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Purification of pharmaceuticals and nutraceutical compounds by sub- and supercritical chromatography and extraction

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ACADEMIC DISSERTATION

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Abstract

This thesis discusses the use of sub- and supercritical fluids as the medium in extraction and chromatography.

Super- and subcritical extraction was used to separate essential oils from herbal plant *Angelica archangelica*. The effect of extraction parameters was studied and sensory analyses of the extracts were done by an expert panel. The results of the sensory analyses were compared to the analytically determined contents of the extracts.

Sub- and supercritical fluid chromatography (SFC) was used to separate and purify high-value pharmaceuticals. Chiral SFC was used to separate the enantiomers of racemic mixtures of pharmaceutical compounds. Very low (cryogenic) temperatures were applied to substantially enhance the separation efficiency of chiral SFC. The thermodynamic aspects affecting the resolving ability of chiral stationary phases are briefly reviewed.

The process production rate which is a key factor in industrial chromatography was optimized by empirical multivariate methods. General linear model was used to optimize the separation of omega-3 fatty acid ethyl esters from esterized fish oil by using reversed-phase SFC. Chiral separation of racemic mixtures of guaifenesin and ferulic acid dimer diethyl ester was optimized by using response surface method with three variables per time. It was found that by optimizing four variables (temperature, load, flowate and modifier content) the production rate of the chiral resolution of racemic guaifenesin by cryogenic SFC could be increased severalfold compared to published results of similar application.

A novel pressure-compensated design of industrial high pressure chromatographic column was introduced. A demonstration SFC plant was built and the

immunosuppressant drug cyclosporine A was purified to meet the requirements of US Pharmacopoeia. A smaller semi-pilot size column with similar design was used for cryogenic chiral separation of aromatase inhibitor Finrozole for use in its development phase 2.

Preface

The research described in this thesis was performed in the Chemical Laboratory of the Technical Research Centre of Finland (VTT) during the years 1986–2004. The supercritical extraction work described here was done during the years 1988–1990. Chromatographic purification of polypeptides was carried out as partly Tekes-funded projects during 1993–1997. Chiral work on supercritical fluid chromatography (SFC) started in 1991 and the cryogenic SFC concept was worked out during 1995–2000. The reversed-phase SFC work on fish oil esters was done in 1998–1999 as international collaboration between VTT and Centro Technologico Gaiker, Spain.

I would like to thank Laboratory Director Veikko Komppa for originating the idea of SFC in VTT and assigning me to that research in 1986 which greatly affected my scientific work in the next 15 years. I want to thank my group leader Olli Aaltonen for his long-time support and innovative ideas during these years. He is also the senior author of three out of the five accompanying articles of this thesis. I am most grateful to Salme Koskimies of VTT and prof. Jari Yli-Kauhaluoma of the University of Helsinki for their invaluable guidance and supervision

I thank Sauli Ruohonen of Rauma Ecoplanning Oy for his ingenious engineering in designing the large scale SFC units in this work. Sauli's earlier innovative thinking while leading the design team of the world-famous deep sea submersibles (MIR 1 and 2) led eventually to creative redesigning of industrial high-pressure chromatographic hardware. This, together with the biotechnical skills of Juhani Lundell and his co-workers of Leiras Oy led to fruitful co-operation in developing industrial SFC between VTT, Ecoplanning and Leiras Oy.

I thank my colleagues at VTT and especially laboratory technician Jouko Rakkolainen for his extraordinary skills in building excellent hardware from almost anything. Numerous discussions with my former VTT colleague of twelve years Markku Rantakylä, delighted my day even when things went wrong. In spite of his current busy teaching work, Markku accepted the task of reviewing my thesis. I also thank the other reviewer, Tapani Suortti for his valuable advise in chromatography during many years. My sincere thanks to Lea

Räsänen who took the tedious task of revising the English language of my thesis and complete proof-reading. I also thank Antero Laitinen from our group for advise on how to compile a thesis on supercritical technology. Antero's own thesis formed an excellent model for me to follow. Warm thanks are also due to Riitta Housh of VTT for decades of long friendship and unselfish help on numerous problems when retrieving scientific information

The permission to publish customers' work of Leiras Oy and Hormos Medical is gratefully acknowledged. The Academy of Finland has partly funded this work.

My most sincere thanks are to my family. I would like to dedicate this thesis to my children Sanna and Sini, my wife Paula and Mom.

Espoo, December 27th, 2007

Martti Alkio

List of papers

I Nykänen, I., Nykänen, L. and Alkio, M. Composition of Angelica Root Oils Obtained by Supercritical CO_2 Extraction and Steam Distillation. J. Ess. Oil Res. 3(1991), pp. 229–236.

II Aaltonen, O., Alkio, M., Lundell, J., Ruohonen, S., Parvinen, S. and Suoninen, V. Polypeptide Purification with Industrial-Scale Supercritical Fluid Chromatography. Pharma Tech. Europe, 10(1998), A42–A54.

III Alkio, M., Gonzales, C., Jäntti, M. and Aaltonen, O. Purification of Polyunsaturated Fatty Acid Esters from Tuna Oil with Supercritical Fluid Chromatography. J. Am. Oil Chem. Soc., 77(2000), pp. 315–321.

IV Alkio, M. Chiral, Sub-Critical Chromatography at Cryogenic Temperatures. Proc. ISASF 8th Meeting on Supercritical Fluids, Institute National Polytechnique de Lorraine, Bordeaux, 2002, pp. 754–759.

V Alkio, M., Aaltonen, O. and Setälä, H. Cryogenic Chiral Chromatography for Rapid Resolution of Drug Candidates. Org. Proc. Res. Dev., 9(2005), pp. 782–786.

Contents

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List of abbreviations

CSP	Chiral Stationary Phase
Cy A	Cyclosporine A (also Cyclosporin, Ciclosporin)
DAC	Dynamic Axial Compression
DHA	Docosahexaenoic Acid
DP	Degree of Polymerization
E/F ratio	Extract-to-Feed ratio
E/S ratio	Extract-to-Solvent ratio
EPA	Eicosapentaenoic Acid
FAME	Fatty Acid Methyl Ester
FDA	Food and Drug Administration
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
MW	Molecular Weight
PUFA	Poly-Unsaturated Fatty Acid
S/F ratio	Solvent-to-Feed ratio
SC	Supercritical
SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
sub-SFC	sub-critical Fluid Chromatography

Greek alphabets

α	Separation factor (also selectivity)
δ	Partial difference
ε	Permittivity

ρ	Density
Δ	Difference, Peak distance

Variables

C_d	Weight fraction of the component in starting material
C_m	Solute concentration in mobile phase
C_s	Solute concentration in stationary phase
DC	Dielectric constant
H(gas)	Enthalpy of formation of gaseous compound
H(liq)	Enthalpy of formation of liquid compound
HETP	Height equivalent of theoretical plate
K	Distribution ratio
k'	Capacity factor
L	Length
LR	Load ratio in chromatography
m_m	Amount of mobile phase in column
m_s	Amount of stationary phase in column
M_{sample}	Amount of injected sample in chromatography
$M_{packing}$	Amount of stationary phase in column
N	Theoretical plate number
P	Pressure
P_c	Critical pressure
PR	Production rate
R	General thermodynamic constant
Rs	Resolution
t	Time, time interval
T	Absolute temperature (K)

T_i	Isoentropic temperature
t_a	Residence time of the former peak
t_b	Residence time of the latter peak
T_c	Critical temperature
t_o	Residence time of the mobile phase
W	Width of a peak
W_{avg}	Average width of two peaks
Y	Visual yield of a chromatographic peak
ΔE	Difference of cumulative amount of extract
ΔF	Difference of cumulative amount of feed
ΔG	Difference in Gibbs' energy
ΔH	Difference in enthalpy
ΔS	1. Difference of cumulative amount of solvent
ΔS	2. Difference in entropy

1. Introduction

Since the 1970's numerous industrial and academic research and development laboratories have investigated the fundamentals and process applications of supercritical fluid solvents. This technique offers several advantages over conventional solvents due to the unique properties of supercritical fluids. As illustrated in figure 1 supercritical fluids offer liquid-like density and solvating power while maintaining gas-like viscosity which facilitates good penetration through natural matrices [1]. Furthermore, the separation of the reaction components from the starting material is much simpler and less energy-consuming than those of traditional organic solvents. Supercritical carbon dioxide is definitely the most often used supercritical solvent and it is also considered a 'green solvent' because it is non-toxic and its atmospheric greenhouse capacity is low.

In supercritical extraction the extracted materials are mostly of natural origin. The product can be the extract or raffinate (residue). In extraction, numerous health and other products are manufactured from natural raw materials, including essential oils and aroma compounds [2] food oils [3], fish oils esters [4, 5], small seed oils such as blackcurrant [6] or linen. Their end use are consumer products: nutraceuticals, cosmetics, food additives. End usage include also pharmaceutical raw materials, for example, sterols (cholesterol [7], beta-sitosterol) as well as synthetic and biotechnical materials.

Examples of raffinate end products include food de-fatting [8, 9] and removal of cholesterol from milk fat [10] or egg yolk powder [11]. Raffination is also used in industrial applications [12]. Supercritical CO_2 is used to clean fine mechanical devices, *e.g.* gyroscopes or printed circuits. One major CO_2 application is dry cleaning textiles where liquid CO_2 replaces organic solvents. In the USA there are several CO_2 dry cleaning chains [13**,** 14]. The most important SC process is also a raffination process: the removal of caffeine from green coffee beans that started early in the 1970's [15].

Another group of applications is the use of SC fluids as a reaction medium [16], in materials processing [17] and in chromatography [18]. Supercritical fluids, especially supercritical carbon dioxide offers an elution material whose

properties combine the advantageous properties of liquids (high solvent power) and gases (high diffusitivity and low viscosity) [1].

In chromatography, super- and sub-critical carbon dioxide as the mobile phase possesses outstanding sample/stationary/mobile phase interactions in certain applications that make SFC superior over LC or GC. This is especially the case in chiral or asymmetric separations where the resolving power of commercial chiral stationary phases can be increased substantially by proper adjustment of the process variables [19]. Moreover, the use of very low (cryogenic) temperatures has been found to be an efficient method of enhancing the productivity of chiral SFC.

The aim of this thesis is to solve four problems:

1. Organic solvents are used in the final stages of pharmaceutical and sometimes in food technology. While some solvents (*e.g.* ethanol) are less objectionable, there are also offensive solvents (*e.g.* hydrocarbons, ethyl ether) used in processing lipophilic compounds. In this work the environmentally benign and economically feasible CO_2-based solvent technology was introduced in Scandinavia. The analytical and sensory properties of CO_2 extracted products were evaluated and compared to products obtained by traditional methods. In addition to extraction and raffination, the use of compressed CO_2 as a chromatography solvent (SFC) was investigated comprehensively and new SFC processes were designed.

2. While the basic technology of high pressure extraction is firmly established, the hardware for high pressure chromatography is mainly HPLC-oriented. There was a need to develop SFC hardware for preparative and industrial applications. In designing high-pressure equipment the group members' expertise on deep-sea technology was linked to knowledge of supercritical and chromatographic techniques which led to developing novel SFC hardware.

3. In chiral SFC the resolving power of the stationary phase in some applications was clearly insufficient. Thermodynamic study indicated that resolution could be substantially increased if low temperature or cryogenic conditions are applied. In this thesis the factors affecting chromatographic resolution are reviewed and applications on cryogenic SFC are presented.

4. Optimizing an SFC process is a time-consuming task, albeit computer software by chromatographic vendors can ease things considerably. One complication in optimization is the preparative and analytical work involved. In this thesis rapid non-preparative empirical SFC optimization methods were developed by combining experiment planning with statistical and graphical solutions.

In the accompanying papers, the parameters to extract the essential oils of *Angelica archangelica* with supercritical CO_2 were established and their effect on the sensory properties and analytical compositions were determined [I]. Secondly, super- and subcritical CO_2 was applied in normal, reversed and chiral phase chromatography. An industrial method for the final purification of biotechnically produced immunosuppressive agent *cyclosporine A (also cyclosporin, ciclosporin)* was developed from the laboratory to industrial scale [II]. Reversed phase SFC was used to separate eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) esters made from raw tunafish oil [III]. A statistical method was used to determine the optimum separation conditions. A racemic drug in its development phase 2 Finrozole was resolved in pure enantiomers by enhancing the resolving power of a commercial chiral stationary phase using cryogenic SFC [IV]. Thermodynamical aspects of the cryogenic sub-SFC are discussed. Finally, some potential drug candidates were resolved by low temperature chiral subcritical chromatography and statistical methods were used also here for optimization of the process conditions [V].

2. Supercritical fluids

2.1 Properties of supercritical fluids

A substance is in supercritical state when its temperature and pressure exceed its liquid/vapor supercritical point. At this point the gas and vapor phases unite and their enthalpies of vaporization become zero (figure 2). The solvent power of a supercritical fluid is related to the solvent density in the critical region. As the reduced density increases to values comparable to liquid, the supercritical fluid begins to act as a liquid solvent.

The physical properties of CO_2 and other supercritical fluids are summarized in table 1 and figure 1.

Table 1. Critical properties of some compounds (according to Klesper) [18].

Compound	**Boiling point at 1 atm °C**	**Critical temperature °C**	**Critical pressure bar**	**Critical density kg/L**
CO_2	-78.9 *)	31.3	73.8	0.448
NH_3	-33.4	132.3	112.8	0.240
H_2O	100.0	374.4	227.8	0.344
N_2O	-89.0	36.2	72.4	0.457
Methanol	64.7	240.5	79.9	0.272
Ethanol	78.4	243.4	64.0	0.276
2-propanol	82.5	235.3	48.0	0.273
Ethane	-88.0	32.4	49.3	0.203
n-propane	-44.5	96.8	43.0	0.220
n-Butane	-0.5	152.0	38.5	0.228
n-Pentane	36.3	196.6	34.3	0.232
n-Hexane	69.0	234.2	30.6	0.234

*) sublimation temperature

The temperature ranges in supercritical fluid processes depend on the fluid used and reflect the respective critical values. The majority of supercritical work is done with CO_2 which has a T_c of 31.3 ^oC (304.6 K). For practical reasons water is often used as the heat transfer medium in CO_2 work. This limits the maximum temperature of the process to near 100 ^oC (373 K) which is quite feasible in most applications. At the lower limit, the temperature of CO_2 is clearly limited by its triple point -56.6 ^oC (216.6 K) where carbon dioxide fluid solidifies. The viscosity of liquid high-pressure CO_2 is very low near down to cryogenic temperatures which makes it a favorable solvent for low temperature chromatography. Working with CO_2 at low temperatures is one of the main topics of this thesis.

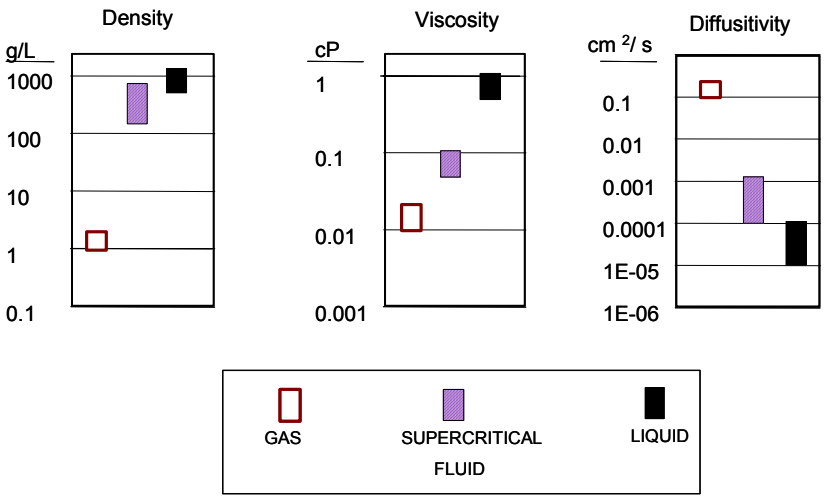

Figure 1. Comparison of physical properties of fluids.

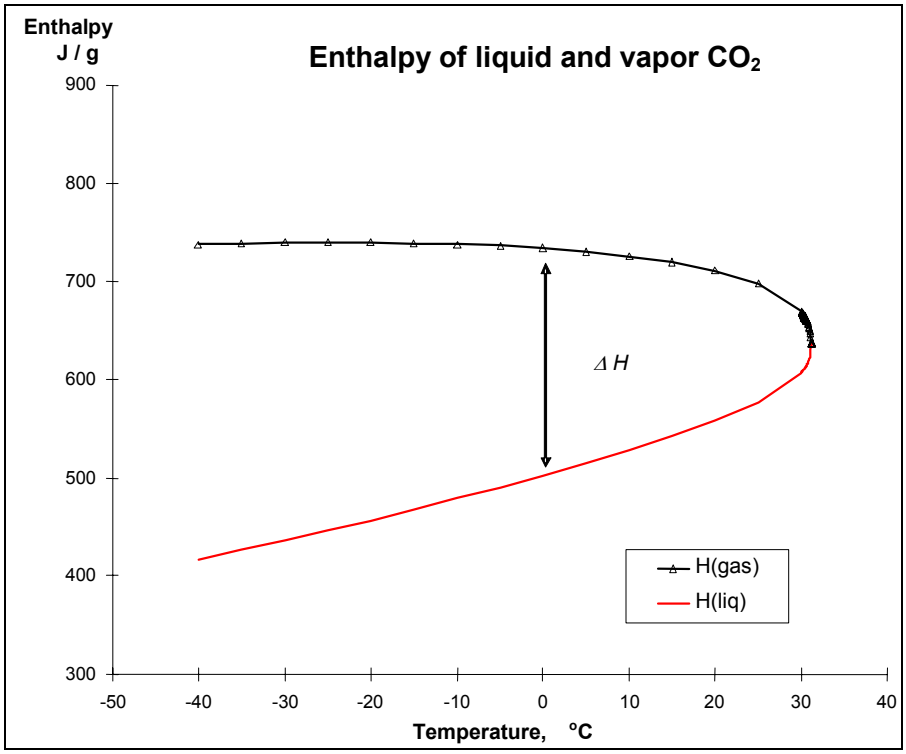

Figure 2. Enthalpies of gaseous and liquid carbon dioxide as a function of temperature. The vertical difference of the curves represents the enthalpy of vaporization at the respective temperature.

Figure 3 depicts the pressure/density isotherms of carbon dioxide. At the two phase region below the critical temperature, the fluid compressibility is infinite (isotherm is vertical) because the liquid and vapor phases are in equilibrium. When T_c is approached, the equilibrium densities of the gas and vapor phases approach each other. At the critical point the discontinuation in the density isotherm disappears. Above the critical temperature, the compressibility near Cp is high. Thus, a small change in pressure results in a large change in density. The fluid is gas-like. When pressure is further increased, the fluid compressibility gradually decreases and it becomes more liquid-like. At high pressures the densities of SC fluids become similar to those of liquids and the fluid begins to act as a liquid solvent.

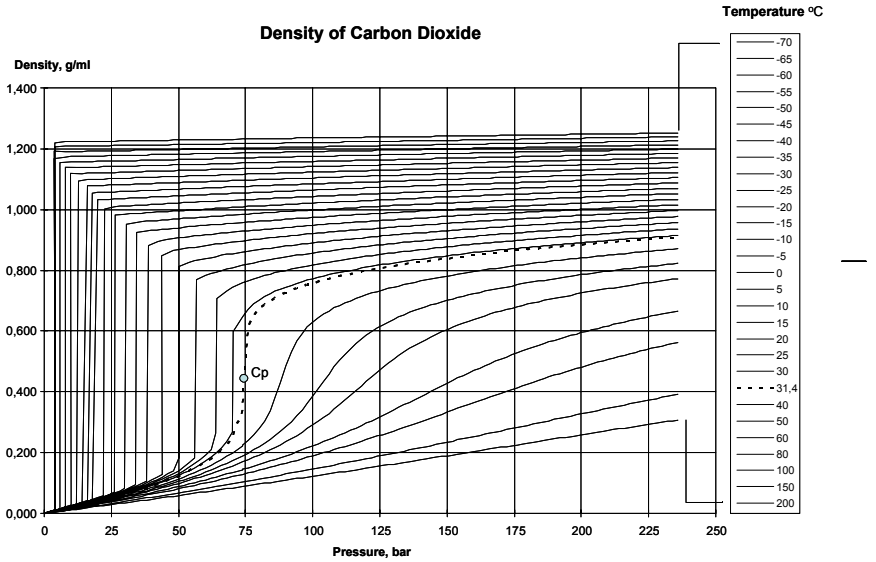

Figure 3. Density isotherms of carbon dioxide. The isotherms are arranged in descending order of temperature. Below critical temperature there are two densities at the same pressure due to the two phases (liquid/vapor or solid/vapor) in equilibrium. Cp = critical point.

Supercritical fluids possess a wide range of solvent characteristics which can be adjusted by the user. Since there is only one solvent phase present in the supercritical state both P and T can be adjusted. The density change vs. pressure change is greatest near the critical region where the solvent properties also change most rapidly. When the density increases the solubility of the less volatile components generally increase. Figure 4 depicts how the density and dielectric constant which is a function of fluid polarity change in a concerted way with isothermal changes in pressure [20].

At high pressures the process economics begins to become an obstacle due to heavy investment costs. In industry, supercritical extraction processes can be categorized as either moderate pressure processes (<350 bars) or high pressure processes (350–600 bars). Pressures in excess of 600 bar are rarely used in industry since the cost of increasing pressure exceeds the advantages obtained at higher pressures.

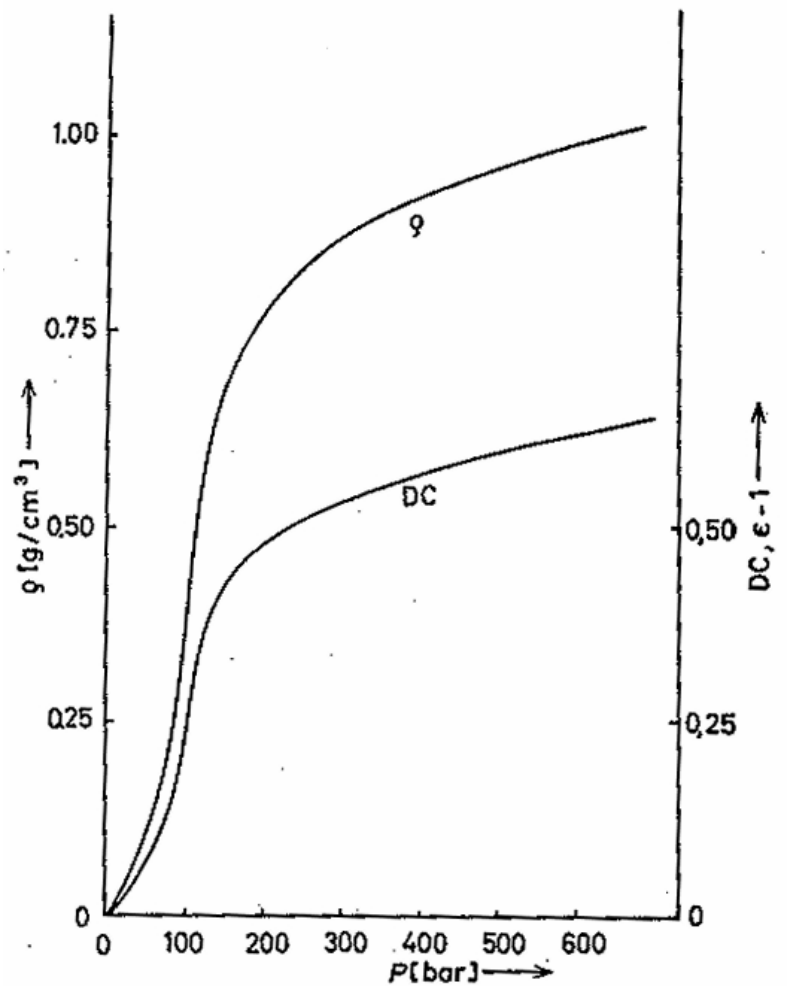

Figure 4. Dielectric constant and density of carbon dioxide as a function of pressure at isothermal temperature of +50 °C [20].

2.2 Other states of fluids

In this thesis, the term *supercritical fluid* is used when both fluid temperature and pressure exceed their respective critical values T_c and P_c. Figure 5 presents the phase diagram of a typical fluid (carbon dioxide) where the fluid states are marked. A fluid at vapor/liquid equilibrium and in the temperature range approaching the critical point is called a *near critical liquid*. An industrially important near critical liquid is water whose hydrolytic properties change

drastically near the critical region [21]. A fluid whose temperature is below T_c but whose pressure is greater than the saturation pressure is simply a *liquid* but is often called a *sub-critical fluid or sub-cooled fluid.* Especially when the temperature of the sub-critical fluid is below the freezing point of water, *i.e.* 273.16 K, it is often called *cryogenic*. A gas whose pressure is below the vapor/liquid saturation line is *superheated or thinned gas*. This is important in steam engines and power plants where water vapor is superheated in order to increase thermodynamic efficiency.

In the sub-critical range there is no point of discontinuity between liquid and supercritical phases when T_c is exceeded. The density and other properties of the liquid change monotonously. This has practical consequences in sub- and supercritical fluid chromatography since at high pressure the temperature can be freely adjusted across the T_c limit.

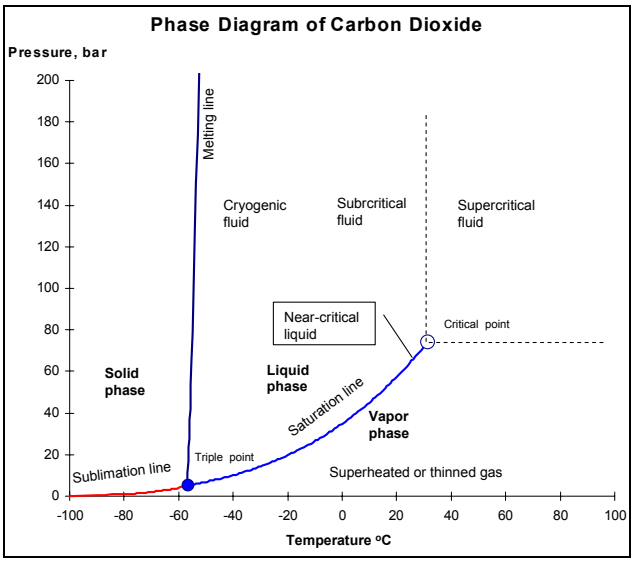

Figure 5. Phase diagram of CO_2. The sublimation, melting and saturation lines are equilibrium curves, i.e. two phases are present in the lines except at the triple point (T -56.6 °C, P 5.2 bar) where three phases coexist and at the critical point (T +31.3 °C, P 73.8 bar) where liquid and vapor phases become one phase. Elsewhere in the diagram there is one phase present. The nomenclature of different fluid states varies in the literature.

2.3 Modification of supercritical fluids

Although there exist many supercritical fluids with different solvent properties (table 1), the majority of applications use carbon dioxide. As discussed previously, the polarity of carbon dioxide can be adjusted to some extent by changing pressure and temperature. Even so, CO_2 is basically non-polar. Its solvent properties are comparable to hexane or chlorinated hydrocarbons such as dichloromethane. Therefore, CO_2 dissolves mostly with lipophilic compounds. Even at high densities pure CO_2 is still relatively non-polar. To enhance its polarity CO_2 can be modified by adding, *e.g.* a short chain alcohol to the fluid. The most common modifiers are methanol, ethanol and 2-propanol. The solubilities of polar compounds in CO_2 increase drastically with modification, typically 1–2 orders in magnitude which would be impossible for an unmodified fluid at any density. This enhancement in solvent power has been attributed to dipole-dipole, dipole-induced dipole hydrogen bonding and other polarity forces [22]. The effect of increasing the solvent density with modification has only a slight effect on increasing the solvent strength.

Low molecular weight alcohols are very soluble in CO_2 as indicated by the phase diagram of the CO_2/ethanol system shown in figure 6. At high pressures ethanol and CO_2 are miscible in practically all proportions, and the intersolubility is enhanced by lowering the temperature. On the other hand, EtOH is only slightly soluble in CO_2 vapor at its vapor/liquid equilibrium pressure which restrains the modifier from recirculating within CO_2 vapor at the solute recovery stage. Therefore, the product from a modified CO_2 process is usually recovered as a modifier solution. This is often a drawback since the recovered modifier dilutes the solution which increases the cost of downstream processing.

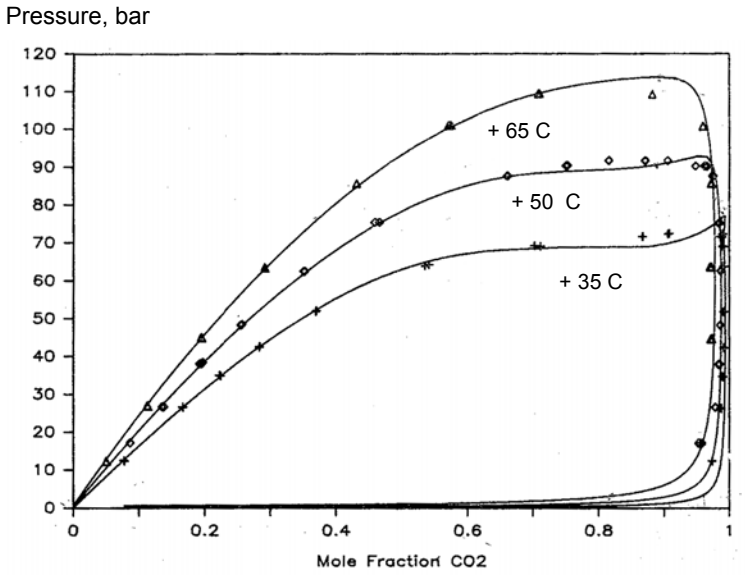

Figure 6. Phase diagram of CO_2/ethanol mixture. The curved lines represent the modeled values and the dots the respective experimental value [23].

In many natural extraction applications the sample contains water which also modifies CO_2. Water forms carbonic acid with CO_2 and changes its polarity. The solubility of water in CO_2 at supercritical conditions is ca. 0.4–0.6% w/w [24]. In many extractive applications modification with water is inadvertent and the inherently lipophilic fluid becomes polar. Instead of the expected oily extract the product may be an aqueous product and at low yield.

