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DIPLOMARBEIT

Titel der Diplomarbeit

Phytonutriments and Chemoprevention:
the Role of Polyphenols in the Prevention of Colorectal Cancer

angestrebter akademischer Grad

Magistra der Pharmazie (Mag.pharm.)

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List of abbreviations

a	acetone
AcOEt	ethyl acetate
APC	adenomatous polyposis coli
C	column
CFR aq	coing ferme rolli aqueous extract (quince)
CFR OPC	coing ferme rolli OPC enriched extract (quince)
CRC	colorectal cancer
cyclo	cyclohexane
EDTA	ethylendiamin-tetra-acetic-acid
EtOH	ethanol
FAP	familial adenomatous polyposis
MEM	Minimum Essential Medium
MeOH	methanol
no.	number
OPCs	oligomeric procyanidins
PFR aq	pomme ferme rolli aqueous extract (apple)
PFR OPC	pomme ferme rolli OPC enriched extract (apple)
RP-HPLC	reversed phase high performance liquid chromatography
TLC	thin layer chromatography
TRAIL	TNF related apoptosis inducing ligand
UV-DAD	ultra-violet diode array detection
w	demineralised water

1 Introduction

1.1 Cancer

Cancer is a serious problem of today's society. According to the World Cancer Report 2008, the global cancer burden is increasing steadily. In 2008, 7,6 million deaths from cancer and 12,4 million new cancer cases were reported. [1]

The incidence (number of new cases occurring) and mortality (number of deaths) in more and less developed countries for women and men are shown in figures 1 and 2.

The most common cancers in men are lung cancer, prostate cancer followed by stomach and colorectal cancer (Figure 1).

In women the most common cancers are breast and cervix uteri cancer, colorectal cancer and lung cancer (Figure 2).

It is estimated that incidence and deaths from cancer will more than double by the year 2030. Factors for the vast increase of this disease are mainly the growth and aging of population. [1]

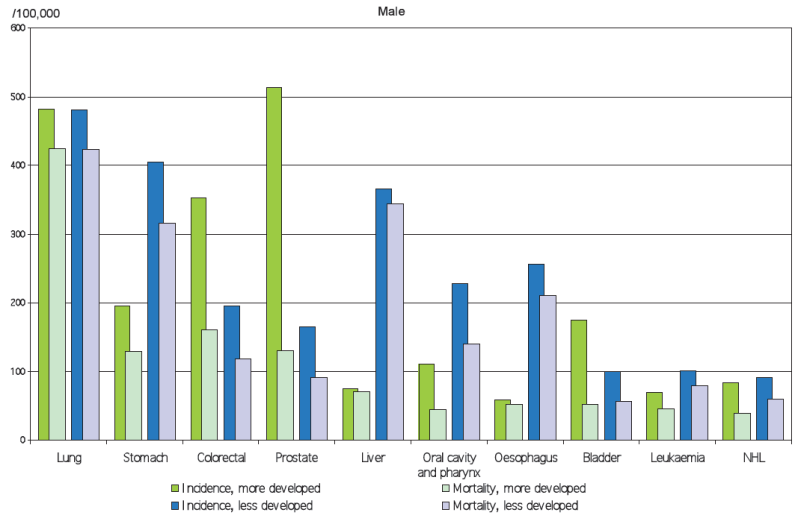


Figure 1: Worldwide cancer burden in men [1]

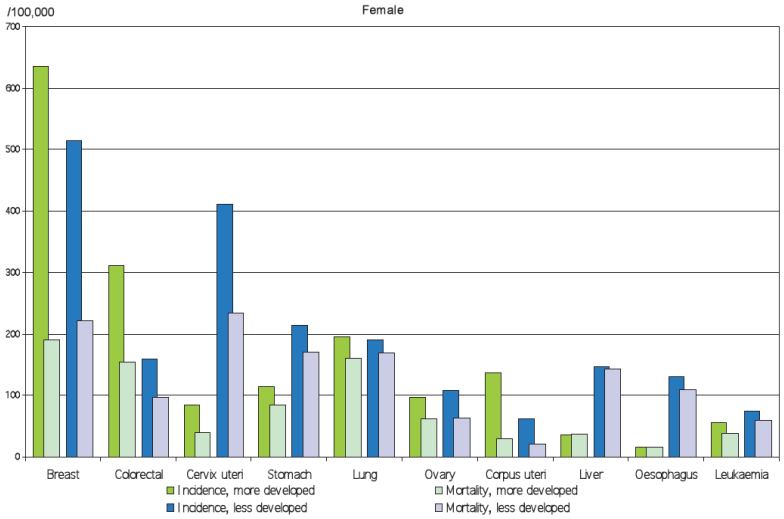


Figure 2: Worldwide cancer burden in women [1]

The prevention of cancer is becoming a more and more important issue due to this development. [1, 2]

1.1.1 Mechanisms of Carcinogenesis

Cancer is a progressive disease whose development can take 1-40 years (Figure 3). [2]

Cancer starts with small lesions in the DNA called mutations.

