7/3/6/4 com caudal 1mL min^{-1}. A pré-concentração foi realizada utilizando uma coluna de cromatografia centrífuga de partição, com o sistema HEMWat 0 em proporção volumétrica 5/5/5/5.

Palavras-chave: xanthohumol (XN), xanthohumol C (XN C), cromatografia contracorrente (CCC), cromatografia centrífuga de partição (CPC), back-extrusion, curva de breakthrough.

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List of abbreviations

Abbreviation	**Meaning**
6-PN	6-prenylnaringenin
8-PN	8-prenylnaringenin
ACN	acetonitrile
ASC	ascending
CC	column chromatography
CCC	counter-current chromatography
CPC	centrifugal partition chromatography
DDQ	2,3-dichloro-5,6-dicyano-p-benzoquinone
DMS	dimethyl sulphate
DMX	desmethylxanthohumol
DSC	descending
EDDA	ethylenediamine diacetate
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
GC	gas chromatography
HPLC	high-performance liquid chromatography
HSCCC	high speed countercurrent chromatography
IX	isoxanthohumol
LAB	lactic acid bacteria
LC-MS	liquid chromatography mass spectrometry
LC-MS/MS	liquid chromatography mass spectrometry mass spectromentry
LLC	liquid-liquid chromatography
LP	lower phase
MOM	methoxymethyl
n-Hex	n-hexane
SP	stationary phase
TBAI	tetrabutylammonium iodide
THF	tetrahydrofuran
TMW	*Technische Mikrobiologie Weihenstephan*
TUM	Technical University Munich
UP	upper phase
UV-Vis	ultraviolet-visible spectroscopy
XF	Xantho-Flav
XN	xanthohumol
XN C	xanthohumol C

1. Aims and scopes

Hop (*Humulus lupulus* Linnaeus) is a dioceious, perennial plant belonging to the Cannabaceae family of the Urticales order [1], which is widely used in the brewing industry. The hydrophobic compounds from hops or hop cones (female inflorescences of hop) are responsible for the bitterness (α- and β-acids) and the characteristic hoppy aroma (essential oils) of beer. Additionally, hops contains many biologically active phenolic compounds and other constituents, that have been attracting much attention. Xanthohumol is the most abundant prenylated flavonoid in hops, with a content of 0.1-1% (dry weight) [2]. Great importance has been placed on this compound due to its cancer chemopreventive properties and strong proliferative activity against certain cancer cell lines [3]. For example, xanthohumol is capable of inhibiting procarcinogens activation, inducing enzymes involved in the destruction of carcinogens, or even preventing tumor growth, both in early and advanced stages of proliferation. Besides cancer-related activities, xanthohumol is also being studied for its anti-inflammatory and antiangiogenic activity, antioxidant activity and anti-infective properties, for example, against Gram-positive bacteria such as *Staphylococcus aureus* [4]. The main method for obtaining xanthohumol is through extraction, isolation, and purification from the female inflorescences [2]. Chen et all., [5] and Chadwick et all., [6] have already reported on the isolation of prenylated phenolics from hops, including xanthohumol, using liquid-liquid chromatography (LLC).

Liquid-liquid chromatography is a separation technique working with a liquid stationary phase and an immiscible liquid mobile phase, mainly oriented towards the purification of compounds [7,8]. This technique is essentially a preparative technique. An essential advantage in LLC is the absence of a solid column and consequently irreversible column adsorption, which means that everything that is injected in a LLC column can be recovered. Furthermore, the two liquid phase interaction process is much gentler for delicate molecules than the solid-liquid interactions involved with a solid stationary phase. In addition, the use of a biphasic liquid system provides a very wide range of possible liquid stationary phases and of polarities. In LLC there are two general column designs: the hydrodynamic columns or countercurrent chromatography (CCC) columns, and the hydrostatic columns or centrifugal partition chromatography columns. In both cases the LLC column volume can be adapted to the desired productivity. Smaller LLC columns allow to quickly find the right biphasic liquid system adapted to the purification, since scaling up to a larger volume column can then be done relatively straightforward as it is mostly linear [8].

Xanthohumol C is a minor chalcone present in the hop cones. This compound has been receiving much attention lately due to its potential as a potent antifungal, and exhibiting antiproliferative, cancer chemopreventive, antimutagenic and antioxidative activities. In addition, it also promotes the differentiation of neuronal precursor cells [9]. However, compared to xanthohumol and other major compounds from hops that are already well characterized in literature having many studies that demonstrate their biological activities, xanthohumol C is still in the process of investigation. Due to its limited availability through isolation methods, alternative approaches must be established in order to obtain quantities of xanthohumol C sufficient for further pharmacological analysis and studies.

The aim of the present work is thus the production and purification of xanthohumol C, and the development of a method for the purification of xanthohumol from a pre-concentrated xanthohumol hop extract through liquid-liquid chromatography. For the production of xanthohumol C, three different methods were thought of: microbial methods (biotransformations), chemical synthesis, and pre-concentration. The hop extract used here was supplied by Hopsteiner, with a xanthohumol concentration range of 65-85%. Initially, a method for the purification of xanthohumol was established at the counter-current chromatograph, and then scaled-up for use at the centrifugal partition chromatograph. The purified xanthohumol was used as the substrate for the production of xanthohumol C in the chemical synthesis and in the biotransformations. As for the pre-concentration, the substrate used was the hop extract itself, that contains a xanthohumol C concentration range between 0.5-2.2%, assayed by HPLC at 290 nm. Finally, the purification of xanthohumol C was established at the countercurrent chromatograph.

This thesis was almost exclusively done at the Biothermodynamic chair at the Technical University of Munich (TUM), under the supervision of PhD candidate Simon Röhrer and Prof. Dr. Mirjana Minceva. All biotransformation experiments were performed at the Technical Microbiology chair (TMW), under the supervision of Dr. Jürgen Behr.

2. Introduction

This chapter consists mainly of two sections. The first section (Section 2.1) introduces the main components from hops and their biological activity, with special emphasis on xanthohumol and xanthohumol C, and the analytical methods for the quantitation of those compounds. The following section (Section 2.2) describes some of the basic concepts behind the separation principle of liquid-liquid chromatography, the structure and operation mode of the involved instruments, and the requirements for the solvent system selection.

2.1. Hops prenylated flavonoids

Hops or hop cones are the female inflorescences (see Figure 2.1) of the hop plant *Humulus lupulus* Linnaeus (Cannabaceae), being well-known worldwide as a raw material in the brewing industry. Hops are used to preserve beer and to add its characteristic bitterness and flavor [3,4]. But hop also has a long history as a medicinal remedy, with the first accountable uses of hop dating back to the 19th century, where hop preparations were recommended for the treatment of sleeping disorders, as a mild sedative, and for the activation of gastric functions. However, nowadays the hop plant is almost exclusively used by the brewing industry, with a global production of 101,000 mt of hop in 2011 [10].

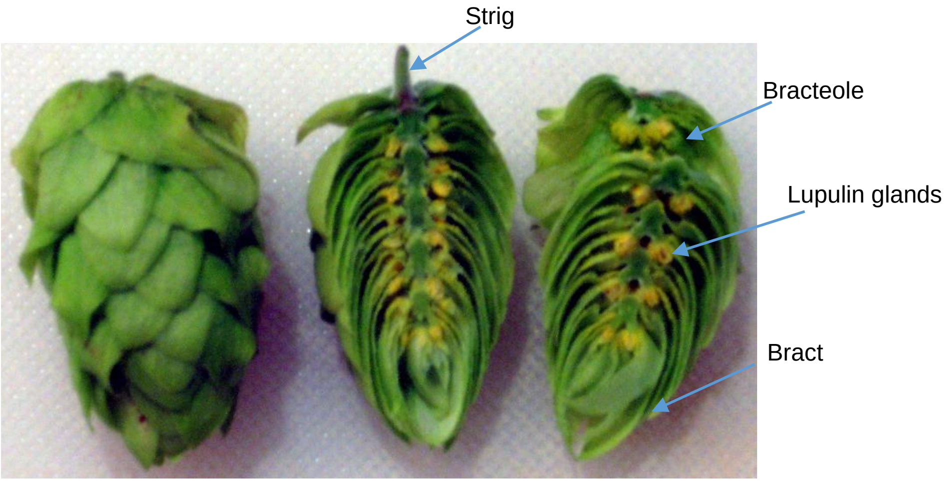


Figure 2.1. Vertical cut on hop cones (hops) showing the lupulin glands, where bitter acids and prenylflavonoids are present. Image source: http://www.hoptomology.com/2011/08/22/picking-and-storing-your-homegrown-hops/.

The yellow lupulin glands seen in Figure 2.1 contain the bitter substances from hop, the essential oils, the polyphenols, proteins, etc. (see Table 2.1). The humulones (α-acids) and the lupulones (β-acids) represent the commercial value of hops as the bittering substances in the beer industry [3]. Recently hops has been under investigation due to its estrogenic properties and its potential cancer chemopreventive activities and some active compounds from hop have been receiving much attention. Research on this topic has grown so much that even Phase I and Phase II clinical trials have already been completed with hop extracts of purified compounds [11].

Table 2.1. Content of the dried lupulin glands from *Humulus lupulus*. Adapted from [12].

Compound	**Content / %**
α-acids (humulone)	2 – 17
β-acids (lupulone)	2 – 10
Essential oils (terpene)	0.5 – 3
Polyphenols (naringenine, chalcone)	3 – 6
Monosaccharides	2
Amino acids	0.1
Proteins	15
Lipids	1 – 5
Pectin	2
Ashes	10
Water	8 – 12
Cellulose, lignin	40 – 50

Among the possible hop active compounds, prenylated flavonoids have been receiving the most attention. Chemically, they can be divided into two groups: prenylated chalcones and prenylated flavanones [11]. Xanthohumol (XN) stands out as the most abundant prenylated chalcone in hop cones. Its content can range from 0.1 to 1% of the cone dry mass. Xanthohumol has been primarily studied for its cancer chemopreventive properties and strong proliferative activity against breast, colon and ovarian cell lines [3,4,13]. In addition, it exhibits anti-inflammatory and antiangiogenic activity, antioxidant activity and anti-infective properties, for example, against Gram-positive bacteria such as *Staphylococcus aureus* [4]. The structure of XN is shown in Figure 2.2.

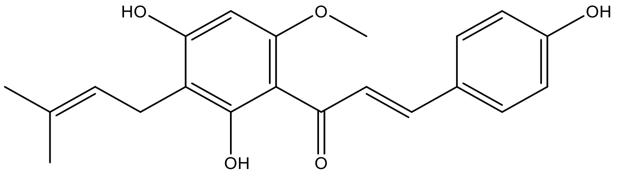
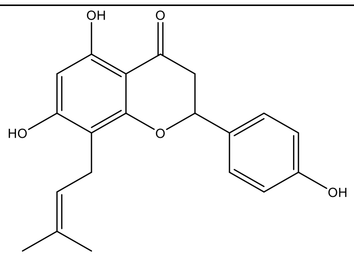
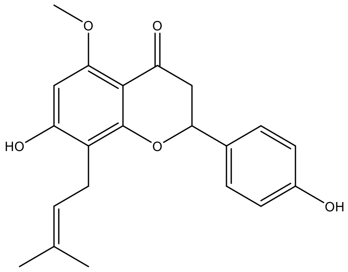
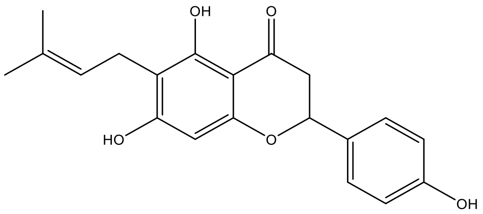
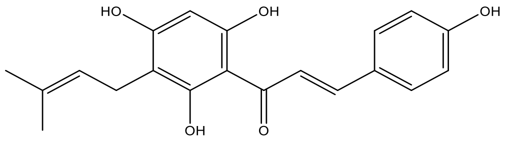


Figure 2.2. Structure of the prenylated chalcone from hops xanthohumol (XN).

Besides xanthohumol there are other important prenylflavonoids present in hops. 8-Prenylnaringenin (8-PN) is the most potent phytoestrogen that has been isolated to date [3,4]. In postmenopausal women 8-prenylnaringenin has decreased the incidence of the hot flushes and of other discomforts that are associated with estrogen deficiency [4]. Isoxanthohumol (IX) is actually the main prenylflavonoid present in beer, as it is the result of the isomerization of xanthohumol as a consequence of the thermal treatment and increase in pH (xanthohumol isomerization happens around pH 5.4 [14]) that occurs during beer production [15]. Isoxanthohumol has a small estrogenic potential mostly due to its conversion to 8-prenylnarigenin. 6-Prenylnaringenin (6-PN) has shown a very weak estrogenic activity, as well as isoxanthohumol, but it has been identified as one of the most potent antifungal agents against *Trychophyton* spp. and *Staphylococcus aureus*, besides xanthohumol. Desmethylxanthohumol (DMX), a proestrogen, is considered as the precursor of the most flavonoids present in hops. Through a chemical isomerization, it gives rise to the 8-prenylnaringenin, along with the racemic 6-prenylnaringenin. The structures of these hops prenylated flavonoids are listed in Table 2.2.

Table 2.2. Structures of prenylated chalcones and flavanones from hops.

Structure	Name
	8-prenylnaringenin
	Isoxanthohumol
	6-Prenylnaringenin
	Desmethylxanthohumol

2.1.1. Xanthohumol C (XN C)

Xanthohumol C (XN C) is a naturally occurring pyranochalcone, an abundant subclass of flavonoids widely distributed in nature [16]. XN C was first isolated from *Humulus lupulus*, where it is a minor compound, and identified in the 90s by Stevens et al. [17] under the name dehydrocycloxanthohumol (DX), later Stevens et al. [18], proposed the name xanthohumol C following the same rational as Tabata et al. (1997), for xanthohumol B. XN C has been shown to have a wide variety of biological activities such as potent antifungal, antiproliferative, cancer chemopreventive, antimutagenic and antioxidative activities [16]. Tests with breast (MCF-7), colon (HT-29) and ovarian (A-2780) cancer cells showed that XN C has quite a significant inhibition capacity for the growth of breast cancer cells, comparable to the one of XN [13]. This cancer cells inhibition is lower in the case of colon and ovarian cancer cells, but similar results are observed with both XN and IX. In a recent study XN C was identified as the most active compound in the differentiation of neuronal precursor cells, from a group of hops-derived prenylflavonoids [9]. In Figure 2.3 it is shown the structure of xanthohumol C.

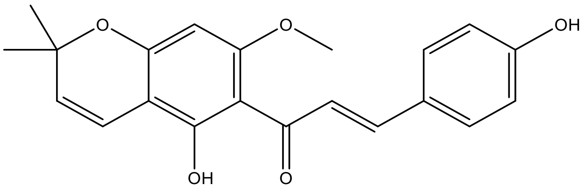


Figure 2.3. Structure of the natural occurring pyranochalcone from hops xanthohumol C (XN C).

While xanthohumol and other major compounds from hops are already well characterized in literature, pharmacological data concerning the minor compounds such as xanthohumol C are scarce due to its limited availability via isolation [19]. Alternative approaches are needed to provide quantities of xanthohumol C available for pharmacological testing. In this work, three different methods were evaluated for the production or concentration of xanthohumol C, chemical (synthesis) and microbial (biotransformations) methods and centrifugal partition chromatography, respectively.

Chemical synthesis

In literature some examples of reactions for the synthesis of xanthohumol C have already been reported. Before explaining the method applied in this work, a general overview of those reactions will be given. In general, these examples involve either a high number of steps, being very time consuming, low global reaction yields, or the use of carcinogenic compounds such as benzene [20].

The total synthesis, i.e. the complete chemical synthesis of a complex molecule from simpler precursors [21], of xanthohumol C, in three steps, is already described in literature [16]. Xanthohumol C (6) was prepared by base-catalyzed aldol reaction of the benzopyran isoevodionol (3) with the methoxymethyl (MOM) ether protected benzaldehyde (4), as shown in Figure 2.4. Isoevodionol (3) is generated from 2,4-dihydroxy-6-methoxyacetophenone (1) with 3-methyl-2-butenal (2) in the presence of 10 mol% of ethylenediamine diacetate (EDDA) in refluxing xylene for 10 hours. Treatment of (3) with (4) in an ethanolic potassium hydroxide solution for 48 hours resulted in 1-(5-hydroxy-7-methoxy-2,2-dimethyl-2H-chromen-6-yl)-3-[4-(methoxymethoxy)phenyl]-2-propen1-on (5). Finnaly, (5) was cleavage of the MOM ether with 3 N HCl in methanol, giving xanthohumol C (6) in 86% yield. The global reaction yield was 65.4%.

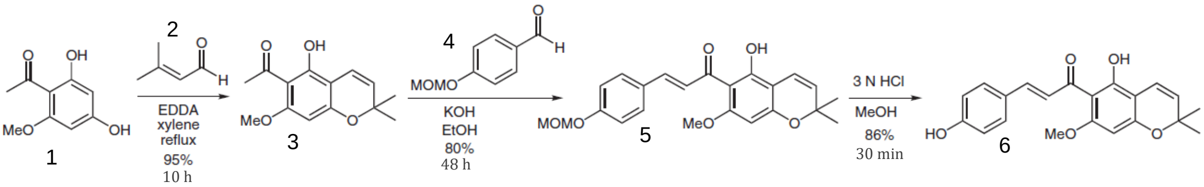


Figure 2.4. Total synthesis of xanthohumol C. 1 = 2,4-dihydroxy-6-methoxyacetophenone, 2 = 3-methyl-2-butenal, 3 = isoevodionol, 4 = MOM ether protected benzaldehyde, 5 = 1-(5-hydroxy-7-methoxy-2,2-dimethyl-2H-chromen-6-yl)-3-[4-(methoxymethoxy)phenyl]-2-propen1-on and 6 = xanthohumol C. EDDA = ethylenediamine diacetate and MOM = methoxymethyl. Adapted from [16].

A different strategy in the synthesis of xanthohumol C was developed by Vogel and Heilmann [19]. In this case it is not a total synthesis as the some of the involved precursors are not commercially available. Here a mixture of 2,4,6-trihydroxyacetophenone, K_2CO_3, and MOM bromide was stirred and refluxed in acetone for 3 hours, affording 6-hydroxy-2,4-dimethoxymethyl-acetophenone (1) in 68% yield. Intermediate (1) was mixed with dimethyl sulfate (DMS), the phase transfer catalyst tetrabutylammonium iodide (TBAI), and NaOH, and stirred in CH_2Cl_2-H_2O at room temperature to give a second protected intermediate (2) in 89% yield. This protected intermediate (2) was then deprotected by dissolution in 5:1 MeOH – 3 N HCl, followed by 15 min reflux, poured into ice – water and extracted three times with dichloromethane and water (CH_2Cl_2-H_2O). The result of the deprotection with a 69% yield, compound 2,4-dihydroxy-6-methoxy-3-prenylacetophenone (3) was dissolved together with 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) in dried benzene and dried dioxane. This reaction mixture was heated under reflux for 3 hours, cooled to room temperature, and filtered, resulting in the protected intermediate (4) with 90% yield. In the last step of Figure 2.5, intermediate (4) was coupled with 4-hydroxybenzaldehyde and deprotected to yield 87% of xanthohumol C, after column chromatography (CC) with petroleum ether-EtOAc, 1:1. The global reaction yield was 32.7%.

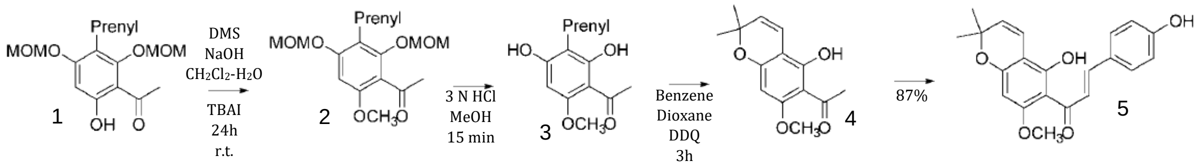


Figure 2.5. Key steps of the synthesis of xanthohumol C. 1 = 6-hydroxy-2,4-dimethoxymethyl-3-prenylacetophenone, 2 = protected intermediate, 3 = 2,4-dihydroxy-6-methoxy-3-prenylacetophenone, 4 = intermediate and 5 = xanthohumol C. DMS = dimethyl sulphate, TBAI = tetrabutylammonium iodide and DDQ = 2,3-dichloro-5,6-dicyano-p-benzoquinone. Adapted from [19].