In supercritical fluid chromatography, modification not only increases the solubility of polar samples but also affects the chromatographic performance by modifying the stationary phase. This is discussed in more detail in chapter (4.1.5). In chiral SFC the sample materials are often synthetic pharmaceutical products with functional groups that are almost invariably somewhat polar. Frequently this requires modification of the fluid.

3. Supercritical fluid extractions

3.1 Batch extraction

Supercritical extraction can be a batch or continuous process. In a batch process (semi-batch in figure 7) the raw material to be extracted is loaded in a pressure vessel(s) at atmospheric pressure. The vessel is closed, pressurized, heated to operation temperature and the fluid is pumped through the extractor. During extraction the most soluble components dissolve first and hence the composition of the sample and extract change during the process. The fluid is directed through into a pressure reducing valve where its pressure is reduced to a predetermined level. There may be several pressure reduction stages in sequence but in the last stage the pressure is reduced to the fluid saturation pressure where in practice adiabatic flash expansion of the fluid splits it into liquid and vapor phase at equilibrium [25]. The expansion results in an aerosol where the liquid phase often forms a mist within the vapor phase. Eventually the heavier liquid phase forms a layer at the bottom of the vessel where most of the precipitated solute also remains [26].

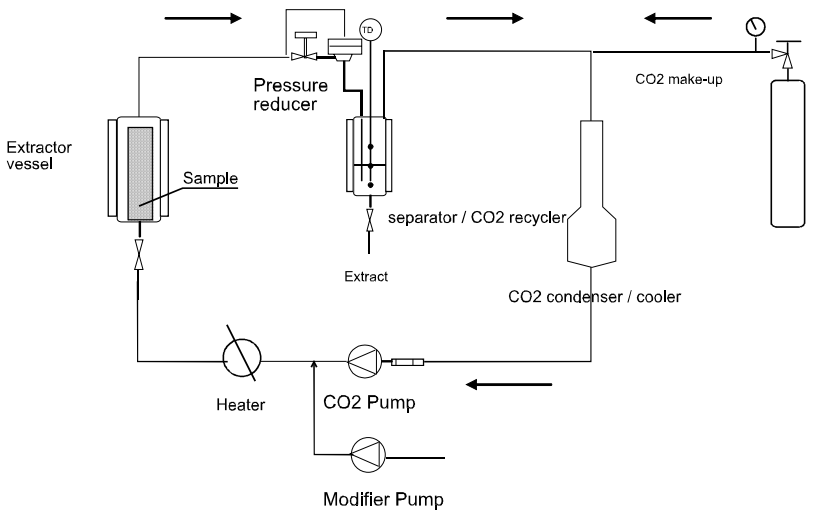

Figure 7. Principle of supercritical batch extraction. There can be several separators arranged in series at decreasing pressures. The process in the figure contains only one separation stage where the depressurized fluid is led via an immersion pipe through the liquid CO_2 pool at the separator bottom. This acts as a demisting device.

In the separation vessel the solvent properties of liquid and vapor CO_2 differ considerably. Solvent power of liquid CO_2 is comparable to that of supercritical CO_2 but the vaporous CO_2 in the separator head-space has only limited solvent capacity. This is also evident from the binary phase diagram in figure 6. Therefore, the product accumulates within the liquid phase at the bottom of the vessel – either as a solution or as slurry. During extraction the liquid CO_2 is gently boiling. The CO_2 vapor is recirculated to the pumps to be repressurized and reheated. Only the most volatile solute components may recirculate within the vapor. If this is not desired, they can be trapped using *e.g.* an activated charcoal line filter.

The scales of supercritical extractions vary:

Laboratory SFE uses 10–1000 mL autoclaves where the sample sizes vary from 1–500 grams. In this scale, recirculation of CO_2 is not usually done, but instead the gas is vented out while the extract is collected at atmospheric pressure. The back-pressure is regulated at the pressure reducing stage with a mechanical or electronic valve. In this thesis electronically controlled varying-orifice pressure reduction valves were used.

Pilot scale SFE uses 1–10 L autoclaves and heavy duty compressors or pumps. The sample sizes may be up to several kilograms per batch. CO_2 is circulated but vented into the atmosphere at the end of the process. The product is collected during extraction or after the collection vessel is exhausted. Pumping rates are 0.5–20 kg CO_2/h. In this thesis, a 4-liter extraction unit was used and the extract was collected in 2 liter separation/recirculation vessel.

Industrial scale SFE Large autoclaves are employed, the sizes ranging from 100 L to 10 cubic meters where sample sizes can be several tons. Industrial pressure vessels are usually locked by quick clamps in order to speed up changes in the batch. CO_2 is recirculated and after extraction CO_2 is recovered in storage vessels. The degree of CO_2 recovery is an economical issue since during system exhaustion CO_2 must be recompressed to saturation pressure from a lower pressure [27]. Pumping rates in industrial SFE vary from 100 kg/h up to several tons/h. In industrial SFE process economizers or heat pumps are used to transfer the latent heat from liquid CO_2 cooling units to CO_2 recyclers.

Figure 7 depicts a typical recirculative pilot-scale batch extraction. To enhance the recovery of the extract, a demisting device is employed. In this case, it is a dip pipe where the gas/liquid/solute mixture is led to the vessel bottom so that the liquid CO_2 pool traps the mist when the gas passes through. The pressure level at the pressure reducing stage is controlled by a back-pressure regulator which also controls the pressure of the collection vessel. Typically the pressure is kept at 50–60 bars where CO_2 boils at approximately room temperature. Since the enthalpy of vaporization decreases near the critical point, it is economical to keep the boiling temperature as near to T_c as possible (figure 2) [1].

During a typical extraction, nothing is removed from the system but the product is collected in the separator(s). In some applications it is possible to collect a liquid extract during the process at atmospheric pressure via a bottom valve. But this is difficult since CO_2 dissolves in the product and when collecting the extract at atmospheric pressure, CO_2 is released as an aerosol. Some applications use closed release vessels in which the product can be collected at an elevated pressure and CO_2 can later be released at a controlled rate. In other applications, there are several small collection pressure vessels in series where the pressure can be released stepwise [28]. The most common method, however, is just to let all extract accumulate within the liquid CO_2 layer at the bottom of the vessel until the process is complete. Then the CO_2 gas is exhausted from the headspace. During exhaust the liquid CO_2 boils away until dryness and the product remains at the bottom of the vessel.

Batch extractions can also be performed in a quasi-counter-current manner in which 2–4 extractors are connected in series. The vessel nearest to the CO_2 inlet is contacted to purest CO_2 and is thus extracted most efficiently. After the extraction of vessel no. 1 is complete, it is refilled with a new sample and the fluid is redirected so that all of the vessels are eventually extracted in a counter-current sequence. The efficiency of serial extraction is clearly greater compared to a system were the same amount of raw material is extracted with the same amount of CO_2. The drawback is the higher cost of multiple vessels and the need to refill the system more often, which also increases the process costs. This setup is used in several processes to remove caffeine [27].

3.2 Continuous extraction

Industry prefers continuous processing methods. In extraction this requires that the sample be pumpable, preferably in a counter-current manner. Usually this applies only to liquid raw materials, *e.g.* various oils. There is one application where dry matter is processed in a continuous way, *i.e.*, the Maxwell House semi-continuous decaffeination process where beans are moved by shocks from the top to bottom of the vessel using lock hoppers [29].

In column extraction, the sample is pumped into a vertical column which contains fillings or a mechanical agitator to enhance the contact between sample and solvent [30]. The extracting solvent is pumped so that sample and solvent move in opposite directions. The continuous phase can be either the oil or CO_2.

A typical counter-current extraction apparatus is depicted in figure 8. Usually oil is the heavier phase and is thus pumped in at the top and removed as a raffinate through a bottom valve at same rate as it is pumped in. CO_2 is pumped in at the column bottom and removed with extract from the top. The extract flow is controlled by the pressure reducing valve. On pressure reducing the pressure is reduced to saturation pressure at which point the extract precipitates in the same manner as in batch extraction. The solvent splits into liquid and gas phases, liquid CO_2 and solute are accumulated at the bottom of the column while CO_2 boils and is recirculated.

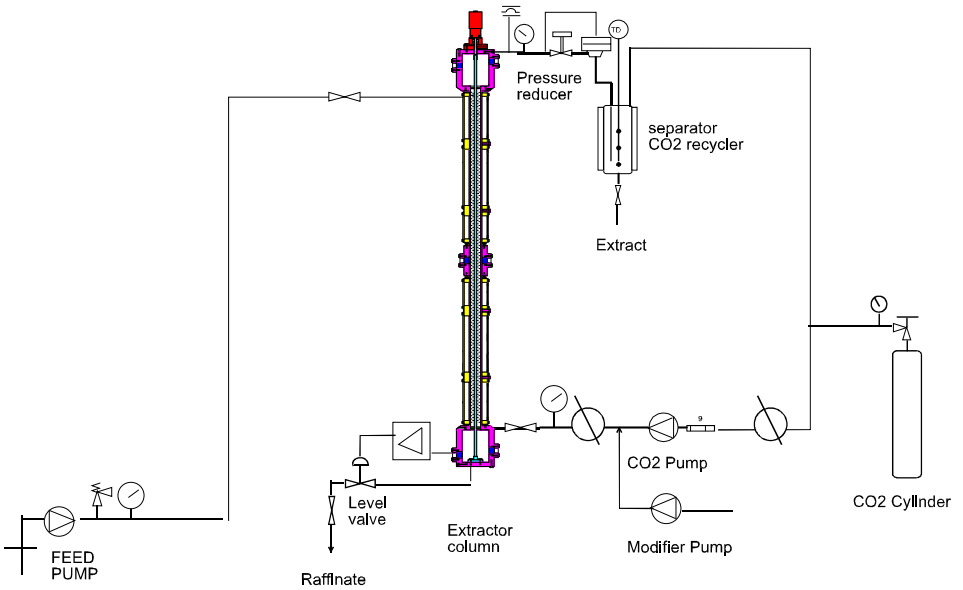

Figure 8. Principle of continuous counter-current supercritical extractor. In this example, the liquid feed is pumped in at the top while the CO_2 solvent is pumped counter-currently from the bottom. The products are collected as the CO_2 extract in the separator and the oily raffinate at the bottom of the column. This column employs an agitated rotating disc (ARD) mixer as the contactor [30].

3.3 Extraction curves and efficiency of the extraction

The fundamentals of supercritical extraction are reported extensively in the literature [31]. In experimental work, the course of a batch extraction is often depicted as an extraction curve, *i.e.*, a diagram showing the recovered product plotted against the amount of solvent used. The data is acquired at time increments so that after a given time, the amount of solvent used and the amount of extract recovered are recorded and the data is plotted as cumulative extract versus cumulative solvent. Instead of absolute values, extraction curves may be scaled for comparison so that the independent variable is the solvent-to-feed ratio (S/F ratio) and the dependent variable is the extract-to-feed ratio (E/F ratio), *i.e.* product yield. An example of such an extraction curve is presented in figure 9.

The derivative of an extraction curve is dimensionless, since the variables are given in the same units

$$\frac{\Delta E}{\Delta F} / \frac{\Delta S}{\Delta F} = \frac{\Delta E}{\Delta S} \tag{1}$$

where

ΔE	difference in cumulative amount of extract, kg
ΔF	difference in amount of feed loaded, kg
ΔS	difference in cumulative amount of supercritical fluid solvent, kg.

The derivative of an extraction curve at any moment corresponds to the dynamic solubility of the sample. Dynamic solubility contains both the effects of extract solubility in the solvent and the mass-transfer rate from the sample matrix to the solvent. The mass transfer or internal friction contains the restraints the supercritical solvent and solute encounter during penetration of the sample. Dynamic solubility is equal to or smaller than the static solubility which is the equilibrium solubility of a component in a solvent. In literature the general term solubility refers to static solubility.

The course of a batch extraction can be divided in three stages as depicted in the extraction curve (figure 9):

1. In the initial phase the solubility is controlled almost exclusively by the static solubility. Mass-transfer rate is not a limiting factor [11]. In this stage, the derivative of the curve corresponds closely to the solubility of the component(s). By definition, the initial stage extraction curve is linear. This is where a majority (80–90%) of the extract yield is recovered.
2. The intermediate stage starts where the linear part of the curve ends. Here the mass-transfer rate becomes significant. This is where process economics in industrial extraction defines the end of the extraction since the gain obtained after this stage is small but the costs go on as long as the process is running.

3. In the final stage the solubility is entirely controlled by mass transfer. Here the dynamic solubility can be several magnitudes smaller than in the initial phase and the extraction in this stage is usually carried only in experimental work. Finally the derivative of the extraction curve approaches zero and no further product is collected.

As a rule of thumb, the S/F ratio should not exceed 10 when process economics are considered. The laboratory extraction depicted in figure 9 shows examples of both economical and less economical extractions.

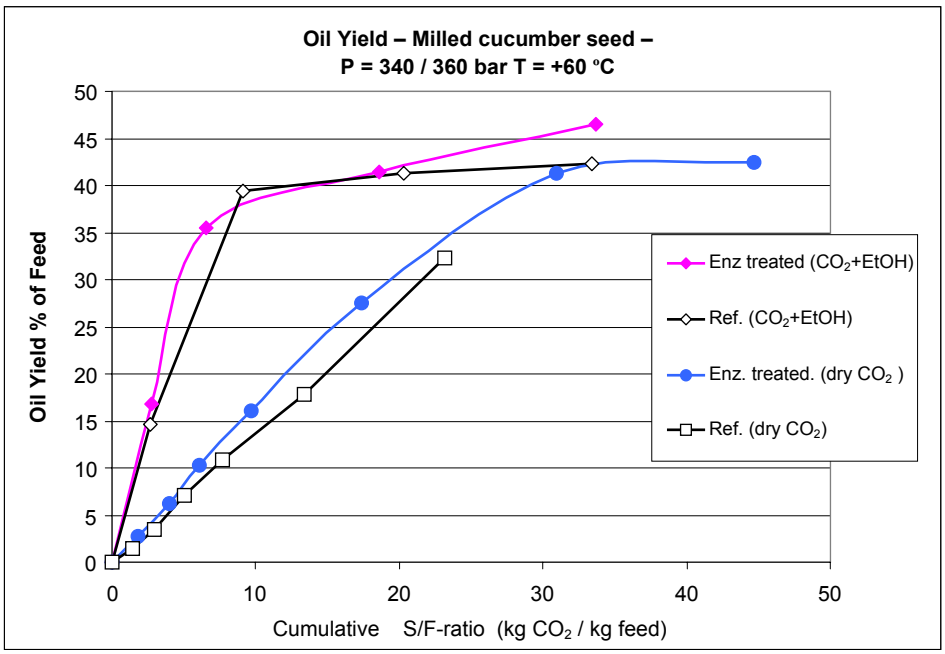

Figure 9. Typical extraction curves in fast and slow extractions. The diagram shows the effect of increasing the inherent solubility by fluid modification. In the figure, cucumber seed oil solubility measured from the slope is ca. 0.05 kg/kg = 5.0% w/w in a modified fluid while in unmodified CO_2 it is only 1.5% w/w. The enzymatic treatment should not affect the static solubility of the oil in CO_2 but it is expected to speed up the mass transfer rates which affect the final stages. From the industrial standpoint, in this case only modified CO_2 could be economical. Data taken from [32].

In continuous extraction, the compositions of extract and raffinate remain constant during steady-state operation. There are no distinct progression stages of extraction. In both continuous and batch extractions, the solvent to feed ratio (S/F) is set by the operator. Since in continuous extraction also E/F ratios are constant, the E/S ratio or yield is also constant [30].

3.4 Experimental results of supercritical extraction of *Angelica archangelica*

The target of this work was to develop supercritical extraction of alcoholic beverage flavor agents. Oy Alko Ab, the Finnish alcohol monopoly, was investigating factors affecting the taste of aromatized wines like vermouth or absinth. In the study presented in Paper I spice materials of the root of Garden Angelica (*Angelica archangelica*) were scrutinized. Carbon dioxide extracts were compared analytically and by sensory evaluation to the product obtained by water vapor distillation. Since the SC extraction process variables are user-adjustable, several extraction pressures were chosen for the study.

The extractions were carried out with the NovaSwiss extraction unit depicted in the cover of this thesis. The set-up is shown in figure 7. The device consists of a 4 liter autoclave operable at pressures up to 600 bars maximum pressure and in the temperature range 10–90 ^{o}C (283–353 K). Pressure reduction was performed at one stage and was arranged using a motorized micrometer needle valve controlled by the back-pressure at the extractor. The rate of extraction (2–10 kg CO_2/h) was controlled by adjusting the two high-pressure compressors.

The work was done as a one step batch extraction, *i.e.*, no intermediate samples were collected during the course of extraction. Therefore, the dynamic solubilities of individual components cannot be determined from the results. The extraction conditions are presented in Paper I.

The dry fragile *Angelica archangelica* root samples were crushed in a hydraulic press without further milling. The extract was collected in a 2 liter high pressure collector. The pressure of the supercritical solution was reduced to collector pressure (50 bars) by the needle valve where the temperature of the fluid dropped below the critical point.

After experiment the CO_2 in the collector vessel was exhausted from the top of the vessel as gas and simultaneously the liquid CO_2 at the bottom boiled to dryness. The semi-solid product in the vessel was dissolved in n-hexane for analysis. Part of the material was collected as such for sensory evaluation. The yield was volatile oils and a white non-volatile component which was recognized as a wax [33]. A total 74 components were quantitatively identified and analyzed as set forth in Paper I. As extraction pressure increased, the proportion of wax increased which is evident in table III of Paper I. At 80 bar extraction pressure all the extracted material were volatiles. At 400 bar extraction pressure only 18% of the extract was volatiles while the rest was wax. However, the total extract yield increased considerably when extraction pressure increased. Thus, the maximum yield of essential oils, 0.6% per dry raw material, was reached at 200 bars when the volatile fraction was 23% of the total extract.

The analytical and sensory evaluations are summarized in figure 10 which illustrates the proportions of volatile oil groups as a function of extraction pressure. As indicated by table II in Paper I, the relative amount of benzopyranoids increased sharply at pressures over 150 bar which coincides with the increase of waxy compounds. The relative amount of mono- and sesquiterpenes decreased as pressure increased. This is not due to a decrease in their solubility but rather to an increase in the yield of less volatile oils and consequently, increase in the total oil yield. The results of the sensory analysis are written on the respective columns in figure 10. According to the evaluations, the compositions at 150 and 200 bar extractions were deemed as acceptable for beverage use. Also the 400 bar extract which resembled that of 150 bar was evaluated as “angelica-distillate like”. The lower sensory score for this extract was attributed to its large wax fraction.

In a follow-up study [34] on temperature effects of SFE at 100 bars extraction pressure and similar setup as in Paper I it was noted that the best sensory ratings were obtained at 20 ^{o}C (293 K) and 40 ^{o}C (313 K) temperatures while at 60 ^{o}C (333 K) and 75 ^{o}C (343 K) the sensory scores were “not acceptable”. At 20 ^{o}C CO_2 was actually liquid, *i.e.* sub-critical. At higher extraction temperatures (60 ^{o}C and 75 ^{o}C) the essential oil yields decreased considerably to ca. 500 mg/kg of root sample while the yields at 20 ^{o}C and 40 ^{o}C were between 2000 to 2500 mg/kg root sample. This is the same order of magnitude as in Paper I. The most

significant difference in composition between the "acceptable" and "not acceptable" extracts was the low benzopyranoid proportion in the latter group.

It might be concluded that a high benzopyranoid content seems to favor an acceptable sensory scoring in the extract while a large percentage of mono- and sesquiterpenes (40–50% of volatiles) gives a weak or thin aroma, regarded as not acceptable. Interestingly the reference standard, steam distillate, contained the lowest benzopyranoid proportion of all samples. As discussed in Paper I some compounds present in the steam distillate but not in the supercritical extracts might be products of reactions between water and the essential oils.

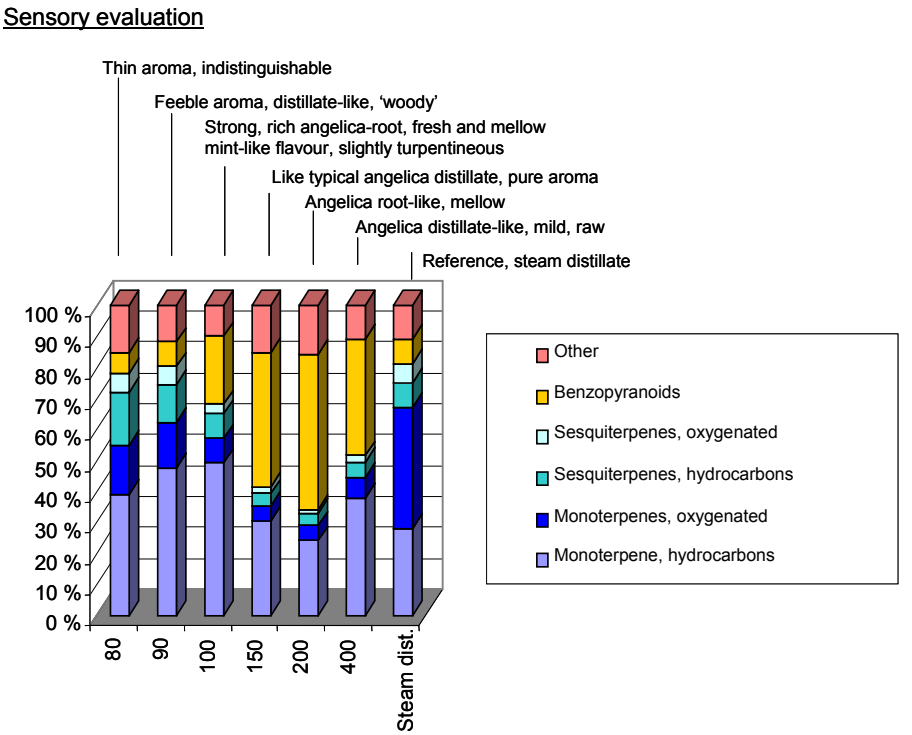

Figure 10. Compositions and sensory evaluations of the supercritical extractions of Angelica archangelica roots. Sensory evaluation was not performed for the steam distillate as it was used as the reference against which the CO_2 extracts were compared.

4. Supercritical fluid chromatography

In all chromatography components are separated by their different affinities to the stationary and mobile phase called eluent. While the mobile phase moves along the column, the solute components are adsorbed and desorbed numerous times between mobile and stationary phases. Eventually individual components distribute within the stationary phase according their respective affinities and at the column exit the components are eluted according to their distributions. Theoretically the solute distributions assume a Gaussian shape.

The use of compressed gases and supercritical fluids as eluent in chromatography was first introduced by Klesper in 1962 [35]. The first applications were capillary analytical chromatographic methods and it was not until 1977 before the technical obstacles of preparative SFC were overcome by Klesper and co-workers [36]. In a series of articles they describe separation of oligostyrenes by molecular weight using porous silica as the stationary phase. As the mobile phase, they used n-pentane modified with 10% of methanol. At $T = 235\ ^{o}C$ (508 K) they separated each styrene oligomer from $DP = 1$ to 49 using MW (average) 2200 as the starting material. More recently, similar separations of polysiloxane oligomers on SFC have been reported by Pyo and Lim [37].

SFC was used mainly as an analytical tool in the laboratory while the industrial development of SFC progressed slowly. The first industrial SFC patents were issued in the early 80's but the first pilot scale commercial prototype was not released until 1992 by Prochrom in France. The production scale SFC work described in Paper II was among the earliest truly industrial applications of SFC.

The properties of SFC resemble both gas chromatography (GC) and high performance liquid chromatography (HPLC). While the solvent capacities of SC fluids are liquid-like, their diffusion coefficients are near those of gases. Therefore, the separation efficiency (resolution, retention times) of SFC generally resembles GC while SFC retains the high loading capacity of HPLC.

The experimental chromatography in this thesis was done with packed columns in which the columns were tightly packed with particulate matter using high pressure. As one of the key targets of this study was the optimization of

production in preparative SFC, the theoretical and practical factors affecting chromatographic performance – especially the factors that affect the chromatographic resolution – are discussed below in detail.

4.1 Resolution and performance

In any chromatography, the practical and economical feasibility is determined by the performance of chromatographic separation. The separation performance is usually measured by the resolution of the most critical pair(s) of components which can be empirically defined as:

$$Rs = \frac{\Delta}{W_{avg}} = \frac{\Delta}{\frac{W_1 + W_2}{2}} \tag{2}$$

where

Rs	resolution of a peak pair
Δ	measure of the separation of the two peaks, min
W_{avg}	average peak width, min
W_1	width of the first peak in a pair, min
W_2	width of the latter peak in a pair, min.

When the peaks' distance (Δ) increases or their widths ($W_{1,2}$) decrease, the resolution increases. Resolution explains the quality of the chromatographic separation more precisely than any other variable. Several analytical equations have been derived to calculate resolution from chromatographical parameters [38]. They are based on various assumptions but the following form for resolution is the one most often applied:

$$Rs = 1/4 * (\alpha^{i} - 1) * \sqrt{N}^{ii} * \left[\frac{k'}{1 + k'}^{iii} \right] \tag{3}$$

where

Rs	resolution of a peak pair
α	separation factor of the peak pair
N	theoretical plate number of the peak
k'	capacity factor of the peak
i, ii, iii	denote the parts of the equation.

The appearance of a chromatogram depends on the resolution and component size ratios. When the size ratio is 1:1 (*e.g.* a racemic mixture), the individual components overlap completely at quite fair resolution. This is because chromatograph is an envelope curve, *i.e.* the sum of the underlying components. This is illustrated in figure 3 of Paper V. Figure 11 below depicts the appearances of ideal resolution curves at size ratios 1:1. The arrowed numbers in the graphs indicate the theoretical purities if the band is split at the middle. According to the figure, even at a fair resolution of 0.5 the peaks look as if there were no separation while the separation would yield 84% purity when split.

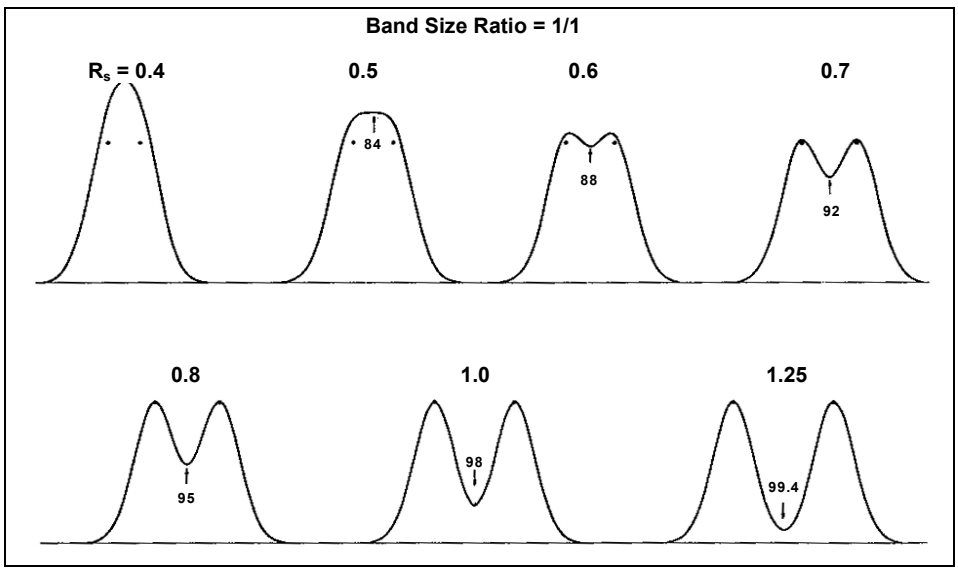

Figure 11. Standardized chromatographic peaks at 1:1 size ratios [39]. The numbers above the graphs indicate peak resolution and the arrowed numbers indicate the theoretical purities of both fractions if the peaks are split at the middle.

Similarly, baseline separation is not required for high purity separation. As indicated in figure 11, at Rs = 1.00 98% purity is yielded without any reject. In industrial work it is economical to load the column as much as possible while maintaining the minimum acceptable resolution. The value of resolution is a function of the three variables marked in equation (3): separation factor *i*, column plate number *ii,* and capacity ratio *iii*. Their properties are described in the chapters below.

4.1.1 Separation factor

Term *i* in equation (3) is derived from the separation factor (α) which is a measure of the selectivity properties of the stationary and mobile phases. Separation factor indicates the relative difference in retention times for a pair of components and it can be empirically measured from the respective retention times

$$\alpha = \frac{t_B - t_0}{t_A - t_0} \tag{4}$$

where

α	separation factor of the peak pair
t_B	retention time of the latter eluting peak, min
t_A	retention time of the first eluting peak, min
t_0	retention time of a non-retaining peak, *i.e.* the residence time of the elute, min.

Separation factor is the most critical parameter for controlling resolution. It can be varied to some extent by adjusting the run conditions and/or by modifying the mobile phase but especially in chiral chromatography the inherent magnitude on a given CSP/analyte pair largely controls the magnitude of the separation factor. In chiral SFC, however, temperature changes affect the separation factor greatly as discussed in chapter 4.1.2 of this thesis.

At $\alpha = 1$, the resolution is zero. A practical minimum for preparative work is about $\alpha = 1.2$ [40], but for excellent separations the separation factor should not be less than 2. When the separation factor is near unity even a small change in α results in a large change in resolution due the subtractive term $(\alpha - 1)$.

4.1.2 Plate number and HETP

In equation (3) the term *ii* defines the separation efficiency which is indicated as the column theoretical plate number N. It is the number of times an average molecule moves between stationary and mobile phase during its time of retention. While the separation factor determines how far the peaks are from each other, the plate number N determines how narrow the peaks are. Together these two define the degree of peak overlap.

In a packed column, N is a measure of the packing quality and is dependent on the particle size. With zero or analytical loads the theoretical upper limit for N is defined by its reciprocal, *i.e.* the height equivalent of the theoretical plate (HETP).

$$HETP = L/N \tag{5}$$

where

HETP	height equivalent of the theoretical plate, mm
L	column length, mm
N	column plate number.

In packed columns the theoretical minimum value for HETP is 2 times the particle diameter. Thus, in a packed column with 5 micron (0.005 mm) particle size, the theoretical maximum of N is 100 plates per millimeter. In practice, the effect of diffusion (4.1.5) and the distribution of particle sizes impair the plate number. The van Deemter curves in figure 14 indicate that by proper adjustment of fluid velocity, nearly theoretical minimum HETP values can be achieved.

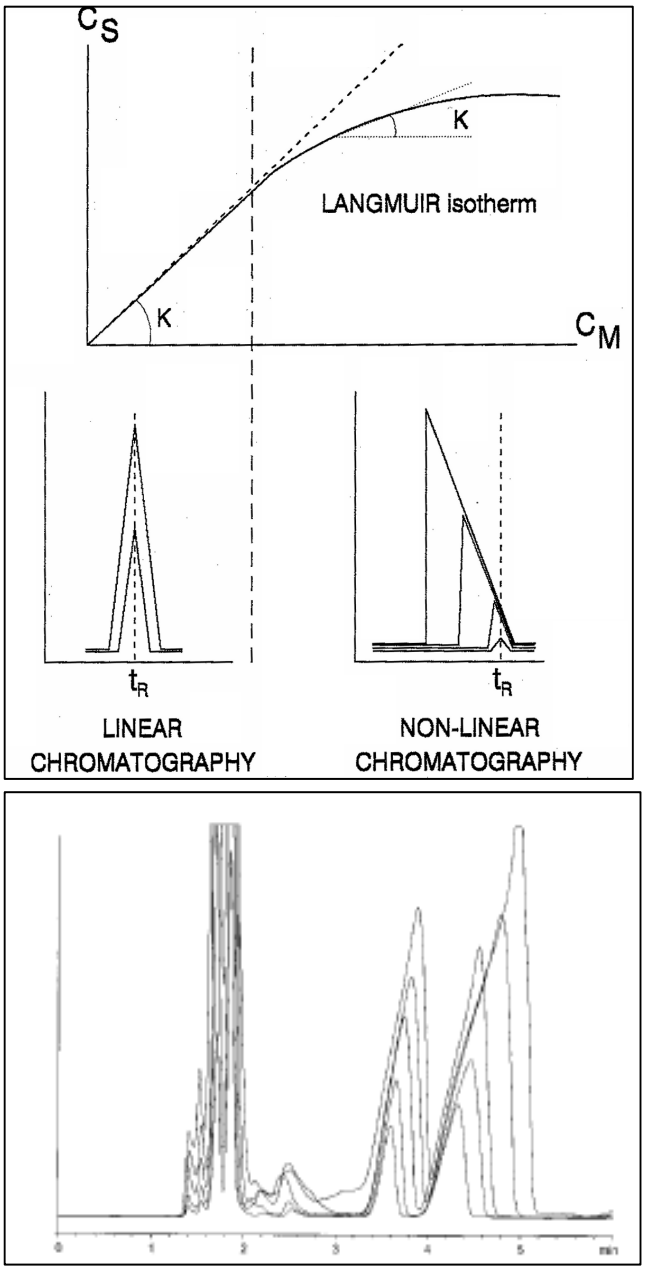

Figure 12. Langmuir adsorption isotherm and its effect on particle shape. Upper graph: The shape of a normal Langmuir isotherm and the respective shapes of a linear and overloaded peak [41] Lower graph: SFC chromatogram for an anti-Langmuir or S-form isotherm [42].

With analytical load, the adsorption isotherm is linear and consequently N is constant. This results in a symmetrical peak of the eluting analyte. With preparative chromatography the plate number depends greatly on the column load since N is a function of the Langmuir adsorption isotherm. Adsorption isotherm (figure 12). presents the solute concentration in the stationary phase as a function of respective concentration in the mobile phase.

In preparative and industrial chromatography the column overload limit is deliberately exceeded and adsorption isotherm is always non-linear. On overloading the adsorption capacity of the stationary phase is consumed and K (the derivative of the adsorption isotherm) starts to decrease, which leads to a decrease in N. In normal overload the Langmuir isotherm is curved downward which leads to a frontal broadening of the peak. The retention time of an overloaded peak is shorter than in an analytical load (figure 12). In supercritical chromatography, anti-Langmuir behavior has also been reported [42] in which the absorption isotherm is concave, *i.e.,* low concentrations migrate faster than high concentrations. One example of this is in figure 12 where phytol is purified with a silica column using 2.5% EtOH modified CO_2.

In chromatography the load is often defined in terms of load ratio which is the amount of injected solute per amount of column packing:

$$LR = M_{sample} / M_{packing} \tag{6}$$

where

LR	load ratio, g/kg
M_{sample}	amount of injected sample on dry basis, g
$M_{packing}$	amount of packing in the column, kg.

For practical reasons the load ratio is expressed as g/kg (or mg/g), *i.e.,* grams of dry injected sample per kilogram of packing. Typical loads for various chromatographies are given in table 2 in chapter 4.3.3.

In overload chromatography the plate number decreases in proportion to the column load. Figure 13 shows an example of the effect of column load ratio on plate number [43]. Due to the large dynamic range involved, it is practical to

present the plate number vs. load ratio in a log-log scale. In this case pure nitrobenzene was injected to test the HPLC column. In the diagram the analytical or linear range extends from zero load to about 0.1 g/kg (log(LR) = -1.0). The onset of the overload range is indicated by the downward curvature of log (N) vs. log (LR) line. With small particle sizes the analytical N is higher but the overload range starts earlier. Coarser particles tolerate higher load before overloading but their plate number starts at a lower level. Above a certain load, N becomes equal for all particle sizes and thus, the higher separation efficiency with more expensive smaller particle seems to be lost. In practice, the chromatographic sample is a mixture and each individual component has a load ratio of its own. In addition, different solutes can have solute-solute interactions which affect their elution. Packing manufacturers emphasize the use of smaller particle size in difficult separations since inexpensive coarse particles often lead to impractical long bed sizes [44].

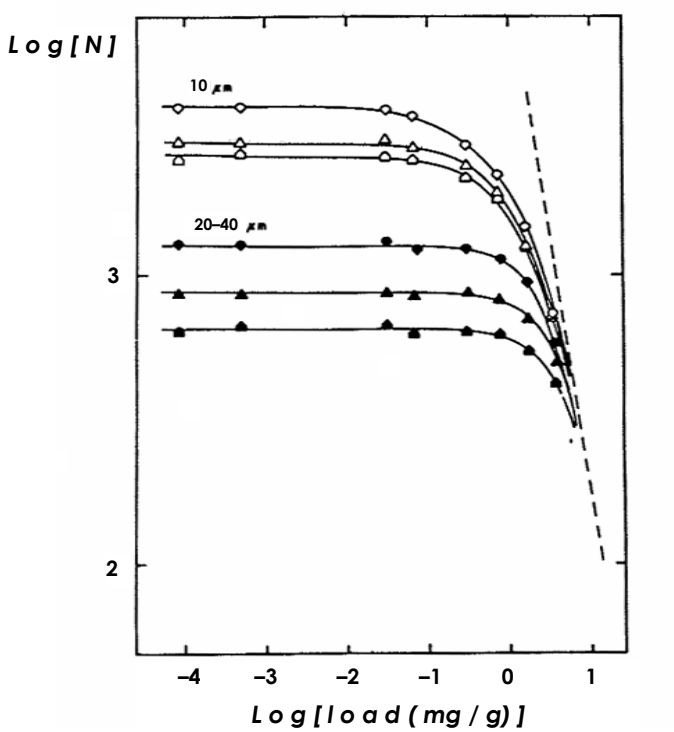

Figure 13. A log-log presentation of the effect of particle size on column plate number (efficiency) in HPLC. Sample nitrobenzene, solvent n-heptane/chloroform, V = 1 mL/min (●), 2.5 mL/min (▲) and 4.0 mL/min (■) [43].

4.1.3 Capacity factor

In the resolution equation (3), the capacity factor k' in term *iii* is the ratio of the amount of solute in the stationary phase to the amount of solute in the mobile phase. It depends on the value of the Langmuir adsorption isotherm and it can be changed by adjusting the solvent strength, especially by modification of the mobile phase. The capacity factor is related to the retention time (equation 8), which should generally be small in order to shorten the injection cycles. There is, however, a minimum range for k', since at very short retention times the peaks overlap.

$$k' = \frac{C_s m_s}{C_m m_m} \tag{7}$$

where

k'	capacity factor
C_s	solute concentration in stationary phase, mg/mL
C_m	solute concentration in mobile phase, mg/mL
m_s	mass of the stationary phase in column, g
m_m	mass of the mobile phase in column, g.

In equation (3) the term *iii* equals the fraction of sample molecules in the stationary phase. When k' increases, resolution also increases but at the expense of run time. The capacity factor is related to the retention times as follows:

$$t = t_o\,(1 + k') \tag{8}$$

where

t	retention time of a peak, min
t_o	retention time of a non-retained peak, min
k'	capacity factor of the peak.

When the capacity factor is zero, *i.e.* when no retention takes place, the retention time is equal to the mobile phase retention time t_o and resolution becomes zero.

4.1.4 Diffusion and van Deemter plot

In chromatography the solute molecules are free to diffuse in any direction as the carrier sweeps them along inside the column. Therefore the linear velocity of the carrier has a considerable effect on column efficiency. The velocity effect is often expressed as a van Deemter plot (figure 14) where theoretical plate height HETP is plotted against the linear velocity of the mobile phase. The plate height of a van Deemter plot is strongly dependent on the diffusion factor of the mobile phase. Thus, in gas chromatography typical optimum velocities are around 200–800 mm/sec, depending on the gas [45]. In SFC the optimum velocity is 10–40 mm/sec and in HPLC about 1–5 mm/sec [46, 47].

In a packed column the optimum velocity also depends on the particle size. Figure 14 compares the van Deemter plots for small particle packings in HPLC and SFC. Due to the smaller self-diffusion of SC fluids, the HETP values are clearly smaller for SFC than for HPLC with 10 μm particles. With smaller 3 μm particles the difference decreases. The authors of figure 14 suggest that the optimum HETP value for a 3 mm SFC is perhaps beyond the plot maximum [48].

According to the figure, the SFC minimum HETP values for 5 μm and 10 μm particle sizes are practically equal to the theoretical minimum values which are 0.010 mm and 0.020 mm, respectively.

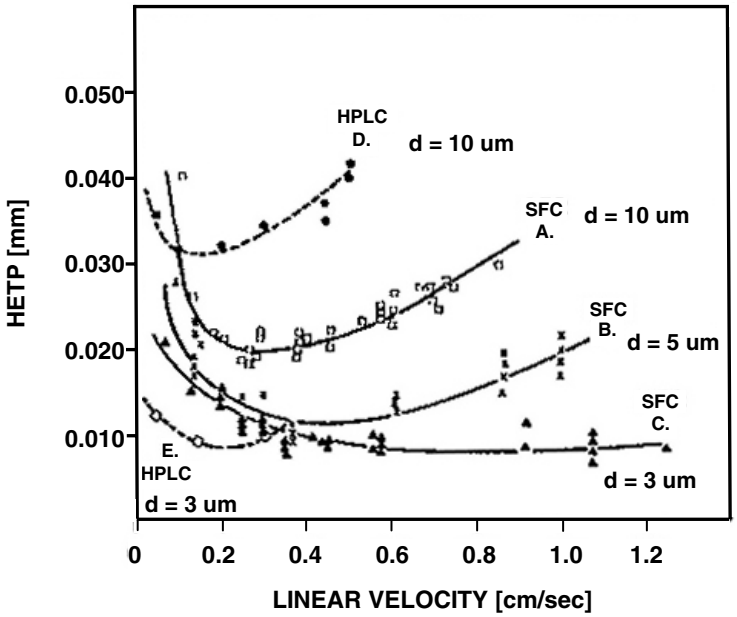

Figure 14. Van Deemter plots for SFC and HPLC runs using various particle sizes. Column: octadecyl silane. [48]

4.1.5 Modification of the mobile phase

In this thesis compressed carbon dioxide was used as the mobile phase. Many applications were for pharmaceutical compounds with polar functional groups which make them only slightly soluble in pure CO_2. To increase their solubility in CO_2 the mobile phase was modified. In modification, light molecular weight alcohol (methanol, ethanol or 2-propanol) is added to the CO_2 fluid by pumping and mixing it as a side stream. The amounts of modifier vary from 1–10% w/w but since they are miscible in nearly all proportions at high pressure (figure 6), there is no actual upper limit. For practical reasons more than 30% w/w modification is seldom used in SFC. Increasing modifier content increases viscosity and the pressure drop across the column (Paper IV). In industrial applications large amounts of modifiers increase cost of recycling and handling – thus annulling the advantage of the 'green' CO_2 solvent. Since modifiers are only slightly soluble in gaseous CO_2 they do not recycle with CO_2 recirculation but are retained in the product recovery stage. Therefore, in applications with modified CO_2 the products are recovered as dilute modifier solutions.

In SFC modifier has a dual function: it increases the mobile phase polarity and the modifier also 'caps' the acid places (partially ionized hydrogen atoms) in stationary phase silica [49, 50]. The magnitude of this effect can be measured by the adsorption isotherm. If uncapped, the acid places tend to retain the solute and cause peak broadening. Modifier interacts with hydrogen and therefore inactivates its effect. Many phase suppliers manufacture also special deactivated silicas where the acid places are substituted by inert compounds such as methyl groups.

4.2 Chiral SFC

Stereochemistry refers to chemistry in three dimensions. Most molecules are three-dimensional but in the evolution of chemical thought the stereochemical view came out relatively late. Stereochemistry can be categorized in several ways but in terms of chromatography, the categorization by Eliel *et al.* as enantiomers and diastereomers [51] is most appropriate. Thus, enantiomers are pairs of isomers related as an object to its mirror image. The difference may stem from configurational difference or conformational differences (*e.g.* sign of torsion angle).

The optically activity of chiral molecules (*gk. cheiron = hands*) is based on one or more asymmetric carbon atoms in the molecule. The most common case is two mirror images or enantiomers. Life is asymmetric because it is based on asymmetric biochemistry which controls physiological processes. In pharmacology the effects of different enantiomers may be subtle or severe. The Food and Drug Administration (FDA) in the USA demands that in chiral pharmaceutical molecules both enantiomers must be scrutinized separately [52]. While most drugs are sold as racemates, about 1/3 of the active pharmaceutical ingredients are single enantiomer drugs.

There are several methods for enantiomeric separation:

- In biotechnical methods the product is asymmetric by nature. An example of this is presented in Paper II.
- In synthesis, the starting materials can be taken from an asymmetric "pool", *i.e.,* they are derived from asymmetric natural sources.

- Producing diastereomers by reaction of the racemic mixture with asymmetric reactant (*e.g*. esterize with *d*-tartaric acid). The resulting pair is not a mirror image pair anymore, but is a diastereomeric pair where the physical properties differ.
- Synthetic reaction can produce symmetric molecules which can further be selectively hydrogenated so that an asymmetric carbon atom forms. A hydrogenation would normally produce a racemic mixture but when an asymmetric hydrogenation catalyst is used, the position of the hydrogen atom favours one position over the other, yielding an asymmetric product. The chiral excess depends on the efficiency of the catalyst. Developing hydrogenation catalysts is one of the most active branches in synthetic stereochemistry.
- Antibody separation [53].
- Chiral chromatography (Papers IV–V) where enantiomers are separated due to their different affinities to the chromatographic phases. These methods are presented below.

4.2.1 Chiral stationary phases CSP

Chromatographic separation is a versatile chiral separation tool since it does not limit the synthesis procedure in any way. The racemic product is produced straightforwardly and separated afterwards. There are numerous CSP's available on the market and in most applications some CSP/run condition resolves the racemate at a sufficient resolution. The drawback in chromatographic separation is its limited capacity, especially in chiral chromatography. The capacities of CSP's are typically an order of magnitude smaller than in non-chiral chromatography. CSP's are also more expensive than achiral phases which increases the investment costs. CSP's are, however reusable and a few standard CSP's can cover most applications.

All chiral SFC applications presented in this thesis are based on chiral stationary phases and non-chiral mobile phases. The molecules in the stationary phases are asymmetric which causes a preference of one enantiomer over the other enantiomer. The difference in the thermodynamic preference between the molecules is small, but with enough repetitions the two enantiomers will

separate. The basic theory of non-chiral SFC also applies in chiral SFC. In chiral separation there are actually two distinctive separations: adsorptive chromatography based on physical preferences and chiral chromatography based on stereomeric properties.

Different CSP's include [54]

- Pirkle-type phases (brush phases)
- cavity phases [55] (*e.g.* cyclodextrins)
- polymer phases (*e.g.* Kromasil CHI phases)
- polysaccharide derivatives [56, 57] (*e.g.* MCA, ChiralCel/ChiralPak)
- ligand exchange phases
- molecular imprinting phases.

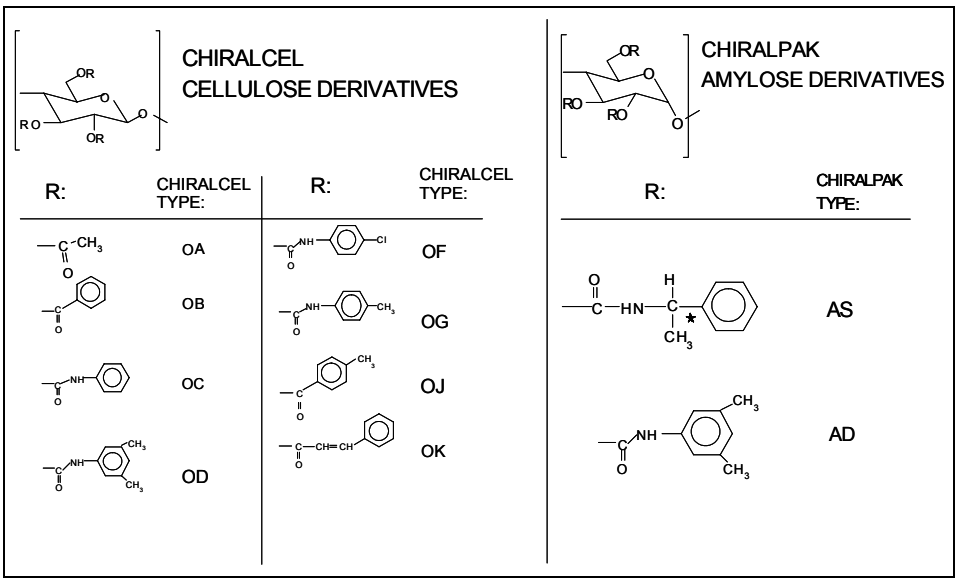

Figure 15. Structures of ChiralCel and ChiralPak CSP's drawn according to Daicel promotion material.

The experimental work in this study was done with polymer and polysaccharide-type CSP's. Figure 15 depicts the structure of ChiralCel and ChiralPak CSP's made by Daicel company of Japan. These phases dominate the CSP market with a nearly 90% share. In the figure the asymmetric centers are derived from

naturally occurring substances, cellulose (ChiralCel) or amylose (ChiralPak). In these CSP's cellulose or amylose are derivatized with a non-chiral substituent, the selector denoted as R in the figure. Selectors can rotate around their axis at the chiral centers but all positions are not steerically equivalent. Therefore the selector induces a preference towards one enantiomer. In ChiralPak and ChiralCel the stationary phase is covalently bonded to a spherical silica-based porous support. The amount of active CSP in these phases is about 20% of total weight.

Another CSP type used in this study was developed by the Kromasil division of Akzo Nobel, Sweden [58]. In these CPS's, an asymmetric polymer derived from L-tartaric acid diamide is covalently bonded to the porous silica matrix. The polymer is esterized with a symmetric selector similar to those of Daicel. In the present study two types of these phases were used, Kromasil CHI-DMB containing 3,5-dimethylbenzoyl selector and CHI-TBB with p-tert-butyl-benzoyl selector.

4.2.2 Temperature effects on chiral SFC

The mechanism of enantiomeric resolution on CSP's generally involves the formation of transient diastereomeric complexes between the optical isomers and the chiral selector. Enantiomeric analytes are equally solvated in the mobile phase but the solvation of the diastereomeric complexes is different, so the eluent composition may play an important role in stabilizing these complexes. According to Gasparrini [19], this complex is more stabilized at a lower temperature which enhances the stereoselectivity in sub-critical conditions. Both solute-solvent interactions and CSP-solvent interactions (dipole-dipole and hydrogen bond) enhance stereoselectivity. Thermodynamically this shows up as differences in the free energy of binding between enantiomers which in turn controls the separation factor. The free energy of binding is:

$$\delta(\Delta G) = -RT\ln(\alpha) = -\delta(\Delta H) + \delta T(\Delta S) \tag{9}$$

$$\ln(\alpha) = -\frac{\delta(\Delta G)}{RT} = \quad -\frac{\delta(\Delta H)}{RT} + \frac{\delta(\Delta S)}{R} \tag{10}$$

where

α	separation factor (selectivity)
ΔG	difference in Gibbs' energy of binding between enantiomers, J/mol
ΔH	difference in enthalpy of binding between enantiomers, J/mol
ΔS	difference in entropy of binding between enantiomers, J/mol·K
R	general thermodynamic constant, m3·Pa/mol·K
T	absolute temperature, K.

From equation (10) it follows that low temperatures will increase the relative importance of the enthalpy term which leads to an increase in the separation factor. Furthermore, there is an isoentropic temperature where both terms in equation (10) are equal and the separation factor $\alpha = 1$, *i.e.,* no separation. Above the isoentropic temperature the elution order is reversed as $\ln(\alpha)$ becomes negative. When $\ln(\alpha)$ is plotted against 1/T, a straight line is expected that crosses the x-axis at the isoentropic temperature. In practice, in the vicinity of the isoentropic point the separation factor cannot be detected accurately since near the isoentropic point both peaks overlap. This is evident also in the similar diagrams published in literature where $\ln(\alpha)$ is presented as zero over a broad temperature range (figure 16) [59]. This agrees with the results presented in Paper IV.

Stringham and Blackwell have published several papers [59, 60, 61] on entropically driven chiral SFC. They conclude that by elevating the temperature above the isoentropic temperature to reverse the elution order, the separation factor is increased. They term this method as "entropically driven" chiral separation. In almost all cases in literature and in this thesis, the isoentropic temperature is high and well above T_c. The authors Stringham and Blackwell list several examples of isoentropic temperatures and corresponding parameters for chiral SFC, and in many cases the isoentropic temperature is more than + 100 ^{o}C (273 K).

The ability to achieve such high isoentropic temperatures is hindering many applications. One major barrier is nonspecific retention increase which is characteristic when the critical temperature of the bulk solvent is traversed. Another difficulty is simply the maximum temperature tolerated by the polymer-based CSP or the solute. The exact isoentropic temperatures in the applications presented in Paper IV could not be determined due to flat range near T_i but no

reversing of elution order occurred below the maximum temperature (+100 °C, 373 K) allowed by the phase manufacturer. Therefore in this thesis, an opposite scheme was employed. Instead of trying to reverse the elution order by high temperature "entropic drive", the separation factor was increased by using cold (cryogenic) modified liquid CO_2. Liquid CO_2 was kept at high pressure to ensure that the mobile phase is monophasic at all temperatures. Paper IV discusses the thermodynamics of the separation of racemic Finrozole by using Kromasil TBB CSP and DMB CSP by Eka Nobel with methanol or ethanol modified liquid CO_2.

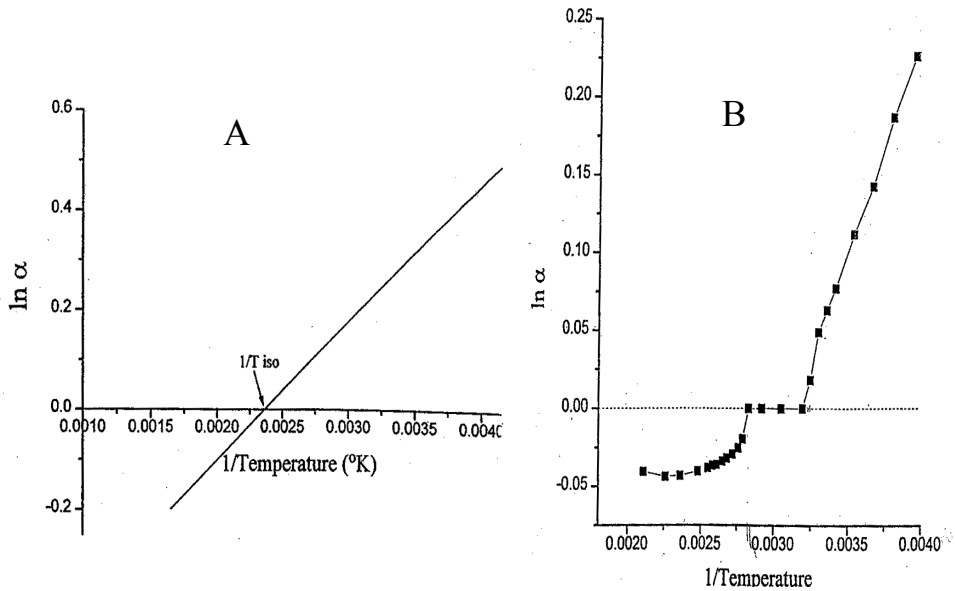

Figure 16. Logarithm of the separation factor as a function of reciprocal temperature [59, 60]. Figure A represents the theoretical plot (according to equation (10)). Figure B represents an actual plot. There is a plateau at ln (α) = 0. This reflects the fact that closely overlapping peaks mask the underlying resolution.

In Paper IV decreasing temperature from ambient to -25 °C (248 K), increased the separation factor from 1.05 to 1.20 as illustrated in figures 2 and 3 in Paper IV. Although the increase in selectivity factor was subtle, its effect on resolution was substantial. The measured resolution increased from Rs = 0.6 at ambient temperature to Rs = 1.0 at -25 °C. In preparative chromatography doubling resolution has a large effect on column loadability and consequently, on process economy. In practice, doubling resolution increases the production rate nearly one order of magnitude.

Similar results for analytical cryogenic SFC are published in literature. Pirkle *et al.* report successful resolution of twelve racemic analytes out of thirteen under cryogenic sub-SFC [62]. The Pirkle research group used temperatures as low as -41 ^{o}C (232 K) and their results for separation factors agree to a great extent with the results presented in Paper IV of this thesis. Cryogenic methods were further applied in resolving guaifenesin and the dimethyl ester of ferulic acid dimer in Paper V of this thesis.

4.3 Columns and capacities in SFC

4.3.1 Column design

In packed chromatography, column lengths at different scales of operation do not differ considerably. Factors that determine column axial dimension are similar irrespective of scale and are discussed in chapter 4.1. The minimum length of the column is determined by the total plate number needed for the separation. The maximum length of the column is limited by the retention time or the maximum feasible mobile phase velocity. In addition, a long column length results in a high pressure drop which in supercritical chromatography is detrimental since a high pressure gradient along the column results also in a high density gradient which further causes peak broadening.

While the column lengths at different scales are uniform, column diameters vary considerably ranging from 1 mm microbore laboratory columns to more than 1000 mm industrial columns. This covers a wide range of capacities but the same basic principles apply with all diameters. With larger diameters, practical problems exist since the mobile phase and injected sample must in all cases be distributed uniformly in radial direction and maintain a plug flow through the whole length of the column. In practice the diffusive motion of solute molecules produce a distribution as illustrated in figure 17. The solute molecules' deviation from ideal plug flow (longitudinal diffusion) broadens the peak which is pulse-shaped when injected.

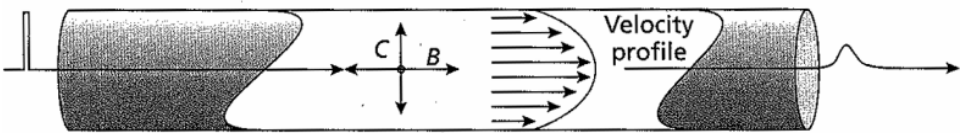

Figure 17. Diffusive motions in packed chromatography column [45]. B = Longitudinal diffusion C = Radial diffusion.

In the laboratory scale, the packed SFC columns are similar to HPLC columns and identical columns are used. At larger column diameters the high pressure and potential hazards of pressurized CO_2 gas/fluid demand different standards than HPLC and the hardware must be designed accordingly.

In industrial applications, the column aspect ratio, *i.e.,* the ratio of column length to column diameter can be much less than 1 despite the difficulty in retaining a uniform radial distribution of mobile phase and sample. The diameter of one of the largest industrial HPLC columns on the market, Novasep LC 1600, is 1600 mm and its indicated maximum bed length is 450 mm. The aspect ratio is thus 0.28.

In the industrial and pilot-size columns of this work (papers II and IV) the radial distribution of sample and mobile phase was solved in a unique way: sample and mobile phase were introduced separately and distributed in the radial direction before the sample made any contact with either phase. Therefore, the extracolumn dead volume of injection was essentially zero. The system is illustrated in figure 18 (chapter 4.3.3) and in more detail in figure 7 of Paper II.

In large scale chromatography, voids can evolve in packing during column lifetime. To compensate, various technical solutions have been developed, most prominently the dynamic axial compression (DAC) technology. In DAC the bed is pressed mechanically (at ca. 20–100 bars) during packing and continuously during column lifetime by using a compression piston which takes up all voids caused by the wall deflections, etc. The DAC columns used in this work were pressure compensated design and developed by one of the authors of Paper II. In this pressure compensation, the pressure is always equal on both sides of the compression piston contrary to ordinary DAC where the piston must be sealed against atmospheric pressure. In pressure compensation, the mobile phase enters the column from the cylinder bottom behind the compression piston and flows through the piston via special channels. Therefore, the pressure is equal on both

sides of the compression piston and chromatographic pressure does not push the piston in any way. Consequently, the particle seals on the piston do not have to withstand the high pressure of SFC against atmospheric pressure. Injection of the sample is done via a capillary tube through the hollow piston shaft.

4.3.2 Column packings

Column volume and thus, the amount of packing is proportional to the square of the column diameter. Column loadability is directly proportional to the amount of packing which is squarely proportional to the column diameter. Therefore, scaling from laboratory chromatography to industrial is straightforward. Examples are presented in table 2.