A one step procedure starting from xanthohumol is described in [22]. In this reaction to a solution of xanthohumol and DDQ in benzene (dry) containing 2 drops of dioxane, was radiated in microwaves at 120°C for 3 min. A yellow solid was obtained after purification with a silica gel CC (ethyl acetate/hexane: 2/3), with a 55% yield. The scheme for this synthesis is shown in Figure 2.6.

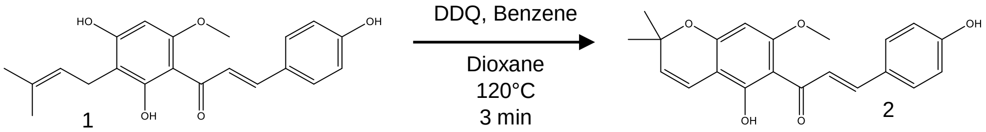


Figure 2.6. Synthesis of xanthohumol C from xanthohumol. 1 = xanthohumol and 2 = xanthohumol C. DDQ = 2,3-dichloro-5,6-dicyano-p-benzoquinone. Adapted from [22].

An alternative approach to the synthesis of xanthohumol C from xanthohumol is here reported. The reaction procedure was based on the synthesis of hyperguinone B [23]. In the published reaction a biosynthetic precursor is treated with (diacetoxyiodo)benzene ($PhI(OAc)_2$) in the presence of 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO), giving hyperguinone B with a yield of 73%, as shown in Figure 2.7.

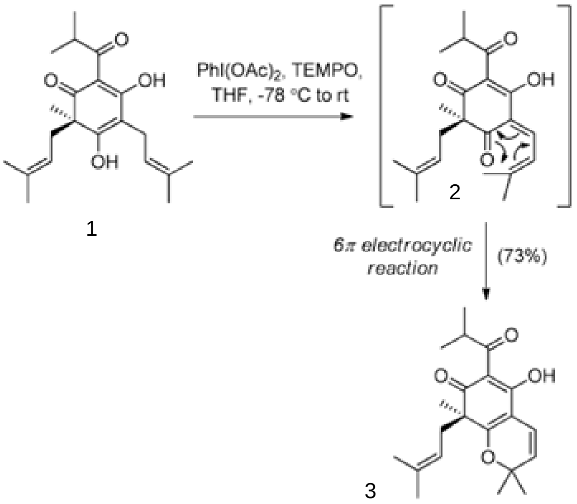


Figure 2.7 Synthesis of hyperguinone B. 1 = monocyclic biosynthetic precursor, 2 = o-quinone methide intermediate ans 3 = hyperguinone B. THF = tetrahydrofuran. Adapted from [23].

The authors presumed that this reaction proceeds via a selective hydride abstract by the *in situ* generated TEMPO cation. This generates an o-quinone methide intermediate that undergoes a 6π-electrocyclization to give the 2,2-dimethyl-2H-pyran ring of hyperguinone B. The ring closing that occurs in this reaction could explain the synthesis of xanthohumol C. Similar to the precursor, xanthohumol has two phenolic oxygen atoms and one prenyl group (see Figure 2.8) that could potentially oxidize with any of the two phenolic oxygens. This hypothesis supposes that not only xanthohumol C can be formed in this conditions, but also a xanthohumol C isomer. The scheme for the synthesis of xanthohumol C and its isomer is shown in Figure 2.8.

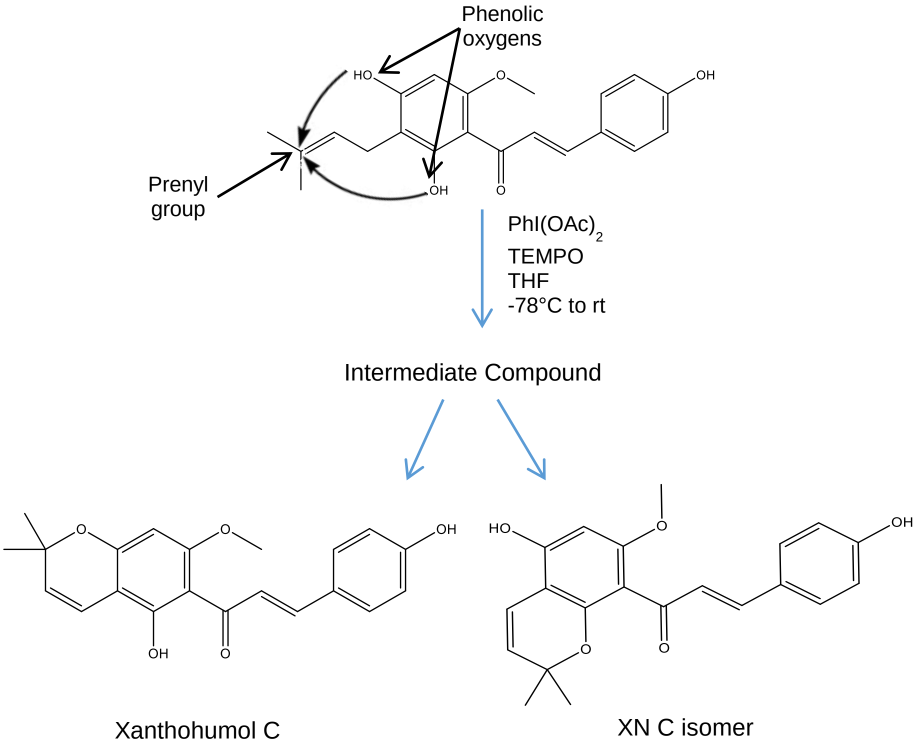


Figure 2.8. Possible synthesis of xanthohumol C and its isomer based on the synthetic route of Hyperguinone B [23].

About the reactants involved, TEMPO or 2,2,6,6-tetramethylpiperidin-1-oxyl is considered to be a very mild oxidation agent [24]. It has already been used in different green and environmentally benign systems. As a stable radical TEMPO has been used in the oxidation of primary alcohols to aldehydes and carboxylic acids [25]. $PhI(OAc)_2$ or (diacetoxyiodo)benzene is a potent, often chemoselective, oxidant widely used in modern organic synthesis [26]. It has been used as a less-toxic alternative compounds such as benzene and lead(IV) derivatives [20]. The structures of both reactants are given in Figure 2.9.

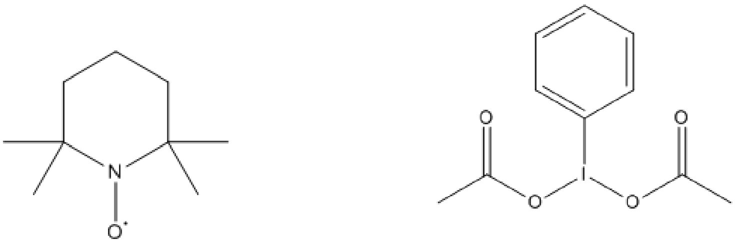


Figure 2.9. Left: molecular structure of 2,2,6,6-Tetramethylpiperidinyloxyl (TEMPO). Right: molecular structure of (Diacetoxyiodo)benzene ($PhI(OAc)_2$).

Biotransformations

When compared to the chemical methods biotransformations offer a number of advantages: they introduce soft modifications that are often stereo- and regio-selective; have mild conditions, and in the majority of cases do not require any protection of other functional groups [27]. Biotransformation have been used with the aim of extending the diversity of hop flavonoids derivatives, with the purpose of finding potential novel drugs.

Both enzymes and microorganisms can be used for biotransformations. But even though enzymes can be obtained from different sources, microorganisms are the most useful due to their short life cycle and simplicity of manipulation and maintenance. Biostransformation studies are also used to mimic the mammalian metabolism through microbial models [15,28,29]. The purpose of these studies is the generation of mammalian metabolites in an easy way, for testing their biological activity potential, and to understand and predict the mechanisms of mammalian systems.

Microbial transformations have already been achieved with hop compounds such as xanthohumol [28], 8-prenylnarigenin [29] and isoxanthohumol [15]. Transformation of IX and 8-PN was performed using the fungi *Beauveria bassiana* AM278, *Absidia glauca* AM177, and *Fusarium equiseti* AM15, and *Absidia coerulea* AM93 and *Beauveria bassiana* AM278, respectively, following screening tests with a large variety of fungi cultures. Xanthohumol transformation was accomplished with the common food spoilage yeast *Pichia membranifaciens*, as it showed the higher transformation efficiency from a total of 20 microorganisms tested. In all experiments, after biotransformation mass spectrometry and ^{1}H and ^{13}C NMR analysis were performed for identification of the corresponding hop compound derivative metabolite produced.

In the present work, the aim was to test a total of 12 bacteria and 12 yeast strains in their ability to metabolize xanthohumol and produce xanthohumol C. The selection of microorganisms was based on two facts, first that all these microorganisms are well characterized by the TMW chair, and second that even though XN is known its antimicrobial activity [4,27] the microorganisms tested are related with beer brewing and ethanol fermentation in wine, thus possibly being in the presence of compounds similar to XN.

2.1.2. Analytical methods for quantitation of hops prenalyted flavonoids

The standard analytical methods for the identification and qualitative analysis of the prenylated flavonoids from hop crude extracts are high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and in LC-tandem mass spectrometry (LC-MS/MS). All three methods are used in combination with UV-Vis absorption for the hop compounds detection. In biological samples mainly due to the complexity of their matrices, mass spectrometry has become the dominant technique for the quantitative analysis of natural products [11]. Because the molecular masses for the pairs XN/IX and DMX/6-,8-PN are identical, a good chromatographic separation is necessary (see Table 2.3). The mass transition values in Table 2.3 were obtained through tandem mass spectrometry analysis, using electrospray ionization (ESI) in negative ion mode $[M\text{-}H]^-$. Q1 is the first quadrupole in MS/MS, that mass-selects the analyte ion of interest (the precursor ion), and Q3 is the third quadrupole mass analyzer, which provides structural information of the molecular ions [30].

Table 2.3. Mass transitions for some hops prenylflavonoids. Adapted from [31].

Prenylflavonoid	Mass transition Q1→ Q3
Xanthohumol	352.9→118.8
Isoxanthohumol	352.9→118.8
Desmethylxanthohumol	339.1→218.9
8-Prenylnaringenin	339.1→218.9
6-Prenylnaringenin	339.1→218.9
Xanthohumol C	351.0→118.9

In previous works, good separations in HPLC have been accomplished using the common C_8 or C_{18} columns, with reversed phase chromatography and gradient elution due to the different polarities of hop flavonoids [11]. In addition, most methods have been carried out in acidic conditions, using either formic or acetic acid, since a lower pH suppresses ionization of phenolic groups which provides a better chromatographic peak shape. For mass spectrometry analysis of the hops prenylated flavonoids, electrospray ionization in negative ion mode seems to be the most popular choice, as seen in Table 2.4.

Table 2.4. Summary of LC-MS and LC-MS/MS methods for analysis of hops prenylated flavonoids. AA = acetic acid, APCI = atmospheric pressure chemical ionization, ESI = electrospray ionization, FA = formic acid, Q = single quadrupole, QQQ = triple quadrupole, SIM = selected ion monitoring, SRM = selected reaction monitoring. Adapted from [11].

Matrix	Analytes	Column	Mobile phase	Ionization	MS
Hop products, beer	8-PN, 6-PN, IX, XN, DMX	C_{18}, 4 x 250 mm, 5 µm	1% FA-MeCN	(+) APCI	QQQ, SRM
Hop products, beer	8-PN	4.6 x 250 mm, 5 µm	0.5% FA-MeOH	(+) ESI	Q, SIM
Dietary supplements	8-PN, 6-PN, IX, XN	2.1 x 150 mm, 5 µm	10mM ammonium acetate-MeCN	(−) APCI	Ion trap
Hop extracts, beer	8-PN, IX, XN, bitter acids	C_8, 4 x 250 mm, 5 µm	Water-MeCN, 0.3% FA	(+) APCI	Ion trap
Human urine, serum	8-PN, IX, XN	C_{18}, 3 x 250 mm, 3.5 µm	Water-MeOH/MeCN (80:20), 0.025% FA	(+) APCI	Q, SIM
Beer	8-PN	C_{18}, 4.6 x 250 mm, 5 µm	Water, MeOH/MeCN	(−) ESI	Q, SIM
Beer	IX, XN, bitter acids	C_{18}, 4.6 x 250 mm, 5 µm	Water-MeCN, 0.5% FA	(−) ESI	Q-linear ion trap, SRM
Rat plasma, tissues	8-PN, IX, metabolites	C_{18}, 2.1 x 100 mm, 3.5 µm	0.1%FA-MeOH	(−) ESI	QQQ, SRM
Human urine, serum, breast tissue	8-PN, IX, XN, metabolites	C_{18}, 2.1 x 100 mm, 5 µm	0.05% AA-MeOH	(−) ESI	QQQ; SRM
Human serum, urine	8-PN, 6-PN, XN, IX	C_{18}, 2.1 x 150 mm, 4 µm	Water-MeCN/MeOH (95:5), 0.01% FA	(−) ESI	Q-linear ion trap, SRM
Rat plasma	8-PN, 6-PN, XN, IX	C_{18}, 2 x 50 mm, 5 µm	Water-MeCN, 0.1% FA	(−) ESI	Q-linear ion trap
Human serum, urine	8-PN, 6-PN, XN, IX	C_{18}, 2 x 50 mm, 1.6 µm	0.1% FA-MeCN	(−) ESI	QQQ, SRM

2.2. Liquid-liquid chromatography (LLC)

Liquid-liquid chromatography (LLC) has become very popular for the isolation and purification of natural products due to its gentle operation conditions, without the risk of decomposition or irreversible adsorption of the often unstable compounds on solid supports as in the conventional chromatographic columns (High Performance Liquid Chromatography (HPLC)) [5,32]. Furthermore, the use of biphasic liquid systems provides a very wide range of polarities, and the separation mechanism is exclusively related to liquid-liquid partitioning [7].

LLC is a preparative separation technique that employs as mobile and stationary phase the two mutually saturated immiscible phases in equilibrium of a multicomponent biphasic liquid system [33]. An example of a ternary diagram of a biphasic liquid system composed of three solvents is given in Figure 2.10. The composition of each liquid phase is determined by following the tie lines in the two phase region until intersection with the binodal curve. In LLC, one of the phases, the stationary phase is kept in a special designed column with the help of a centrifugal field produced by rotation of the column, while the other phase is pumped through the column as the mobile phase [34].

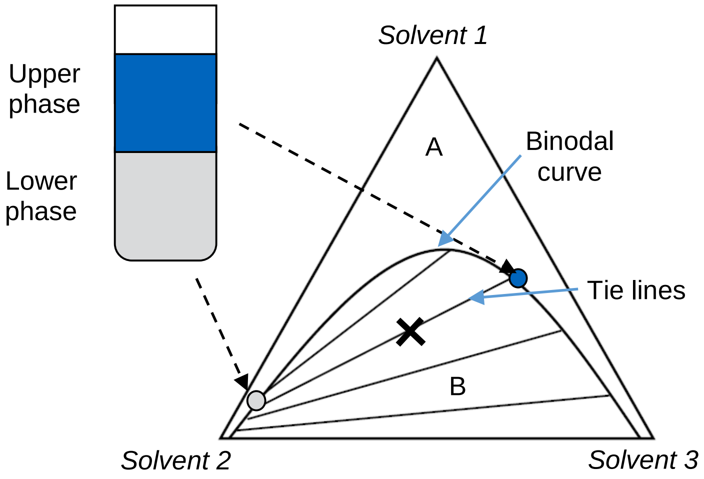


Figure 2.10. Ternary diagram of a biphasic liquid system composed of three solvents. A = single phase region, B = two phase region and X = initial volumetric ratio of each solvent in the mixture.

The set up used to perform liquid-liquid chromatographic separations is identical to the set up used in liquid-solid chromatography. The only difference is that instead of the classical column a specially designed column mounted on the axis of a centrifuge is used. Once the biphasic system is prepared and equilibrated the phases are placed in separated reservoirs. At the beginning the column is filed with the phase intended to be used as stationary phase. The rotation is started and as soon as the set rotation speed is achieved the mobile phase is pumped in the column using the second pump. The mobile phase will start replacing part of the stationary phase. After hydrodynamic equilibrium is achieved no more stationary phase comes out from the column and the feed sample solution can be injected. A scheme for the set-up used in LLC is presented in Figure 2.11. The LLC technique can be run as a batch, a semi-continuous or a continuous process [33,34].

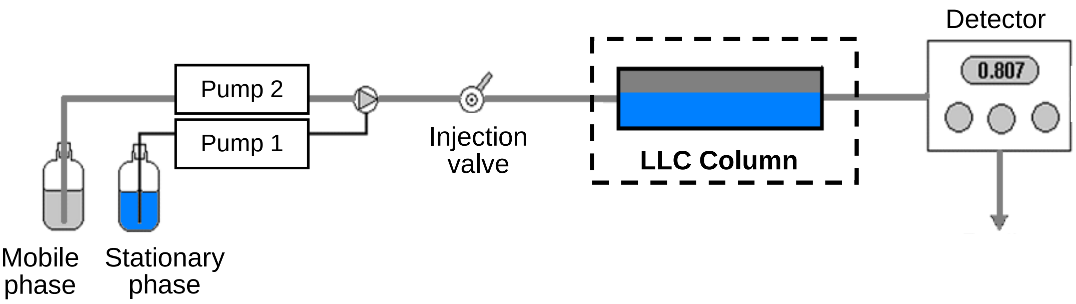


Figure 2.11. Schematic set-up for a liquid-liquid chromatographic separation, including the reservoirs containing mobile and stationary phase connected to the respective pump, the injection valve for the feed injection, the LLC column and the detector for analysis of the eluting products.

In LLC, the liquid-liquid solute partitioning between the two phases is the only exchange responsible for solute retention [7], and it is characterized by the partition coefficient, P_i for component i, where:

$$P_i = \frac{c_{SP,i}}{c_{MP,i}} \tag{2.1}$$

and $c_{SP,i}$ and $c_{MP,i}$ are the concentrations of the component i in the stationary phase and the mobile phase of the biphasic system, respectively. As the volumetric content, i.e. the column volume, V_C, of a LLC column is just the mobile phase volume, V_{MP} and the stationary phase volume, V_{SP}, it can be described by a simple equation:

$$V_C = V_{MP} + V_{SP} \tag{2.2}$$

The retention volume of a solute, $V_{R,i}$, is then calculated by the retention equation:

$$V_{R,i} = V_{MP} + P_i V_{SP} \tag{2.3}$$

By substitution of Equation 2.2 in the retention equation [33]:

$$V_{R,i} = V_C - V_{SP}(P_i - 1) \tag{2.4}$$

From Equation 2.4 it becomes clear that, for a constant P value and column volume, a change in the stationary phase volume will affect the retention volume. The relationship between the volume of the liquid stationary phase inside the LLC column, and the volume of the column itself is important for a good resolution between two components/peaks, that a special parameter was introduced in order to quantify it:

$$S_f = \frac{V_{SP}}{V_C} \tag{2.5}$$

The stationary phase retention depends on several factors, such as, column geometry (type and dimensions) and the hydrodynamic behavior of the liquid phases inside, the physical properties of the two liquid phases (differences of density, viscosity and interfacial tension between the phases), the rotational speed, or created centrifugal force, and last the mobile phase flow rate [34].