The most common packing materials in CO_2 chromatography are normal and reversed phase silicas. Normal phases are polar and they are best suited for general SFC. In reversed phases the stationary phase is grafted with lipophilic groups of C8–C18 hydrocarbons. Contrary to HPLC, in SFC a similar mobile phase is used both with reversed and normal stationary phases; only the degree of modification is changed. Reversed phase SFC is used in the separation of lipophilic natural materials (*e.g.* carotenes, FAME's) which in a normal phase SFC would elute too early due to low k' values.

Both spherical and irregular stationary phases are manufactured. Due to their higher cost spherical phases are used mainly in the laboratory. In industrial routine separations, inexpensive irregular silica is preferred wherever possible and spherical silica is used in difficult and final stage purifications.

4.3.3 Columns in different scales of operation

Laboratory columns

Typical laboratory HPLC/SFC columns range from 1–10 mm in internal diameter. They are prepacked with a slurry technique and used in laboratory and bench-scale devices. 1–2 mm microbore columns are used in analytical SFC only. The standard column size in laboratory SFC is 4.6 x 250 mm which

contains ca. 2 g stationary phase. This is enough for milligram-scale preparative SFC where fractions are collected at atmospheric pressure after the pressure releasing valve.

Preparative columns

Preparative columns are prepacked tubes of 10–20 mm in diameter. At this scale of work, recycling CO_2 is not feasible due to the small scale and therefore the spent CO_2 is released into the atmosphere and fractions are collected at atmospheric pressure.

Industrial columns

Industrial columns are self-packing devices with diameters in the range of 50–1000 mm. Although many column packing methods have been developed, the dynamic axial column (DAC) is practically a standard technique in the industry. DAC consists of a column cylinder into which the packing material is poured as a solvent slurry. A hydraulic or pneumatic piston is activated and the piston presses the slurry at a preset pressure against the top frit whereby the solvent is expelled while the particle material is pressed as a tight pack against the cover.

In operation the fractions are collected in pressure vessels at CO_2 saturation pressure and CO_2 is recirculated similarly to supercritical extraction.

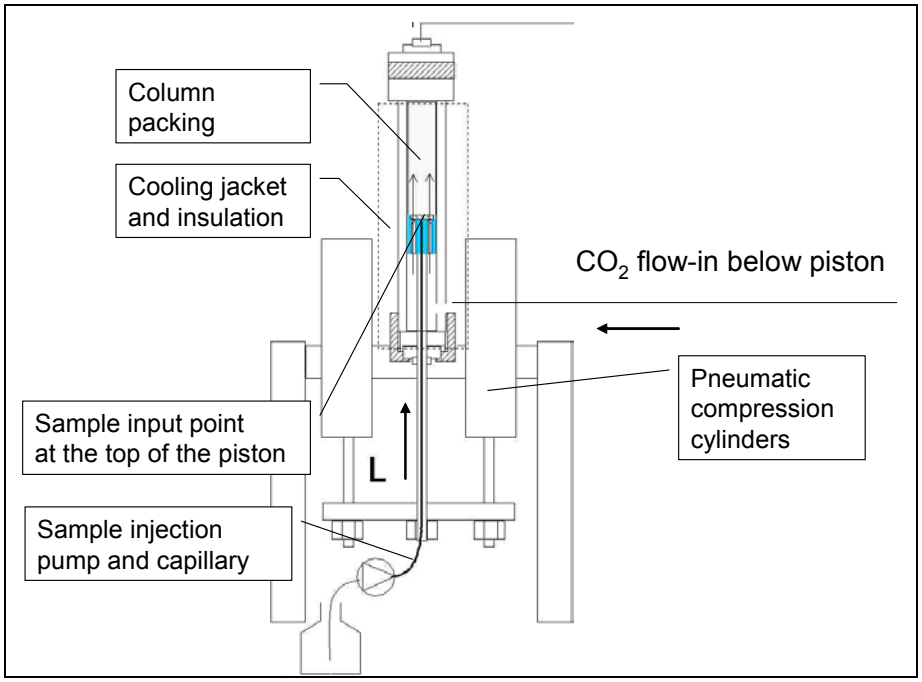

Figure 18. 50 mm pressure compensated DAC column for cryogenic sub-SFC built at VTT.

Table 2. Sizes and typical capacities of SFC columns of equal lengths.

Column diameter	**Column volume (L) at 300 mm packing length**	**Amount of stationary phase in kilograms *)**	**Typical injection size in grams at 10 g/kg load ratio**
Laboratory			
4.6	0.005	0.003	0.030
10	0.024	0.014	0.140
20	0.094	0.057	0.570
Industrial			
50	0.59	0.35	3.5
100	2.4	2.8	14
200	9.4	11.3	56
400	38	22.6	226
800	150	90.5	904
1200	339	203	2035

*) at 0.6 kg/L packing density

In this thesis, column diameters varied from 4.6–10 mm in the laboratory (papers II–V) and from 50–200 mm in the pilot work (papers II and IV). The largest plant presented in this work was equipped with a 200 mm experimental DAC column designed by Rauma Ecoplanning. It contained ca. 10 kg stationary phase and the largest injection volumes were about 250 mL, corresponding ca. 75 grams solute per injection.

4.4 Experimental SFC – Results

The equipment, working procedures, separation and calculation methods of Papers II–V are presented in this section.

Papers II and III present non-chiral SFC where separation was based on normal phase SFC and reversed phase SFC, respectively. Paper III demonstrates a statistical method to determine the optimum run conditions for maximum production rate from a set of laboratory SFC runs and visual assessment of the chromatographs. Preparative verification of the statistical results was performed.

Papers IV and V present chiral sub-SFC of a racemic mixture using cryogenic conditions. Paper IV describes cryogenic sub-SFC in the enantiomeric separation of a synthetic drug racemate. In Paper V a statistical method was used to optimize separation of chiral enantiomers in two cases.

Experimental SFC work was performed in several working scales which are summarized in table 3.

Table 3. Summary of the experimental SFC work in Papers II–V.

Paper	Chapter in this thesis	Aim of the research	Target of separation	Column sizes internal diameter x bed length in millimeters	Stationary phase types used	Results in preparative SFC or sub-SFC
II	4.4.1	To develop the final purification stages for production of pure cyclosporine A	Separation of cyclosporine A from a mixture of cyclosporines	Laboratory 4.6 x 250 Preparative 10 x 250 Production 200 x 300	Normal phase, spherical and irregular silica, cyano- and diol phases	76% pure Cy A intermediate; 98.5% pure final product
III	4.4.2	To produce EPA and DHA from commercial tuna fish oil	Separation of the ethyl esters of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from ethyl esterized tuna fish oil. Statistical optimization of process variables.	Laboratory 4.6 x 250 Preparative 10 x 250	C18 reversed phase, spherical	DHA ester 87–100% purity; EPA ester 54% purity
IV	4.4.3	To produce pure enantiomers for animal tests in phase 2 drug development	Chiral separation of racemic aromatase inhibitor Finrozole	Laboratory, 4.6 x 250 Preparative 10 x 250 Pilot 50 x 300	N,N' diallyl-L-tartramide based chiral synthetic polymer bonded to spherical silica	Finrozole [a] 100% enantiomeric purity; Finrozole [d] 93% enantiomeric purity
V	4.4.4	To synthesize pharmaceutical materials	Chiral purification of racemic guaifenesin and racemic ferulic acid dimer diethyl ester racemate. Statistical optimization of process variables.	Preparative 10 x 250 Laboratory 4.6 x 250	cellulose derivative bonded to spherical silica	not preparative

4.4.1 Polypeptide purification on SFC

Cyclosporines are eleven amino acid polypeptides that are used clinically as immunosuppressants in human transplant surgery. The drug was developed by Sandoz in the 1990's and 25 cyclosporine variants are known. The desired variant is denoted as cyclosporine A. The traditional purification method by low pressure liquid chromatography is slow and solvent-extensive. White *et al.* [63] had previously published a blood analysis method where cyclosporines were analyzed by capillary SFC using pure CO_2 at 150 ^{o}C (423 K) and 200–300 bar pressure with gradient pressure programming. The standard analytical assay of cyclosporines is based on HPLC and in production, cyclosporines are purified using medium-pressure liquid chromatography (LC). Paper II describes a work on SFC based purification of cyclosporines made biotechnically from a new high yield strain of *Topylocladium inflatum* by Leiras Oy, Finland. The cyclosporine A separation method was developed from the laboratory to industrial demonstration plant and used modified CO_2 solvent recycling, normal phase silica and moderate temperatures.

In the laboratory experiments two main equipment setups were used. One setup included Jasco Super 200 preparative SFC with a maximum CO_2 pumping capacity of ca. 10 g/min at 200 bars. It was modified so that recovery of the pharmacologically active (immunosuppressant) compounds could be done safely. CO_2 and modifier were both precooled, pumped as liquids and then mixed, conditioned and tempered. The raw material was injected as a 1–30% toluene solution using Rheodyne 7000 loop injector valve. The loop sizes varied according to application. The column was located in a thermostatted oven and a variable wavelength UV detector was placed after the column and used to monitor the elution. The fluid was led to Jasco's proprietary vibrating back-pressure valve 880–81 where the system pressure was lowered to atmospheric pressure [64]. The valve was located in a transparent and vented collector chamber and was equipped with a 6-position Rheodyne switching valve to fractionate the product. Small volume metal capillary tubes led the product to tared collector glass bottles equipped with mechanical mist separators. The products yielded clear modifier solutions of cyclosporines and by-materials which were analyzed at Leiras Oy research laboratory.

Analytical and non-preparative SFC work was carried out with Hewlett-Packard 1205 A SFC unit equipped with a HP 1100 diode array UV/VIS detector and HP ChemStation SFC software. Its maximum CO_2 pumping capacity was ca. 5 g/min at 400 bar. The runs were recorded by ChemStation software and processed later off-line. This unit was also used for the longevity testing of the silica columns where ca. 1800 subsequent injections per column were processed.

In the industrial work, Rauma Oceanics (later Rauma Ecoplanning Oy, Pori, Finland; currently a subsidiary of Chematur Engineering Ab, Sweden) constructed a full-scale demonstration SFC plant capable of producing ca. 1000 kg pure cyclosporine A annually. The unit was developed jointly by Rauma Oceanics, VTT and Leiras Oy, then a part of the Huhtamäki Company. The unit consisted of two identical 200 mm ID dynamical axial compression (DAC) columns using the pressure compensation technology developed by Rauma Oceanics' submarine division. The details of the unit are illustrated in figures 7 and 8 of Paper II.

The column was packed on-site with 10 kg silica whose loading capacity was ca. 75 grams dry raw material per injection. The maximum pumping capacity was 800 kg CO_2 per hour at 200 bars but only half of this capacity was used in the work. A high pressure modifier pump added modifier (2-propanol) at ca. 100 kg/h. The products were collected in a 30 liter pressure vessel guided by the signal from a side stream UV detector. The collected samples were drained into bottles after releasing collector pressure. Carbon dioxide was boiled and recycled in the same manner as in SC fluid extraction. About one-tenth of the modifier stream recirculated within the CO_2 vapor. The rest of the modifier was recovered as liquid with the products.

Results

The starting material was a toluene extract of the cyclosporine fermentation broth. Due to the large UV absorption of toluene, solvent swap to methanol was done to prevent the increase in background UV absorption in the recirculating fluid. In overload SFC chromatography, the injection volume must be as small as possible, therefore the high load injections were done with concentrated solutions [43]. The maximum practical injection concentration was ca. 30% w/v. The injection solvent, methanol was stronger solvent than the mobile phase

which was CO_2 modified with 20% 2-propanol. This did not, however affect to the peak shapes.

The starting material contained ca. 23% of the desired material, cyclosporine A (Cy A) on a dry basis. The rest was a mixture of other cyclosporines. US Pharmacopoeia demands that the content of Cy A must be at least 98.5% and the contribution of any other cyclosporine must be less than 0.7%. In preparative laboratory SFC runs at ca 5.5 g/kg load ratio, 97.7% pure Cy A could be produced in a single run at nearly 100% yield. However, the column longevity tests revealed that some impurities accumulated within the silica causing changes in stationary phase capacity. Therefore a two-stage process was designed where in the first stage an inexpensive consumable silica was used to prepurify the solution to yield an intermediate Cy A concentrate.

In the final setup the industrial SFC prototype unit yielded Cy A at ca. 58% purity in the first stage which used DuPont Spherisorb 40 μm silica as the stationary phase. In the second stage the column was packed with Kromasil 10 μm spherical silica. This stage yielded ca. 98–99% pure Cy A which after recrystallization met the requirements of US Pharmacopoeia.

According to the economic calculations in Paper II, in 1999 the non-optimized purification costs using the pilot plant described above were 1000 DM/kg product which corresponds to ca. 500 e/kg. The current market bulk price of cyclosporine (CIF Main Indian Ports) is USD 5500/kg corresponding to 4100 e/kg as of August 2007 [65].

4.4.2 Purification of fatty acid monoesters from tuna fish oil

Tuna fish oil contains several omega-3 polyunsaturated fatty acids which are often sold as triglycerides, *e.g.,* cod liver oil or other low value products. Some pure ω-3 acids, most notably eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are quite valuable as pure components. Paper III describes a method where commercial tuna fish oil was transesterified with ethyl alcohol and the resulting ethyl monoesters were separated using reversed phase SFC. Since fatty acid monoesters are only weakly polar, their retention (the k' value) in normal phase silica is too weak to make SFC possible. Therefore,

octadecylsilane (ODS) reversed phase was used as the stationary phase. The mobile phase was unmodified CO_2.

The laboratory hardware was similar to the HP 1205 SFC presented in the previous chapter, but it was modified to adapt the JASCO 880-81 vibrating pressure reducing valve at the fraction collector instead of HP's own variable orifice valve. In the preparative runs, an ethanol flush was added after decompression in order to quantitatively wash the semi-solid products from the fractionating valve. The setup is depicted in Paper III, Scheme I.

In this work the process variables were optimized using a statistical method. It differs from the traditional procedures used by chromatography vendors where simulations are usually applied. In the present work, there were initially 4 main independent variables: column load, CO_2 pumping rate (~ linear velocity), column temperature and column pressure. The dependent variable to be maximized was the production rate of EPA or DHA. The production rate was defined as grams of EPA or DHA ester, respectively, that one kilogram of stationary phase can yield to specifications in one hour.

The target variable was defined as:

$$PR = LR * C_d \frac{Y/100\%}{t} \tag{11}$$

where

PR	Production rate, g pure product per kg stationary phase per hour
LR	column load ratio, grams injected per kg stationary phase
C_d	weight fraction of the desired component in the starting material
Y	visually determined yield of the cut area, as percent of the total chromatogram area
t	interval between successive injections, hours.

In the statistical runs no preparations or analyses were done. The production rates were calculated by estimating the recoverable yields from each peak

visually as shown in Paper V, figure 3. A general linear model of the four independent variables was fitted to the experimental data. The model was optimized in two steps using multivariate methods and the optimum values for the independent variables were established. The optimized values were verified by preparative SFC runs and by analyzing the preparative products by standard GC methods. In the preparative verification runs, column load was varied in order to verify the target variable's sensitivity to load.

Results

The optimum conditions for maximum simultaneous EPA and DHA production are summarized in Paper III. According to the statistical results 2.46 g purified DHA ethyl ester and 0.16 g purified EPA ethyl ester were expected per kg stationary phase per hour at 4.95 g/kg load ratio. However, the preparative verification yielded EPA ester at only ca. 50% purity while DHA ester was collected at 90–100% purity. At optimized conditions DHA ester was yielded as 80% pure product at 1.90 g /kg/h production rate. At a lower load (2.5 g/kg) DHA ester was collected at more than 90% purity at 0.85 g/kg/h production rate. Simultaneously 40% pure EPA ester concentrate was collected at 0.23 g/kg/h production rate.

The production rate was strongly dependent on the required product purity. 80% pure DHA concentrate could be produced at nearly 2 g/kg/h. This corresponds to ca. 1 kg production in 24 h when using a pilot plant of similar size to was used for the cyclosporine separation in Paper I. If 95% minimum purity is desired, then the pilot plant production rate would drop to ca. 0.25 kg/day.

The results indicate that there is a source of error in the visual determination. Obviously other underlying polyunsaturated esters co-elute with EPA and DHA and remain undetected in a purely visual analysis. Figure 19 illustrates some of the partially co-eluting compounds of EPA and DHA ester peaks at various preparative loads.

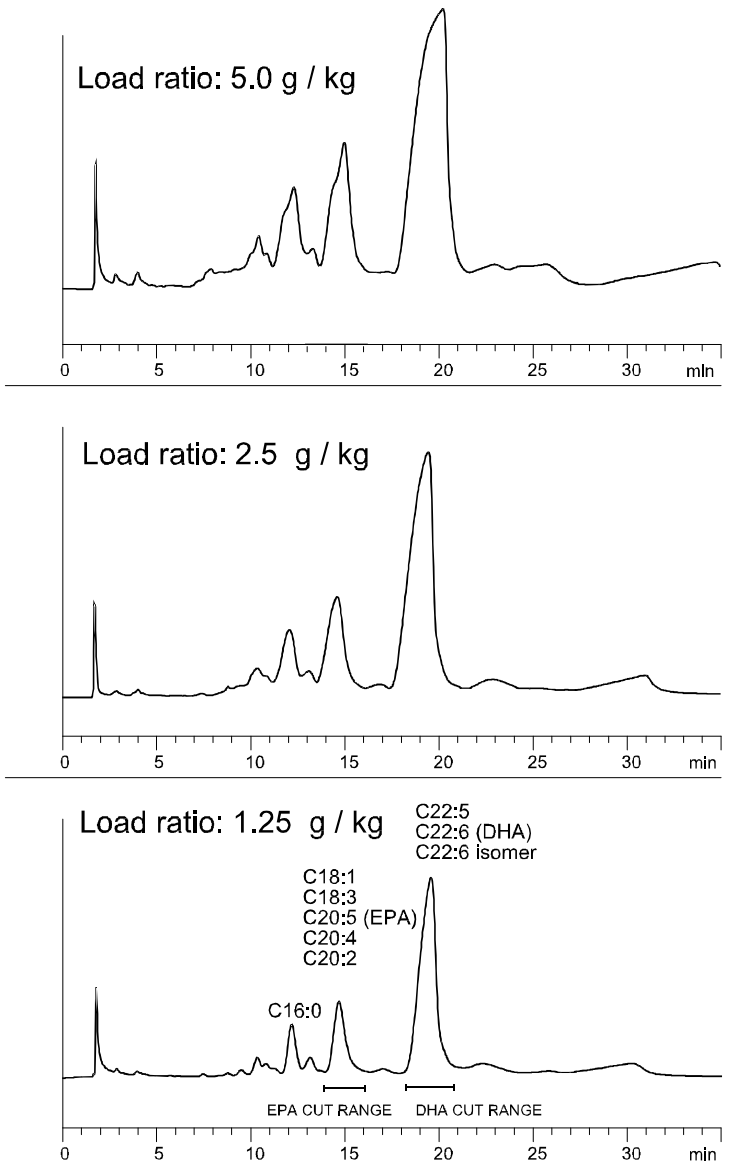

Figure 19. Preparative SFC chromatograms at various loads. In the figure with the lowest load, the partial co-elution of some other poly-unsaturated (PUFA) esters with DHA and EPA are marked (from Paper III).

It is concluded that a visual-statistical SFC method is applicable for determining the optimum in multi-component SFC only if caution is taken in the

interpretation of the chromatographic raw data. A preparative check run and analysis at optimized conditions is necessary. The visual method is clearly more appropriate in binary separations, *e.g.* in separation of racemates, as the racemates generally do not contain any other significant compounds than the enantiomers themselves. This is demonstrated in chapters 4.4.3 and 4.4.4 and the respective papers IV and V.

Tuna fish is not a very favorable raw material for EPA production since its EPA content is relatively low (5.3% of all fatty acids). For DHA tuna fish oil is much more favorable. In the present study its DHA content was 23.7% of all fatty acids which rates tuna fish oil among the richest DHA sources in marine industry [66].

Despite the lower-than-expected yield of PUFA esters in this thesis, SFC is still quite a competitive method in the purification marine fatty acid esters as described in the economic calculations in Paper III. The purification costs of DHA and EPA concentrates were estimated at USD 550/kg which conforms with the 200–500 USD/kg reported by K.D. Pharma Company [67] in 1994. Indicative is also a relatively recent decision by the Board of Appeal of the European Patent Office regarding a patent dispute over SFC purification of polyunsaturated fatty acids [68].

4.4.3 Chiral sub-SFC of aromatase inhibitor

Paper IV describes the chiral separation of a pharmaceutical product in its phase 2 development. The resolved compound was an aromatase inhibitor, Finrozole by Hormos Medical Oy, Turku, Finland. Its indicated use is male urinary dysfunction but also use as a veterinary indication has been patented [69]. There are two chiral centers in Finrozole, so two diastereomeric pairs exist. The samples in the present work were purified to racemic (RS' and R'S) enantiomers are denoted as *a* and *d*.

Paper IV summarizes the work on cryogenic sub-SFC which was developed to enhance resolving of Finrozole enantiomers. The resolution was initially unfeasible on all tested CSP's. When the research started in 1995 cryogenic sub-SFC was a novel method and not mentioned in literature.

The laboratory SFC equipment employed was modified for cryogenic use from the units described in Paper III. The column was placed in an insulated cooling jacket where the cooling fluid was circulated. The fluid exit temperature was measured by a sensor and this was considered as the column temperature. The refrigeration was done with a Lauda F6 cryostat having a minimum operating temperature of ca. -30 ^{o}C (243 K). With this setup, about one milligram per injection could be processed.

For pilot scale work, a high pressure 50 mm SFC column with dynamic axial compression (DAC) mechanism and adaptation to cryogenic work was constructed. The column was made using the same pressure compensated design as in the industrial DAC device presented in Paper II. The incoming mobile phase was precooled with a Lauda F6 laboratory cryostat and the DAC column unit was cooled with a Lauda 40 large scale refrigerator. The unit's minimum operation temperature was below -40 ^{o}C (233 K). The CO_2 flow was ca. 7–8 kg/h at 200 bars. The sample was injected as a toluene/THF solution via a HPLC pump directly under the frit in piston. A side stream UV detector was located after the column across the flow control valve. Column inlet pressure was controlled by the CO_2 circulation pump's own pressure control. The column is depicted in figure 18 and the whole unit in figure 20.

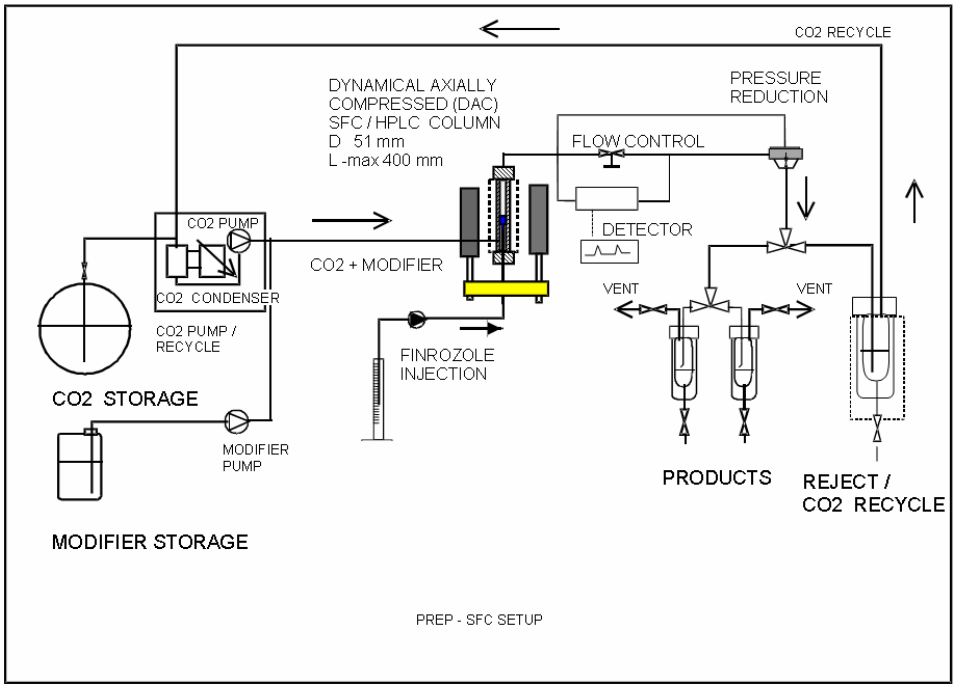

Figure 20. The schematic layout of the experimental unit used in the multi-gram scale chiral SFC of Finrozole (from Paper IV).

The mobile phase was liquid CO_2 modified with 5–10% w/w ethanol. After the column, the fluid was led to the CO_2 recirculation vessel where high pressure liquid CO_2 was split into vapor/liquid CO_2 boiling at equilibrium. The reject together with a majority of the modifier was collected at the bottom of the vessel while CO_2 vapor was recirculated with part of the modifier.

The enantiomer fractions were cut into high pressure vessels via manual valves and the CO_2 recirculation was omitted. To ensure system purity, there was no recirculation in the collectors but the vessels slowly filled up as the fractions were collected. Before the next injection, the CO_2 in the collector vessel was exhausted from the headspace and vented while the collected solute and modifier remained at the vessel bottom.

Results

In the laboratory experiments at supercritical and ambient temperatures, the racemic Finrozole resolved only slightly with Eka Nobel chiral phases Kromasil CHI-DMB and Kromasil CHI-TBB. It did not resolve with Daicel ChiralCel phases in the preliminary tests. The largest SFC separation factor was ca. 1.05 with Kromasil CHI-TBB when methanol was used to modify CO_2. This was too close to unity for practical separation. Using the principles outlined in chapter 4.2.2, the separation factor could be increased substantially when the temperature was lowered to cryogenic temperatures. Even though this decreased the mobile phase diffusion rate which resulted in a broadening of the peaks, the maximum resolution was achieved at ca. -30…-25 °C (243–248 K). At this temperature the separation factor was about 1.20 which was sufficient for preparative work. The thermodynamic results presented in Paper IV agree with those published in literature [59, 60, 62].

Table 4 summarizes the enantiomeric purities and yields in different chromatographic projects. Typically, one project consisted of 100–150 injections. In laboratory sub-SFC, both enantiomers could be purified to requirement (roughly 93–95%). In pilot sub-SFC, *a*-form was recovered at 95–100% purity but the second peak yielded only ca. 80% pure Finrozole *d* when cut points were adjusted at a reasonably economical level. This was due to the excessive peak tailing of the *a*-peak at low temperatures. Therefore the latter *d*-fraction was purified twice. For safety reasons, methanol was replaced with ethanol as modifier in pilot scale runs. The analyses were done with laboratory SFC at 0 °C (273 K) using methanol as the modifier and Kromasil CHI-TBB as the stationary phase.

Table 4. Summary of preparative resolutions of racemic Finrozole. In all cases, the CSP was Akzo Nobel Kromasil CHI-TBB.

Year	Column id x length in mm	Typical separation temperature	Modifier	Total yield mg	Purity of Finrozole *a*	Purity of Finrozole *d*
1996	10 x 250	-13 °C	9.0% MeOH	130	100.0	97.2
1998	10 x 250	-25 °C	7.0% MeOH	135	99,5	93.8
1999	10 x 250	-28 °C	7.2% MeOH	300	100.0	95.1
2000	50 x 270 DAC	-13 °C	6.0% EtOH	3100	100.0	80.0 / 93.1
2001	50 x 270 DAC	-21 °C	7.4% EtOH	3285	100.0	79.0 / 92.9

In summary, preparative separation of Finrozole enantiomers was possible with cryogenic sub-SFC using Kromasil CHI-TBB stationary phase. The loadability was low and numerous injections were required for gram scale work on a 50 mm column. For industrial production, the need for a more selective CSP is obvious.

4.4.4 Optimization of chiral SFC using a rapid method

In industrial chromatography optimization of chromatographic conditions is critical. In optimization, the values of user-controlled variables are established and their validity define the economical and practical feasibility of the process. The procedures of method development in analytical chromatography are not practical for optimization in production-scale as the targets are different. There are commercial optimization software especially by chromatography vendors, but these are often related to the companies' own phase materials or hardware. Moreover, these software are usually made for HPLC, while SFC and especially cryogenic SFC applications are rare.

Paper V describes a quick empirical method to determine the optimum conditions for chiral SFC separation of racemic drug materials using only

standard statistical software. This procedure is further derived from the statistical 3-step method presented in Paper III. Since the starting material is a purified racemate, there are only two components present. Therefore, one can judge the yields visually from the chromatographs with only minimal uncertainty. Thus, if a single peak was visible, then the yield was defined as zero. If both peaks were visible, the yield was measured as illustrated in Paper V. The proper cut points were determined from the chromatographic curve by using the peak parameters (unsymmetry, width and distance) as a guide. When cut points were established, the respective peak areas were integrated by computer chromatography software. The minimum time interval between successive injections was also determined from the curves. The total daily production rate of both enantiomers was calculated similarly to the method described in chapter 4.4.2 and the same target function was to be maximized.

Guaifenesin and ferulic acid dimer diethyl ester (FADDE) were selected as two examples to demonstrate the optimization method. Guaifenesin is an anti-inflammatory agent and FADDE is an experimental intermediate of the lignan group.

Three levels of four independent variables (modifier %, column temperature, fluid linear velocity and load ratio) were determined by preliminary tests. A full experimental matrix would require at least $3^4 = 81$ tests to cover all possible combinations. This number was reduced by using matrix orthogonization where the 4-dimensional test space is covered only partially. The ortogonization was done by the Modde 5.0 experimental design software which reduced the number of experiments to 19 for guaifenesin and to 25 for ferulic acid dimer diethyl ester which had four temperature levels.

Results

Two types of chiral columns, ChiralCel OD by Daicel and Kromasil CHI-TBB by Akzo-Nobel, were tested for resolving guaifenesin and ferulic acid dimer diethyl ester. In both applications, ChiralCel was superior to Kromasil, so it was chosen for the statistical runs. The factorial analysis revealed that the principal determining variables for production rate were column load ratio and column temperature. The effect of the two other variables (modifier % and fluid linear velocity) was less.

To determine the optimum values for the variables, geometric response surface analysis was used. The surface analysis was calculated with two independent variables at a time using the Stanford Graphics 1.01 statistical 3D software. It features a distance-weighted interpolating algorithm to construct a "best fit" surface for two independent variables. The advantage of geometric surface analysis over general linear regression analysis (as in Paper III) is that local optima are easily detected. The interpolating surface follows the local changes of the target variable smoothly while a general linear model follows the analytical equation in an averaged fashion. The disadvantage of the interpolating surface method is that only two variables and their interactions at a time can be analyzed. The effect of the other variables cannot be eliminated since the dependent variable is a function of all four variables. To eliminate this, a stepwise method was used. First, the two most important variables (temperature and column load ratio) were processed. Then, similar graphs were made, all with temperature as one variable and one of the remaining variables (*i.e.* fluid velocity and modifier %) as the other variable. The other variable's optimum value was deduced from the corresponding surface graph. The software allows rotating the surfaces which facilitates determining the locations of the optima from different projections. This way a set of optimum values for all four dependent variables was determined. Figure 21 illustrates the effects of the four independent variables in a concerted view on the processing racemic guaifenesin.

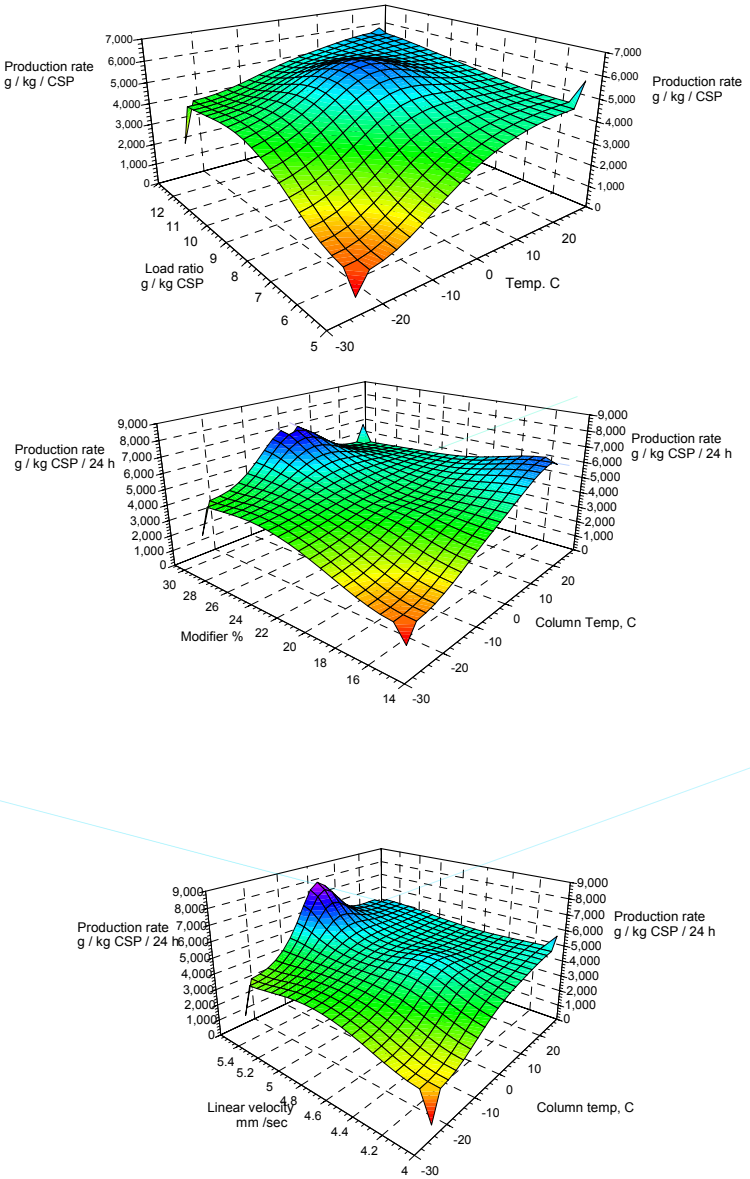

Figure 21. Interpolating surface analysis of the production rate for guaifenesin. The input data is presented in table 3 of Paper V. Upper graph: calculated production rate as a function of column temperature and load ratio. Middle graph: calculated production rate as a function of column temperature and modifier %. Lower graph: calculated production rate as a function of column temperature and mobile phase velocity. The spikes at the corners are computing artefacts.

In the surface analyses the detected optimum temperature was roughly the same in all independent variable pairs. Thus the optimum of the respective other variables could be read from the graph with reasonable certainty. In the separation of guaifenesin and FADDE the optimum production rates were in both cases near 0 °C (273 K). This suggests that in these applications band broadening at the lower temperatures eliminates the advantage that is attained by runs performed at cryogenic temperatures.

The location of the optimum temperature depends on various chromatographic factors. The optimum temperature for maximum production rate is not necessarily the temperature of largest separation factor since band broadening is significant at low temperatures. Band broadening is clearly evident in figures 4 and 5 of Paper V. Resolution is a proper indicator for the optimum temperature but resolution alone can predict neither the location of variables nor maximum production rate. In the studied cases, there were individual runs and respective combinations where calculated production rate was greater than the statistical overall maxima. This is because a statistical analysis smoothens out individual points and the largest data points do not necessarily coincide with statistical data maximum.

Optimization of the variables can enhance the process economy substantially. Table 5 compares the above optimized production rates for guaifenesin with values calculated for the same application from published data.

Table 5. Comparison of chiral SFC of racemic guifenesin from published data and this thesis, Paper V.

	Calculated from Jusforgues *et al.* [70]	**This thesis, Paper V**
Column	Chiralcel OD 10 x 250	Chiralcel OD 4.5 x 250
Load ratio, g/kg CSP	8.8	10.0
Mobile phase	CO_2 + 10.0% EtOH	CO_2 + 30% EtOH
Cycle time, min	5	2.3
Temperature, °C	+10	0
Pressure, bar	250	210/150 *)
Separation factor	1.8	2.3
Production rate, g/kg CSP/d	2400 **)	6400

*) inlet /outlet pressure
**) calculated from the published data

Comparison of the variables shows how optimization increased the processing capacity to nearly threefold. Obviously, the main source of difference is the degree of modification which speeds up the cycle time substantially. The temperature effect is also obvious which can be concluded from the increase of separation factor.

5. Concluding remarks

The need to find environmentally benign solvents is widely recognized and there have been several research programs within EU framework on green chemistry. Dense carbon dioxide has been commonly used for extraction since the early 1970's when the emphasis was to find a substitute for chlorinated and other hydrocarbons. Extraction of pharmaceuticals and essential oils with CO_2 is currently a firmly established method in industry. In central Europe health products and natural extracts industries have been a considerable business for decades and were based earlier on organic (mainly ethanol) solvents. Since the 1980's companies have offered a large line of natural CO_2 extracts.

In this thesis the effect of process variables in the CO_2 extraction of flavoring materials was studied. When extracting *Angelica archangelica* the sensory ratings of CO_2 extracts were clearly attributed to the extract composition which, in turn, was dependent on extraction conditions. Especially large benzopyranoid content was found to have a favourable effect on the aroma and this could be accomplished by using properly selected extraction pressure.

In chromatography, SFC matured as a viable analytical technique throughout the 1980's and 1990's as described by King and others [71, 72]. Still, the companies have been slow to adopt SFC in favour of the more traditional HPLC. Only a few companies [73, 74, 75, 76] are known to actively offer and employ anything more than laboratory scale SFC. This is reflected in column sizes. While the largest industrial HPLC offered by the industry market leader, the German-French Novasep, is huge, 1600 mm in diameter, their largest SFC application uses only a 100 mm column which can be regarded as a research tool.

In this thesis, supercritical chromatography was applied using dense CO_2 mixed with light alcohols to separate and purify the immunosuppressant agent cyclosporine A. The separation method was developed from laboratory to industrial scale. The results indicate that normal phase silica could be used and supercritical carbon dioxide modified with 2-propanol was the most suitable mobile phase. For technical and economical reasons, SFC purification must to be done in two stages. This way, the final product met the cyclosporine A purity requirements of US Pharmacopoeia. The process was substantially faster and

less solvent-extensive than the industrial standard LC method. Also the process economy seems feasible.

Novel hardware for industrial chromatography was introduced and tested. Two pilot systems were built in which a pressure compensation technique was applied in a DAC column. With this pressure compensation technique, some common problems in industrial HPLC were almost completely eliminated, namely the wall-deflection, the compression piston sealing problems and the extra-column volume at the injection line.

The chromatographic separation of chiral enantiomers could be enhanced substantially when the process was performed at very low temperatures. The low viscosity of carbon dioxide at cryogenic temperatures enabled its use at temperatures and velocities that are not possible with ordinary solvent based LC methods. In many SFC applications, subzero or cryogenic temperature increased the separation factor of the chiral stationary phase so that the column load could be increased by an order of magnitude. This required that the other process variables be matched properly with temperature. While the key factors affecting chromatographic performance are well known and discussed in this thesis, it was found that semi-empirical optimization is also feasible when statistical analysis is applied.

The target of an industrial chromatographic process is to produce the maximum amount of product at a minimum cost. Chromatographic phase manufacturers offer many HPLC optimization services (*e.g*. "Kroma Guide" by Akzo Nobel [77), but these do not generally apply to SFC. In this thesis statistical methods were used in SFC for maximizing the resulting production rate. In resolving the enantiomers of racemic mixtures, chromatogram alone could be used as an indication of the yield thus eliminating the need for preparation and analysis of the products. As a result of statistical optimization the production rate in a chiral SFC application could be increased substantially when compared to published results of the same application.

In addition to chiral SFC, statistical method was also used in non-chiral applications for optimizing the production rate of the ethyl esters of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from tuna fish oil. These omega-3 fatty acid esters were prepared from raw tuna fish oil and purified with SFC

using a reversed stationary phase. In a one stage process, 90–95% pure DHA ethyl ester could be produced at a reasonable production rate, while EPA ethyl ester concentrate was recovered at 50% purity.

Extensive research and development on supercritical technologies has been carried out worldwide for more than 25 years but still, supercritical fluid applications have been limited to only a few areas. Most applications are extraction of natural products, the largest single application being decaffeination of coffee. High pressure techniques are often considered as expensive and exotic, but as demonstrated in this thesis and elsewhere [78], the production costs with high pressure processes are very often the same order of magnitude as with conventional processes.

According to Perrut [79] By by the year 2000 more than 70 industrial plants plants employing supercritical technology were installed by the industry leader Separex S.A., France alone. In Finland, the first and so far the only industrial SFE plant Aromtech Oy designed by Chematur Ecoplanning was launched in 1999 in Tornio where it specializes in arctic flavors.

In SFC not many industrial applications are reported in the literature. The most prominent applications are separation of omega-3 fatty acids from marine oils [67]. Rauma Ecoplanning (later Chematur Ecoplanning) demonstrated how the technological skills developed in designing deep sea submersibles MIR 1 & 2 could be applied in a very innovative way in an entirely different field of technology, namely in supercritical extraction and chromatography.

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PAPER I

Composition of Angelica Root Oils Obtained by Supercritical CO_2 Extraction and Steam Distillation

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RESEARCH REPORT *J. Ess. Oil Res.*, **3**, 229-236 (Jul/Aug 1991)

Composition of Angelica Root Oils Obtained by Supercritical CO_2 Extraction and Steam Distillation

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ABSTRACT: Supercritical CO_2 extraction was used to isolate flavor compounds from angelica root (*Angelica archangelica* L.). The extractions were conducted at 40°C at pressures ranging from 80-400 bar. The extracts were analyzed by capillary gas chromatography and mass spectrometry. For comparison, flavor compounds were also isolated from angelica root by steam distillation.

A total of 76 components were identified and determined quantitatively. α-Pinene, sabinene, myrcene, α-phellandrene, δ-3-carene, p-cymene, β-phellandrene, chrysanthenyl acetate, α-copaene, 15-pentadecanolide, 7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one, and a furanocoumarin tentatively identified as oxypeucedanin were among the major volatile components in the supercritical extracts.

The total yield of volatiles obtained by supercritical CO_2 extraction at various pressures ranged from 0.13-0.55%. The highest yield was obtained at 200 bar, particularly because of the large amount of 7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one. For monoterpenoids, maximum yield was obtained already at 100 bar.

KEY WORD INDEX: Angelica root, Umbelliferae, supercritical CO_2 extraction, essential oil composition, 7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one.

INTRODUCTION: Supercritical fluid extraction (SFE) is a promising method for isolating labile flavor compounds from plant material (1-3). Compared with traditional steam distillation, yields are higher in SFE (3). Aldehydes, ketones, esters, alcohols and ethers, as well as aliphatic hydrocarbons up to a chain length of 20 carbons, are considered to be very soluble in supercritical CO_2, whereas acids, phenols and compounds with a molecular weight of over 500 show limited solubility (3).

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Supercritical extraction can be performed under less extreme conditions than steam distillation, and thus product degradation, e.g., due to high temperature, can be avoided. The purpose of this study was to examine the effect on flavor compound yield of the separation conditions, viz. the extraction of the essential oil of angelica root with supercritical CO_2 at various pressures. For comparison, the essential oil was separated also by steam distillation.

EXPERIMENTAL: *Samples*—Dry angelica root was purchased from Heinrich Ambrosius GmbH, Hamburg, FRG. Immediately before the SFE experiments, the roots were crushed in a hydraulic press. For steam distillation, the roots were ground in a Moulinex chopper.

SFE—The supercritical extractions were performed as isothermal batch experiments at 40°C at constant pressures of 80, 90, 100, 150, 200 or 400 bar. The extraction apparatus, manufactured by Nova Werke AG, Effretikon, Switzerland, was equipped with extraction and separation vessels of 4 L and 2 L, respectively. The maximum extraction pressure of the apparatus was 700 bar, with the extraction temperature adjustable between ambient temperature and 100°C. The pressure was microprocessor controlled by means of an electric back-pressure fine metering valve. In each experiment about 500 g of angelica root was extracted; the total mass of carbon dioxide flowed through the sample was 15 times the weight of the sample.

Steam distillation—The volatile compounds of angelica root were isolated by steam distillation followed by liquid-liquid extraction with a mixture of dichloromethane and pentane (1:9) as described elsewhere (4).

GC and GC/MS—A Hewlett-Packard 5880A gas chromatograph coupled to a Hewlett-Packard 3550 Data System was used for the qualitative and quantitative determinations. The mass spectra were run on a Finnigan MAT 212 mass spectrometer coupled to a Varian 3700 gas chromatograph and a PDP 11/34 Data System. The ionization mode was EI and the electron energy 70 eV. For the GC and GC/MS determinations, the compounds were chromatographed on a 50 m x 0.3 mm i.d. fused silica column coated with bonded phase OV-101. The oven temperature was kept at 40°C for 5 min., then heated to 220°C at the rate of 4°C/min.

To quantify the compounds obtained by supercritical extraction, a known amount of anisole (phenyl methyl ether) was added into the oil as an internal standard, while dimethyl malonate was used as the internal standard to analyze the extracts obtained by steam distillation. The different responses of the two internal standards were taken into account by using different response factors, 1 for anisole and 0.25 for dimethyl malonate, when calculating the concentrations of the volatile compounds in the extracts.

Authentic reference compounds, as well as published mass spectra (5-7) and retention indices (8-10) were used as a basis for the identification of compounds.

RESULTS AND DISCUSSION: The amounts of various components in the steam distilled oil and in the supercritical extracts are listed in Table I. α-Pinene, sabinene, myrcene, α-phellandrene, δ-3-carene, p-cymene, β-phellandrene, chrysanthenyl acetate, α-copaene, 15-pentadecanolide, 7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one, 4-methoxy-7H-furo[3,2-g][1]benzopyran-7-one, and a furanocoumarin tentatively identified as oxypeucedanin were the major volatile components in the supercritical extracts. Monoterpenes have previously been reported to be the main components of angelica root oil isolated by steam distillation (11,12) and subcritical carbon dioxide extraction (13).

Depending on the extraction pressure, the yields of some compounds by supercritical extraction were up to 50 times those obtained by steam distillation. In particular, chrysanthenyl acetate, which was a minor component with a relative concentration of 0.1% in the steam distilled oil, was found to be a prominent component with a relative concentration ranging from 1.7-5.5% in the supercritical extracts. Furthermore, oxypeucedanin, which was present in all supercritical extracts, could not be isolated by steam distillation.

Conversely, the data in Table I show that α-terpinene, 2-pentylfuran, cis-piperitol, terpinen-4-ol, α-terpineol, cis- and trans-p-menth-2-en-1-ol, limonene-1,2-oxide, and sabinol, with relative concentrations of 0.6-3.5% in the essential oil isolated by steam distillation could not be detected in the supercritical extracts.

Depending on the extraction conditions, the appearance of the product varied from pale, yellow, mobile oil to dark yellow, viscous liquid. Regarding odor and taste, the extracts obtained at a temperature of 40°C and pressures of 100-200 bar were acceptable. The relative proportions of various classes of compounds in the oils obtained either by steam distillation and solvent extraction or by SFE at various pressures are presented in Table II. It is interesting that the relative amount of oxygenated monoterpenoids was clearly higher in the steam distilled sample than in any of the samples obtained by SFE. Furthermore, the amount of benzopyranoids was markedly increased when the supercritical extraction was done at pressures of 200-400 bar.

The differences in the oils obtained by steam distillation and supercritical extraction might be explained by the lower relative solubility of polar components, such as oxygenated monoterpenoids, in supercritical carbon dioxide. Moreover, the occurrence in the steam distilled oil of some compounds not found in the supercritical extracts may be partly explained by reactions taking place in the presence of water at elevated temperature.

Table II. Relative proportions (%) of various classes of compounds in volatile oil obtained either by SFE at various pressures or by steam distillation and solvent extraction

	Supercritical Extraction						
Compound Class	80 bar	90 bar	100 bar	150 bar	200 bar	400 bar	Steam Dist.
Monoterpenes	39	48	49	30	24	38	28
Oxygenated monoterpenoids	16	15	8	5	5	7	39
Sesquiterpenes	17	12	8	4	4	5	8
Oxygenated sesquiterpenes	6	6	3	2	1	2	6
Benzopyranoids	7	8	22	43	50	38	8
Miscellaneous compounds	15	12	10	15	16	11	11

Table I. Contents of various volatile compounds in angelica root (mg/kg) obtained by steam distillation and supercritical extraction with carbon dioxide at various pressures

			Supercritical Extraction						
RI	Compound	Steam distillation	80 bar	90 bar	100 bar	150 bar	200 bar	400 bar	Means of identification
927	α-thujene	1.0	3.4	6.7	12.9	2.7	1.3	11.9	MS,RI
936	α-pinene	36.9	84.3	228.3	386.5	333.4	312.6	377.2	MS[a],RI[a]
949	camphene	2.5	3.9	14.4	18.7	17.7	19.0	20.8	MS,RI
971	sabinene	2.6	15.8	26.8	91.7	42.6	34.1	95.6	MS,RI
976	β-pinene	2.3	4.6	13.2	22.7	19.0	18.4	22.6	MS[a],RI[a]
983	2-pentylfuran[b] + MW 152	8.2	0.0	0.0	0.0	0.0	0.0	0.0	MS,RI
984	myrcene	7.2	23.8	35.8	84.2	67.1	51.6	68.1	MS[a],RI[a]
999	α-phellandrene	14.3	56.4	62.7	206.5	175.9	151.6	116.0	MS[a],RI[a]
1008	δ-3-carene	29.0	68.8	126.1	266.9	222.9	194.7	228.9	MS,RI
1013	α-terpinene	5.8	0.0	0.0	0.0	0.0	0.0	0.0	MS[a],RI[a]
1015	p-cymene	48.0	39.0	88.0	121.7	117.2	104.4	111.1	MS[a],RI[a]
1022	limonene	0.0	1.1	1.8	0.0	0.0	0.0	0.0	MS[a],RI[a]
1026	β-phellandrene	49.3	152.2	217.4	432.3	412.7	359.1	353.6	MS,RI
1028	cis-ocimene	3.8	9.0	8.7	27.1	21.0	17.8	18.5	MS,RI
1040	trans-ocimene	9.6	23.4	25.0	67.5	51.0	43.1	45.3	MS,RI
1054	γ-terpinene	8.2	4.1	7.2	12.1	2.0	1.3	1.1	MS[a],RI[a]
1081	terpinolene	2.3	2.9	3.3	9.1	0.0	0.0	0.8	MS,RI
1083	linalool	11.7	3.6	5.3	13.6	1.5	1.2	1.0	MS[a],RI[a]
1112	trans-p-menth-2-en-1-ol	24.8	0.0	0.0	0.0	0.0	0.0	0.0	MS[a],RI[a]
1129	limonene-1,2-oxide[b]	15.5	0.0	0.0	0.0	0.0	0.0	0.0	MS,RI
1131	cis-p-menth-2-en-1-ol	13.0	0.0	0.0	0.0	0.0	0.0	0.0	MS[a],RI[a]
1159	p-cymen-8-ol	21.5	4.4	8.8	7.6	1.4	1.3	2.0	MS,RI
1162	5-isopropylbicyclo-(3.1.0)-hexan-2-one[b]	28.4	5.4	10.7	9.0	15.7	17.6	14.2	MS

Table I (Cont).

1165	5-undecen-3-yne[b]	15.2	7.3	8.4	14.1	1.9	14.6	12.0	MS
1169	terpinen-4-ol	10.2	0.0	0.0	0.0	0.0	0.0	0.0	MS[a],RI[a]
1178	α-terpineol[b]	11.9	0.0	0.0	0.0	0.0	0.0	0.0	MS[a],RI[a]
1187	trans-piperitol	6.6	12.3	20.3	22.9	25.4	30.0	20.2	MS,(RI[c])
1188	sabinol[b]	33.7	0.0	0.0	0.0	0.0	0.0	0.0	MS
1194	cis-piperitol	7.7	0.0	0.0	0.0	0.0	0.0	0.0	MS,(RI[c])
1200	trans-carveol	4.4	1.7	4.1	0.0	0.0	0.0	0.0	MS[a],RI[a]
1221	carvone	2.0	0.0	0.0	0.0	0.0	0.0	0.0	MS[a],RI[a]
1234	piperitone[b]	2.8	0.0	0.0	0.0	0.0	0.0	0.0	MS[a],RI[a]
1259	phellandral[t,b]	4.3	0.0	0.0	0.0	0.0	0.0	0.0	MS
1265	trans-anethole[b]	8.9*	2.1	2.7	7.7	2.0	13.4	1.8	MS[a],RI[a]
1271	chrysanthenyl acetate isomer 1	2.7	6.2	6.7	6.7	0.0	1.2	1.2	MS
1276	bornyl acetate	28.5	26.8	30.3	37.4	33.9	36.7	35.1	MS[a],RI[a]
1279	chrysanthenyl acetate isomer 2[b]	1.3	73.5	81.3	94.3	81.4	93.5	91.6	MS
1312	sabinyl acetate[b]	1.8	3.6	5.4	5.0	0.0	0.0	1.0	MS
1382	a sesquiterpene with MS identical to that of α-muurolene	3.4	10.9	10.6	15.5	13.9	16.0	13.9	MS
1387	α-copaene	10.8	46.7	44.3	70.0	61.6	66.0	58.2	MS,RI
1396	β-elemene	1.2	5.6	4.2	7.4	0.0	0.0	1.2	MS,RI
1398	tetradecane[b]	0.0	2.0	2.1	0.0	0.0	0.0	0.0	MS[a],RI[a]
1429	β-sesquiphellandrene[b]	1.6	5.5	5.2	7.3	0.0	0.0	1.0	MS
1432	γ-caryophyllene[b]	1.6	5.0	4.7	5.7	0.0	0.0	0.7	MS
1440	a sesquiterpene with MS identical to that of germacrene D	2.1	8.1	8.5	12.1	1.6	1.5	1.3	MS
1449	trans-β-farnesene	2.1	7.5	8.8	10.5	0.0	0.0	1.2	MS,RI
1465	α-humulene	9.9	33.3	29.0	42.3	33.2	38.5	33.2	MS[a],RI[a]
1476	α-curcumene	0.0	2.4	3.1	0.0	0.0	0.0	0.0	MS,RI
1481	α-cubebene[b]	3.5	4.6	4.9	0.0	0.0	0.0	0.8	MS
1489	germacrene D	2.2	13.0	10.0	22.0	12.5	12.8	15.3	MS,RI
1494	zingiberene[b]	1.6	3.8	4.7	0.0	0.0	0.0	0.0	MS,RI
1498	β-himachalene[m,b]	0.0	4.5	6.9	0.0	0.0	0.0	11.6	MS,RI

Table I (Cont.).

RI	Compound	Steam distillation	Supercritical Extraction 80 bar	90 bar	100 bar	150 bar	200 bar	400 bar	Means of identification
1503	α-muurolene	9.0	19.8	20.0	27.7	24.2	25.4	22.4	MS,RI
1506	β-bisabolene	8.9	34.6	31.5	41.9	35.9	33.7	37.2	MS,RI
1513	bornyl isovalerate	2.3	2.6	4.0	0.0	0.0	0.0	0.0	MS,RI
1520	γ-cadinene[b]	2.3	8.2	12.1	14.3	13.9	12.2	13.9	MS,RI
1524	δ-cadinene	7.4	9.2	8.2	12.2	2.3	1.8	1.1	MS,RI
1540	α-copaen-11-ol	15.3	19.6	26.1	33.6	36.0	31.4	31.4	MS
1545	elemol	3.4	5.4	6.6	7.1	0.0	0.0	0.9	MS,RI
1562	α-copaen-8-ol	5.9	10.7	13.7	17.8	19.7	17.6	15.9	MS
1598	hexadecane[b]	0.0	7.0	7.2	5.6	1.4	0.0	0.8	MS[a],RI[a]
1629	13-tridecanolide	14.3	25.0	28.3	34.1	49.2	35.4	33.7	MS
1654	β-eudesmol[m]	3.2	5.2	11.0	6.4	2.1	2.0	1.1	MS
1685	12-methyl-13-tridecanolide	3.4	4.9	6.6	6.2	11.9	1.5	0.9	MS
1698	heptadecane[b]	0.0	4.8	6.6	0.0	0.0	0.0	0.8	MS[a],RI[a]
1735	psoralen[b]	10.6	2.3	2.4	27.1	78.7	137.2	46.8	MS
1797	octadecane[b]	0.0	4.6	5.4	0.0	0.0	0.0	0.0	MS[a],RI[a]
1812	a hydrocarbon	0.0	3.7	3.2	0.0	0.0	0.0	0.0	MS
1835	15-pentadecanolide	15.8	38.0	29.1	50.0	73.2	59.8	50.9	MS
1897	nonadecane[b]	0.0	4.5	4.0	0.0	0.0	0.0	0.0	MS[a],RI[a]
1997	eicosane[b]	0.0	3.9	4.7	0.0	0.0	0.0	0.0	MS[a],RI[a]
2019	4-methoxy-7H-furo[3,2-g] [1]benzopyran-7-one[b]	1.1	5.2	6.0	39.0	151.3	226.4	78.7	MS
2035	17-heptadecanolide[t]	2.0	8.7	6.2	15.2	16.4	18.6	18.3	MS
2091	7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one[b]	51.5	76.4	108.8	597.1	1479.0	2004.0	898.1	MS
2411	oxypeucedanin[t,b]	0.0	7.8	17.6	114.1	291.2	257.4	385.9	MS

MS = mass spectrum; RI = retention index; [a] = comparison with authentic sample; [b] = not previously reported as a volatile component of angelic root; [c] = elution order of cis- and trans-isomers according to Brosche et al. 1981 (14); [t] = tentative identification; [m] = mixed peak, major compound identified; * = mostly cuminyl alcohol.

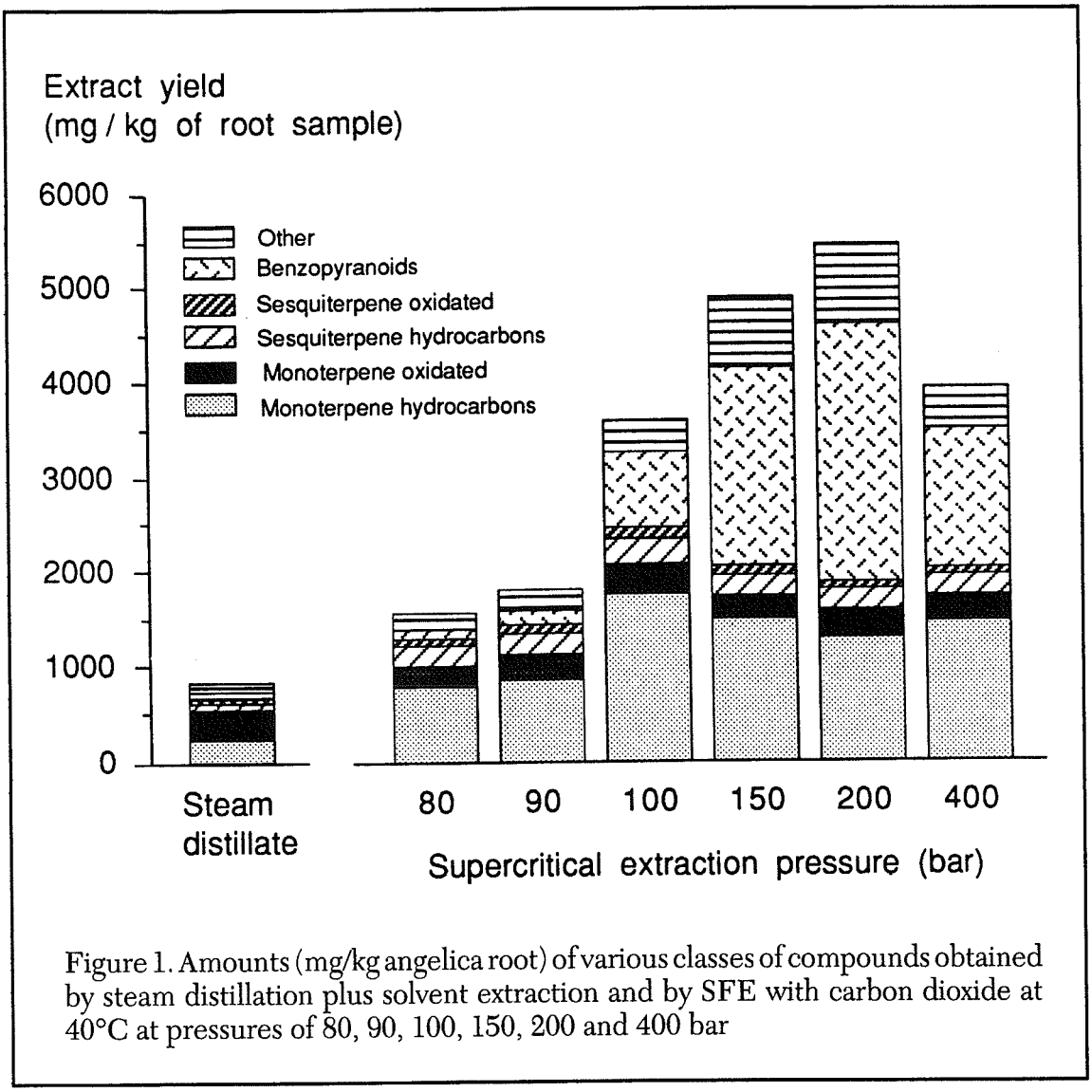

Figure 1. Amounts (mg/kg angelica root) of various classes of compounds obtained by steam distillation plus solvent extraction and by SFE with carbon dioxide at 40°C at pressures of 80, 90, 100, 150, 200 and 400 bar

The total amount of volatiles obtained by supercritical CO_2 extraction at various pressures ranged from 1.3-5.5 g/kg of dry angelica root (Figure 1). At the lower pressures, the supercritical extraction yields were similar to that obtained by steam distillation. The highest total yield was obtained at 200 bar, especially because of the high amount of 7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one (Figure 2). For monoterpenoids, e.g., α-pinene, β-phellandrene, δ-3-carene, and chrysanthenyl acetate, maximum yield was obtained already at 100 bar.