2.2.1. Design and function of a liquid-liquid chromatographic system

Over the years, two general types of chromatographic columns have been established in LLC, which will be explained in more detail in the following paragraphs: on the one hand, the hydrodynamic column, the counter-current chromatography (CCC) column, in which a tube is rolled over an axis. The structure and operating principle of a hydrodynamic column, as developed by Yoichiro Ito in the 70s, is shown in Figure 2.12. On the other hand, the hydrostatic column, the Centrifugal Partition Chromatography (CPC) column, which consists of several small chambers arranged on a metal plate, which are connected to one another via fine channels [7].

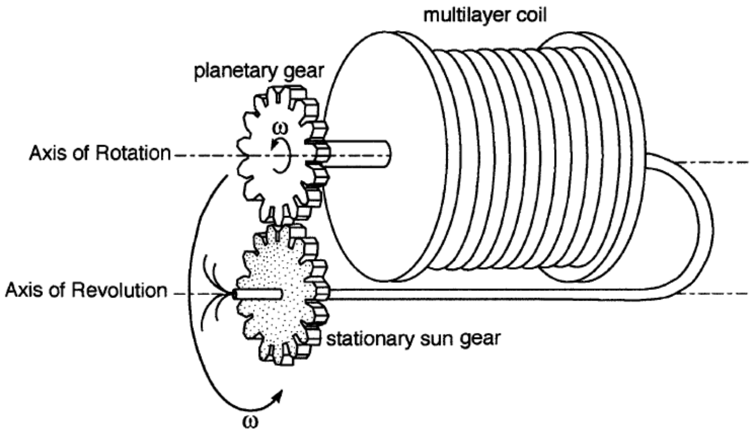


Figure 2.12. Design and function of a Type-J planetary motion of a multilayer coil separation column, developed by Yoichiro Ito, for the use in high-speed counter-current chromatography (HSCCC) [35].

The type of column depicted in Figure 2.12 consists of a column holder rotating about its own rotation axis and revolving around a second horizontal axis, the centrifugal axis, at the same angular velocity in the same direction. The two axis are connected by means of a planetary gear. The result is an inhomogeneous centrifugal field that periodically changes its intensity and direction during rotation along the tube. As a result, the mixing zone and the decantation zone also change periodically along the axis [7,35]. This periodic change is evidenced in the schematic coil of Figure 2.13 [8].

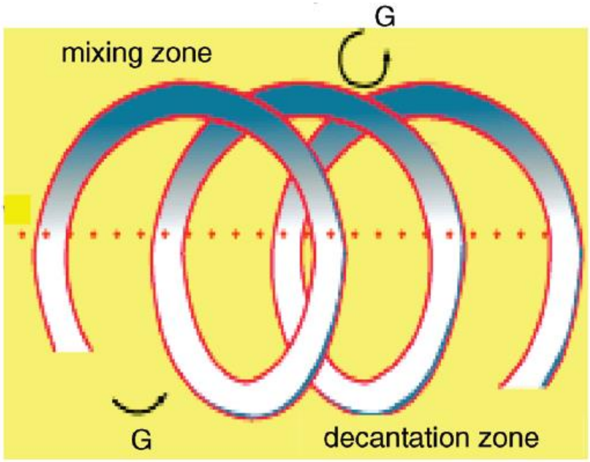


Figure 2.13. Schematic representation of a hydrodynamic CCC column coiled tube with the mixing and decantation zone [8].

This inhomogeneous centrifugal field leads to a mixing process and thus to a material exchange between the stationary and the mobile phase (mixing zone). On the other side, in the decantation zone, phase separation is taking place. The Archimedean force, in similarity to the principle of gravity, created by the thread of the coiled tubing pushes the lighter liquid phase towards one end of the coil called “head”. The mobile phase is pumped through the column, while the stationary phase is retained in the plant by the centrifugal field. In the case that the mobile phase is the lower denser liquid, it will have to enter in the coil through the head side, in a head-to-tail-direction, against the Archimedean force. This head-to-tail-direction is referred in this work as descending (DSC) mode. Otherwise, the mobile phase has to enter the CCC column through the “tail” in a tail-to-head direction [7,35]. Here, referred as ascending (ASC) mode. The wrong operation mode of the mobile phase will result in precluding of the separation, as the stationary phase will not be retained inside the CCC column. The two CCC operation modes are illustrated in Figure 2.14.

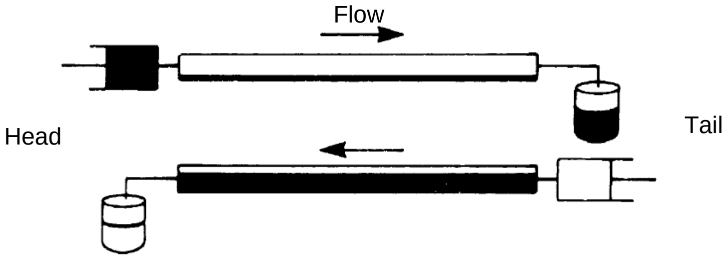


Figure 2.14 One-way elution mode of both the lighter and heavier phases through the coiled tubing column. The white phase is the head phase moving in the tail-to-head direction, and the black the phase is the tail phase moving in the head-to-tail direction. Adapted from [35].

Unlike the two-axis hydrodynamic columns, the CPC columns are uniaxial hydrostatic apparatuses and therefore are subjected to a constant centrifugal field. CPC columns have a modular structure. The main component of a hydrostatic column is an annular plate, the engraved disk, in which a certain number of milled chambers are connected by small channels, the ducts, (Figure 2.15. a)). The column consists of several such identical disks placed one on top of the other (Figure 2.16). An annular Teflon sheet is then placed between two adjacent disks (Figure 2.15. b)). Through the disk small openings, the last chamber of each disk is connected to the first chamber of the next. The amount of disks present will determine the volume of the entire column. The column is mounted on a rotor of a centrifuge. The mobile phase enters or leaves the column via mechanical seals. The rotary seal joints make it possible for leak-poof connections between the mobile phase inlet/outlet tubes and the column even at high rotational speeds.

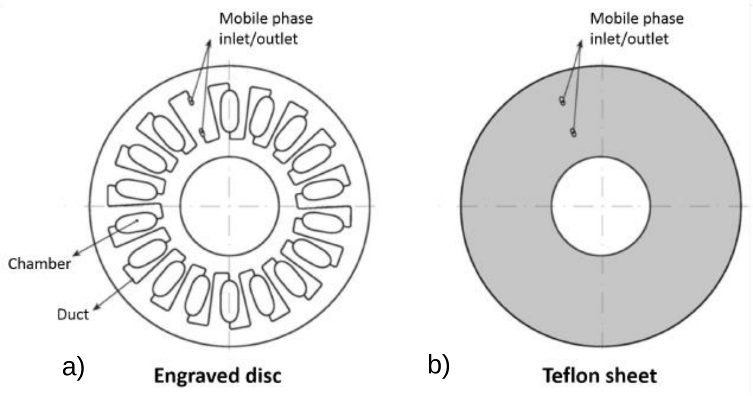


Figure 2.15. Schematic view of a CPC: a) engraved disk and b) Teflon sheet. Adapted from [33].

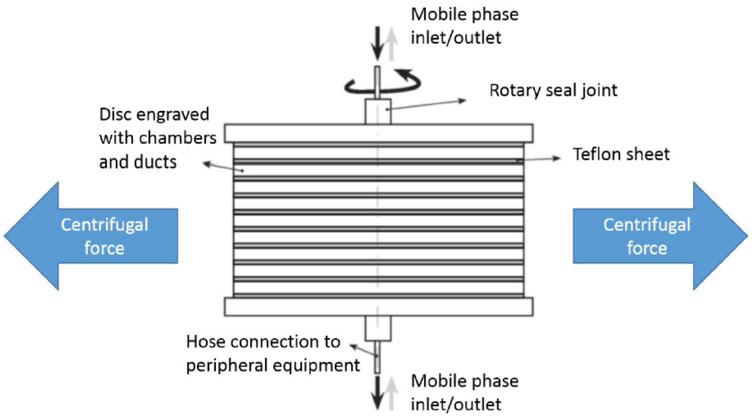


Figure 2.16. Schematic view of a CPC column design, including the engraved disks, the Teflon sheets, the rotary seal joint, the hose connection to the peripheral equipment and the mobile phase inlets and outlets [33].

The working principle of such a hydrostatic column is shown in Figure 2.17. In this case, the small chambers connected through the ducts, are under a constant centrifugal field [7]. During operation, the ducts are filled with only the mobile phase, the blue phase, and mass exchange will only take place inside the chambers, by passage of the mobile phase through the stationary phase, the white phase. The connecting ducts are a major disadvantage of this type of construction, as they do not contribute for the mass transfer, since they are only filled with mobile phase.

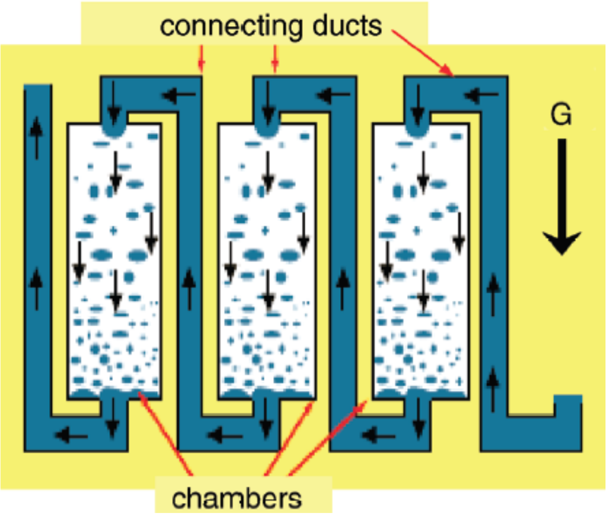


Figure 2.17. Hydrostatic CPC column design with interconnected chambers by the connecting ducts in an engraved disk. The blue phase is the mobile phase and the white phase is the stationary phase. The gravitational field, G, acts vertically downward. Adapted from [8].

A comparison of the properties for the two types of LLC column designs is summarized in Table 2.5. Both systems can be used in practical applications, but according to the desired separation or purification it is important to determine whether a hydrostatic or a hydrodynamic system is to be preferred [33]. For example, hydrostatic machines have a better stationary phase retention compared to the hydrodynamic ones. However, CPC columns can have significant pressure drops during operation, while hydrodynamic machines on the other hand have a higher efficiency.

Table 2.5. Summary of properties for the comparison of the two types of LLC columns. Adapted from [7].

LLC column	**Hydrodynamic (CCC)**	**Hydrostatic (CPC)**
Liquid stationary phase retained by/in	Coiled PTFE tubing	Channels or locules
N° of axis	2 with planetary motion obtained by a gear system	1 with simple rotation
Gravitational field	Highly variable, planetary motion	Constant, simple rotation
Efficiency	Good	Average
Pressure	Low (1 – 10 atm)	Average (10 – 95 atm)
Maintenance	Flying connecting leads	Rotating seals
Noise	Very noisy	Quiet

2.2.2. Requirements of the solvent system

In contrast to the chromatographic separations using a solid support as the stationary phase, HPLC columns, liquid-liquid chromatography requires not a single-phase, but a two-phase solvent system, in which the two phases formation is interdependent. A suitable solvent system for use in LLC must meet some physical and chemical requirements. The solvent system prepared generally from at least three solvents which form two phases at a particular composition, must still be able to keep a liquid two-phase system when mixing at the plant scale [36]. The selection of the two-phase solvent system is one of the most important steps in LLC, as there are so many different possibilities and the solvent system has to be tuned to the substances which are to be separated. This selection may account for up to 90% of the time for the design of a LLC separation process [35]. In distinction to an extraction process, in which the substance to be separated should only dissolve in one of the two phases, it is important for a chromatographic separation that both phases dissolve the substances to be separated in a similar manner in order to enable a good separation of the sample. This solution property is characterized by the partition coefficient P_i and is therefore very important for the choice of the system. This can theoretically be determined with different models, or experimentally through shake flask experiments. The partition coefficient should be in the optimal range for operation, also called best operating region or "sweet spot" [33] (see Figure 2.18).

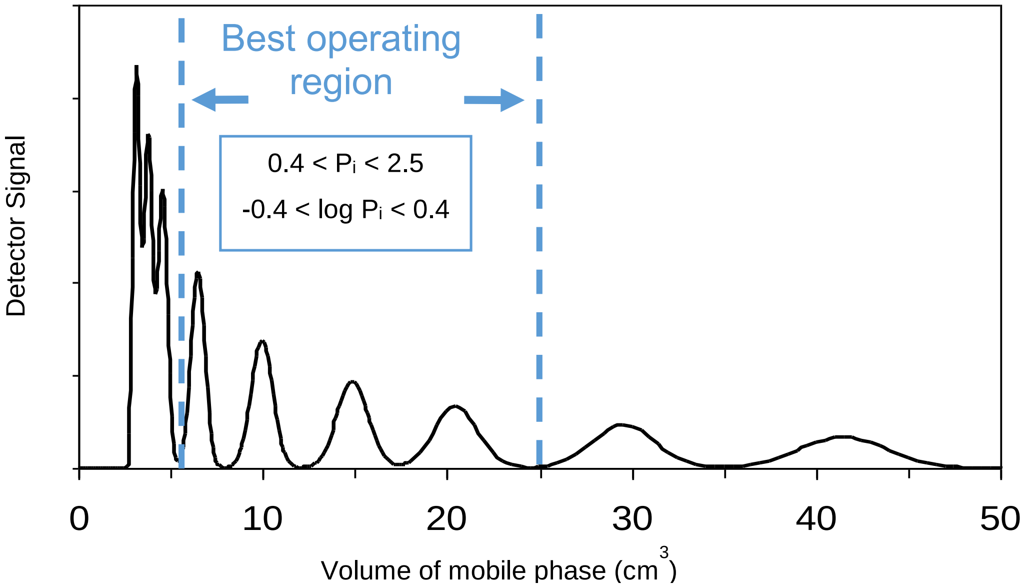


Figure 2.18. Illustration of the partition coefficient best operating region for effective LLC separation processes. The best operating region or "sweet spot" befalls between $0.4 < P_i < 2.5$ (or logarithmic between $-0.4 < \log P_i < 0.4$) as shown in the chromatogram of detector signal over volume of mobile phase (cm^3). The last could also be replaced by the separation run time, as both are related through the flow rate.

As observed in Figure 2.18, if substances with small partition coefficients are to be separated, it can result in an overlap of the peaks, for partition coefficients which are smaller than 0.4 ($\log P = -0.4$). This elutes the substance near the solvent front and thus results in a low resolution of the separation. At a partition coefficient greater than 2.5 ($\log P = 0.4$), the peaks become very shallow and wide. In this case, the dilution rate of the target substances in the mobile phase is thus very large. Due to the resulting high solvent consumption, these separation processes can become uneconomical. Sill for each separation the cost of the process must always be evaluated. The optimal range to be strived in order to achieve a particularly good separation is therefore between a partition coefficient of $0.4 < P_i < 2.5$ or logarithmic between $-0.4 < \log P_i < 0.4$ [37].

Another important parameter is the density of the two phases. The difference in density between the upper and lower phases should be at least 0.1 g cm^{-3}, so that the stationary phase is retained in the column and not discharged with the mobile phase. In addition, the settling time (i.e., the time required for both phases to be completely separated into two layers) of the phases should not be longer than 60 seconds to ensure effective mixing and demixing within the chambers of the hydrostatic CCC [36].

Not least, the interfacial tension, $\frac{\gamma}{\Delta\rho}$, plays an important role. If the ratio of interfacial tension and density difference (between the two phases is very small, then an emulsion is produced from the two phases, but the stationary phase is carried out with the mobile phase.) This can be minimized by the plant with a low flow rate If the quotient is too large, too little mass transfer takes place between the two phases because, due to the high interfacial tension, the drops are too large and stable, and thus they pass through the column too quickly, thus rendering the separation ineffective. For the ideal case a compromise must be found, the droplets should be small enough to allow an adequate mass transfer between the two phases, but at the same time it must be ensured that a sufficient quantity of stationary phase is retained in the plant. A range of $7 < \frac{\gamma}{\Delta\rho}, < 60$ cm^3 s^{-2} was found to be stable [36].

Finally, in the case of a separation, the ratio of the substances to be separated must also be considered, being the separation factor α of great importance. In commercial liquid-liquid chromatography, the separation factor should be higher than 1.5 [35]. The separation factor, α, is expressed as the quotient of the partition coefficients of the substances under consideration:

$$\alpha = \frac{P_1}{P_2} \tag{2.6}$$

2.2.3. Typical solvent systems

The two families of solvent systems mostly used in liquid-liquid chromatography are the HEMWat family for systems composed of different volumetric ratios of n-hexane (n-Hex), ethyl acetate (EtOAc), methanol (MeOH) and water, and the Arizona family, for systems composed of different volumetric ratios of ethyl acetate, n-heptane, methanol and water. With these solvent systems, a large number of different natural mixtures can be separated through LLC separation processes [38]. The HEMWat solvent system family is presented in Table 2.6, as all solvent systems tested in this work belonged to the HEMWat family.

Table 2.6. Solvent system numbering and volumetric ratio of the hexane/ethyl acetate/methanol/water (HEMWat) solvent system family [37]. The polarity of the system increases with increasing number of the systems.

HEMWat solvent system n°	**n-Hex**	**EtOAc**	**MeOH**	**Water**
-8	10	0	10	0
-7	9	1	9	1
-6	8	2	8	2
-5	7	3	7	3

Table 2.6. Continued.

HEMWat solvent system n°	n-Hex	EtOAc	MeOH	Water
-4	7	3	6	4
-3	6	4	6	4
-2	7	3	5	5
-1	6	4	5	5
0	5	5	5	5
+1	4	6	5	5
+2	3	7	5	5
+3	4	6	4	6
+4	3	7	4	6
+5	3	7	3	7
+6	2	8	2	8
+7	1	9	1	9
+8	0	10	0	10

2.2.4. Operating modes

In LLC there is a variety of operating modes that can be applied at both CCC and CPC. In this work two different modes were employed: batch mode or pulse injections were performed in the CCC and CPC machines, and back extrusion was performed only at the CPC apparatus. Batch mode can be considered the simplest mode, and as such not much explanation is required. At one end of the column, and according to the mode (ASC or DSC), the sample is injected and elution is carried out with either the UP or LP, respectively, of the biphasic system as the mobile phase [33]. Pulse injections can also be performed semi-continuously, by injecting the sample at regular intervals, timed in a way that the last peak of the previous injection does not overlap with the first peak of the current injection (see Figure 2.19).

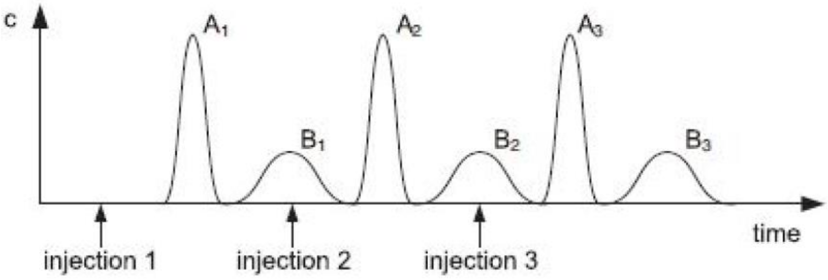


Figure 2.19. A series of three stacked batch injections with complete separation of components A and B [33].

The back-extrusion method consists in the fact that the liquid stationary phase can be easily moved [39]. By switching the column inlet and outlet ports, i.e. varying between ascending and descending mode, without changing the liquid phase that is initially used as the mobile phase will cause the rapid collapse of the two immiscible liquid phases inside the column. This results in the previously stationary phase gathering at the new column outlet. Then this previously stationary liquid phase is extruded outside the LLC column carrying all the retained solutes that did not elute in the first mode. Essentially, in back-extrusion the same mobile phase is kept in both ASC and DSC mode, while the way that the mobile phase circulates in the column is reversed. In Figure 2.20 a scheme for the behavior of two solutes with different partition coefficient values in a back-extrusion separation is illustrated.

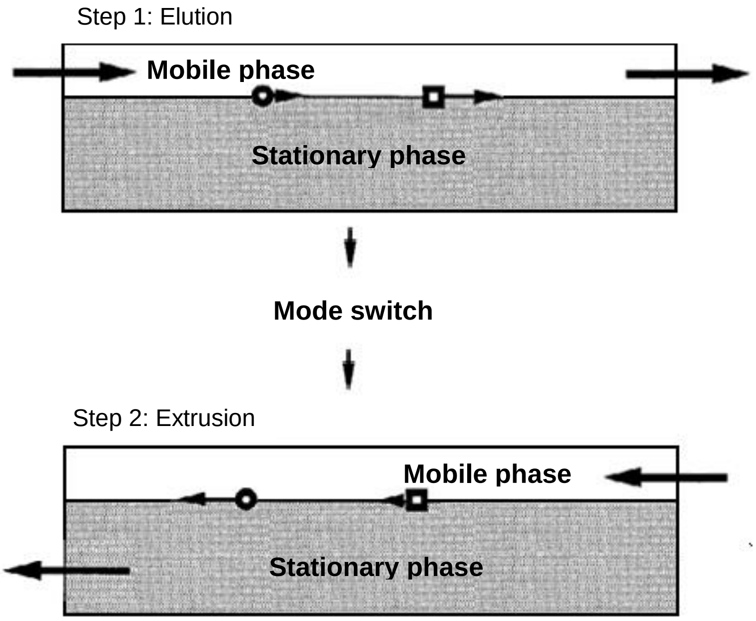


Figure 2.20. Behavior of two solutes during elution (step 1) and extrusion (step 2) of a back-extrusion LLC separation. Adapted from [40].

3. Materials and methods

This chapter is divided in three main sections. The first (Section 3.1), describes the LLC separations used for the purification of XN and XN C and for the pre-concentration of XN C. The following section (Section 3.2), describes the microbiological and chemical methods evaluated for the production of XN C. The last section of this chapter (Section 3.3) describes the different analytical methods developed for the analysis of hop compounds.

3.1. LLC separations

The LLC separations used in this work can be divided in two types: pulse injections or batch separation at both CCC and CPC, applied to the purification of XN and XN C, and back extrusion or elution extrusion at the CPC applied to the pre-concentration of XN C. The method for the purification of XN was initially developed at the CCC and then scaled-up to the CPC, while the methods for the purification and pre-concentration of XN C were developed only for the CCC and CPC, respectively. A brief explanation of the procedure of each apparatus will be given, followed by the specific parameters used in each of the previous conditions.

3.1.1. Materials

The pre-concentrated xanthohumol crude extract, Xantho-Flav (XF), was supplied by the company Hopsteiner (Mainburg, Germany). Depending on the hop batch used this mixture can have a XN concentration between 65 and 85%, based on a HPLC analysis at 370 nm. N-hexane (n-Hex), GC, ≥95% was purchased from Honeywell (Seelze, Germany). Ethyl acetate (EtOAc) for liquid chromatography, ≥98%, and methanol (MeOH) for analysis, ≥99.9%, were purchased from Merck KGaA (Darmstadt, Germany). The deionized water was obtained from an in-house network system.

3.1.2. CCC apparatus

All CCC experiments were carried out on a Mini Centrifuge from Dynamic Extractions (Gwent, Wales). This CCC set-up consists of a column with a total volume of 18.2 mL and two 306 pumps from Gilson (Middleton, USA), for delivering both mobile and stationary phase. The detector is a 171 Diode Array Detector (DAD), from Gilson (Middleton, USA). The heat generated during operation is cooled by a FL300 cooler from Julabo (Seelbach, Germany). A photograph of the former laboratorial CCC set up is available in Figure 3.1.

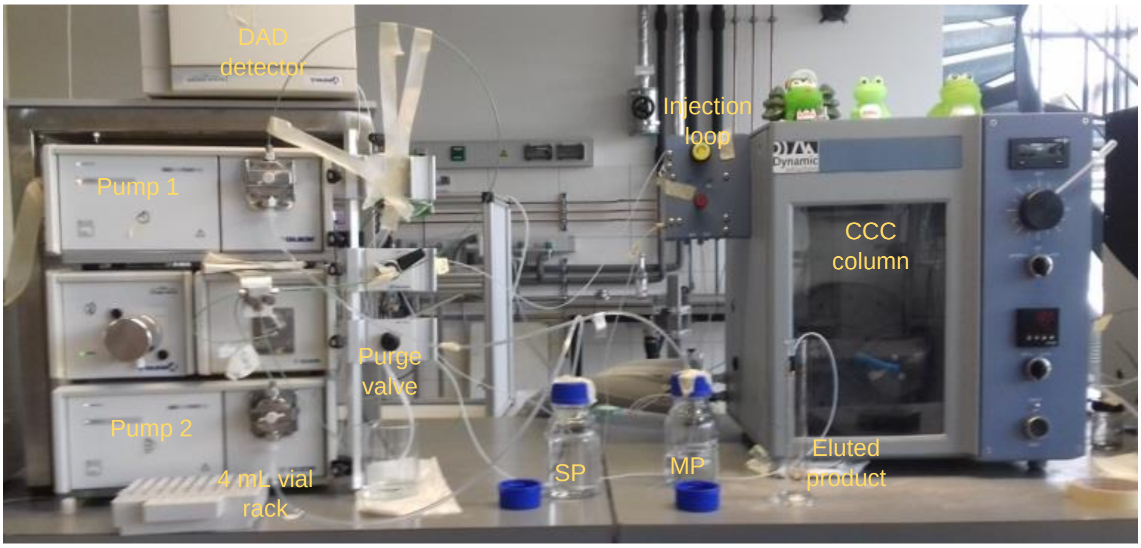


Figure 3.1. CCC apparatus (Dionex Extractions) with a column volume of 18.2 mL, a DAD detector and two HPLC pumps from Gilson (Middleton, USA).

CCC pulse injections or batch separation

The experiments were performed at room temperature. Initially both phases are purged in order to make sure that there is no air inside the system. The column was filled with the phase intended to be the stationary phase (SP). The rotation is started and as soon as the set rotation speed of 1900 rpm is achieved the mobile phase (MP), is pumped in the column using the second pump. The flow rate of the mobile phase was set to 1 mL min^{-1}. Once the hydrodynamic equilibrium has been established between the two phases and no more SP elutes from the column after one column volume of pumping, the feed solution is injected. An injection loop with a volume of 0.5 mL was used for all experiments. A syringe is filled with circa 0.7 mL of feed solution to make sure that no air enters the system, and the feed solution inserted in the injection loop. The CCC run after injection is then manually fractionated, at every 30 s or 1 min, into 4 mL glass vials, and each fraction analysed. The purification of XN was performed in ASC mode with the solvent system HEMWat -3, and the purification of XN C in DSC with the solvent system HEMWat -4. The cooling temperature of the cooling unit was set to 4°C so that during each CCC run the column temperature would not exceed the 23°C.

3.1.3. CPC apparatus

All CPC experiments were carried out with Model Armen SCPC-250 from Armen Instrument (Saint-Avé, France). The apparatus consisted of two rotating chromatography columns connected in series, each with a capacity of 125 mL, thus making a total of 250 mL of column capacity. Four HPLC gradient pumps deliver feed and mobile phase to the column during the ASC and DSC modes. Two ECOM DAD600 2WL 200-600 nm UV detectors (Prague, Czech Republic) monitor the product streams at both ends of the column according to the mode in use. Fractions were collected by means of two LS 5600 fraction collectors from Armen Instrument (Saint-Avé, France). A photograph of the former laboratorial CPC set up is available in Figure 3.2.

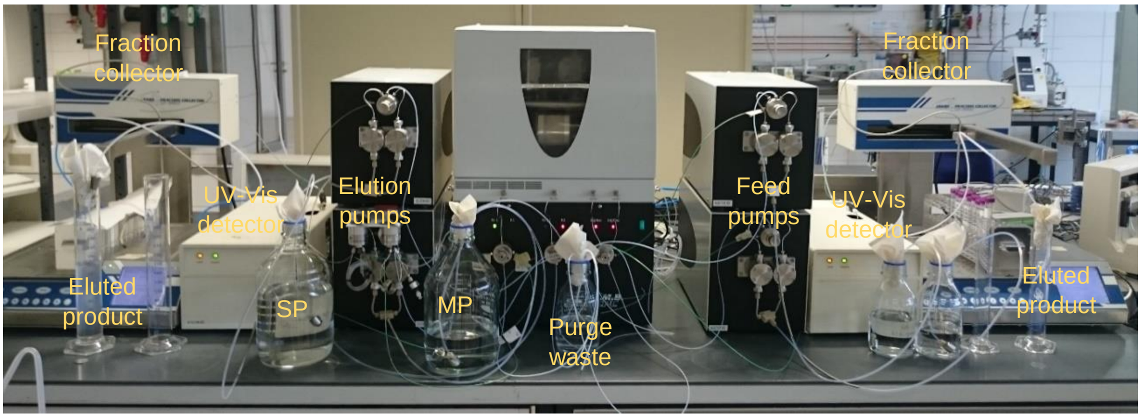


Figure 3.2. CPC apparatus with column volume of 250 mL, feed and elution pumps, two UV-Vis detectors and two fraction collectors from Armen Instrument (Saint-Avé, France).

CPC pulse injections

The procedure for the pulse injections at the CPC is similar to the one used at the CCC. The system must be initially purged. The column is then filled with SP until 1.5 column volumes of solvent have eluted from the column. Rotation is set to 1700 rpm, and when achieved the MP is pumped to the column using the ASC mode pump. The flow rate used for all CPC experiments was 14 mL min^{-1}, and the injection loop had a volume of 5 mL. At the CPC, the fractionation of the run after injection was done with the liquid handler, in intervals of 30 s or 1 min, into 15 mL falcon tubes, and all fractions were analyzed afterwards.

CPC fractions are combined, according to the analysis results, and evaporated using a rotary evaporator, and lyophilized in a Free Zone Plus 2.5 freeze dry system from Labconco (Kansas City, MO, USA). The obtained powder of purified xanthohumol was then stored for later use.

CPC back extrusion

In the back extrusion experiments at the CPC for the pre-concentration of XN C, after reaching hydrodynamic equilibrium and no more SP comes out, a three door valve is connected to the DSC mode pump, connecting two reservoirs to this pump, the reservoir containing MP, and the reservoir containing the XF feed solution. Initially only MP is pumped inside the column, but once a good base line has been obtained, the valve is turned, and the feeding initiated. The injection time, t_{inj}, or feed duration was 65 min. Finished this time, the valve is again turned so only MP is pump into the column until the end of the CPC run. Before the breakthrough of the compound of interest, the mode is switched, and the extrusion of the stationary phase initiated. Fractions are collected manually in 30 s intervals into 15 mL falcon tubes, and analyzed at the LC-MS. The pre-concentration of XN C was performed in DSC with a flow rate of 14 mL min^{-1} and solvent system HEMWat 0.

3.1.4. Biphasic systems and samples for feed solution preparation

The preparation of both the biphasic system and the sample for feed solution at the CCC and the CPC is similar. For preparing the biphasic solvent systems HEMWat -4 (7/3/6/4 v/v/v/v), HEMWat -3 (6/4/6/4 v/v/v/v) and HEMWat 0 (5/5/5/5 v/v/v/v), the volumes of n-hexane, ethyl acetate, methanol and water are measured in a gradient cylinder according to the volumetric ratio of each in the system. The biphasic system formed is stirred for two hours at room temperature, for phase equilibration. The two phases are then separated using a separation funnel into two reservoirs, and degassed for 5 min at 20°C on a Sonorex digitec ultrasonic bath (Bandelin, Berlin). The pre-concentration of XN C involved the preparation of a third reservoir containing MP for the 65 min feeding.

For the purification of XN and the pre-concentration of XN C the XF extract samples were prepared in 15 mL falcon tubes. In the purification of XN the feed concentration used in the CCC experiments was 40 mg mL^{-1}, while in the CPC experiments two concentrations were used, 55.6 and 27.8 mg mL^{-1}. After dissolving the weighted mass of XF in the LP of the biphasic system. Due to the low solubility of XF in the UP of the solvent systems, even when using ASC mode (UP is the mobile phase) the XF extract was still dissolved in the LP (SP in ASC mode). The sample was then centrifuged for 10 min at 5000 rpm and 23°C, in a Sigma 3-16 KL centrifuge. The pellet was discarded, and the supernatant transferred to a glass vial. Whenever after centrifugation there was still turbidity in the solution it would also be filtered through a 0.2 µm Nylon filter before injection at the CCC and CPC.

In the pre-concentration of XN C at the CPC, the total mass of XF weighted was divided into various falcon tubes, and to each falcon tube it was added circa 10 mL of MP. As before, the falcon tubes were centrifuged, the pellet discarded, and all supernatants added to the extra reservoir prepared containing MP, after filtration through a 0.2 µm Nylon filter. The XF feed concentrations used were 1 mg mL^{-1} and 0.88 mg mL^{-1}.

The purification of XN C at the CCC was performed using as feed sample a XN C chemical synthesis product in standard conditions (see Section 3.2.2). After evaporation and lyophilisation of the synthesis product, this was dissolved in the MP of the HEMWat -4 solvent system at a concentration of 5 mg mL^{-1} and 13 mg mL^{-1}. For the higher concentration filtration of the feed solution was necessary.

3.1.5. Shake flask experiments

The solvent system selection for use in liquid-liquid chromatography is done based on shake flask experiments. In these experiments, after preparing the biphasic system, after phase equilibration by stirring for 2h the biphasic system, the sample is added. After agitation, the two phases are separated at the separation funnel. Once the phases have been separated, they are analyzed at the HPLC for determination of the partition coefficients, P_i, of the different compounds, i, in the sample, based on Equation 2.1. As no calibration equation for the hop compounds was determined, the calculation of P_i is done by using the peak areas of the compound, i, in each phase.

3.2. Xanthohumol C microbiological and chemical production methods

The two methods here described for the production of XN C used as substrate the purified XN obtained from LLC (see Section 3.1.3), unlike the XN C pre-concentration method, at the CPC, that had XF as the feed solution.

3.2.1. Biotransformations

Materials

Twelve yeast and twelve bacteria strains for the screening tests were supplied by the *Technische Mikrobiologie* (engl. Technical Microbiology) chair at the Technical University of Munich (TUM) (Freising, Germany). The complete list of microorganisms can be found in Table 3.1. Casein peptone, yeast extract, glucose and agar-agar were from Sigma-Aldrich (St.Louis, MO). NBB$^{®}$-B ready to use media was obtained from Döhler (Darmstadt, Germany).

Table 3.1. List of yeasts and bacteria used for the biotransformation screening tests. All microorganisms were provided by the Technische Mikrobiologie Weihenstephan (TMW) chair.

List of yeasts	List of bacteria
Dekkera bruxellensis TMW 3.0680	*Lactobacillus backi* TMW 1.1989 and 1.1991
Hanseniaspora uvarum TMW 3.0457	*Lactobacillus brevis* TMW 1.213 and 1.465
Pichia norvegensis TMW 3.0317	*Lactobacillus lindneri* TMW 1.481 and 1.1993
Saccharomyces cerevisiae TMW 3.0256, 3.0250, 3.0343 and 3.0261	*Lactobacillus paracollinoides* TMW 1.1994 and 1.1995
Saccharomyces pastorianus TMW 3.0275 and 3.0285	*Pediococcus claussenii* TMW 2.340 and 2.54
Schizosaccharomyces pombe TMW 3.0022	*Pediococcus damnosus* TMW 2.1535 and 2.1533
Torulaspora delbrueckii TMW 3.0685	
Zygosaccharomyces bailii TMW 3.0058	

Yeast extract-peptone-glucose (YPG) medium preparation for yeast growth

In 1600 mL of deionized water, 10 g of yeast extract and 20 g of peptone from casein were dissolved, mixed and titrated to pH 6.5 at room temperature, with 1M HCl. The solution was divided into two 1 L bottles. To only one of them 15 g of agar-agar were added. 40 g of glucose were dissolved in 400 mL of deionized water, and divided into two 250 mL bottles. All four containers were autoclaved at 121°C for 15 min. After sterilization each 200 mL of glucose solution was added to each of the 800 mL solution, one with agar, and one without, thus making the final volume of 1 L.

Pre-sterilized and sealed in plastic packaging petri dishes were used for yeast solid medium. Inside a hood, each petri dish was removed from its packaging and the two halves separated. The still warm agar solution was poured carefully into the bottom half of the petri dish, just enough to form a layer over the bottom of the dish. In total twelve petri dishes were prepared, for each of the yeast, and set aside until the next day in order for the agar solution to cool and harden.

Cultivation of yeast in solid and liquid YPG medium

The yeasts were transferred to the respective petri dish containing the solid YPG medium, inside the hood, using a wire loop that must be sterilized between each colony by passing it through a flame. The yeasts were then incubated at room temperature, with the exception of *S.pombe* and *D.bruxellensis* that were incubated at 30°C, between 3 days to one week.

Once new colonies have formed on each petri dish, and using the same procedure as above, each yeast is transferred to a 15 mL pre-sterilized falcon tube containing circa 10 mL of the liquid YPG medium (without agar-agar). The incubation proceeded as before.

Cultivation of bacteria

The bacteria previously kept in petri dishes containing NBB® medium and under reduced oxygen, were transferred to 15 mL pre-sterilized falcon tubes, containing circa 10 mL of the same medium, using sterile toothpicks, which are discarded after each use. The bacteria were incubated at 25°C for one week.

Preparation of propionate buffer

To prepare 1 L of 0.01 M propionate buffer at pH 5, 0.74 g of propionic acid were dissolved in approximately 900 mL of deionized water. To which 6.977 g of potassium chloride, and 5 g of glucose were added. The mixture was stirred and titrated to pH 5 at room temperature with a monovalent strong base (1 M NaOH) or acid (1 M HCl) as needed. Finally, the volume was made up to 1 L with deionized water, and the buffer was autoclaved at 121°C for 15 min.

Screening tests

After incubation, all microorganisms were centrifuged for 5 min at 5000 rpm and 20°C, the supernatant removed, and 10 mL of propionate buffer were added to the falcon tubes followed by mixing at the vortex mixer for cell resuspension. The process was repeated two more times, until all media had been cleared out.

The purified xanthohumol (see Section 3.1), was first dissolved in 1 to 2 mL of absolute ethanol, and then in 250 mL of the prepared propionate buffer, with a final concentration of 1 mM. Due to the low solubility of xanthohumol in the buffer, the surfactant Tween 80 was previously added to the last, at a concentration of 0.8% (v/v).

To each falcon tube containing a different strain and after the washing step with only buffer, 10 mL of the 1 mM xanthohumol solution were added. After a week of incubation on a rotary shaker at room temperature, all falcon tubes were centrifuged for 5 min at 5000 rpm and 20°C. The supernatant was transferred to a 50 mL falcon tube, to which 10 mL of absolute ethanol were added, and the result was analyzed. The remaining pellet was dissolved in 3 mL of chloroform in order to permeate the cell membrane and allow for intracellular metabolites extraction. After 5 min centrifugation at 5000 rpm and 20°C, the chloroform supernatant was evaporated, the sample resuspended in 1 mL of methanol and analyzed.

3.2.2. Chemical synthesis of xanthohumol C

Materials

(Diacetoxyiodo)benzene ($PhI(OAc)_2$), 98% and 2,2,6,6-Tetramethylpiperidinyloxyl (TEMPO), 98% were purchased from Sigma-Aldrich (St.Louis, MO).Tetrahydrofuran (THF), 99%, anhydrous, was purchased from Alfa Aesar (Karlsruhe, Germany).

Reaction procedure

The reaction procedure was followed according to the literature [23]. In brief, to a solution of purified xanthohumol (102.4 mg, 0.289 mmol) in 10 mL of THF, in an ethanol bath at -80°C and under a nitrogen atmosphere, TEMPO (90 mg, 0.578 mmol) and $PhI(OAc)_2$ (102.4 mg, 0.137 mmol) were added sequentially, each previously dissolved in 5 mL of THF. The three THF solutions were left overnight at -80°C, together with the ethanol bath.