It is worth noting that the relation between pressure and solubility is not the same for volatile and nonvolatile compounds. This may explain why pressure had a greater effect on the total amount of extract, which also includes the nonvolatile compounds, than on the amount of the volatile essential oil (Table III). The relative proportion of the volatile fraction in the supercritical extracts of angelica root decreased when the extractions were done at higher pressures. The low proportion of volatile material in extracts obtained at higher pressures may partly explain the poor sensory rating of the extract obtained at 400 bar.

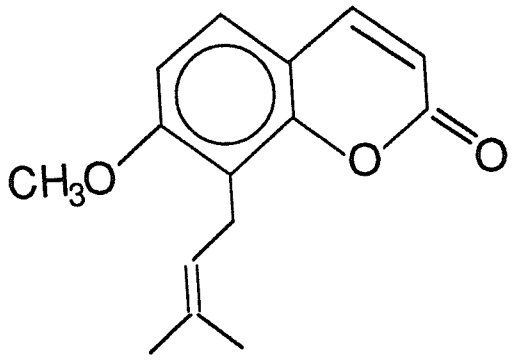

Figure 2. Structure of 7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzo-pyran-2-one

Table III. Relative proportion of volatile fraction in oil obtained by supercritical extraction at various pressures

Pressure (bar)	Volatile Fraction
80	102 %
90	57
100	48
150	29
200	23
400	18

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PAPER II

Polypeptide Purification with Industrial-Scale Supercritical Fluid Chromatography

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Polypeptide Purification with Industrial-Scale Supercritical Fluid Chromatography

This article describes a supercritical fluid chromatography (SFC) process for separating a polypeptide from a mycelial extract, in this case, the purification of cyclosporin A obtained by fermentation. The chromatographic challenge was to separate a number of very similar cyclosporin forms to produce a product for final crystallization, and the technological challenge was to develop a compact, rapid process that would not use chlorinated or hydrocarbon solvents.

Image courtesy of VTT Chemical Technology.

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Cyclosporin is a cyclic polypeptide with 11 amino acid residues (Figure 1). It is produced by fermentation of the fungus *Tolypocladium inflatum*, which simultaneously produces several cyclosporin forms that differ from each other only by their amino acid profile and sequence. At least 25 cyclosporin forms have been identified, including the A-form (CyA), which is used clinically as an immunosuppressant following transplant surgery. The drug was developed during the 1990s by Sandoz and it is sold under the trade name Sandimmune. The *United States Pharmacopeia* (*USP*) specifications for CyA are given in Table I.

A traditional liquid chromatography process for CyA purification consumes approximately 1 ton of toluene, hexane and methanol per kg of purified product. The specific productivity is also low — more than 1 ton of silica is needed per kg product per hour. A conventional process for producing a cyclosporin product for final crystallization is shown in Figure 2.

A project consortium of three Finnish organizations was formed to develop an improved, non-conventional purification process for cyclosporin A. The consortium consisted of Leiras Oy (to develop a new fermentation method),[1] Chematur Ecoplanning Oy (SFC process designer and hardware manufacturer) and VTT Chemical Technology (laboratory-scale SFC process developer).

Supercritical fluid chromatography (SFC) was selected as the technology candidate because of its potential for rapid processing, economical recycling of eluent and reduced use of organic solvents. Preparative SFC (P-SFC) is not, however, a novelty — its development was initiated in 1982 by Professor Michel Perrut at ENSIC (Nancy, France).[2] Pilot plants with columns up to 100 mm i.d. have been available for some years.

Process development

Fractionation of the crude material. The crude material for SFC purification is a mycelial extract. The composition of the extract includes CyA (22% w/w), a number of other cyclosporin forms (9–11% w/w), other peptides (39% w/w), various lipids from the mycelia (28% w/w), colour substances and fermentation additives. A high performance liquid chromatography (HPLC) chromatogram of the extract is shown in Figure 3a.

Cyclosporin is sparingly soluble in pure, dense carbon dioxide (CO_2). It is therefore necessary to use an additive in the CO_2 eluent to increase cyclosporin's solubility and decrease its retention time on a silica column.

The specific production rate of pure CyA was used as the criterion to evaluate the effect of process parameters. The specific production rate was expressed as the amount of purified CyA (g) per amount of packing material (kg) per hour. Expressed in this way, the specific production rate takes into account factors that affect the production costs and, also, the investment costs.

Screening was done with laboratory-scale packed columns. The CyA yields were calculated from chromatograms and also by analysing collected fractions. The screened parameters were

- stationary phases: cyano, diol and silica
- particle size of packing: 5 and 10 μm
- modifier concentration: 4–27% w/w CO_2
- eluents: CO_2 containing MeOH, EtOH, i-PrOH or acetonitrile modifier
- pressure: 150–300 bar

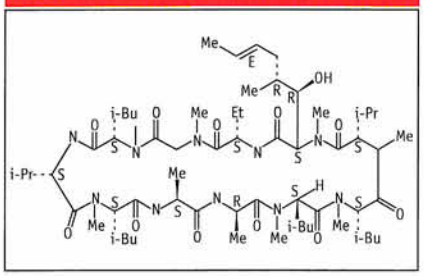

Figure 1: *The structure of cyclosporin A.*

- temperature: 40–70 °C
- eluent flow rate: 1–11 mm/s
- column load: 0.01–16 g crude per kg stationary phase per injection.

The screening and calculating of CyA yields from chromatograms indicated that it may be possible to produce purified CyA at economically feasible rates by choosing optimal parameters. Silica stationary phases gave the highest production rates. They required strongly modified CO_2 to avoid excessive retention times. Methanol or isopropanol were found to be suitable modifiers. It was also found that the specific production rate of pure CyA was most sensitive to the modifier concentration and column load. An example of the effect of parameters in the preparative chromatographic purification of the crude material is shown in Figure 4. Preparative runs showed that the highest yields of purified cyclosporin were obtained by using isopropanol as the modifier.

Repeated injections of the crude material to the same silica column caused considerable shortening of CyA retention times. When injections were repeated 1800 times at constant conditions, the retention time decreased from an initial 23 to 8 min. Retention times decreased because a component in the crude material modifies the silica. In an industrial process this would require frequent regeneration or replacement of expensive, spherical silica. Furthermore, cyclosporin production directly from the crude material in one chromatographic step uses the silica inefficiently. The collection time of the product would only be a very short fraction of the total elution cycle.

A two-step SFC purification process was adopted to use the silica more efficiently, to reduce column volumes and to reduce the amount of recirculating eluent. The purpose of the first step is to separate the polypeptides from the other crude components. It is therefore not a difficult chromatographic separation. The principle of the two-step SFC process is shown in Figure 5. In the first step, almost all CyA from the crude material is collected in an intermediate product. The conditions are chosen so that each extract injection is eluted at a high rate.

Table I: *US Pharmacopeia specifications for cyclosporin A.*

Identification		Cyclosporin A by HPLC
Tests	Loss on drying Heavy metals	Not more than 2.0% Not more than 0.002%
Assay	Cyclosporin A	98.5–101.5% on dried basis
Related compounds	Individual impurity Sum of impurities	Not more than 0.7% Not more than 1.5%

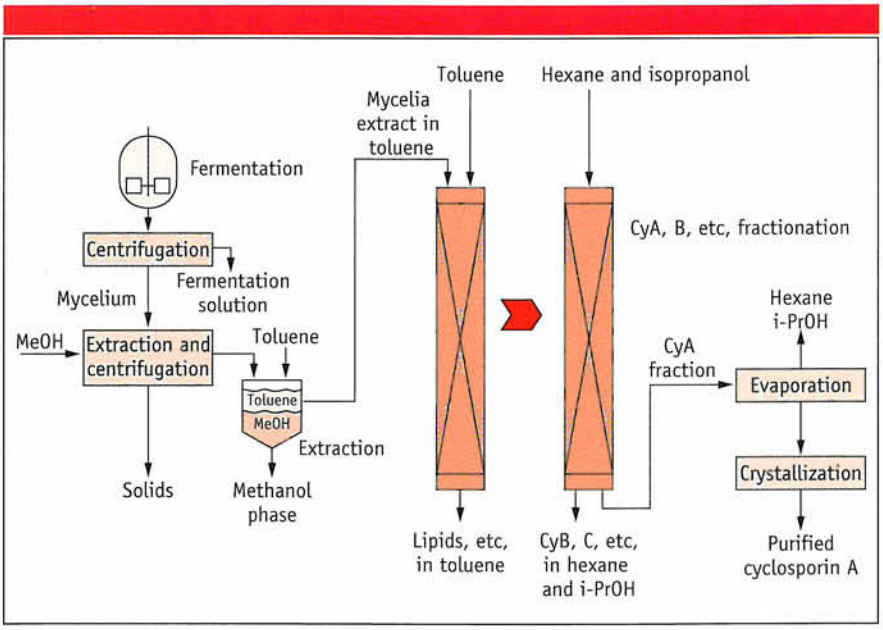

Figure 2: *Traditional LC process for producing cyclosporin A.*

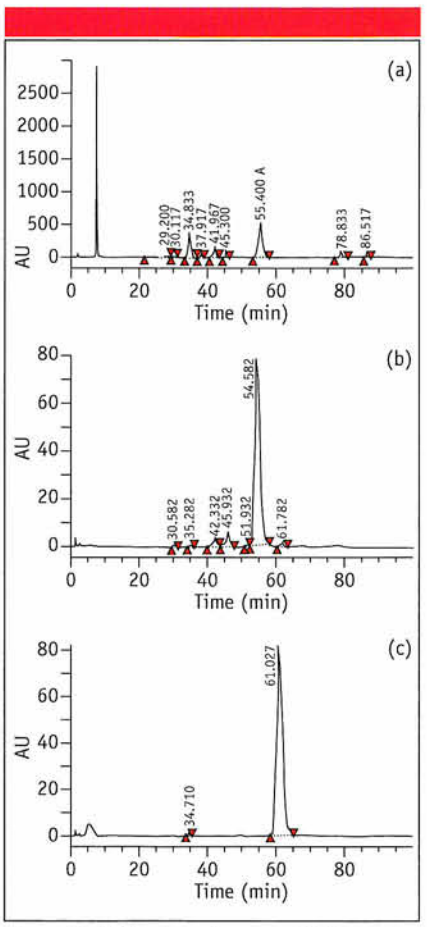

Figure 3: *HPLC chromatograms of (a) the crude (22% w/w CyA), (b) the intermediate SFC product (58% w/w CyA) and (c) the final SFC product (98% w/w CyA).*

The first step — fractionation of the crude material — can be done using a coarse, irregular silica. Several silica candidates for the first SFC step were evaluated at laboratory scale. The particle sizes of the tested silicas ranged from 5 to 40 μm.

The solvent in which the crude material is injected onto the first chromatographic column has a marked effect on the peak form of CyA. The crude material was injected as a toluene solution in the preparative laboratory columns and produced sharper CyA peaks than with other crude solvents. Toluene, however, is not suitable for use in industrial SFC for cyclosporin purification because it forms an azeotrope with the alcohol modifier, making it impossible to recycle completely. Furthermore, toluene has strong ultraviolet (UV) adsorption properties, which disturb the detection of eluting cyclosporin — a fraction of the feed toluene is always recycling with the eluent, creating an increasing background in the ultraviolet (UV) detector. It is therefore necessary to remove all the toluene from the mycelium extract and redissolve the crude material in alcohol for SFC purification. At high SFC column loads, both methanol and isopropanol were suitable solvents for the crude material.

Intermediate CyA products with purities of 86–96% (*USP* assay) were obtained from the crude material at a yield of more than 99%, using all the tested, non-spherical silicas. The acceptable column load per injection was 12–20 g crude material/kg silica.

Purification of intermediate. CyA fractions with purities greater than 98.5% (*USP* assay) were obtained from a 76% pure intermediate in laboratory SFC preparations with fine, spherical silica packing. The intermediate product for optimizing the final chromatographic step was obtained from an industrial-scale SFC column, which is described later.

Kromasil (5 and 10 μm; Eka Chemicals AB, Bohus, Sweden) and NovaPrep (6 μm; Waters, Milford, Massachusetts, USA) silicas were evaluated for the final purification step. Both methanol and isopropanol were tested as the CO_2 eluent modifier and also as the solvent for the injected intermediate. The use of Kromasil 10 silica, with isopropanol as modifier and solvent for the injection, produced the required 98.5% pure fractions of CyA directly from chromatographic fractionation, without crystallization. The critical separation occurred between CyA, CyE and an unrecognized cyclosporin form.

Given that a major portion of the critical impurities can be removed by crystallization, the target purity of the final product from industrial SFC could be approximately 97–98% CyA.

The industrial process

A pilot plant for testing the process concept was designed and built by Chematur Ecoplanning Oy (Tampere, Finland). The capacity of the pilot was designed for producing up to 1000 kg of purified cyclosporin per year.

A schematic flowsheet of the two-stage SFC purification process for cyclosporin is shown in Figure 6. The process could be augmented with parallel columns for increased capacity.

SFC column design. Columns of 200 mm i.d. with maximum packing lengths of up to 600 mm were built. The basic differences between an HPLC and an SFC column are the operating pressure and the pressure drop across the packed bed. Supercritical columns typically operate at 100–300 bar pressures. The eluent in an SFC process experiences a pressure drop of only a few bars as it passes through the packed column.

The SFC column design that was used in the pilot plant is schematically presented in Figure 7. The packing is held under a constant mechanical pressure using a piston, which is pushed against the packing material by a rod connected to two hydraulic cylinders. This arrangement is known as DAC (dynamic axial compression). The hydraulic cylinders are placed along the side of the chromatographic column to keep the total length of the cylinder–column combination less than 1.5 m. This should facilitate installation, operating and column packing procedures. The eluent is transported to the

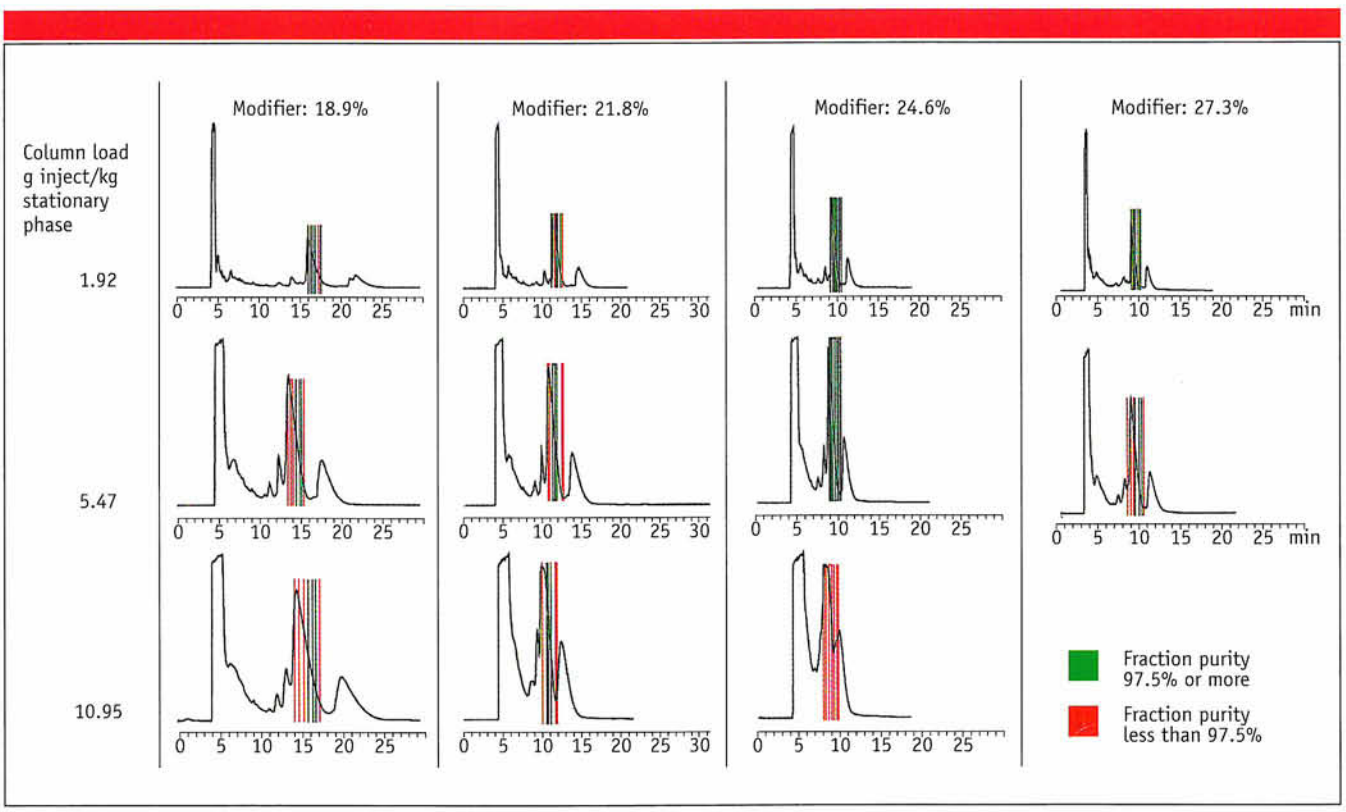

Figure 4: *An example of the effect of column load and modifier concentration on the laboratory SFC chromatograms.*

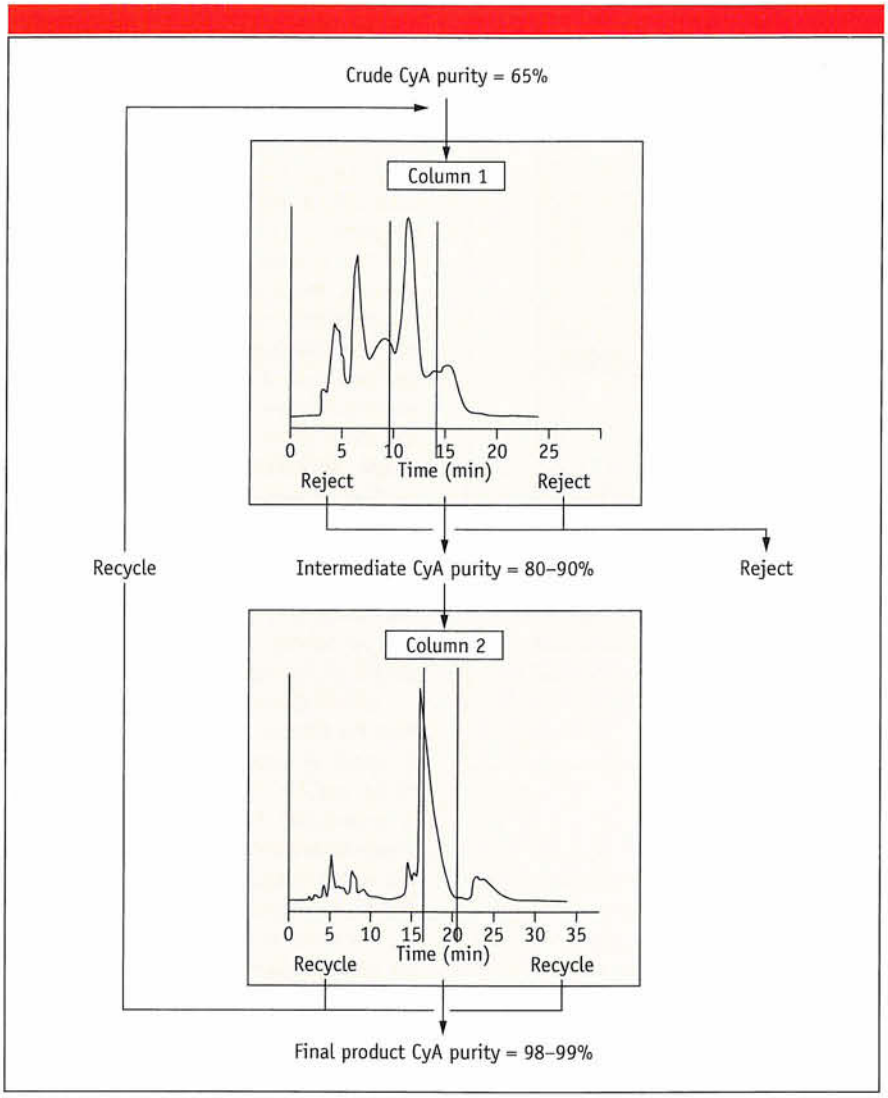

Figure 5: *Two-stage SFC process concept.*

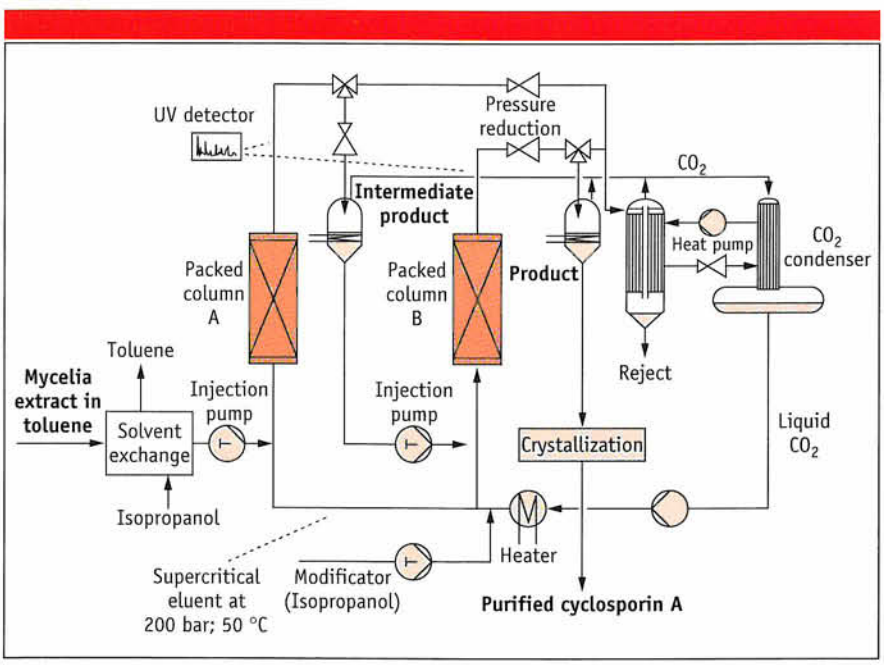

Figure 6: *Schematic diagram of the industrial SFC process.*

packing through the piston from the space below the piston. In this arrangement, there is only a small (positive) pressure difference across the piston. The only task of the piston seals, therefore, is to keep the packing material above the piston. The pressure seal is placed between the piston shaft and the bottom plate of the column cylinder. In this design, the pressure seal is in contact with pure eluent only.

The SFC column design also includes a pressure-compensated column wall. The diameter of the pressure wall of the SFC column changes slightly with temperature. The thermal expansion coefficients between the packing material and the pressure wall are quite different, which may cause dislocations in the packing if it is in direct contact with the pressure wall. In the design that was used in the pilot, there is an inner tube within the pressure column, with the column packing inside the inner tube. Pure eluent is present in the small space between the pressure wall and the inner tube. Any changes in the pressure and temperature of the eluent are therefore compensated so that the diameter of the packing remains constant.

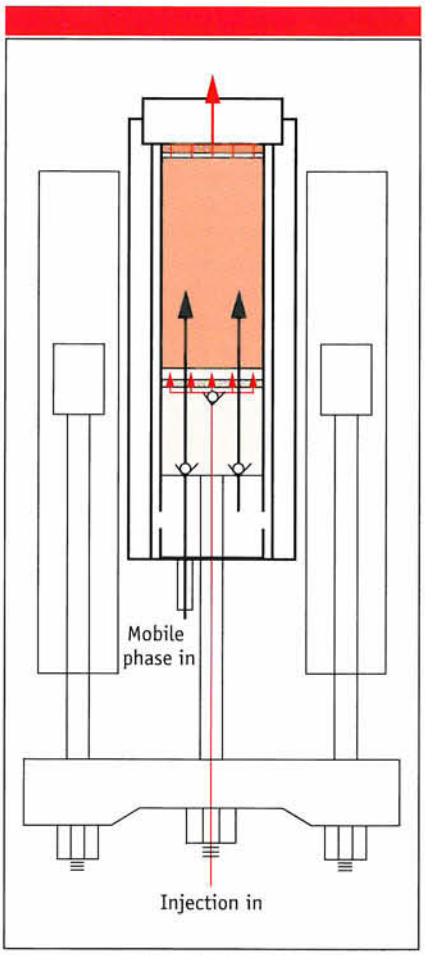

Figure 7: *Operating principle of the axially compressed SFC column.*

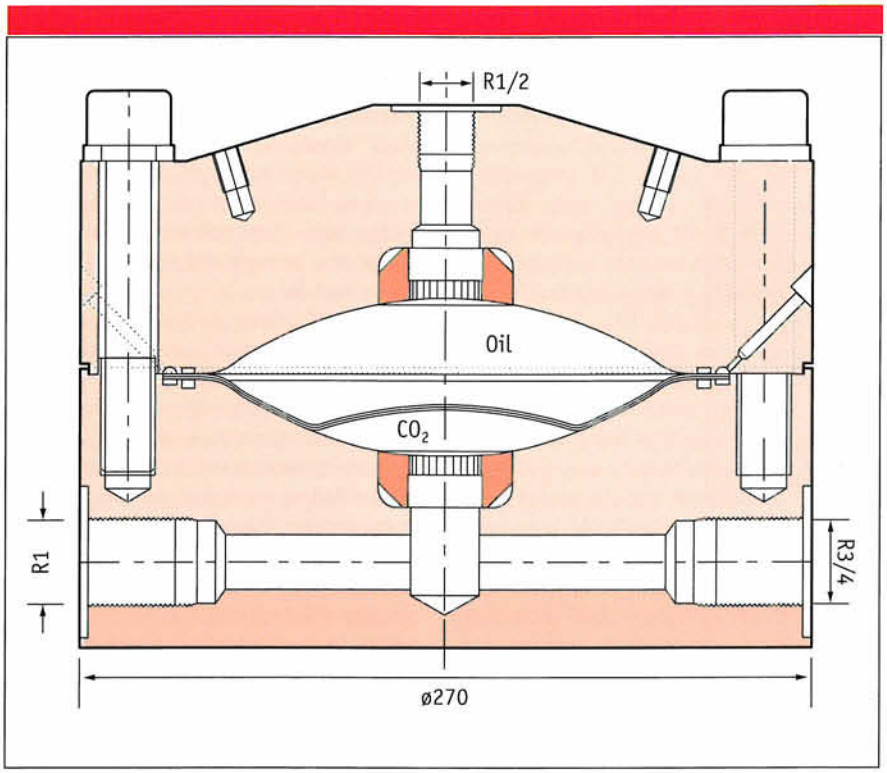

Figure 8: *Carbon dioxide eluent pump.*

Injections. The crude material and the intermediate product are injected into the eluent with a piston pump. They are injected as isopropanol solutions in which the concentration of solids may be as high as 30% w/w. The prototype plant has an injection pump that can deliver injection volumes of up to 0.5 L. The injection pump and the main CO_2 pumps are connected to the same hydraulic system, so that the main CO_2 flow is not interrupted while the liquid injection takes place. In this way, the pressure and the flow rate through the column are kept constant. The injected solution flows through a bore inside the piston shaft and through the piston to the bottom of the column packing where it first meets the CO_2 eluent (see Figure 7).

Collection of fractions. The eluent from the top of the column is directed through a pressure reduction valve into one of the collection vessels. The eluted components are monitored by a high-pressure UV detector, which is installed in a side stream. The pressure reduction valve in the prototype plant operates in constant flow mode.

The gas–liquid mixture, which is formed at the pressure reduction valve, is directed to either the product, recycle or reject vessel. The pressure in these vessels is approximately 50 bar. At this pressure, the density of the CO_2 eluent becomes so low that the dissolved components precipitate in the collection vessels.

The valves are operated according to the signal from the UV detector, so that a required sample from the product containing peak is obtained. The product is collected in a cylindrical vessel where the eluent enters tangentially. After collecting the purified product from several injections, the valve at the bottom of the collection vessel is opened and the product is collected at ambient pressure. The product is collected with the simultaneously precipitated alcohol modifier and it is free from the CO_2 eluent. Most of the flow goes to the reject line where the unwanted material is collected in the same way as the product.

Eluent recirculation. The eluent for CyA purification consists of CO_2 and isopropanol, which are recycled separately. To prevent the recycling process becoming excessively complex, it is important that the modifier in both chromatographic steps is the same. The solvent for injections should also be the same alcohol as that used as the eluent modifier.