After 5 min of continuous stirring at -80°C, the ethanol bath was removed and the reaction mixture left to gradually warm up until room temperature over 30 min, after which 10 mL of water were added for quenching. In Figure 3.3 the reaction set-up at this point is presented. This set-up is composed by a three-neck glass round-bottom flask as the reactor with a stir bar, a magnetic stirrer, an in-line condenser for a nitrogen stream and a graduated flow control adapter.

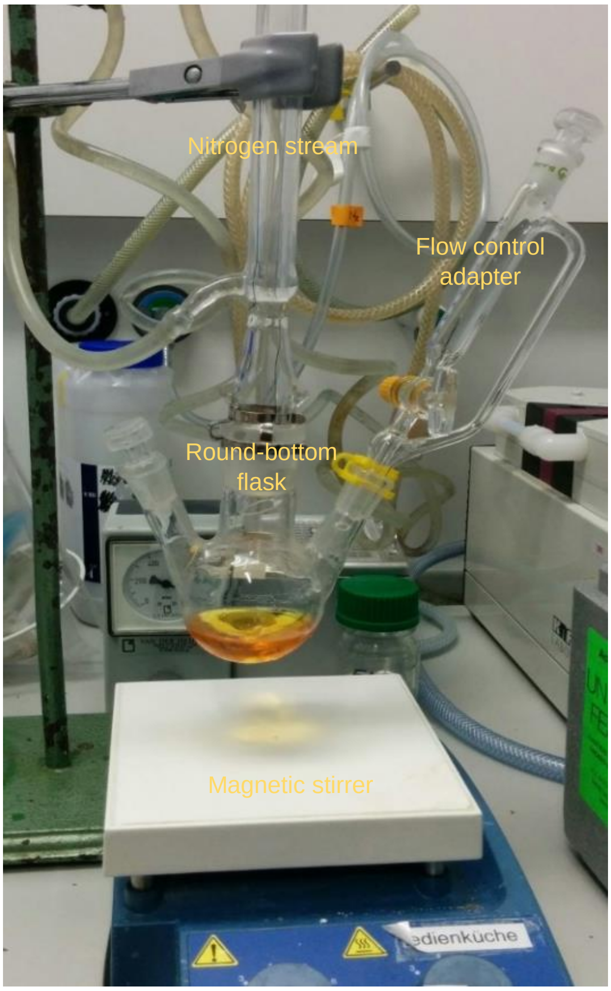


Figure 3.3. Xanthohumol C synthesis reaction set-up. After removing the ethanol bath, the reaction mixture is left to warm up to room temperature for 30 min.

Re-extraction was performed by adding 10 mL of diethylether, separating the two phases and discarding the lower phase. This step is repeated three times. Deionized water can be added if no biphasic system is formed after addition of diethylether. Magnesium sulfate salt was added to the extract product, for drying. The salt is added until crystal aggregation stops. The dried extract was then filtered through a 0.2 µm Nylon filter, the solvents were evaporated using a rotary evaporator at 27°C and 10 mbar (the pressure gradient initiates at 150 mbar, and gradually decreases until 10 mbar), and subsequent lyophilisation in the FreeZone freeze dry.

Besides the standard conditions described, that is, using the purified XN obtained from LLC with a concentration of 10.2 mg mL^{-1} (102.4 mg of XN in 10 mL of THF), and a 5 min duration of the -80°C ethanol bath, other conditions were tested, such as:

i) Half concentration of purified XN, 5 mg mL^{-1}.
ii) Using XF with an 80% XN concentration as substrate. XN concentration in the reaction remains at 10.2 mg mL^{-1}.
iii) Half concentration of XF (80% in XN). XN concentration in the reaction is now 5 mg mL^{-1}.
iv) The same as ii) but with a longer time for the -80°C ethanol bath (2h instead of the 5 min).

3.3. Analytical Methods

For the identification and analysis of the various hop compounds present in the hop crude extract, Xantho-Flav, two analytical methods were used, at three different analytical apparatus, High Performance Liquid Chromatograph (HPLC) with UV-Vis detector, Liquid Chromatograph-Mass Spectrometer (LC-MS) and Triple Stage Quadrupole Mass Spectrometer (TSQ-MS or LC-MS/MS). The method used in LC-MS/MS was developed by the TMW chair.

3.3.1. HPLC

An HPLC system from Gilson (Middleton, USA) was used to perform the initial analysis to the hop crude extract, XF, and its components. This system includes two HPLC pumps (Pump 321 and a Syringe Pump 402) with a dynamic mixing unit, a thermostatted column compartment, a temperature controlled sample rack with a Rack Code 34 (for 4 mL glass vials), a liquid handler GX-271 with an injection unit, and for data acquisition an UV-VIS-Detector 155 with two channels.

As stationary phase a reversed-phase silica Acclaim 120, C18, 5 µm Analytical (4.6 x 250 mm) from Dionex (Dionex, Idstein, Germany) column was used for the separations. As mobile phase Milli-Q water and acetonitrile with circa 0.05% formic acid (FA) were used with flow rate 1 mL min^{-1} (gradient: 0 min 20% B, 1 min 20% B, 21.75 min 90 % B, 28.75 min 90% B, 29.75 min 20% B, 34 min 20 % B). The water was provided from a Milli-Q Direct Water Purification System from Merck Millipore (Darmstadt, Germany). Two wavelengths were used for analysis of the hop compounds, 290 nm and 370 nm. The injection volume of the sample was 10 µL.

The mobile phase solvent A was prepared by pipetting small volumes of FA to the total volume of Milli-Q water, until obtaining a pH of 2.878, for data reproducibility. The same concentration of FA was then applied to the mobile phase solvent B, containing ACN. Once both solvents have been stirred for 30 min, they are degassed for 5 min at 20°C on a Sonorex digitec ultrasonic bath (Bandelin, Berlin).

When sample dilution was necessary, HPLC-grade methanol from VWR Chemicals (Darmstadt, Germany) was used. An example is the XF feed solution, with a concentration of 40 mg mL^{-1} or 55 mg mL^{-1} at the CCC and CPC, respectively, that must be dissolved to a maximum concentration of 10 mg mL^{-1} before the analysis at the HPLC.

3.3.2. LC-MS

For the identification of the different hop compounds, mass spectrometry was implemented, and a LC-MS equipment was used. The LCMS-2020 Single Quadrupole Liquid Chromatograph Mass Spectrometer from Shimadzu (Kyoto, Japan) is composed by two main modules, a Prominence series HPLC and a 2020 MS. The first module consists of an auto-sampler SIL-20 A, a solvent delivery module LC-20 AB Prominence and a photodiode array UV-VIS detector SPD-M20 A. Between the two main modules a flow rate splitter was necessary, as the MS module maximum flow rate is 0.2 mL min^{-1}, and the HPLC method has a flow rate of 1 mL min^{-1}. Both the HPLC method and the mobile and stationary phases were kept the same as for the Gilson HPLC (see Section 3.3.1). Mass spectrometric data were obtained from the MS module, equipped with a Dual Ionization Source, and analyzed using the software LabSolutions LCMS from Shimadzu. Data were collected over a m/z range of 10-800 in the negative (NI) mode. Mobile phases preparation was the same as for the Gilson HPLC.

3.3.3. LC-MS/MS

For LC-MS/MS a Dionex UltiMate 3000 HPLC system (Dionex, Idstein, Germany) with UV mediated detection at 210 nm, 270 nm, 314 nm and 370 nm wavelength, was coupled with a Triple Stage Quadrupole (TSQ) Quantum System from Thermo Fisher Scientific (Waltham, USA), including a syringe pump, a divert/inject valve, a mass spectrometer and the Xcalibur^{TM} data system.

An Aeris-PEPTIDE 3.6u XB-C18, 150x4.6 mm, column from Phenomenex (Aschaffenburg, Germany) column and mobile phases A (0.1% FA in HPLC-grade water) and B (0.1% FA in ACN) were used with a flow rate of 0.4 mL min^{-1} and a 60 min gradient (-1 min 20% B, 1 min 20% B, 35 min 65% B, 47.75 min 100% B, 54.75 min 100% B, 55.75 min 20% B, 60 min 20% B). Column temperature was set to 25°C.

4. Results and discussion

4.1. Xanthohumol purification through LLC

4.1.1. Hop extract Xantho-Flav (XF)

The pre-concentrated xanthohumol extract from Hopsteiner, XF, is a mixture containing innumerous hop compounds besides the target compound xanthohumol. This mixture, being a natural mixture, can have different compositions depending not only on the extraction method used, but also on the characteristics of the hop cultures itself at each batch. The concentration of xanthohumol in this extract variates between 65 and 85%. In Figure 4.1, it is presented the HPLC chromatogram of the XF extract at two wavelengths, 290 nm and 370 nm. These wavelengths are routinely used in hops analysis [31]. This chromatogram was zoomed in, so that even the compounds with very low concentrations in the mixture can also be perceived. From the many compounds present, a few were identified, and will be referred by their names, others simply as impurities.

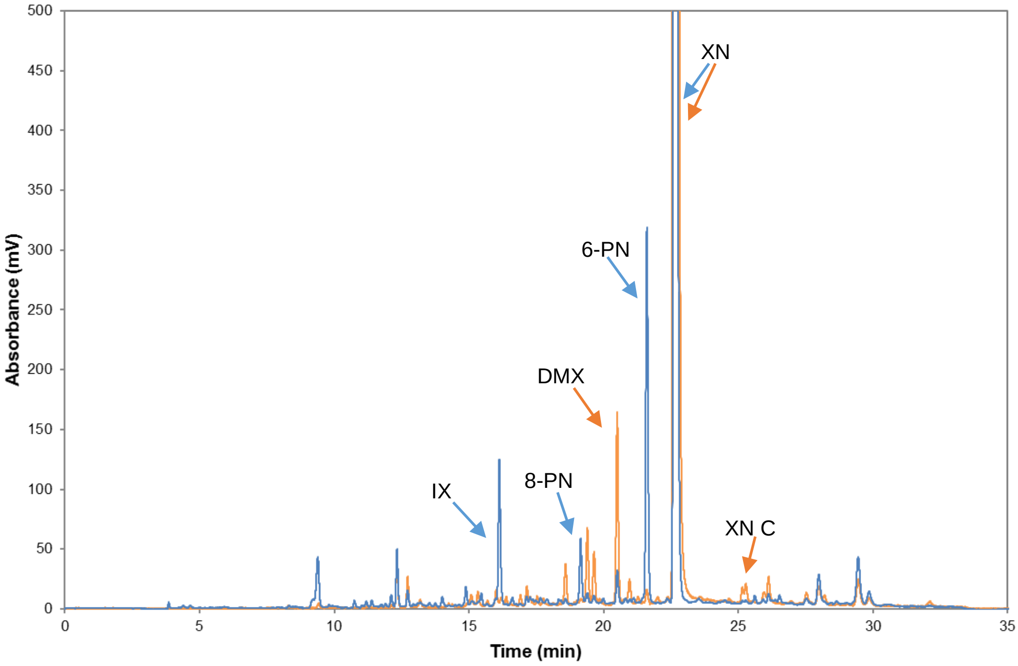


Figure 4.1. HPLC chromatogram of absorbance over HPLC run time for the hop crude extract, XF, at two wavelengths. — = 290 nm, — = 370 nm. Zoom factor of absorbance is 5x.

4.1.2. Solvent system selection

For the purification of xanthohumol from the complex natural mixture, a solvent selection of an applicable biphasic system for the use in liquid-liquid chromatography had to be done. This selection was done by shake flask experiments in the group of Biothermodynamics (TUM). Due to the fact that Chadwick et al., [6] already showed the applicability of HEMWat for the preparative isolation of hop compounds with LLC, the HEMWat solvent family was selected for further investigations. Therefore, shake flask experiments with different systems were done in order to select a solvent system that enables the separation of different hop compounds in XF extract. In addition, the objective was to proof whether an increase in the separation factor of different hop compounds is possible and thus optimize the resolution, separation efficiency and productivity for later preparative separations. Following the procedure for the shake flask experiments described in Section 3.1.5, the partition coefficients for xanthohumol in the different systems were calculated (see Table 4.1). In Table 4.1 is still included the partition coefficient calculated for the HEMWat system 5/5/4/3 v/v/v/v, that even though does not belong to the HEMWat family (see Table 2.6), has already been employed for the isolation of xanthohumol, obtaining a final purity of over 95% assayed by HPLC [5].

Table 4.1. Comparison of partition coefficient, P-value, of xanthohumol in different solvent systems from the HEMWat (hexane, ethyl acetate, methanol, water) solvent family. The calculation was based on HPLC analysis at 290 nm.

HEMWat system n°	**System Composition (v/v/v/v)**	**P_{XN}**
-6	8/2/8/2	0.02
-5	7/3/7/3	0.08
-4	7/3/6/4	0.28
-3	6/4/6/4	0.55
Chen et al., [5]	5/5/4/3	1.87
-1	6/4/5/5	3.01
0	5/5/5/5	5.10
1	4/6/5/5	6.83
3	4/6/4/6	66.43

Even though the partition coefficient for the HEMWat system from Chen et al., presented a better value (in the best operating region) compared to the rest of the systems, this system was discarded based on the separation factors between xanthohumol, 8-prenylnaringenin and 6-prenylnaringenin. These two compounds elute the closest to xanthohumol (see Figure 4.2), and thus were used as an element for the selection of the solvent system. The separation factors, α, (as expressed by Equation 2.6) for the different HEMWat systems are given in Table 4.2. For a good peak resolution the separation factor must be above 1.5.

Table 4.2. Separation factors between xanthohumol and 8-prenylnaringenin, $\alpha_{XN/\ 8\text{-}PN}$, and 6-prenylnaringenin, $\alpha_{XN/\ 6\text{-}PN}$, in the HEMWat solvent system family. [a)]Not determined.

HEMWat system n°	$\alpha_{XN/\ 8\text{-}PN}$	$\alpha_{XN/\ 6\text{-}PN}$
-6	[a)]	0.91
-5	1.62	0.98
-4	1.52	0.85
-3	1.31	0.84
Chen et al., [5]	0.87	0.63
-1	1.06	0.61
0	0.85	0.61
1	0.84	0.58
3	2.78	0.73

From Table 4.2 the system HEMWat -5 has simultaneously high values for both 8-PN and 6-PN separation factors, but selecting this system for the isolation of xanthohumol would still result in a low resolution of the separation, due to having a very low P-value (see Table 4.1).

Thus attending to Table 4.1 and Table 4.2, the solvent systems selected for the preliminary tests at CCC and CPC, were HEMWat -4 (7/3/6/4 v/v/v/v) and HEMWat -3 (6/4/6/4 v/v/v/v), due to having both a partition coefficient close or in the “sweet spot” for effective separations in LLC (see section 2.2.2), and a high separation factor, and as a result obtaining XN with a higher purity.

4.1.3. LLC experiments

Solvent system HEMWat -4

A method for the purification of xanthohumol through LLC was initially tested at the CCC with the solvent system HEMWat -4, in ASC mode, using a XF feed concentration of 40 mg mL^{-1}, and a flow rate of 1 mL min^{-1}. Performing the LLC experiments in DSC mode resulted in all compounds eluting at the same time. In those conditions the average stationary phase retention, S_f, was 0.66. The scale-up between CCC and CPC was done linearly, by keeping constant the mass load, m_{load}, that is the XF feed mass per column volume, and the stationary phase retention, S_f. Attending to the CCC column volume of 18.2 mL, a feed concentration of 40 mg mL^{-1} and an injection loop of 0.5 mL, the m_{load} at the CCC was 1.1 mg mL^{-1} of column volume. Since the CPC has a column volume of 250 mL and the injection loop used a volume of 5 mL, the concentration of XF feed at the CPC was 55.6 mg mL^{-1}.

For determining the flow rate to use at the CPC, a compromise was done between stationary phase loss and CPC run time. The different flow rates tested for CPC and their respective S_f value are reunited in Table 4.3. In order to decrease the runtime of the experiment and still keeping the S_f close to the one at the CCC, the flow rate of 14 mL min^{-1} was selected for the following experiments.

Table 4.3. Values of stationary phase retention, S_f, at different flow rates for the CPC using the solvent system HEMWat -4.

Flow rate / mL min^{-1}	**S_f**
10	0.67
12	0.67
14	0.63
16	0.61

The CPC run was fractionated, and each fraction analyzed in order to form a fraction pool for obtaining purified xanthohumol with a final purity of over 98%. The fractions containing a concentration of xanthohumol equal or higher than 95%, based on HPLC (UV-Vis) analysis at 290 nm, were put together into a pool, evaporated and freeze dried, obtaining a yellow powder. The purity of the sample was also determined by HPLC analysis at 290 nm, with a purity range of 98.2 to 98.7%. The parameters used at the CCC and CPC experiments were collected in Table 4.4.

Table 4.4. Values of parameters used at CCC and CPC experiments, F = flow rate, C_{feed} = feed concentration, rotation, m_{load} = mass load, and mode.

Parameter	**CCC**	**CPC**
F / mL min^{-1}	1	14
C_{feed} / mg mL^{-1}	40	55.6
Rotation / rpm	1900	1700
m_{load} / mg mL$^{-1}_{\text{column volume}}$	1.1	1.1
Mode	ASC	ASC

Solvent system HEMWat -3

The solvent system HEMWat -3 was also tested at both CCC and CPC for the purification of xanthohumol. The experimental parameters at the two apparatuses were the same as used for HEMWat -4 (see Table 4.4). Again flow rate experiments were conducted using the HEMWAt -3 system (see Table 4.5). After sample injection at the CPC, the eluent was fractionated, the factions analyzed at the HPLC, and put together into a fraction pool according to the concentration of xanthohumol present in the sample. Only fractions with a concentration equal or higher than 95% formed the fraction pool. The pool was then evaporated and freeze dried, and again analyzed for xanthohumol purity, being it over 98%.

Table 4.5 Values of stationary phase retention, S_f, at different flow rates for the CPC using the solvent system HEMWat -3.

Flow rate / mL min^{-1}	**S_f**
10	0.69
12	0.68
14	0.64

The comparison of the CPC experiments using the solvent systems HEMWat -4 and HEMWat -3 is summarized in Table 4.6. Here, yield represents the ratio between the sum of the xanthohumol peak areas present in the fractions that constitute the pool and the sum xanthohumol peak areas present in all fractions. It is calculated based on the assumption that the xanthohumol concentration is in the linear range, being the area of the peak proportional to the compound concentration, and consequently the ratio of the areas equal to the ratio of the concentrations. The stationary phase, $\bar{S}_f$, is the average between the stationary phase retention at the beginning of the experiment (see Table 4.3 and Table 4.5), and the retention at the end of the experiment. These two values are different due to the loss of stationary phase that occurs after sample injection.

Table 4.6. Comparison of the XN partition coefficient, P_{XN} from shake flask experiments, yield, XN purity and average stationary phase retention, $\bar{S}_f$, between the solvent systems HEMWat -4 and HEMWat -3 at the CPC. Yield and purity were determined based on HPLC analysis at 290 nm.

Solvent system	**Shake flask P_{XN}**	**Yield** / %	**XN purity** / %	**$\bar{S}_f$**
HEMWat -4	0.28	62.2 – 67.1	98.2 – 98.7	0.41
HEMWat -3	0.55	86.5 – 90.6	98.5 – 98.6	0.47

Attending to Table 4.6, and to the values of yield and $\bar{S}_f$ for the two systems, HEMWat -3 was selected as the solvent system for the purification of xanthohumol using LLC. In Figure 4.2, two chromatograms have been combined, on one side is the CPC chromatogram of the pre-concentrated xanthohumol extract, XF, and on the other the HPLC chromatogram of the analysis of the fractions collected from the same run. This allows to better understand which components are eluting at the same time as xanthohumol.

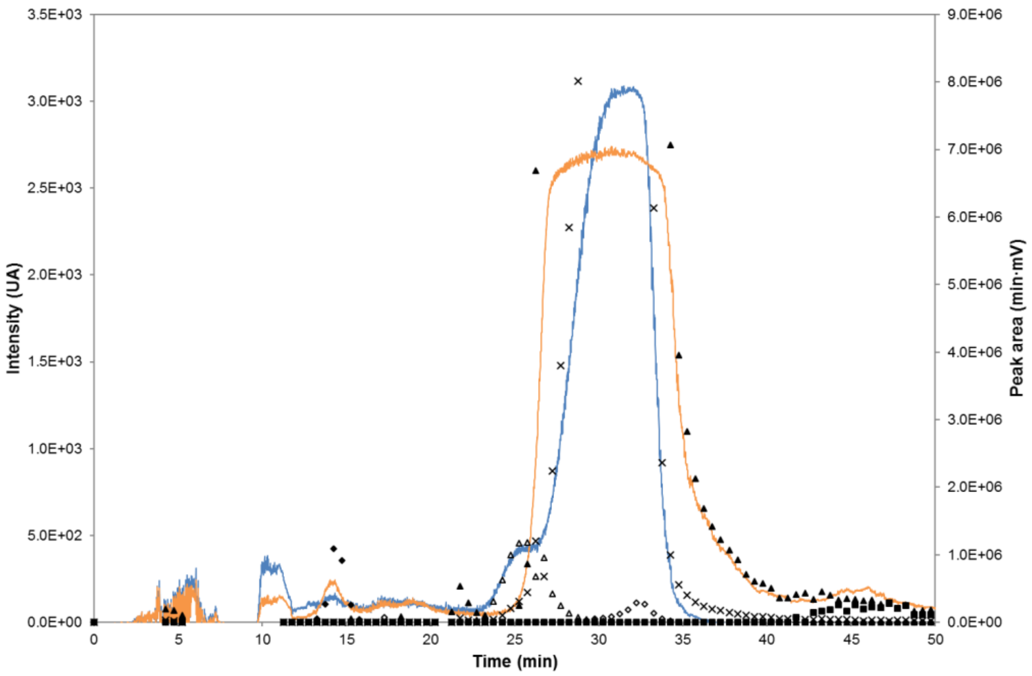


Figure 4.2. Overlap of the CPC chromatogram at two wavelengths: — = 290 nm and — = 370 nm, with the profile of the different compounds in the XF extract, based on UV-Vis analysis at 290 and 370 nm: Δ = 6-PN at 290 nm, ◆ = impurity at 370 nm, ■ = impurity at 370 nm, ◇ = 8-PN at 290 nm, ▲ = XN at 370 nm and × = XN at 290 nm.

Eluting at the same time as XN are 8-PN and 6-PN, as seen in Figure 4.2. The fractions analysis allows to choose the fractions with the lowest concentration of the two compounds, before forming the pool fraction with the purified XN.

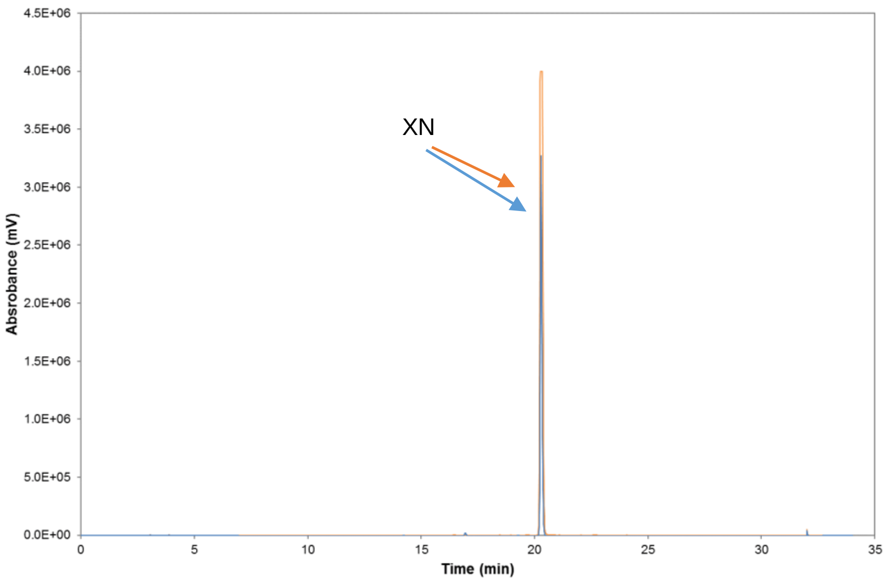


Figure 4.3. HPLC chromatogram of the purified xanthohumol obtained by CPC separation, after evaporation and freeze drying steps. — = 290 nm and — = 370 nm. Final purity of XN ranges between 98.5 and 98.6%, assayed at 290 nm.

The result of the purification of xanthohumol from the Xantho-Flav crude extract using LLC is shown in Figure 4.3. The purified XN sample was later used as the substrate for the biotransformation and chemical synthesis of xanthohumol C. The obtained xanthohumol remained stable even after more than three months, after HPLC analysis.

A recurring problem in the purification of XN was the high stationary phase losses observed at the CPC experiments. As this could be caused by a high concentration of the feed solution, since in the CCC experiments with a concentration of 40 mg mL^{-1}, the stationary phase loss was only 10% (with HEMWat -3), a lower concentration was also tested at the CPC. For a concentration of 27.8 mg mL^{-1}, there was no significant reduction in the stationary phase loss to justify reducing the feed concentration. The average value for the stationary phase in these conditions was 0.48, with an initial S_f of 0.63. The XF feed concentration remained at 55.6 mg mL^{-1} for the following separations, since in order to not risk more SP losses, this value was also not increased.

In order to decrease the amount of HEMWat -3 lower phase discarded at the end of each CPC run, the two phases were prepared separately according to each phase composition, as shown in Table 4.7 [40]. This also did not have a significant influence in the stationary phase loss, which can be explained by the fact that the two phases were equilibrated and separated again before using, in order to avoid any changes in the thermodynamic equilibrium of the phases during the run. The average and initial values of S_f, respectively, 0.42 and 0.56. The XN purity obtained in these conditions remained in the range of 98.5 to 98.6%.

Table 4.7. Composition of the upper and lower phases of the solvent system HEMWat -3: measurements by gas chromatography (GC) analysis (vol%), mean total percentage 100.46% vol% and 101.82 vol%. Adapted from [41].

Upper phase composition / %				**Lower phase composition / %**			
n-Hex	EtOAc	MeOH	Water	n-Hex	EtOAc	MeOH	Water
74.33	23.42	2.29	0.17	1.01	19.60	48.27	33.93

4.2. Xanthohumol C production methods and purification

4.2.1. Biotransformations

Biotransformation was the first method tested for the production of XN C, the objective was to identify one or more microorganisms capable of metabolizing XN into XN C. For that a total of twenty-four organisms (12 yeasts and 12 bacteria) were used (see Table 3.1). The brewing environment is considered to be unfavorable to microbial growth, mostly due to the presence of hop compounds that are added for bitterness and flavor and that can prevent some gram-positive bacteria from growing in beer [42,43]. Lactic acid bacteria (LAB) are commonly isolated from spoiled beer, being the majority of these isolates from the Lactobacillus and Pediococcus genera [44,45]. The hop resistance mechanisms for some species of these LAB genera are already reported in literature [42,43.44,46]. All strains selected for the biotransformation screening tests belonged to the previous genera. As for the selected yeast, most have been isolated from traditional beer productions, being responsible for the ethanol fermentation that occurs in beer brewing [47]. In addition, all microorganisms used are well characterized by the Technical Microbiology chair at TUM, where screening tests were performed.

In these experiments two tests were performed. In the first test, only the yeasts were incubated in the presence of XN, as the bacteria had not grown by then. After the addition of 1 mL XN in absolute EtOH to 250 mL of propionate buffer at a concentration of 1 mM, it was observed that the XN had not dissolved, being completely in suspension. The falcon tubes were incubated for three days at room temperature and attached to a rotary mixer in an attempt to help dissolve the XN. After that time, absolute EtOH was added to the falcon tubes, the samples centrifuged, the pellet discarded and the supernatant analyzed at the LC-MS/MS. The LC-MS/MS chromatogram for the yeast case *S.cerevisiae* 3.0256 is shown in Figure 4.4.

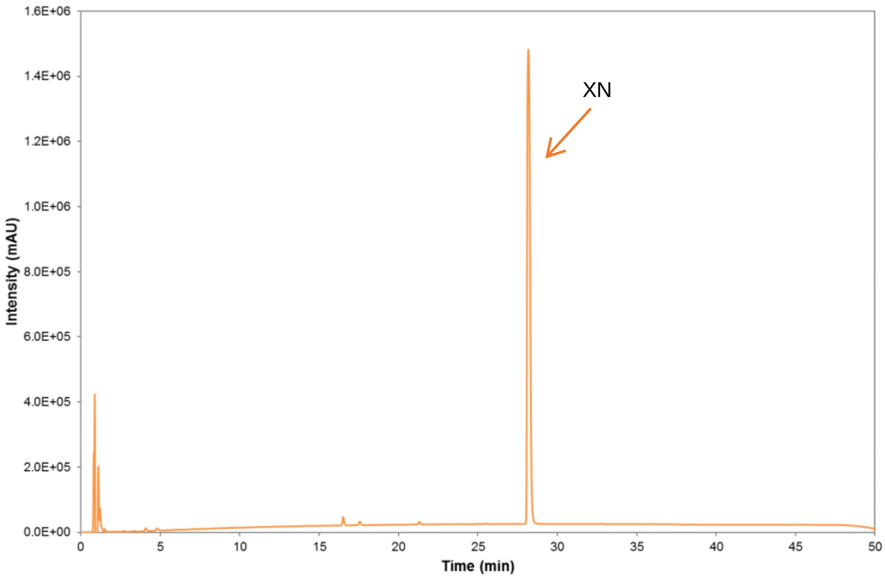


Figure 4.4. LC-MS/MS chromatogram for the yeast *S.cerevisiae* 3.0256. UV-Vis analysis at 370 nm.

In these conditions there was no transformation of XN into any derivative, being this the only compound present at 370 nm (see Figure 4.4). The comparison between the XN peak area before and after incubation with the yeasts suggests that there was no consumption of XN by the microorganisms as the area decreased by a factor of 2, as expected after dilution with absolute EtOH. In other microbial experiments at the TMW chair using hop compounds it was observed that the yeast cells would turn yellow after incubation. This was not the case, as all the cells remained in their original color. This could be explained by the low solubility of XN in the buffer solution, thus not making it available for the microorganisms. Another hypothesis is the time of contact between the cells and the solution, that could have been too short (3 days) for any metabolism to take place.

In the second test for increasing the solubility of XN in the propionate buffer the nonionic surfactant Tween 80 was added at a concentration inferior to 2% as per FDA (Food and Drug Association) [48]. Solubility tests with Tween 80 were performed until determining the concentration of the surfactant for the dissolution of 1 mM XN in the buffer, achieved at a concentration of 0.8% of Tween 80. The incubation time in the presence of XN was also increased from the three days to one week. After centrifugation the supernatant, here on referred as the extracellular supernatant, was analyzed. For cell membrane permeation chloroform was added to the pellet, the samples vortexed and again centrifuged. The new pellet formed was discarded and the supernatant, here on referred as the intracellular supernatant, also analyzed. The LC-MS profiles of both supernatants for the case of the yeast *S.cerevisiae* 3.0256 are presented in Figure 4.5.

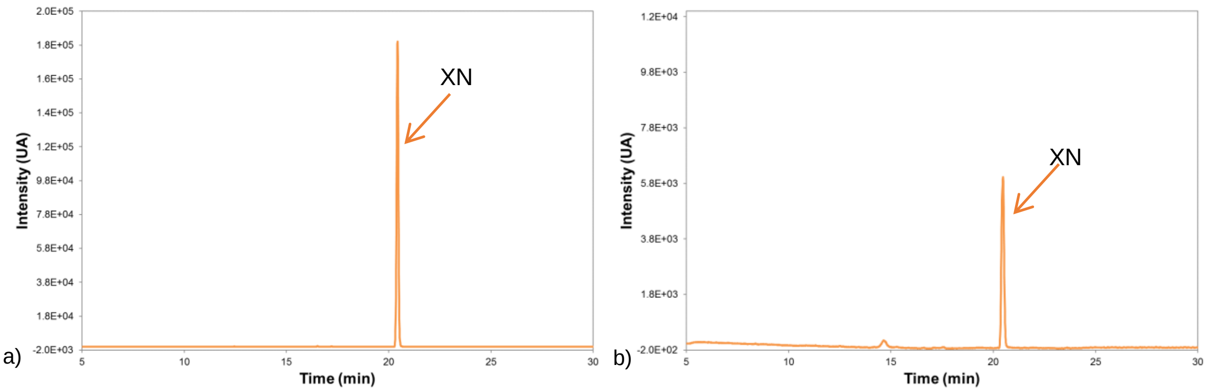


Figure 4.5. LC-MS chromatogram of the a) extracellular supernatant and b) intracellular supernatant of *S.cerevisiae* 3.0256, by UV-Vis analysis at 370 nm.

In the first chromatogram (extracellular supernatant) again XN is the only compound present in the sample. Even after increasing the solubility of XN and the contact time of the cells with the buffer solution, there was no metabolizing of the first by the microorganisms. The entrapment of XN by the surfactant Tween 80 could have rendered it difficult for the cells to access the substrate, or the contact time still remained too short for any changes to take place. The presence of XN in the intracellular supernatant could suggest that in fact the cells did incorporate the XN, but did not metabolize it. Another explanation, and at this point the one that seems the most likely is the presence of residual XN in the cell solution, due to the absence of a washing step before cell membrane permeation with chloroform. Still in the second chromatogram, the LC-MS analysis of the second peak present (at circa 15 min) did not provide a conclusion on the identification of this compound, as there was no m/z value associated to this peak.

These experiments concluded that in these conditions the metabolizing of XN for the production of XN C is not possible. Different approaches on the availability and solubility of XN should still be tested, including the use of a different type of method for increasing solubility of XN in the buffer solution, for example, a xanthohumol-cyclodextrin complex [49].

4.2.2. Xanthohumol C pre-concentration

Alternatively, to the chemical and microbial production methods, a different approach was tested for obtaining pure XN C, through pre-concentration at the CPC column. In this method, the substrate is the original XF extract (with a XN C concentration range of 0.55 to 2.2%, by UV-Vis analysis at 370 nm), used as the feed solution for CPC. The first step of the pre-concentration method was the validation of a simulation based on shake flask experiments, by a Breakthrough curve of XN C at the CPC, in order to then perform the pre-concentration itself with the help of back extrusion of the stationary phase. In this chapter the rational for the solvent system selection differed from the one used in the purification of xanthohumol (e.g., P-value in the best operating region). In order to enable a long retention of xanthohumol C in the stationary phase and to achieve a high injection volume, and consequently a high mass load of the target compound, the value of the partition coefficient must be as high as possible. The goal here is to get the highest mass load of xanthohumol C in the stationary phase and then to have it only in a relatively low volume, namely the stationary phase volume (see Section 2.2.4). To recover this volume, the stationary phase is extruded from the column by changing the mode (ASC or DSC) in use, but keeping the same mobile phase as during elution.

Breakthrough curve

In order to obtain the breakthrough curve of xanthohumol C and determine the parameters to use for its pre-concentration at the CPC, simulations were done with the software gPROMS using the cell model, that modulates the behavior of complex systems based on given process parameters and partition coefficient of the single compounds [33,50]. From these simulations the best separation of xanthohumol C from three other impurities, including xanthohumol, were obtained with the parameters present in Table 4.8. As it has already been mentioned the solvent system selection was done based on the partition coefficient value for xanthohumol C, obtained from shake flask experiments. From this experiments HEMWat 0 was selected as the solvent system to use for the pre-concentration at the CPC, and therefore was implemented also in the XN C breakthrough curve simulation. The time of injection, t_{inj}, is selected in a way that the XN is eluted completely and before the breakthrough of the target component XN C. Both the number of theoretical plates (N = 500) and the concentration of each compound (C_i = 0.05 mg mL^{-1}) in the feed solution were the same for all compounds simulated. This assumption was done on one hand because the exact values for N are not available, but also because for N higher than 500 it will have no significant influence on the breakthrough volume of the compounds, as found in previous works. The breakthrough volume can be defined as the mobile phase volume at which the breakthrough curve of the compound starts [51]. Therefore, it is not necessary to determine the exact N for each compound. Concentration was also kept the same to make it simpler for analysis, since in reality concentration of XN would be so high that the concentration of XN C and other impurities would not be visible in the chromatogram. And as long as the concentration is in the linear range it also has no influence on the breakthrough volume of the single compounds. Another reason why concentration was kept the same for all compounds in the simulation has to do with the very low concentration of XN C in the XF extract (between 0.5 and 2.2%). Keeping the real ratios between the different compounds in the simulation would result in a very small peak (in terms of peak height) for XN C in Figure 4.6 compared to the one from XN, for example, making the visualization of its breakthrough curve more difficult.

The breakthrough curve of xanthohumol C obtained from gPROMS simulation with the mentioned parameters is shown in Figure 4.6, where concentration refers to the concentration of each compound in the mobile phase of the system. In Figure 4.6, the compounds order is the order at which they are eluting from the column.

Table 4.8. Values for the parameters used in the gPROMS simulation, S_f, stationary phase retention, V_c, column volume, F, flow rate, P_1 and P_4, partition coefficients of impurities, P_2, partition coefficient of xanthohumol C, P_3, partition coefficient of xanthohumol, N, number of theoretical plates, C_{feed}, concentration of the compounds in the feed solution.

Parameter	**Value**
S_f	0.6
V_c / mL	247.09
t_{inj} / min	65
F / mL min^{-1}	14
P_1	27.10
P_2	10.87
P_3	2.13
P_4	2.12
N	500
C_{feed} / mg mL^{-1}	0.05

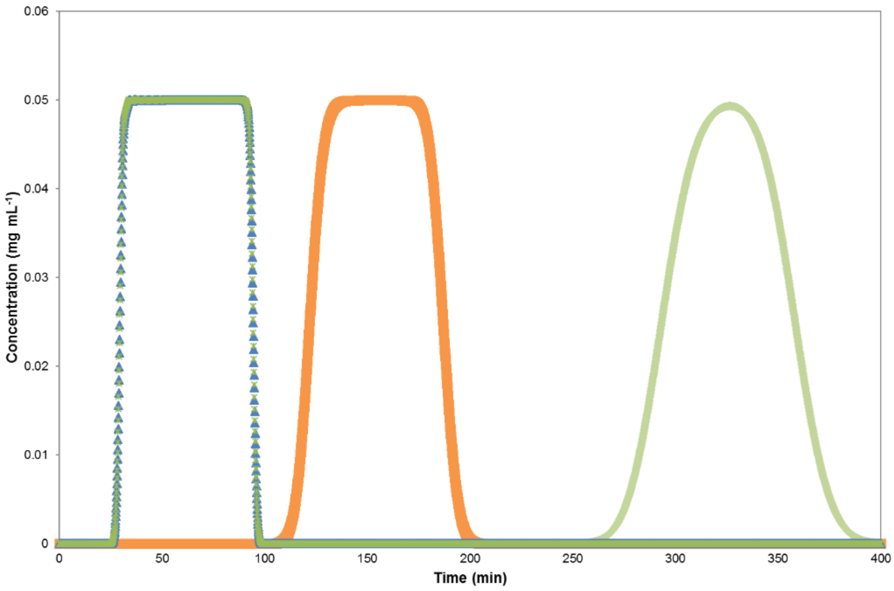


Figure 4.6. Breakthrough curve for xanthohumol C obtained from the software gPROMS. ■ = xanthohumol C (P_2), ▲ = xanthohumol (P_3), ✳ = impurity 2 (P_4) and ▲ = impurity 1 (P_1).