Carbon dioxide is withdrawn as a liquid from the bottom of the condenser, where it is stored at vapour–liquid equilibrium, at 50 bar and 21 °C (see Figure 6). Liquid CO_2 is supercooled before entering the main pumps to avoid cavitation and performance loss in the pumps. The slowly reciprocating diaphragm pumps are oil driven (Figure 8).

The modifier is continuously pumped to the pressurized, heated CO_2 eluent using a piston pump. In the prototype plant, the CO_2 flow rate is approximately 400 kg/h and the modifier is circulated at approximately 80 kg/h.

After passing through the column, the eluent is fed through a pressure reduction valve to the different collection vessels. Because CO_2 partially condenses during pressure reduction, the collection vessels are heated to evaporate all liquid CO_2. The CO_2 vapour is then condensed and stored as a

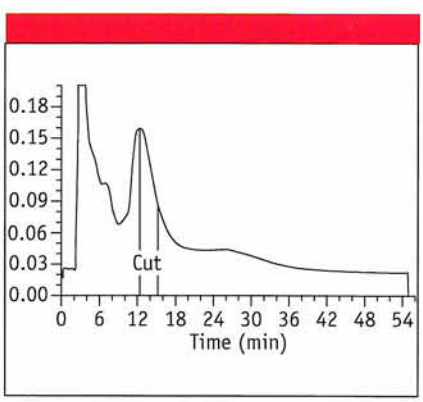

Figure 9: *An example of a pilot-plant SFC chromatogram of the crude fractionation.*

Table II: *The compositions of the crude material, the intermediate SFC product and the final SFC product obtained from pilot runs.*

	Crude material	Product from SFC stage 1	Product from SFC stage 2	Composition units
Cyclosporin A assay	56–65	80–93	98	Area (%)
Composition				
Cyclosporin A	22	58	98	Weight (%)
Other cyclosporins	9–11	18	<1.5	Weight (%)
Other peptides	39			Weight (%)
Glycerides, etc	28	24		Weight (%)

liquid at the bottom of the condenser. The CO_2 evaporator and condenser are connected to each other with a standard heat pump, so there is very little net energy consumed owing to evaporation and condensing operations. The alcohol modifier precipitates as a liquid during the pressure reduction of the eluent. It is removed from the product, and the recycled and rejected materials by evaporation for reuse.

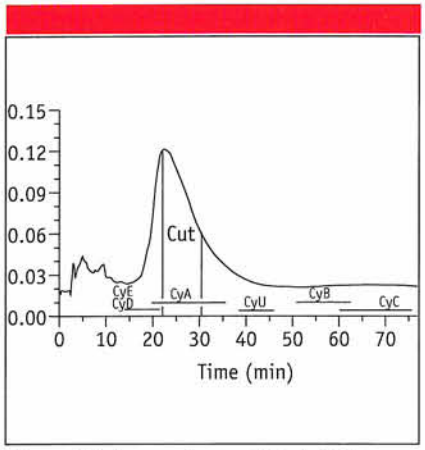

Figure 10: *An example of a pilot-plant SFC chromatogram of the final purification. The elution of different cyclosporin forms is indicated.*

Table III: *Costs of industrial SFC purification, based on non-optimized pilot plant, which used a two-stage process with four DAC columns.*

Assumptions		
Annual production		630 kg/yr
Operating rate		6500 h/yr
CO_2 circulation rate		450 kg/h
Modifier circulation rate		68 kg/h
Investment to process equipment and working capital		2.5 MDM
Operating costs		**kDM/year**
CO_2 make-up	6.9 kg/h	45
Modifier make-up	1.4 kg/h	12
Coarse silica	59 kg/yr	14
Spherical silica	18 kg/yr	90
Electricity	25 kW	16
Staff	1 person/shift	
	3 shift/day	395
Waste treatment	2.5 kg/h	16
Insurances	0.6% of equipment	13
Service, repairs	2% of equipment	44
Total operating costs	645 kDM/yr	
	1.0 kDM/kg product	

Performance. A two-step SFC purification with the industrial-scale plant yielded a CyA product which, after crystallization, met the *USP* purity specifications.

A column of 200 mm i.d. was packed with Zorbax LC 40 spherical silica to 240 mm bed length and was used to fractionate an intermediate product from the mycelial extract. An example of the SFC chromatogram and the collection time of the intermediate product is shown in Figure 9. The compositions of the crude material, the intermediate product and the final product are given in Table II. HPLC chromatograms of the intermediate and final products are shown in Figures 3b and c.

The intermediate product was purified to the final product using the 200 mm i.d. column, packed to a bed length of 280 mm with Kromasil 10-SIL 60 spherical silica. The SFC chromatogram, with approximate elution intervals of the different cyclosporin forms, is shown in Figure 10. The collected fraction contained approximately 98% (w/w) CyA. After removing isopropanol from the product, the CyA was dissolved in methanol and recrystallized. A *USP* assay of the crystallized CyA showed the purity to be 98.5% — the sum of impurities totalled 0.9% with the largest single impurity contributing 0.3%. These values are in compliance with the *USP* specifications for CyA.

Processing costs

The SFC purification cost for cyclosporin is approximately 1 kDM per kg product (1 DM = US$0.56). A cost breakdown for purifying a mycelial extract with SFC is given in Table III. The estimate is based on the non-optimized conditions used in the pilot-plant runs. The use of a relatively large amount of modifier alcohol in the CO_2 eluent is not a big cost factor. Instead, injection interval has an almost linear effect on purification cost. The simultaneous separation of a compound from closely eluting and more slowly eluting compounds leads to long injection intervals. A more complete removal of differently eluting compounds in the first SFC step would reduce the total SFC purification costs. Purification cost is also sensitive to the lifetime of the spherical silica.

Conclusions

SFC is a technically feasible process for purifying a polypeptide from other peptides and from other constituents obtained from fermentation. Cyclosporin A can be separated from mycelial extracts by SFC and purified by subsequent crystallization to meet the *US Pharmacopeia* purity specifications. An industrial-scale SFC plant for the production of up to 1000 kg purified CyA was built and successfully tested. The purification costs are in the order of 1000 DM per kg of purified product.

In summary, the developed SFC process uses CO_2 as the bulk eluent and normal phase silicas as stationary phases. Isopropanol is used as the modifier in CO_2. The industrial-scale process prototype was capable of producing a CyA product with 98% purity (*USP* assay), at a yield of more than 80%. Total purification costs are an order of magnitude less than those of traditional elution chromatography.

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PAPER III

Purification of Polyunsaturated Fatty Acid Esters from Tuna Oil with Supercritical Fluid Chromatography

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Purification of Polyunsaturated Fatty Acid Esters from Tuna Oil with Supercritical Fluid Chromatography

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ABSTRACT: The technical and economic feasibility of producing docosahexaenoic acid (DHA)- and eicosapentaenoic acid (EPA)-ethyl ester concentrates from transesterified tuna oil using supercritical fluid chromatography (SFC) was studied. A systematic experimental procedure was used to find the optimal values for process parameters and the maximal production rate. DHA ester concentrates up to 95 wt% purity were obtained in one chromatographic step with SFC, using CO_2 as the mobile phase at 65°C and 145 bar and octadecyl silane-type reversed-phase silica as the stationary phase. DHA ester, 0.85 g/(kg stationary phase · h) and 0.23 g EPA ester/(kg stationary phase · h) can be simultaneously produced at the respective purities of 90 and 50 wt%. The process for producing 1,000 kg DHA concentrate and 410 kg EPA concentrate per year requires 160 kg stationary phase and 2.6 tons/h carbon dioxide eluant recycle. The SFC operating cost is U.S. $550/kg DHA and EPA ethyl ester concentrate.

Paper no. J9256 in *JAOCS 77*, 315–321 (March 2000).

KEY WORDS: Docosahexaenoic acid, eicosapentaenoic acid, polyunsaturated fatty acids, supercritical fluid chromatography, tuna oil.

Fish oils are a rich source of polyunsaturated fatty acids (PUFA). The interest is particularly in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) because of their reported beneficial physiological activities. The EPA content of fish oil varies from 5 to 26 wt%, and DHA from 6 to 26 wt% of total fatty acids (1).

More concentrated n-3 fatty acids are needed to study their physiological effects and metabolism. PUFA concentrates have a market in pharmaceutical products, food additives, and in health supplements.

In fish oil, the EPA and DHA are preferentially located in the middle carbon of the glycerol backbone. However, triglycerides where two such long-chain fatty acids would be in the same backbone are expected to be minor constituents (2). It is therefore not possible to obtain fish oil products with very high EPA and DHA concentrations if the oil is fractionated as triglycerides. To make fractionation possible, the oil may first be transesterified with an alcohol to fatty acid monoesters. A number of methods have been developed to isolate EPA and DHA from transesterified fish oil, including molecular distillation, liquid chromatography, supercritical fluid extraction, and supercritical fluid chromatography (SFC) with carbon dioxide eluant (3,4). KD-Pharma GmbH, a German company based in Bexbach, is already operating a large-scale SFC plant in Tarragona, Spain. The company produces n-3 fatty acid esters from fish oil at over 95 wt% purity.

It is also possible to eliminate all saturates and the bulk of mono- and dienes from the re-esterified oil by mixing the oil with hot urea dissolved in ethanol. Upon cooling, the urea crystallizes, forming solid adducts with the saturates. The remaining solution can then be fractionated with supercritical (SC) CO_2 extraction to obtain polyunsaturates in high concentration (2). Table 1 presents the composition of urea-adducted menhaden oil. Although technically advantageous as a pretreatment, one should be aware that authors from the U.S. Food and Drug Administration recently called attention to the danger of the formation of the animal carcinogen ethyl carbamate by this method (5).

Fatty acid ethyl esters (FAEE) are soluble up to 10 wt% concentration in dense CO_2 at 230 bars and 80°C (6). Therefore, chromatography with SC CO_2 offers an attractive method to produce EPA and DHA esters at high purity using a nonflammable and nontoxic eluant. Using a SC eluant also brings the potential advantage of increased separation rate and therefore more compact equipment compared to using a liquid eluant. The reason for this is that mass-transfer rate is generally much higher in a SC fluid than in a liquid. SC CO_2 is essentially a nonpolar eluant that can be used with straight (normal) stationary phases like silica. However, due to fatty acid esters' low polarity, silicas retain them too weakly, leading to only partial separation between EPA and DHA esters (7). Reversed phases such as octadecylsilane-grafted silica (ODS) yield a good resolution of fatty acid esters with CO_2 eluant. Berger and co-workers separated n-3 fatty acid methyl esters using reversed-phase C18-silica with a CO_2 eluant (8). Starting from a concentrate containing 14.8 wt% EPA and 73.0 wt% DHA, they obtained three fractions whose respective EPA/DHA concentrations in weight percent were: 54.8/26.7, 2.7/78.0 and 18.7/78.8. Perrut and Breivik (9) obtained EPA and DHA in up to 55.5 and 77.7 wt% purities with CO_2 chromatography. Their starting material contained 28.5 wt% EPA and 26.9 wt% DHA.

Reichmann and Brunner (10) investigated SFC separation

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TABLE 1
Fatty Acid Compositions of Various Fish Oils (wt%)[a]

Fatty acid	Codfish oil ester (14)	Menhaden oil (15)	Urea-adducted menhaden oil (2)	Baltic herring oil (16)	Herring liver oil (17)	Sand launce ethyl ester (18)	Present study	
							Tunafish oil	Tunafish oil ethyl ester
14:0	5.8	10.8		6.8	6.9	6.2	3.1	
16:0	12.9	23.2		22.3	12.4	16.3	22.8	15.1
16:1	9.8	11.4		9.5	11.6	11.7	3.9	3.1
16:2		1.5		0.8		0.6		
16:3		2.2	5.2					
18:0	2.7	4.2		1.8	1.8	2.2	6.7	4.4
18:1	23.3	22.6		24.5	22.6	9.9	17.7	12.5
18:2	0.2	1.8		4.3	1.4	4.3	1.6	1.8
18:3		1.7		3.4	1.3	0	2.3	1.9
18:4	2.2	2.1		2.6	1.9	4.4	0.3	
20:0		0.4		0.1	0.1	0.1	0.3	
20:1	11.4	1.3		1.1	7.6	4.9	0.3	
20:2		0.6		0.6	0.5	0.3	0.3	1.7
20:4	0.5	2.3	1.4	0.8	0.5	1.2	1.5	
20:5 (EPA)	14.5	11.9	48.9	7.2	12.6	11.1	4.6	5.3
22:0		0.1					0.2	
22:1	8.6	0.2		0.1	5.2	7.5	0.8	
22:2								0.3
22:4	0.4	0.2						1.2
22:6 (DHA)	5.7	9.8	22.5	6.4	10.6	11.3	18.3	23.7
24:1						0.9	0.8	
C_{18} total	28.4	32.4		36.6	29.0	20.8	28.6	20.6
C_{20} total	26.4	16.5	50.3	9.8	21.3	17.6	7.0	7.0
C_{22} total	14.7	9.2	22.5	6.5	15.8	18.8	19.9	25.2
Percent EPA of all C_{20}	54.9	72.1	97.2	73.5	59.2	63.1	65.7	75.7
Percent DHA of all C_{22}	38.8	95.7	100.0	98.5	67.1	60.1	92.0	94.0

[a]DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

of fish oil ethyl esters with several types of reversed and normal stationary phases, using the separation of ester pairs ($C_{20:5}$/$C_{22:6}$) and ($C_{22:5}$/$C_{22:6}$) as a reference. Alumina stationary phase showed an especially good selectivity by the degree of saturation and chain length. They report that alkali treatment of the Al_2O_3 phase leads to substantial improvements in resolution.

The published reports and the operations of KD-Pharma GmbH clearly show that the separation of EPA and DHA ethyl esters from transesterified fish oil is technically possible using SC CO_2 eluant. However, the design of the economically most favorable purification process requires that the process variables be optimized for maximum productivity.

This paper describes a systematic procedure for developing an SFC separation method for producing EPA and DHA ethyl ester concentrates. The development was done in preparative, laboratory scale using the specific production rate of EPA and DHA ethyl esters as the target function, which was maximized. The specific production rate is the hourly production in grams of the desired compound per kilogram of stationary phase. The starting material was tuna oil, which is a low-value by-product of the fish meal industry. Statistical analysis of production rates calculated from nonpreparative SFC runs was first used to estimate the best operating conditions. A series of preparative SFC runs was then carried out at estimated optimal conditions to obtain the real production rate. The objective of this work was to study the technical and economic feasibility of producing EPA and DHA ethyl ester concentrates from by-product fish oil with SFC using a commercially available stationary phase and CO_2 eluant without co-solvents.

EXPERIMENTAL PROCEDURES

Materials. EPA and the ethyl ester of DHA were used as PUFA standards and purchased from Sigma-Aldrich Chemie (Deisenhofen). Their reported purities were 99%. EPA was esterified into the methyl ester by diazotization.

Carbon dioxide was food-grade quality (99.7% pure) from AGA Oy (Espoo, Finland). Absolute ethanol was from Primalco Oy (Helsinki, Finland). Anhydrous sodium sulfate was from Merck (Darmstadt, Germany).

Tuna (*Thunnus thynnus*) oil was obtained from Centro Technologico Gaiker (Zaimudio, Spain) from a Spanish fish-canning company. The oil contained 10.5 wt% water as determined with Karl Fischer titration. Its fatty acid composition was analyzed by first hydrolyzing the oil in NaOH-containing methanol, adding BF_3 in methanol to form methyl esters, extracting the methyl esters with *n*-heptane, drying the heptane phase with Na_2SO_4, and then analyzing the fatty acid ester mixture with gas chromatography–mass spectrometry (GC–MS) using the GC method described below. Commercial ethyl ester

made from sand launce (*Ammodytes lancea*) obtained from Grinsted Products (Aarhus, Denmark), was used in step 1 SFC runs where process variable levels were selected. Its reported fatty acid composition is presented in Table1.

Fish oil ethyl esters. The fatty acids in the tuna oil were converted to ethyl esters by transesterification with absolute ethyl alcohol. In the transesterification, 50 g tuna fish oil was first dried with anhydrous Na_2SO_4. Its water content after drying was 0.24 wt%. Dried oil was filtered and refluxed for 1.5 h with 350 g absolute ethyl alcohol. Freshly made sodium alcoholate was used as catalyst. The mixture was then extracted with *n*-hexane to obtain the fatty acid esters. The composition of the resulting fish oil ethyl ester mixture was analyzed by GC–MS and GC–flame-ionization detector (FID). The fatty acid compositions of the crude tuna oil and the transesterified oil are presented in Table 1. After transesterification, the DHA content of the oil was higher, the EPA content unchanged, and the oleic and palmitic acid contents lower than in the original oil. The authors suspect that the repeated extraction of the ethanol-containing aqueous layer with hexane did not remove all the lighter fatty acid esters. However, the slight change of the fatty acid composition during transesterification does not influence the SFC process development.

GC analysis. The fatty acids in the tuna oil, the transesterified oil, and the fractions collected from SFC runs were analyzed as ethanol solutions with a Hewlett-Packard (Palo Alto, CA) 9633 gas chromatograph, using an FID. The column was from J&W Scientific (Folsom, CA), DB-22, 30 m × 0.254 mm. Temperature program: 150°C; 5°C/min to 180°C, hold 25–30 min; 10°C/min to 250°C, hold 7 min. Injector: 250°C. Column flow 1 mL/min. Helium was used as the carrier gas. EPA and DHA ester GC peaks were located using the retention times of pure EPA and DHA esters.

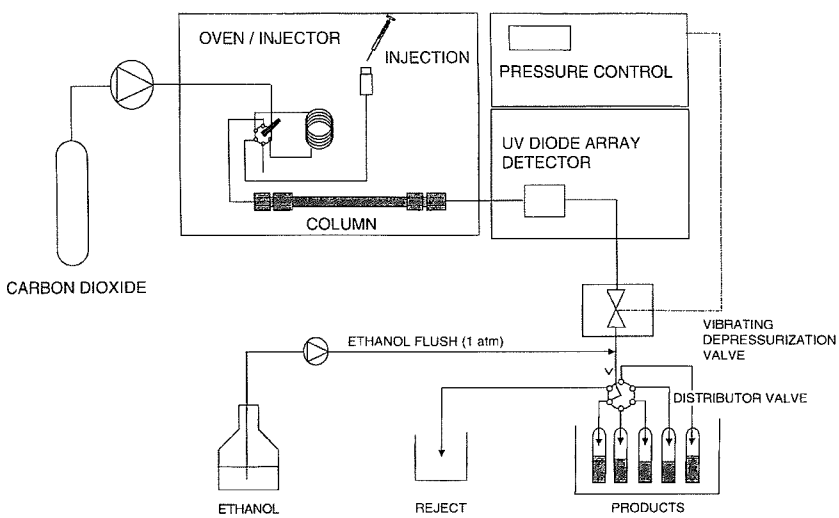
GC–MS analysis was done with the aforementioned method using a JEOL (Tokyo, Japan) SX-102 mass spectrometer. Fatty acid identification was done using a spectrum library and verified by using a known mixture of fatty acids. Margaric acid was used as internal standard.

Preparative supercritical chromatography. Preparative supercritical chromatography in milligram scale was done using modified Hewlett-Packard 1250-G SFC equipment. The columns were Kromasil 10-C18, 10 × 250 mm, and Kromasil 5-C18, 10 × 250 mm, obtained from Eka Chemicals AB (Bohus, Sweden). CO_2 as received was used as the mobile phase.

Transesterified fish oil was dissolved in ethanol at 10–50 wt% concentration. The ethanol solution was injected in the CO_2 eluant flow. To accommodate the large amount injected, the original automatic injector was replaced with a manual needle injection loop filler. Also, the original Hewlett-Packard restrictor valve was replaced with a JASCO (Tokyo, Japan) PB-880 vibrating restrictor valve system. From the restrictor valve the fluid was directed through a Rheodyne 7000 (Cotati, CA), six-port valve to vials that were immersed in an ice-water bath. From each injection, several successive fractions were collected and analyzed. The fractions were collected using the sequential-cut shaving technique, described previously (11). To flush the small, oily fractions quantitatively from the 1/16″ pipings, a small sidestream of ethyl alcohol was pumped continuously to a point in the piping immediately after the restrictor valve. The fractions were thus obtained as ethanol solutions. This is not necessary in industrial-scale SFC. The preparative SFC setup is depicted in Scheme 1.

RESULTS AND DISCUSSION

Selection of process variables. In industrial chromatography, much larger amounts of solutes must be injected per kilogram stationary phase than is customary in analytical work in order to achieve feasible production rates. However, increasing the injected amount decreases column efficiency, leading to decreasing resolution. At some column loads the required product purity cannot be achieved with any sacrifices in yield. For



SCHEME 1

a cost-effective chromatographic process, the process variables should be optimized so that the production rate per mass of stationary phase is at maximum while product purity is maintained at the required level.

The specific production rate in repetitive injection chromatography can be expressed in terms of the column load ratio:

$$PR = LR \cdot C_d \cdot \frac{Y/100\%}{t} \quad [1]$$

where PR is specific production rate [g of pure product/ (kg stationary phase · h)]; LR is load ratio (g injected solute/kg stationary phase); C_d is weight fraction of the desired component in starting material; Y is yield of the desired component of the injected amount (%); and t is injection interval (h).

Y in Equation 1 depends on column resolution, which is a function of column dimensions, stationary phase, and process variables in the following way:

$$R_s = 1/4(\alpha - 1)\sqrt{N}\left(\frac{k'}{1+k'}\right) \quad [2]$$

where R_s is resolution (dimensionless); α is separation factor (dimensionless); N is column plate number (dimensionless); and k' is retention factor (dimensionless).

Separation factor α is varied by changing the stationary and mobile phases and temperature. Plate number N is determined by column length, particle size, quality of packing, mobile phase, and linear velocity. In preparative chromatography, the plate number is strongly dependent on the column load ratio.

The retention factor k' depends on solvent strength, which in SFC can be varied by changing the pressure and density of the mobile phase. With a given column length, particle size, and stationary and mobile phases the resolution in SFC can thus be adjusted by changing the eluant linear velocity, pressure, temperature, and load ratio. To optimize these SFC process variables, a two-step statistical approach was used.

Step 1. Selection of process variable levels. Initial values for the four SFC process variables were selected based on the authors' previous experience in SFC studies. The variables and their ranges were: u, mobile phase linear velocity in packing (1.5–4.5 mm/s); p, column pressure (130–200 bar); T, column temperature (40–65°C); LR, column load ratio (1–10 g solute injected/kg stationary phase).

Three levels were given to each variable. A fractional factorial design was used to find process variable values for 20 SFC experiments. The experiments were done and the resulting SFC chromatograms were evaluated qualitatively and classified in three categories: good, fair, and not feasible.

It was found that 11 of the 20 combinations of process variable values resulted in chromatograms that would not be feasible in PUFA purification. The unacceptable values were identified, and reduced ranges for each of the four variables were set to avoid a large number of unacceptable value combinations in the next SFC experiments.

Step 2. Optimization of process variables. A new set of 20 SFC experiments was planned through fractional factorial design.The peaks that contained EPA and DHA esters were identified by injecting pure EPA and DHA esters separately and using their retention times for identification. Proper fraction collection intervals for obtaining pure EPA and DHA esters were visually estimated from each SFC chromatogram. The imaginary fraction collection times were selected so that there should be no overlapping of neighboring peaks during collection. Product yields were estimated from the segments of peak areas that were obtained during the imaginary fraction collection. The production rates for EPA and DHA esters were then calculated from Equation 1 using the data shown in Table 2. The calculated production rates are also shown in Table 2.

In order to optimize the conditions and to select the parameter values for the preparative work, a general linear model was fitted to the experimental data shown in Table 2. The best equation form was found by adding and removing first-order, second-order, and combination terms and fitting each equation to the data by the partial least squares (PLS) method. The best equation form was selected by comparing the fits using standard statistical tests. Principal component analysis (PCA) revealed that LR and temperature had the strongest effect on DHA and EPA production rates. Mobile- phase linear velocity and column pressure had lesser effects on the production rates.

The best-fitting general linear model for DHA and EPA PR was found to be the form of Equation 3:

$$PR = a + b \cdot p + c \cdot p^2 + d \cdot u^2 + e \cdot \exp(LR) + f \cdot T^2 + g \cdot p \cdot \exp(LR) + h \cdot p * T + i \cdot u \cdot T + j \cdot \exp(LR) \cdot T \quad [3]$$

where a through j are constant regression coefficients (PR of DHA/PR of EPA, respectively): *a,* –32.1/–3.4; *b,* 0.44/0.047; *c,* –0.002/–0.00023; *d,* –0.56/–0.025; *e,* –1.66E-10/0; *f,* –0.006/–0.0003; *g,* –4.24E-08/–2.84E-09; *h,* 0.005/0.00028; *i,* 0.039/0.002; *j,* 5.57E-07/2.06E-08.

The numerical values of the coefficients apply when the previously shown units are used for the variables. The standard error of the highest PR estimate from Equation 3 was ±0.67 g product/(kg stationary phase · h) for DHA and 0.05 g product/(kg stationary phase · h) for EPA. The standard errors of the coefficients were generally quite large, indicating a lot of noise in the data of Table 2. However, visual inspection of the calculated response surfaces from Equation 3 revealed clear trends that could be used for selecting the parameter ranges for preparative experiments in step 3.

Response surfaces were calculated from Equation 3 covering the range of process variables that were used in the step 2 experiments. Several response surfaces were drawn by alternatively changing two parameters and keeping the other two constant. The maximal production rates, calculated from Equation 3, were then found and the corresponding optimal process parameter values discovered. The best estimated SFC conditions for fish oil ester fractionation are u = 1.9 mm/s; p = 145 bar; column temperature = 65°C; LR = 4.95 g/kg.

Under optimal conditions, the estimated (Eq. 3) maximal PR for pure DHA ethyl esters was 2.46 g DHA-ethyl ester/(kg stationary phase · h). For pure EPA, the estimated maximal pro-

TABLE 2
Process Parameters and Calculated Resulting DHA and EPA Production Rates from Step 2 SFC Experiments

Run no.	Pressure (bar)	Temperature (°C)	Fluid linear velocity (mm/s)	Column load ratio (g/kg)	Injection interval (min)	Visual yield of DHA (%)	Calculated prod. rate of DHA [g/(kg·h)]	Visual yield of EPA (%)	Calculated prod. rate of EPA [g/(kg·h)]
1	130	55	1.5	1.0	36	90	0.35	20	0.02
2	130	55	2.5	1.0	14	85	0.84	0	0.00
3	160	55	1.5	1.0	15	50	0.46	0	0.00
4	160	65	2.5	1.0	11	85	1.07	0	0.00
5	160	65	1.5	1.0	22	90	0.56	10	0.01
6	130	65	2.5	1.0	33	95	0.40	50	0.05
7	130	55	1.5	5.0	30	60	1.38	75	0.38
8	160	55	1.5	5.0	20	40	1.38	0	0.00
9	160	55	2.5	5.0	11	0	0.00	0	0.00
10	130	65	1.5	5.0	90	100	0.77	100	0.17
11	130	65	2.5	5.0	42	100	1.64	100	0.36
12	160	65	2.5	5.0	15	50	2.30	40	0.40
13	145	60	1.5	3.0	30	75	1.04	75	0.23
14	145	60	2.5	3.0	15	60	1.66	50	0.30
15	130	60	2.0	3.0	38	50	0.54	90	0.21
16	160	60	2.0	3.0	14	40	1.18	0	0.00
17	145	55	2.0	3.0	15	0	0.00	0	0.00
18	145	65	2.0	3.0	24	90	1.55	80	0.30
19	145	60	2.0	1.0	19	80	0.58	10	0.02
20	145	60	2.0	5.0	20	80	2.76	50	0.38

[a]SFC, supercritical fluid chromatography. See Table 1 for other abbreviations.

duction rate was 0.16 g EPA-ethyl ester/(kg stationary phase · h).

Step 3. Preparative verification of estimated PR. Transesterified tuna oil was injected in the SFC column at three load ratios: 1.25, 2.5, and 5.0 g crude ester mixture/kg stationary phase. The other process variables were kept at the estimated optimum conditions. The obtained SFC chromatograms at each column LR are depicted in Figure 1. At the highest load ratio of 5 g/kg the peaks became distorted and peak doubling was visible. This was apparently caused by overloading and not by column irregularities since the retention times were repeatable and at lower loading the peaks were symmetrical. Five fractions were collected from each EPA- and DHA-containing peak. The purities of EPA and DHA in each fraction are shown in Table 3.

It was observed that the PR was a strong function of the LR. At 5.0 g crude/kg stationary phase, the purest obtained fraction contained 87 wt% DHA. At 2.5 g/kg load ratio, more than 90 wt% pure DHA-ethyl ester fractions were collected. At the smallest, 1.25 g/kg load, the three purest fractions could be combined to obtain a product that constituted most of the peak and contained more than 95 wt% DHA. The corresponding production rates are shown in Figure 2.

The most critical impurities in obtaining pure DHA were other C_{22} esters. SFC separation between 22:5 and 22:6 was incomplete, but due to the marginal amount of 22:5 in the starting material, 22:5 did not interfere with DHA purification. At all load ratio levels, the first DHA fractions contained also 18:0 and 18:1 esters. This indicates that the low unsaturated C_{18} esters tend to tail. This was not observed with C_{20} esters nor with C_{18} esters of higher degree of unsaturation. The separation of 20:5 (EPA) from 22:6 (DHA), judged feasible by Reichmann and Brunner (10), was complete at each load ratio. The calcu-

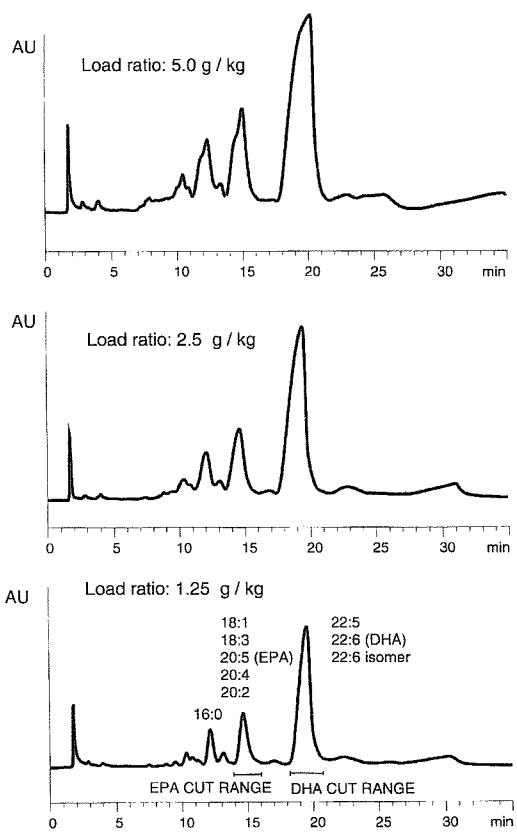


FIG. 1. Supercritical fluid chromatograms from preparative fish oil ethyl ester injections at different column load ratios. Fraction collection intervals for docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) concentrates are indicated. Kromasil 10-C18, 10 × 250 mm column. Pressure, 145 bar. Temperature 65°C. Mobile-phase linear velocity 1.9 mm/s. AU refers to absorbance units from the ultraviolet detector.