Attending to Figure 4.6 the first compounds to elute from the column will be XN and impurity 2, as they have similarly low P-values. The next compound to elute is XN C, and finally impurity 1, which has the highest P-value. The two impurities simulated along with XN and XN C were chosen based on the fact that in ASC mode impurity 1 was eluting at the same time as XN C, and impurity 2 as XN. In DSC mode and in these conditions is now possible to separate XN C from impurity 1.

For the validation of the previously simulated and optimized elution profile based on partition coefficients from shake flask experiments, and for the validation of the duration of injection (column loading), the breakthrough curve was performed at the CPC. The CPC experiment was done with the same parameters as the simulation (see Table 4.8), with the exception of the feed concentration that was 1 mg mL^{-1}, in order to increase the XN C mass load in the column. The average stationary phase retention calculated at the CPC was 0.46. According to the XN C breakthrough curve in Figure 4.6, at 94 min of the run, fractionation was initiated. The first fractions were collected in volume fractions of 14 mL, and starting at 110 min, five fractions of 250 mL each were collected. The compounds eluting after XN C (see Figure 4.6) will be retained inside the column. All fractions were analyzed at the LC-MS, in order to confirm the presence of xanthohumol C through its value of m/z. As expected in the first fractions there was no xanthohumol C, as this compound only eluted after 110 min of the run. The 250 mL fractions had to be evaporated and dissolved in 1 mL of MeOH before analysis, as they were to diluted for analysis at the LC-MS. The LC-MS chromatogram for the analysis of the second 250 mL fraction is shown in Figure 4.7.

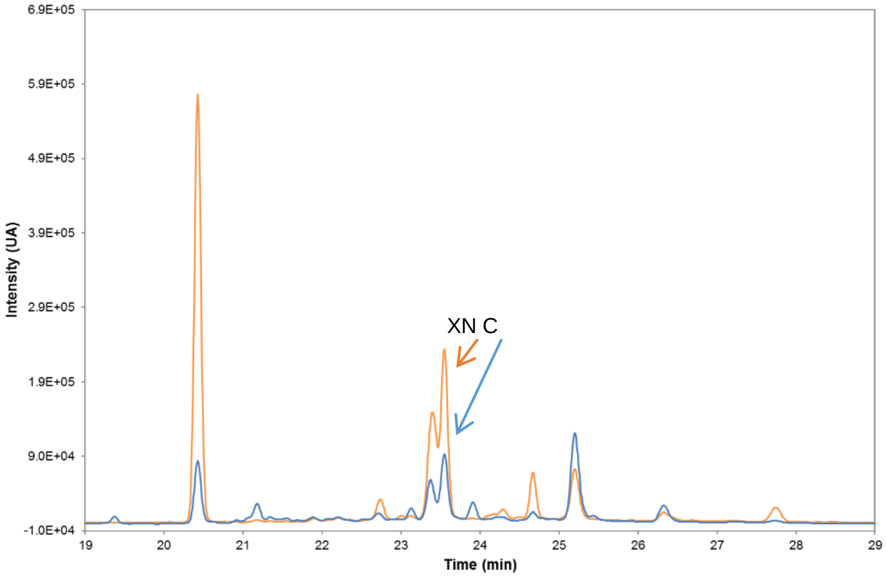


Figure 4.7. LC-MS chromatogram of a pre-concentrated xanthohumol C fraction. — = 290 nm and — = 370 nm.

Back extrusion

The concept for the back extrusion experiment is slightly different from the previous breakthrough curve, now instead of switching after the elution of XN C, the switch happens before, and XN C will elute with the stationary phase (extrusion). The moment of switching was selected from the simulation and experimentally validated by the breakthrough curve experiment, in a way that XN has completely eluted from the column and before the breakthrough of XN C, staying this inside the column. That happens after 110 min of run. For this experiment all CPC parameters were the same as before (see Table 4.8), except the feed solution concentration that was 0.88 mg mL^{-1}. Both the last 10 minutes of the elution and the entire extrusion were fractionated in 1 min intervals (14 mL fractions), and the fractions analyzed at the LC-MS. Again as expected there was no XN C present in the elution (DSC mode) fractions, only in the extrusion (ASC mode). The CPC chromatogram of the back extrusion including the moment of switch is shown in Figure 4.8.

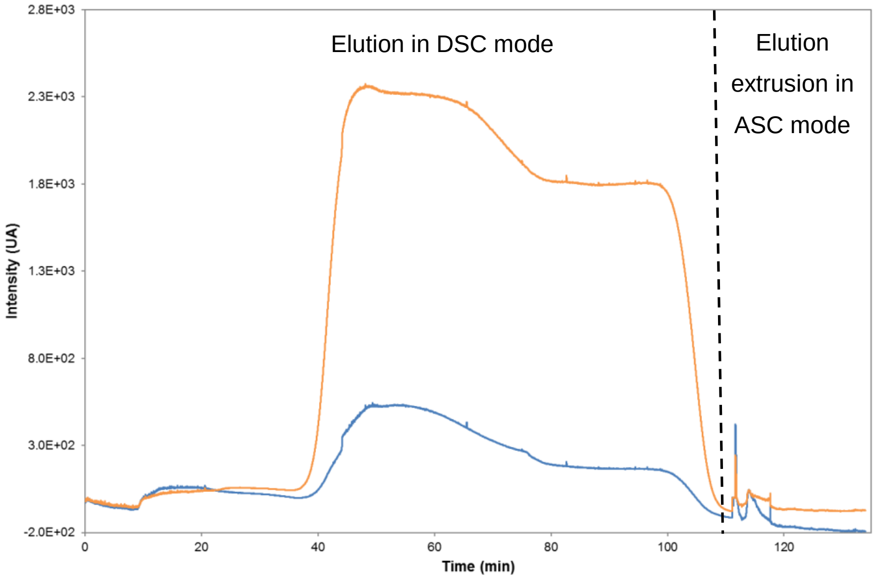


Figure 4.8. Back extrusion CPC chromatogram for the pre-concentration of XN C. The dotted line marks the moment of switching between the elution in DSC mode and the elution extrusion in ASC mode. — = 290 nm and — = 370 nm.

As mentioned before fractions were collected in the last minutes of the elution, in which no XN C was found, and in the entire extrusion, where XN C elutes with the stationary phase (ASC mode). Based on the analysis of these fractions, the profile for XN C in the extrusion over time was put together (see Figure 4.9). This profile was done based on the peak area of XN C in each of the analyzed fractions, and the moment at which the respective fraction is collected during the elution extrusion. The impurities still present in each fraction (see Figure 4.10) that compose the XN C profile, are not shown here, for simplicity.

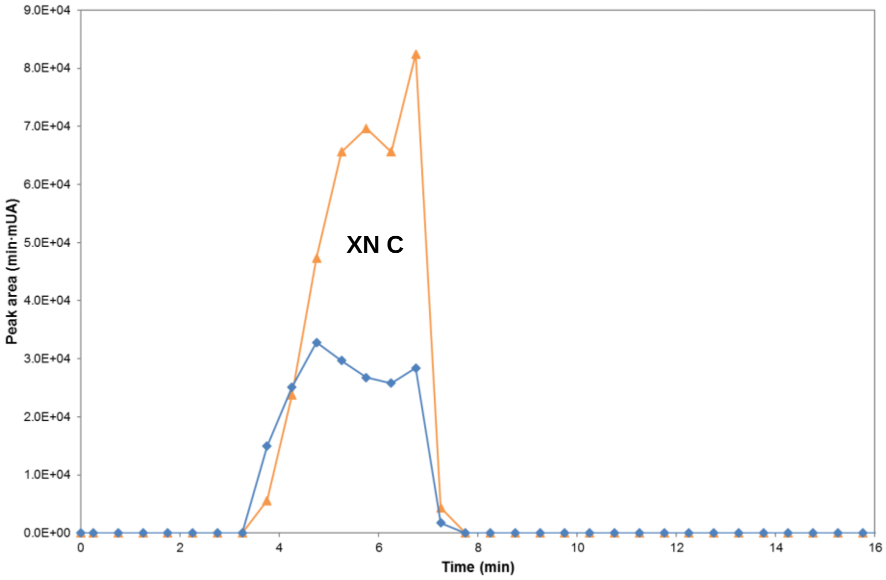


Figure 4.9. Xanthohumol C extrusion profile of peak area over time. ▲ = 290 nm and ◆ = 370 nm.

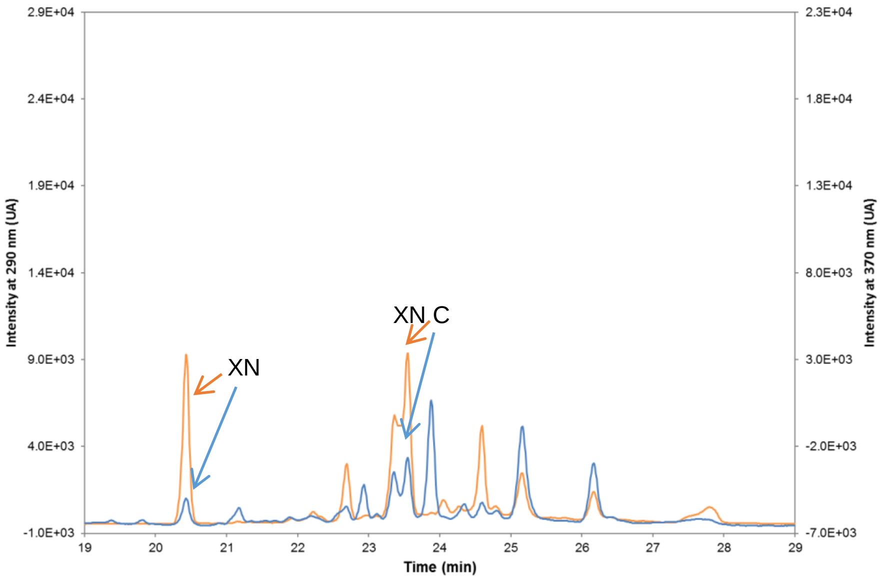


Figure 4.10. LC-MS chromatogram of pre-concentrated xanthohumol C fraction 14 (6.75 min) after back extrusion at the CPC. — = 290 nm and — = 370 nm.

In Figure 4.10 the result of the pre-concentration of XN C at the CPC is presented, using fraction 14, collected at 6.75 min, as example. Through analysis at the LC-MS of the m/z value for the compounds present in the concentrated mixture of XN C and comparison with the values in [31], it was possible to have an idea of the identification of some of those compounds besides XN C and XN (see Table 4.9). For the rest of the peaks, there was no matching between their m/z value and the ones found in literature [31].

Table 4.9. Possible identification for some of the peaks in the XN C pre-concentrated fractions, through comparison with the m/z values from LC-MS analysis and [31].

Mass transition Q1→ Q3	Compound	$t_{retention}$ / min
347→278	co-Humulon	25.1
361→292	n- and ad-Humulon	26.1 26.4
421→119	1”,2”-Dihydroxanthohumol F, 4’-Hydroxytunicatachalcon and 5’-Prenylxanthohumol	24.5

Through the gPROMS simulation it was possible to define the best set of parameters for operation at the CPC. The breakthrough curve experiment confirmed the previous simulation and determined the moment at which XN C starts eluting, in order to perform the switching in the back extrusion. In this chapter it was shown that the concentrating of XN C using a CPC column is possible. Even though the breakthrough curve experiment seems to have less impurities in the XN C fractions (see Figure 4.7) as the back extrusion (see Figure 4.10), in both cases at least a separation step using LLC will be needed. Shake flask experiments using the pre-concentrated fractions have to be done for new solvent system selection. The advantage of performing back extrusion is the shorter run time when compared with the breakthrough curve experiment and consequently less solvent consumption. In preparative scale the use of solvent system with a higher partition coefficient value of xanthohumol C would be even more advantageous, as it would make it possible to increase the feed mass load. This would result in a higher concentration of XN C, as the volume of the stationary phase remains approximately constant. In this work, for proof of concept, the used solvent system, HEMWat 0, was selected to be able to perform the experiment in a convenient experimental time.

4.2.3. Chemical synthesis

The chemical synthesis reaction used for the production of XN C was based on the synthesis of Hyperguinone B from a biosynthetic precursor [23]. As explained before (see Section 2.1.1), the treatment of XN with $PhI(OAc)_2$ in the presence of TEMPO, should perform the ring closure necessary for the formation of XN C. Due to the presence of two phenolic oxygen atoms that could cyclize, two possible compounds can be formed, XN C and its isomer (see Figure 2.8). The different steps used for the chemical synthesis of XN C are summarized in Figure 4.11.

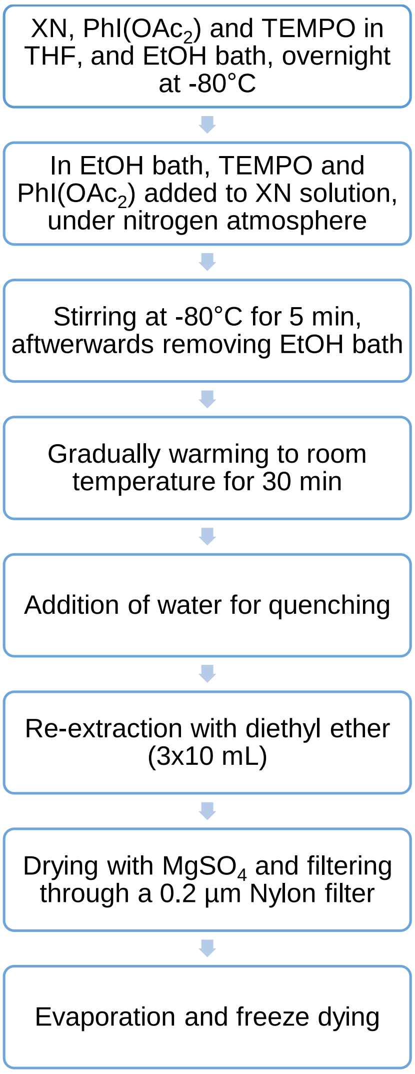


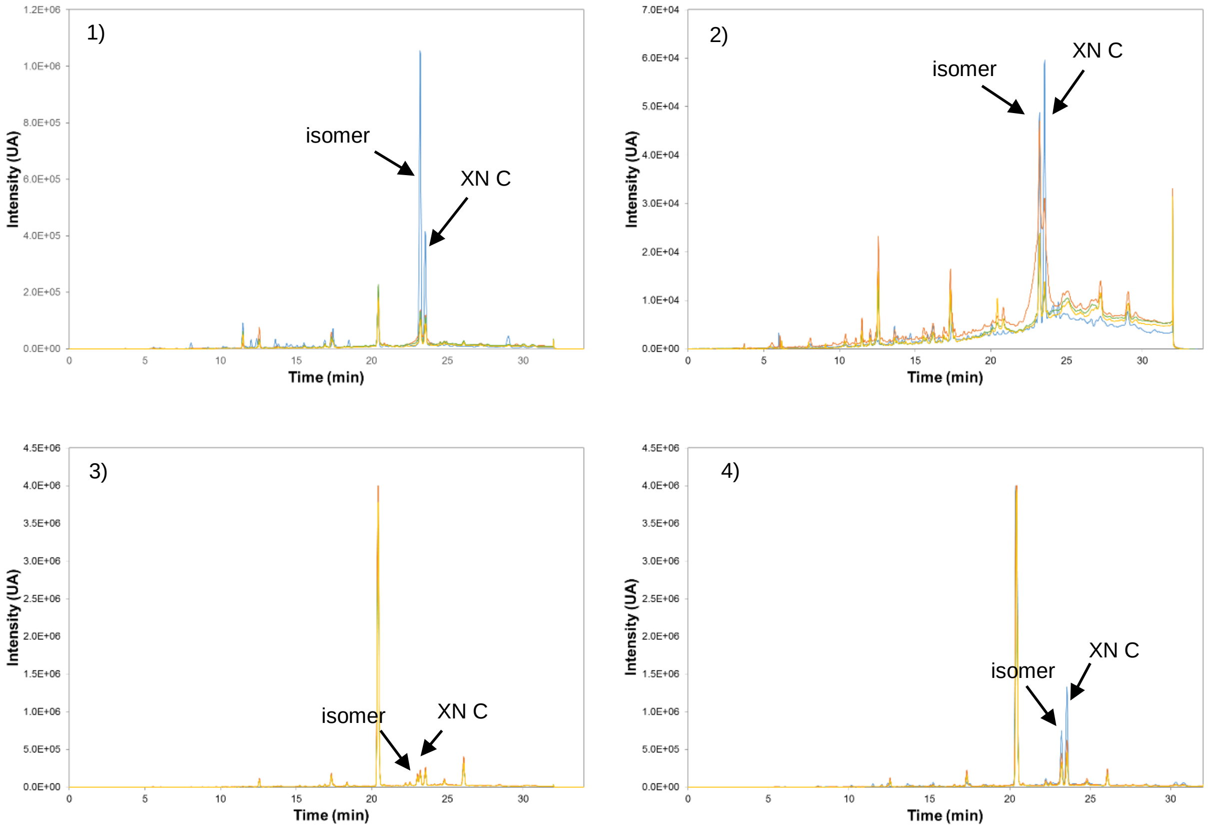
Figure 4.11. Flow chart illustrating the different steps in the chemical synthesis of XN C.

Five different experiments were done by changing the reaction conditions, as shown in Table 4.10, where experiment 1 is done at the standard conditions. When XF was the substrate used for the synthesis reaction, the mass to weight was calculated knowing that the XF sample has an 80% concentration of XN. The substrate concentration refers to the initial XN substrate in the 10 mL of THF.

Table 4.10. The five different experiments done for the chemical synthesis of XN C, by changing, the substrate, the initial XN concentration and the ethanol bath duration.

Experiment	**Substrate**	**XN concentration / mg mL^{-1}**	**Ethanol bath duration / min**
1	XN	10	5
2	XN	5	5
3	XF	10	5
4	XF	10	120
5	XF	5	5

For each of the five experiments 1 mL samples were taken at four different steps of the process: before (blue) and after quenching with water (orange), after extraction (green) and after drying and filtering (yellow). A total of 19 samples were analyzed at the LC-MS (UV-Vis analysis at 370 nm) for identification and mass comparison of XN C and its isomer. In Figure 4.12 are shown the five LC-MS chromatograms at 370 nm of all five experiments. In each chromatogram the different colors represent the step at which the sample was analyzed as above-mentioned in parentheses.



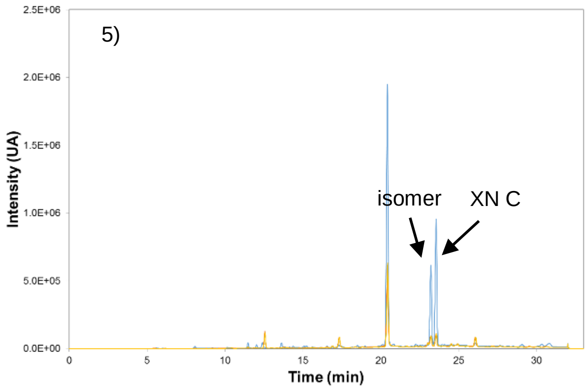


Figure 4.12. Overview of the different steps analysis at the LC-MS for each of the five conditions used in the synthesis of XN C. — = before quenching, — = after quenching, — = after extraction with diethyl ether and — after drying with $MgSO_4$ and filtration with a 0.2 µm Nylon filter. UV-Vis analysis at 370 nm. 1) experiment 1 in standard conditions, 2) experiment 2, 3) experiment 3, 4) experiment 4 and 5) experiment 5.