TABLE 3
DHA- and EPA-Ethyl Ester Fraction Purities (wt%) Obtained from Step 3 Preparative SFC Experiments[a]

	Load ratio (1.25 g/kg)		Load ratio (2.5 g/kg)		Load ratio (5.0 g/kg)	
Fraction	DHA purity	EPA purity	DHA purity	EPA purity	DHA purity	EPA purity
1	0.0	0.0	0.0	0.0	58.9	0.0
2	57.3	17.8	45.9	6.9	52.9	13.6
3	93.7	43.6	91.1	53.8	86.6	25.0
4	100.0	31.8	76.1	51.2	68.1	33.1
5	100.0	37.5	55.8	53.5	55.2	11.0

[a]For abbreviations see Tables 1 and 2.

lated SFC separation factor between EPA and DHA was good, $\alpha = 1.40$.

In the preparative runs, the measured production rate at 90 wt% purity was 0.85 g DHA-ethyl ester/(kg stationary phase · h). At 80 wt% purity the production rate was 1.9 g DHA ethyl ester/(kg stationary phase · h). These are lower than the estimated (Eq. 3) production rate, which was based on visual estimates of collection intervals from chromatograms. Visual estimation obviously does not reveal the elution of minor impurities within the main DHA peak. Therefore, it is imperative that fractions from the main peaks be collected and analyzed so that the real production rates can be calculated.

Purification of EPA-ethyl ester was far more demanding. The purest EPA fractions at 5.0 and 2.5 g/kg load ratios contained, respectively, 33.0 and 53.8 wt% EPA. Reducing the load to1.25 g/kg did not increase EPA purity. Similarly to the preparation of DHA, also in the separation of EPA the main impurities were stearic (18:0) and oleic (18:1) acid esters. At 50 wt% purity, the specific production rate of EPA was 0.23 g/(kg · h) while the calculated PR maximum from Equation 3 was 0.16 g / (kg · h).

The FAEE eluted primarily in the order of increasing carbon number with CO_2 mobile phase and ODS stationary phase. Within each carbon number, the most unsaturated esters eluted first.

The effect of LR on the experimentally verified PR at different purity levels is shown in Figure 2. The PR, calculated as pure PUFA, increase as column loading increases. However, when the LR exeeds a certain value at constant purity the production rate starts to decrease because the amount of collected fraction that meets the purity level decreases. When the required purity of the PUFA is set there is always an optimal column LR that gives the highest specific production rate.

Process design and purification costs. The process design and cost estimate are based on the results from the preparative SFC experiments carried out in Step 3. The production of DHA- and EPA-ethyl ester concentrates from transesterified tuna oil at 80–95 and 50 wt% respective purities requires only one supercritical chromatographic step. The flowsheet of an industrial SFC process, with descriptions of main equipment, has been described previously (12). An SFC process, which produces 1,000 kg DHA-ethyl ester and 400 kg EPA-ethyl

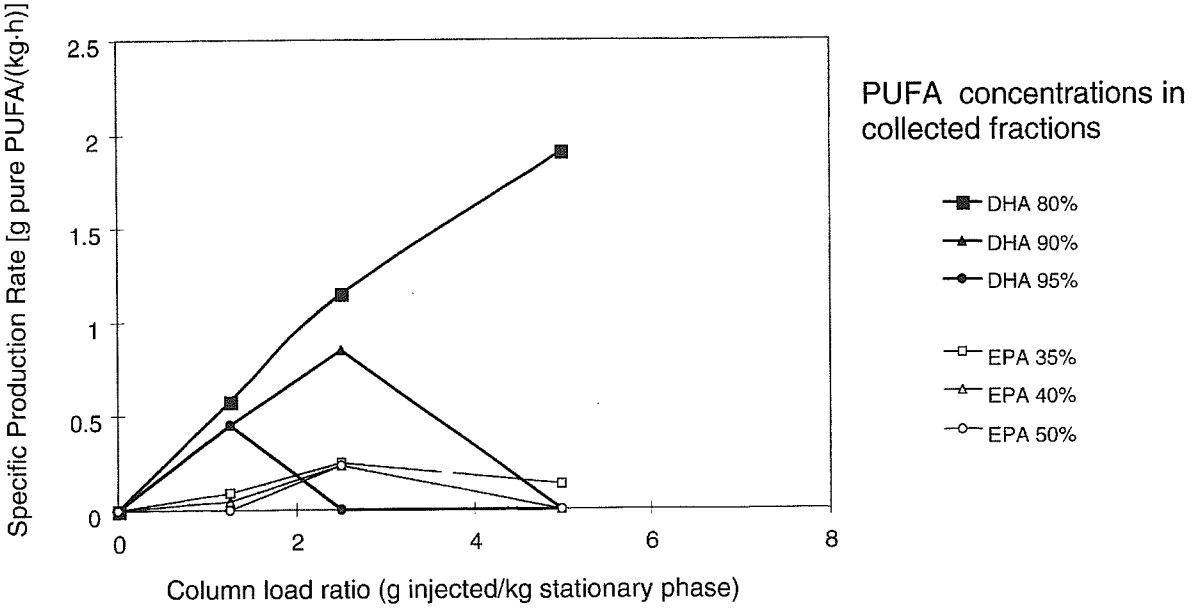


FIG. 2. The effect of column loading on the specific production rate of polyunsaturated fatty acids (PUFA) at different purities . Calculated from results obtained in Step 2 preparative SFC experiments. See Figure 1 for abbreviation.

ester concentrates per year requires that 2.6 tons of CO_2 per hour is circulated in the process. The ODS stationary phase requirement is 160 kg, which would preferably be packed in four parallel 600-mm i.d. columns. Main equipment for such an SFC process costs about U.S. $2 million. In assuming that the stationary phase would have to be replaced once a year, the total SFC operating costs are U.S. $550/kg DHA and EPA concentrate. The purification cost is sensitive to the lifetime of the stationary phase. The cost almost equals the US $200–500 range reported in 1994 by KD Pharma (4) and is considerably less than the US $ 4,000/DHA concentrate (95%) reported by Shisheido Corp. in 1996 (13) where a proprietary, silver-containing stationary phase was used.

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PAPER IV

Chiral, Sub-Critical Chromatography at Cryogenic Temperatures

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Chiral, Sub-Critical Chromatography at Cryogenic Temperatures

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1. Introduction

Nearly 50% of active pharmaceutical ingredients (API) are chiral. Since different enantiomers have different physiological properties US FDA demands that the effect of both enantiomers are to be scrutinized separately. More than one-third of API's produced globally, are single-enantiomer drugs and their proportion is increasing almost 10% annually.

Single-enantiomer drugs are tried to synthesize selectively by using non-symmetrical starting material, low temperature (- 60 C) and chiral catalysts. This is not possible or economical in all cases and the synthesis then yields a racemic mixture which is resolved by *e.g.* crystallization or chiral chromatography. Chiral chromatography is often regarded as expensive, due to high solvent consumption and fairly expensive stationary phase (CSP). The economics of chiral chromatography can be improved by shortening the throughput time and increasing the load ratio.

Increasing column capacity is possible only if the separation factor is sufficiently large because at high loads the peaks tend to overlap. In some cases of fluid chromatography (SFC and HPLC) the separation factor is inversely proportional to column temperature. Therefore, the separation factor is greatly increased when the separation takes place at cryogenic temperature.

Liquid carbon dioxide is especially suitable as cryogenic solvent due to its very low viscosity even at low temperatures. The low viscosity is retained also when 5-10% polar modifier (MeOH, EtOH) is added. At low temperatures, ethanol is soluble to CO_2 in all proportions if only pressure is more than ca. 100 bars [1, 2]. Moving phase viscosity is essential in chromatography, since the pressure-drop across the column may easily become excessive at low temperatures. In that respect HPLC is much more problematic than SFC.

In this paper the abbreviation SFC is used both for supercritical and sub-critical fluid chromatography that is, all chromatography where pressurized CO_2 is used as the bulk solvent. The objective of this work was to study how the use of low temperature improves the separation factor and to calculate the possible productivity increase. This paper describes preparative chiral SFC of the enantiomers of a synthetic racemic aromatase inhibitor at sub-critical temperatures. The eluent was liquid carbon dioxide, modified with methanol or ethanol. The stationary phases were two new commercial chiral stationary phases (CSP) N,N'-diallyl-L-tartramide (DATD) [3] by Akzo Nobel , Sweden. In these CSP's, the chiral moiety is cross-linked directly into silica matrix.

2. Materials and methods

Materials

Chiral laboratory columns were HPLC columns packed with Kromasil CHI-TBB and Kromasil CHI-DMB CPS by Akzo Nobel Ab, Bohus Sweden. Laboratory runs were done with 4.6 x 250 mm columns of both type and preparative runs were performed with 10 x 250 mm column CHI-TBB

Chiral pilot column was packed on-site from Kromasil CHI-TBB 5 um bulk phase material using pneumatically compressed 50 mm dynamic axial compression (DAC) column.

Sample: The racemic Finrozole (TM) aromatase inhibitor was from Hormos Medical Oy, Turku, Finland. The molecular structure of Finrozole is presented in [4]. With 2 chiral centers, it contains 2 diastereomeric enantiomer pairs. The starting material was pre-purified, containing only one of enantiomeric pair, forms *a* and *d* . Injectons were done as THF / Toluene solutions, 25 mg/ml.

Carbon dioxide was food grade (99.7% pure) from Oy AGA Ab, Espoo Finland

Methanol, THF and toluene were reagent grade from Rathburn Chemicals, England

Ethanol was absoluted technical grade (min 99.5%) made by Primalco Oy, Rajamäki Finland

Experimental setup

Laboratory runs

A modified Jasco Super 200 SFC system was used in laboratory and preparative runs. The detailed description is published elsewhere [5]. The column was placed in a cooling jacket connected to cryostat whose minimum temperature was - 30 C.

Pilot runs

The pilot setup is depicted in figure 1. Liquid CO_2 and modifier is mixed and pumped through pre-cooler to the cryostat-cooled dynamic axial compression column. The structure of this column is described previously [6]

Analysis

The purified products were analyzed with Hewlett-Packard G-1205 SFC at 0 C using Kromasil CHI-DMB 4.6 x 250 mm column.

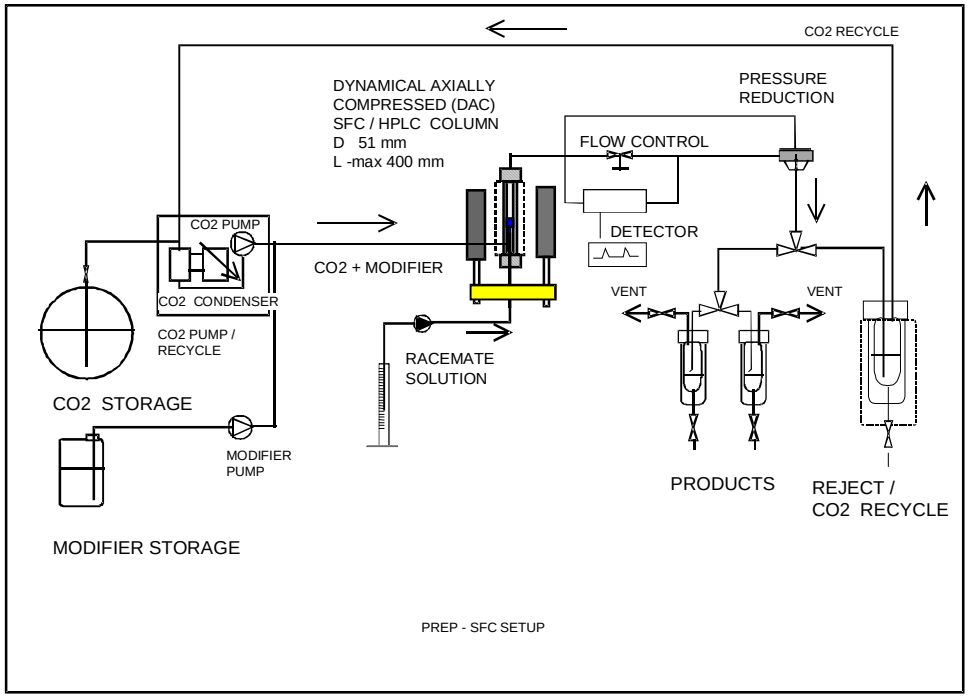

Figure 1. Pilot-scale setup of Finrozole separation

3. Results and discussion.

3.1 Effect of column temperature

When racemic Finrozole is separated with chiral SFC , the single peak gradually resolves in two when temperature is decreased This indicates that the separation factor increases when lowering the temperature. (fig. 2).

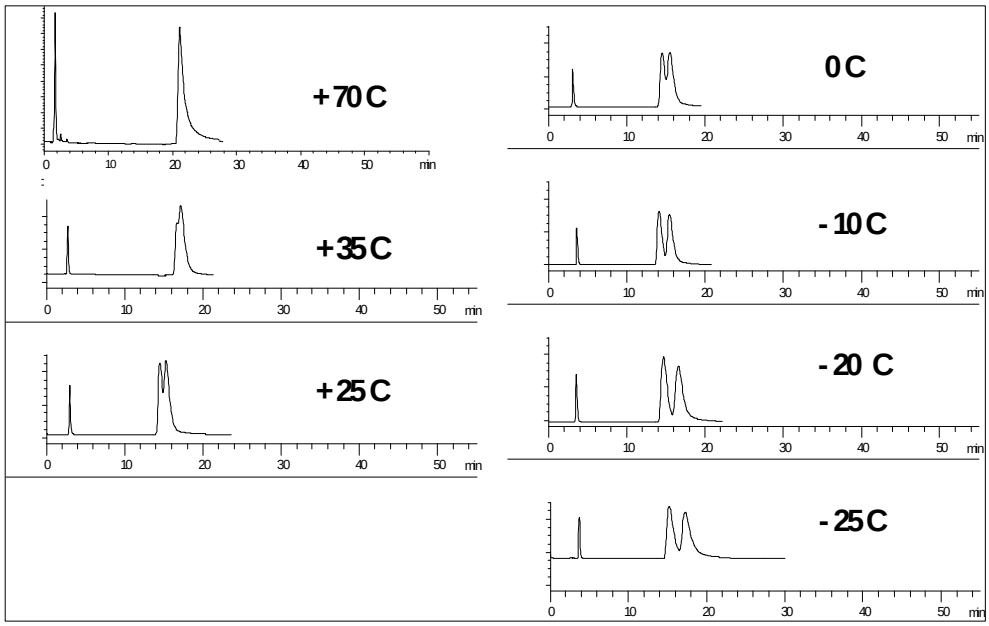

Figure 2.
Change of enantioselectivity of Finrozole separation when changing temperature.

The enantioselective separation factor α is related to the free energy of binding [7] :

$$\delta(\Delta G) = -RT\ln(\alpha) = -\delta(\Delta H) + \delta T(\Delta S) \qquad (1)$$

$$\ln(\alpha) = -\frac{\delta(\Delta G)}{RT} = \quad -\frac{\delta(\Delta H)}{RT} + \frac{\delta(\Delta S)}{R} \qquad (2)$$

Where $\delta\Delta H$ and $\delta\Delta S$ are the differences in enthalpy and entropy of binding between the enantiomers. From equation (2) it follows that low temperatures will increase the relative importance of the enthalpy term, which then increases the separation factor. Both terms are negative under these circumstances. Stringham and Blackwell [8] have pointed out that when varying the elution temperature there is an isoentropic temperature where both terms of equations (1) and (2) are equal. Thus, at isoentropic temperature $\alpha = 1$ and both enantiomers elute simultaneously. Above this temperature the elution order is reversed ($\ln\alpha < 0$; $\alpha < 1$) and when temperature is further increased the reversed separation factor increases. Similarly, when decreasing temperature at less than isoentropic point, separation factor increases but with different elution order

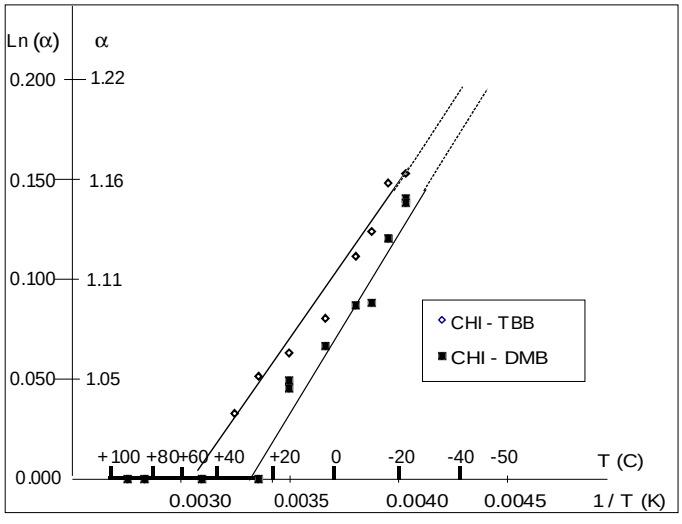

Figure 3.
The temperature dependence of the logarithm of separation factor (α) in separation of Finrozole

From practical point of view the location of isoentropic temperature is essential. The isoentropic point in typical SFC is often located at temperature range +40…+80 C and the reversing of elution order may become apparent only at temperatures much over +100 C. Similarly, at the lower end the resolved peaks may become visible only at temperatures well below the isoentropic temperature. Figure 3 depicts the measured separation factor of the system Finrozole / CHI-TBB and CHI-DMB / CO2 / MeOH as a function of elution temperature. According to equation 1, there should be a linear relationship between 1/T and ln(α).

According to figure 3, the isoentropic temperature of this system lies between 40...60 C on CHI-TBB and about +25 on CHI-DMB. When temperature decreases the separation factor increases. The separation factors were higher on Kromasil CHI-TBB than with CHI-DMB. The lowest experimental points on both CSP's were at - 25 C where the separation factors were at 1.18 and 1.14 on CHI-TBB and CHI-DMB, respectively. Resolution (eq. 3) is very sensitive to separation factor, since it contains the term (α-1):

$$Rs = 1/4 \times (\alpha - 1) \times \frac{k'}{1+k'} \sqrt{N} \qquad (3)$$

R_s column resolution
N column plate number
k' capacity factor
α separation factor

3.2 Comparison of SFC and HPLC in low temperature chromatography

Table 1 summarises a series of test runs with Kromasil CHI-TBB. The column pressures were up-stream controlled that is, the UV-detector exit pressure was kept constant via computer controlled back-pressure valve and the eluent pumping rate was constant. The column inlet pressure was thus, controlled mainly by the column's internal pressure drop caused by viscosity. The bulk solvents were carbon dioxide and n-hexane. With CO_2 the column exit pressure was kept at 190 bar. Therefore, the chromatography type was SFC. With n-hexane the column exit pressure was kept at 10 bars (to prevent bubble formation in detector). This corresponds HPLC albeit the detector back-pressure was slightly larger than usual.

Table 1. Comparison of separation of Finrozole at different temperatures using HPLC and SFC. Column: Kromasil CHI-TBB 4.6 x 250 mm. Too slow elution time means that no peaks eluted within the 45 min run time.

Moving phase	Temp C	Flow ml/min	P-in bar	P-out bar	t-ret Finrozole a	t-ret Finrozole d	sepa-ration factor
CO2 + 6% MeOH	+22	1.5	199	180	14.99	15.43	1.05
CO2 + 8% MeOH	+22	1.5	201	180	10.41	10.85	1.04
CO2 + 10% MeOH	+35	1.5	205	190	16.70	17.16	1.03
CO2 + 10% MeOH	0	1.5	202	180	7.71	8.16	1.06
CO2 + 10% MeOH	-10	1.5	205	190	14.12	15.45	1.12
CO2 + 10% MeOH	-15	1.5	214	190	14.59	16.15	1.13
CO2 + 10% MeOH	-20	1.5	220	190	14.70	16.60	1.16
CO2 + 10% MeOH	-25	1.5	222	190	15.24	17.31	1.17
Hexane/IPA 95:5	+22	1.0	67	9	too slow	too slow	-
Hexane/IPA 90:10	+ 22	1.0	66	9	19.7	21.83	1.11
Hexane/IPA 90:10	0	1.0	64	9	too slow	too slow	-
Hexane/IPA 80:20	+22	1.0	95	10	9.56	10.36	1.08
Hexane/IPA 80:20	0	1.0	81	10	12.23	13.63	1.11
Hexane/IPA 50:50	+22	0.8	83	10	5.10	5.10	1
Hexane/IPA 50:50	0	0.8	>110	10	Exceeded the pressure drop limit 100 bar		

In SFC the moving phase was CO_2, modified with MeOH and in HPLC, the moving phase was Hexane/2-propanol mixture in various proportions. The main chromatographic parameters are summarized in table 1.

In HPLC mode, the effect of temperature on elution time was substantial. With 95:5 and 90:10 hexane/2-propanol mixtures the elution time at 0 C was too long. At least 20% polar component was needed for practicable elution time at 0 C. In SFC mode, the ambient temperature separation factor was slightly smaller than in corresponding HPLC but it was possible to increase separation factor in SFC substantially just by lowering temperature down to -25 C. In HPLC mode this was not possible, since the amount of polar component that was needed to limit the elution time, eventually increased the column back-pressure over the recommended values.

3.3 Productivity of cryogenic SFC

In all chromatographic types the economics is roughly determined by a few basic parameters: productivity , solvent consumption and investment costs [9]. The productivity gives an indication on how large process is needed to handle starting material at a given hourly rate. The productivity in ordinary pulse chromatography is given in equation 4.

$$P = M_{sample} / (M_{CSP} * t_{CYCLE}) = LR / h \qquad (4)$$

P	Productivity (g sample/ kg CSP / h)
M_{SAMPLE}	Size of injection in grams
M_{CSP}	Size of column packing in kilograms
t_{CYCLE}	Injection interval in hours
LR	Load ratio (g injected sample / kg packing material)

In simulated moving bed (SMB) chromatography, the productivity can be calculated in similar way, if hourly feed rate and total column sizes are known.

The most crucial variable in productivity is the column load ratio LR, whose values in preparative chromatography vary broadly, typically at 0.1...20 g/kg. In chiral chromatography the LR values are smaller than in non-chiral chromatography. The feasible LR and thus, the productivity depends on the separation factor in a strongly non-linear way.

Table 2 summarizes the productivities and solvent consumptions in cryogenic SFC separation of Finrozole enantiomers. In bench and in pilot scale the respective columns were 10 mm pre-packed and 50 mm ID on-site packed DAC. In both cases the work was done at -25...-30 C temperature, using methanol and ethanol as modifiers. For comparison, the table includes three other chiral chromatographic separations from the literature. The first two cases (Jusforques *et al* [10]) present separation of guiafenecine with SFC at +10C and HPLC at ambient temperature. In the last case (Denet *et al* [11]) the enantioseparation of tetralol with SMB-SFC has been calculated in comparable terms.

The load ratio of Finrozole was modest at 0.30…0.40 g sample / kg CSP resulting in 3.0...4.0 g/kg/h productivity. This is somewhat higher than the calculated productivity from the SMB-SFC data of Denet *et al* in separation of tetralol using Daicel OD phase. On the other case, Jusforques *et al* loaded guaifenecine more than 8 g/kg, using same stationary and moving phases. Their productivity was ca. 100 g/kg/h. The difference arises from the separation factors: on guaifenecine / Chiracel OD separation factor was $\alpha = 1.80$ while on Finrozole/ Kromasil CHI-TBB the separation factor was $\alpha = 1.20$ This difference increases resolution (eq. 3) by factor of 4 which according to our measurements corresponds to about 10 – 20 times increase in productivity.

Table 2. Comparison of productivities and solvent consumptions on some chiral SFC and HPLC separations. All solute values are indicated as dry matter.

Sample	**Finrozole**	**Finrozole,**	**Guaifenecine Jusforques et al**	**Guaifenecine Jusforques et al**	**Tetralol Denet et al**	
Mode	**SFC pulse**	**SFC pulse**	**HPLC**	**SFC-pulse**	**SMB-SFC**	
Stat. phase	**Kromasil CHI-TBB**	**Kromasil CHI-TBB**	**Daicel Chiralcel OD**	**Daicel Chiralcel OD**	**Daicel Chiralcel OD**	
Bulk solvent	**CO2**	**CO2**	**EtOH/Hex**	**CO2**	**CO2**	
Modifier	**MeOH**	**EtOH**		**EtOH**	**EtOH**	
Column ID	10	50	4.6	10	33	x 8 columns
Column Lenght	250	230	250	250	200	
Temperature, C	-25	-25	+10	ambient	+40	
Separation factor	1.18	1.20	1.80	1.80	1.10	
Load, g	0.004	0.075	0.020	0.104	0.061	per cycle
Phase, kg	0.011	0.25	0.0026	0.013	0.109	per column
Flowrate, kg/h	0.27	7.3	0.048	0.96	6.0	all columns
Modifier-%	8.0	7.4		10.0	5.7	
Cycle time, min	6	6	4	5	4.6	
modifier consumption, kg/h	0.022	0.540		0.096	0.34	all columns
Load Ratio (LR), g/kg	0.40	0.30	7.56	8.32	0.56	all columns
Productivity , g/kg/h	4.0	3.0	113.4	99.8	0.92	all columns
Productivity , g/kg/ 24 h	80	60	2268	1997	18	
Feed rate, g/h	0.04	0.75	0.30	1.25	0.80	all columns
modifier consumption, g/g feed	500	720		76.92	428	all columns
CO2 g/g feed	6 250	9 733		769	7 500	all columns
HPLC bulk solvent g/g feed			160			

4. Conclusions

The use of cryogenic sub-critical chromatography increased the separation factor of Finrozole from 1.05 at ambient temperature to 1.20 at – 25 C. The maximum productivity was ca. 4 g / kg/h which was more than one order of magnitude higher than the same at room temperature. Literature data suggests that if the separation factor could be increased to 1.80 then about 100 g/kg/h productivity could be achieved. Further decreasing of temperature would increase separation factor. This can be deduced from published data [12] where comparable SFC had been performed at near –50 C. In the present case (figure 3) separation factor at -50 C would be ca. 1.25 which by rough estimation, would double Finrozole separation productivity from the present maximum value.

Minor revision on Table 1 was done by Martti Alkio on October 24, 2007

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Title

Purification of pharmaceuticals and nutraceutical compounds by sub- and supercritical chromatography and extraction

Abstract

This thesis discusses the use of sub- and supercritical fluids as the medium in extraction and chromatography.

Super- and subcritical extraction was used to separate essential oils from herbal plant *Angelica archangelica*. The effect of extraction parameters was studied and sensory analyses of the extracts were done by an expert panel. The results of the sensory analyses were compared to the analytically determined contents of the extracts.

Sub- and supercritical fluid chromatography (SFC) was used to separate and purify high-value pharmaceuticals. Chiral SFC was used to separate the enantiomers of racemic mixtures of pharmaceutical compounds. Very low (cryogenic) temperatures were applied to substantially enhance the separation efficiency of chiral SFC. The thermodynamic aspects affecting the resolving ability of chiral stationary phases are briefly reviewed.

The process production rate which is a key factor in industrial chromatography was optimized by empirical multivariate methods. General linear model was used to optimize the separation of omega-3 fatty acid ethyl esters from esterized fish oil by using reversed-phase SFC. Chiral separation of racemic mixtures of guaifenesin and ferulic acid dimer ethyl ester was optimized by using response surface method with three variables per time. It was found that by optimizing four variables (temperature, load, flowate and modifier content) the production rate of the chiral resolution of racemic guaifenesin by cryogenic SFC could be increased severalfold compared to published results of similar application.

A novel pressure-compensated design of industrial high pressure chromatographic column was introduced. A demonstration SFC plant was built and the immunosuppressant drug cyclosporine A was purified to meet the requirements of US Pharmacopoeia. A smaller semi-pilot size column with similar design was used for cryogenic chiral separation of aromatase inhibitor Finrozole for use in its development phase 2.

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Supercritical technology enables the use of environmentally green solvents like carbon dioxide in producing pharmaceutical and nutraceutical compounds. Unlike many organic solvents, carbon dioxide is relatively inexpensive, non-toxic and easily recirculated. This thesis focuses on the use of compressed dense carbon dioxide as a solvent in extractive and chromatographic separation of high-value compounds. Carbon dioxide can be used as such in processing lipophilic substances like essential oils or fatty acid monoesters and triglycerides. It can be easily modified with light molecular weight alcohols which makes it compatible with many pharmaceutical compounds.

Carbon dioxide is a good extraction solvent for essential oils of plants, like Angelica, Juniper or Bitter orange. It can also be used in removing cholesterol from dairy products and egg yolk. In this thesis the effect of extraction conditions on the quality of Angelica root extracts is discussed.

Carbon dioxide was also studied as the mobile phase in high-pressure chromatography, either as such or modified. Using silica and C18-grafted silica as the stationary phases cyclosporines and omega-3 fatty acid monoesters were purified. Chiral enantiomers of racemic mixtures were separated by using liquid CO_2 based mobile phase in very cold conditions. Empirical optimization based on statistical methods was used to maximize the production rates. Novel pressure-compensated design of high-pressure column was introduced and used in industrial demonstration sized chromatographic plant.

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