Through the LC-MS analysis the two conformations were identified in all five conditions and in each step, as both have the same m/z of 351. This confirms the hypothesis presented by Figure 2.8 XN C was first identified using both its m/z value and its retention time. The second peak found with a m/z value of 351 was then identified as being the XN C isomer. In general, from Figure 4.12 it is observable that when purified xanthohumol (retention time of xanthohumol is 20.4 min) is the substrate, its conversion is higher, than when XF is used. In experiments 1 and 2 almost the totality off xanthohumol has been consumed, while in experiments 3, 4 and 5 the big majority of xanthohumol is still present in the samples. For experiment 3 the sample before quenching was not collected, so here only 3 steps (colors) are present. After the quenching step it is expected that all peaks in the different experiments decrease, since quenching implies adding water to the samples and thus dilution of the sample will happen. This reduction of the peaks should happen in the same proportion for all compounds present in the sample. Therefore, the ratios of xanthohumol C and of its isomer should remain constant before and after quenching. That is not the case though for all experiments as shown in Figure 4.13. Here the ratios of xanthohumol C and of its isomer were calculated based on the peak areas from the chromatograms in Figure 4.12. The peak areas of xanthohumol C and its isomer were divided by the total sum of all peak areas in each sample. For xanthohumol C the ratio always decreases after the quenching step, while for XN C isomer it decreases in experiments 1, 4 and 5, but increases in experiment 2. As referred in experiment 3 no sample was collected before quenching so no comparison can be done in this conditions. A low stability of the compounds in the reaction mixture could be a reason as to why the ratios are not constant before and after quenching with water. Increasing the ethanol duration time to 2 hours (experiment 4), instead of the 30 min, does not seem to have a significant influence in the conversion of xanthohumol. Still for comparison purposes this condition should be repeated but now using xanthohumol as the substrate. In experiments 2 and 5, i.e., when using half substrate concentration, the conversion of xanthohumol was higher when compared to experiments 1 and 3, respectively. This result suggests the reaction can be extended when xanthohumol is the limited reactant, and TEMPO and $PhI(OAc)_2$ are the excess reactants. In overall, experiment 1 presents the highest ratio of xanthohumol C between the five experiments.

However, it also has the highest ratio for XN C isomer. Experiment 3, that is, keeping everything the same, but using Xantho-Flav as substrate, showed the lowest ratio of the two compounds. This results suggest that the best conditions for performing the chemical synthesis of xanthohumol C are with xanthohumol as the substrate, increased time, and standard concentration in XN.

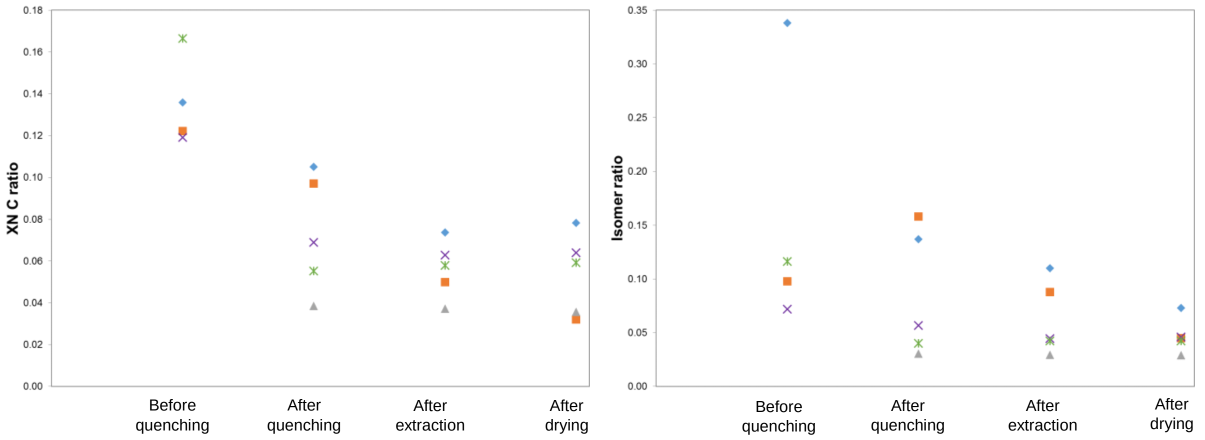


Figure 4.13. Comparison of the ratios of xanthohumol C (left) and its isomer (right) in the different steps, for each of the five experiments. ◆ = experiment 1, ■ = experiment 2, ▲ = experiment 3, × = experiment 4 and ✳ = experiment 5. The ratios of both XN C and its isomer are calculated based on the peak areas of the two compounds in each experiment from Figure 4.12. For each step in the respective experiment, the peak area of XN C or of its isomer is divided by the sum of the peak areas of all compounds present in the that step sample.

The last step in the chemical synthesis of xanthohumol C was the evaporation and lyophilization of the products. After lyophilization, the solid product obtained was dissolved in MeOH and analyzed on LC-MS at 370 nm. The mass spectrometry analysis showed that only one compound with a m/z value of 351 was now present in the sample, in all the five experiments performed. By comparison with the analysis of a xanthohumol C standard supplied by the TMW chair it was concluded that this compound was in fact xanthohumol C. Based on this results, it seems that the XN C isomer is not sable at the conditions needed for the lyophilization (sample freezing at -80ºC). In Figure 4.14 the chromatogram, at 370 nm, of the freeze dried product from experiment 1 is given. Even though the XN C isomer is no longer present in the sample, purification step of the product will still be necessary due to the other impurities present, being the residual xanthohumol the major impurity here. The advantage of using the purified xanthohumol as substrate instead of the hop extract, is the reduced amount of impurities in the final product in the first case.

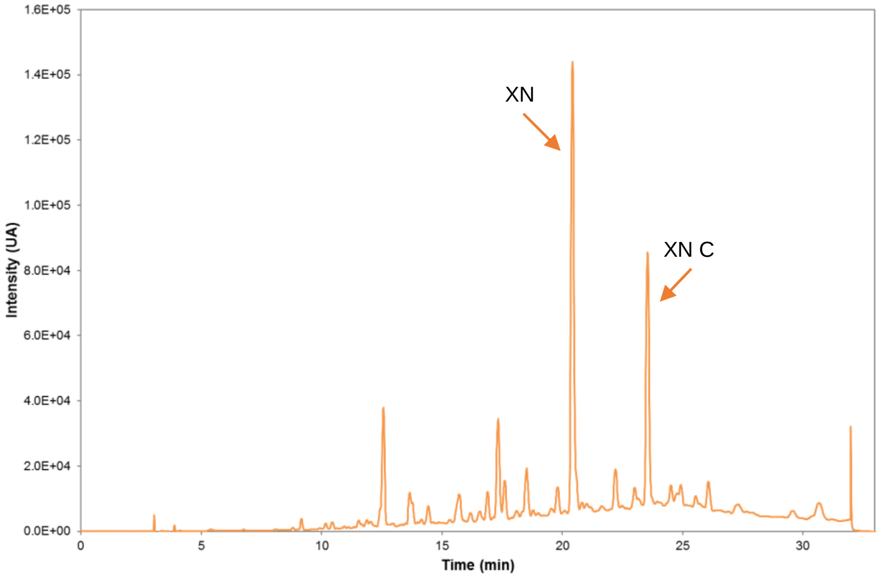


Figure 4.14. LC-MS chromatogram of experiment 1 in standard conditions after evaporation and freeze drying. UV-Vis analysis at 370 nm.

4.2.4. Xanthohumol C purification

The final step in the production of XN C after chemical synthesis, was the purification of the synthesis product obtained from the purified XN in standard conditions (see Section 4.2.3). Following sample evaporation and lyophilisation, shake flask experiments were done for solvent system selection. Four different solvents were tested and the results are gathered in Table 4.11.

Table 4.11. Values of partition coefficient for xanthohumol and xanthohumol C, P_{XN} and $P_{XN\ C}$, respectively, and separation factor between xanthohumol C and xanthohumol, $\alpha_{XN\ C\ /\ XN}$, according to solvent system.

Solvent system	**P_{XN}**	**$P_{XN\ C}$**	**$\alpha_{XN\ C/XN}$**
HEMWat -4	0.46	1.81	3.92
HEMWat -3	0.65	2.32	3.57
HEMWat 0	2.91	15.92	-
HEMWat 1	3.14	13.94	-

It should be noted that the values obtained for the xanthohumol partition coefficients in these systems are quite different from the ones obtained in the selection of a solvent system for the purification of xanthohumol (see Table 4.1). The partition coefficient depends on diverse factors, such as temperature, pH, concentration and hydrodynamics [52]. Concerning pH, temperature and concentration, there was no significant variance in these factors that could have caused such disparity of the P-values for these systems. A possible explanation for this phenomenon is the nature of each sample, i.e., the composition of the sample. In the first shake flask experiments a natural hop extract was used (see Figure 4.1), while in the second it was the product of the chemical synthesis of xanthohumol C, where different reacts such as $PhI(OAc)_2$ and TEMPO were implemented. This suggests that the presence of certain compounds can have an influence in the properties and behavior of the solvent system and therefore the partitioning of the target compounds. Attending to the standard deviation of the P-values for each HEMWat system: 0.09, 0.05, 1.095 and 1.845, for HEMWat -4, HEMWat -3. HEMWat 0 and HEMWat 1, respectively, this influence appears to increase with increasing polarity of the solvent system.

Taking into account the P-value for XN C in the four systems, both HEMWat 0 and 1 were discarded for presenting high values, which means the CCC run would be too long, and the solvent consumption very high. Such an effect could be avoided by operating with those solvent systems in ASC mode, but then the same overlapping would happen with the impurities that elute at the same time as XN C. Between solvent systems HEMWat -4 and -3, the first was selected as the solvent system for the purification of XN C at the CCC, for presenting a higher value for the separation factor, α, than HEMWat -3. Two CCC experiments were done for the purification of XN C, the first with a feed concentration of 5 mg mL^{-1}, and the second with 13 mg mL^{-1}. The lyophilized synthesis product was dissolved in the LP of the solvent system, as the experiments were done in DSC mode. A flow rate of 1 mL min^{-1} and an injection loop of 0.5 mL were used. From the partition coefficient of XN C in HEMWat -4, and from a stationary phase retention of 0.62, determined at the CCC, the expected retention volume of XN C was calculated based on Equation 2.3, being circa 27 mL. The relationship between retention volume and retention time is described in Equation 4.1. So, for a flow rate of 1 mL min^{-1}, the retention time of xanthohumol C is 27 min. The experimental values of retention time and partition coefficient are gathered in Table 4.12 together with the ones obtained from the shake flask experiments. The difference between the values is not significant, having the shake flask experiments a good prediction of the CCC parameters in the purification of XN C.

$$\mathrm{t_R = V_R \cdot F} \tag{4.1}$$

Table 4.12. Comparison of partition coefficient, $P_{XN\ C}$, and retention time, t_R, for XN C between shake flask experiments and the CCC run.

	Shake flask experiment	**CCC run**
$P_{XN\ C}$	1.81	1.56
t_R / min	27	25.1

For the two CCC runs performed fractions were collected starting after 24 min of run time, and analyzed at the LC-MS. In Figure 4.15 the CCC chromatogram from the second run using a feed concentration of 13 mg mL^{-1}, was overlapped with the XN C profile obtained from the fractions UV-Vis analysis at 370 nm (see Figure 4.16). The gradient is based on the peak area of XN C in each fraction.

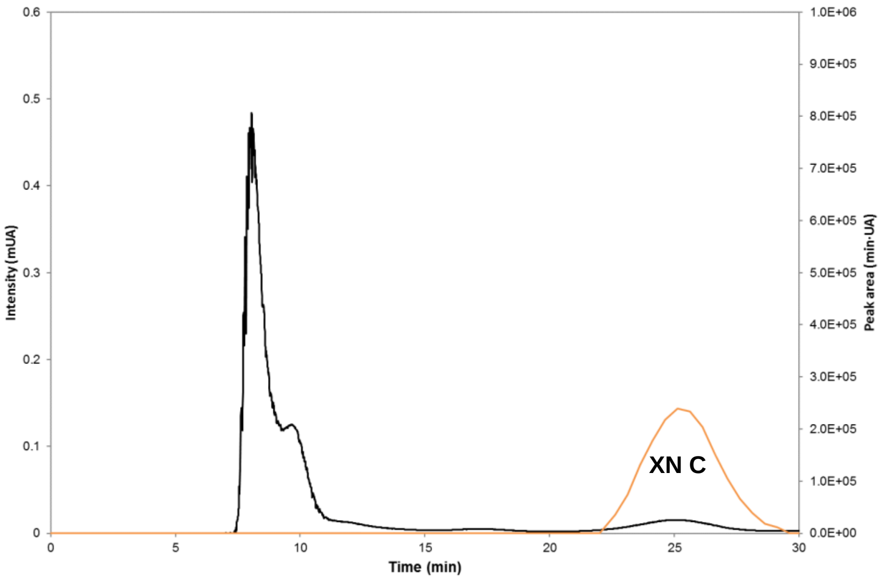


Figure 4.15. Overlap of CCC chromatogram with XN C profile. — = CCC chromatogram at 370 nm (Channel 8, reference 700 nm) and — = XN C profile with UV-Vis analysis at 370 nm.

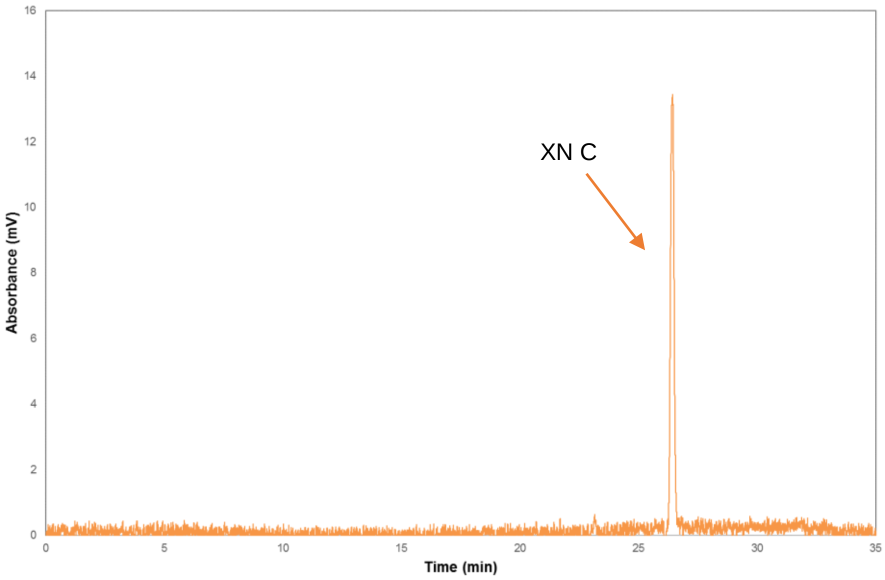


Figure 4.16. HPLC chromatogram of fraction 12 (25.62 min) from XN C purification at the CCC with solvent system HEMWat -4 and feed concentration 13 mg mL^{-1}. UV-Vis analysis at 370 nm.

Attending to Figure 4.15 and Figure 4.16, it was possible to purify XN C from the chemical synthesis product 1 mixture using CCC with solvent system HEMWat -4 in DSC mode. Due to the high separation factor a baseline separation could be performed and a purity of over 99% (based on HPLC at 370nm) achieved, as shown in Figure 4.16 for fraction 12.

5. Conclusion

The current work focused on two main topics. First the evaluation of the use of liquid-liquid chromatography in the isolation of xanthohumol, the major prenylated flavonoid present in hops, from a hop crude extract. Second, the development of a method for the production of xanthohumol C, a minor chalcone in hops, and subsequent purification.

Xanthohumol, was successfully purified by means of centrifugal partition chromatography. The hop extract pre-concentrated in xanthohumol, Xantho-Flav, used as the feed solution was supplied by the company Hopsteiner. In this extract the concentration of xanthohumol can range between 65 to 85% (assayed by UV-Vis analysis at 370 nm). Selection of the solvent system for xanthohumol purification was based on the values of partition coefficient and separation factors obtained through shake flask experiments, for the HEMWat (n-hexane-ethyl acetate-methanol-water) solvent system family. Two solvent systems were initially selected, HEMWat -4 (7/3/6/4 v/v/v/v) and HEMWat -3 (6/4/6/4 v/v/v/v), for use at the countercurrent chromatograph. The method developed in this apparatus was linearly scaled-up to the CPC machine. Here, HEMWat -3 showed the highest efficiency for the separation of xanthohumol, based on the values of stationary phase retention and xanthohumol yield. The obtained xanthohumol, after purification by CPC using the biphasic solvent system HEMWat -3 had a final purity of over 98% assayed by UV-Vis analysis at 290 nm. The purified xanthohumol was used as substrate in the chemical synthesis and in the biotransformation experiments for the production of xanthohumol C, without additional processing.

As mentioned varied times through this work, the availability of xanthohumol C through isolation methods is limited due to its low concentration in natural hop extracts. In the specific case of Xantho-Flav, the concentration of xanthohumol C ranges between 0.5 and 2.2% in the extract. Therefore, the development of alternative methods for obtaining xanthohumol in significant amounts that allow further pharmacological studies, is necessary. In this work, three different methods were proposed with that purpose.

The first method in the production of xanthohumol C were the biotransformations. Here, a total of 12 yeast and 12 bacteria were tested on their ability to metabolize xanthohumol, and produce xanthohumol C. To overcome the poor dissolution of xanthohumol in the aqueous propionate buffer the surfactant Tween 80 was added to the last. However, even after adding this surfactant, xanthohumol was the only compound present in both the extracellular surfactant and the intracellular surfactant analyzed at the LC-MS/MS. Although the presence of xanthohumol in the intracellular surfactant could suggest that in fact the microorganisms were able to incorporate xanthohumol, the most reasonable explanation is for residual xanthohumol to have remained attached to the cell surface, as no cleaning step was performed before cell permeation. These results led to the conclusion that no meaningful information regarding the metabolism of xanthohumol by the selected microorganism can be from here extrapolated. Therefore, the use of biotransformation experiments as a method to obtain xanthohumol C remains to be ascertained.

The second method here reported for obtaining xanthohumol C, was the pre-concentration of xanthohumol C using centrifugal partition chromatography, and the original hop extract, Xantho-Flav, as the feed solution. The first step of the pre-concentration method was the validation of a simulation based on shake flask experiments, by performing a breakthrough curve of xanthohumol C at the CPC, in order to then pre-concentrate the last with the help of back-extrusion of the stationary phase. Comparison of the xanthohumol C breakthrough curve obtained at the CPC with the one from the gProms simulation, confirms that the last is a useful tool in the prediction of the behavior of complex systems. For the CPC experiments the solvent system HEMWat 0 (5/5/5/5 v/v/v/v) was employed together with a flow rate of 14 mL min^{-1}, and a 65 min injection time. In this work it was shown that the concentrating of xanthohumol C using a CPC column is possible. However, a subsequent separation step using LLC is still necessary in order to obtain pure xanthohumol C.

The third and final method for production of xanthohumol C was through chemical synthesis. This synthesis was based on the synthetic route for hyperguinone B, as described in [23]. The presence of two phenolic oxygen atoms in the B ring of xanthohumol suggested that the ring closure could occur in two ways, resulting in the formation of xanthohumol C or of its isomer. In fact, in all the different five experiments performed by changing the reaction conditions, both compounds were synthetized. The ratio of xanthohumol C and of its isomer in the different steps of each experiment show that using xanthohumol as the substrate, with half concentration and an ethanol bath duration of 30 min, should present the best set of conditions for the synthesis of xanthohumol C. The synthesis product obtained from experiment 1, i.e., in the standard conditions was employed for the purification of xanthohumol C at the countercurrent chromatograph. Selection of the solvent system for use at the CCC was done based on shake flask experiments, from which HEMWat -4 was selected based on both the partition coefficient and the separation factor for xanthohumol C and xanthohumol. Fractions with a xanthohumol C purity of over 99% (assayed by UV-Vis analysis at 370 nm) were obtained.

In conclusion, in this work a method for the separation of xanthohumol and purification of xanthohumol C using LLC was successfully developed; production of xanthohumol C was concluded through both chemical synthesis and pre-concentration at the CPC; the use of biotransformations for obtaining xanthohumol C still needs further investigation; and LLC needs to be employed in the purification of the back extrusion product. Furthermore, a calibration with the purified xanthohumol C, for quantification of productivity and yield, will allow for a comparison with the other literature methodologies.

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