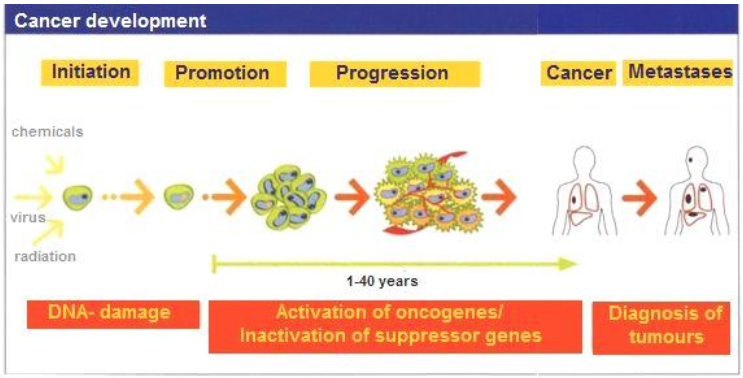


Figure 3: The stages of cancer development. Adapted from Beliveau and Gringas (2005) [2]

Mutations can appear as DNA base modifications, covalent bridges between DNA strands or single or double strand breaks. [1]

Many risk factors for DNA damage and carcinogens have already been defined. Béliveau and Gringas accord nutritional deficits and tobacco with 30% each, genetic susceptibility with 15%, infections (*Hepatitis B and C virus, Human Papilloma Virus*), professional risks and obesity/lack of physical activity with 5% each, alcohol with 3%, UV-radiation, drugs, pollution with 2% each and other reasons with 1% risk of cancer development (Figure 4). [2]

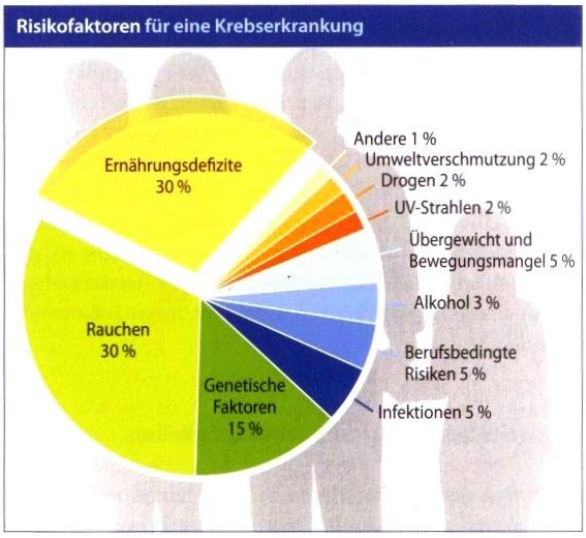


Figure 4: Risk factors for cancer development. From Beliveau and Gringas (2005) [2]

Normally those DNA-damages are detected during the cell cycle and through the activation of checkpoints at the G_1/S-phase or the G_2/M-phase DNA damage can be repaired (Figure 5). Therefore a mutation does not imperatively cause cancer. [3]

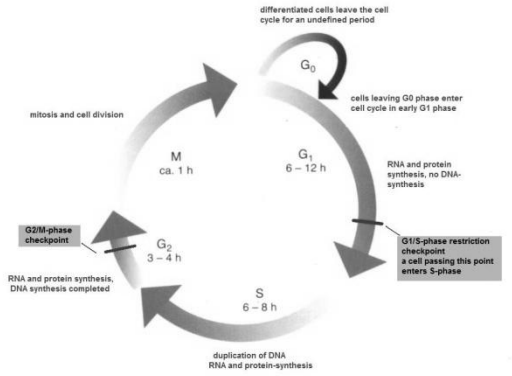


Figure 5: Cell cycle. Adapted from Dingermann et al. (2002) [4]

Cells with DNA damages beyond repair enter programmed cell death (apoptosis). Numerous endogenous and exogenous factors for starting the apoptotic signal transduction pathways have been identified (Figure 6). [1]

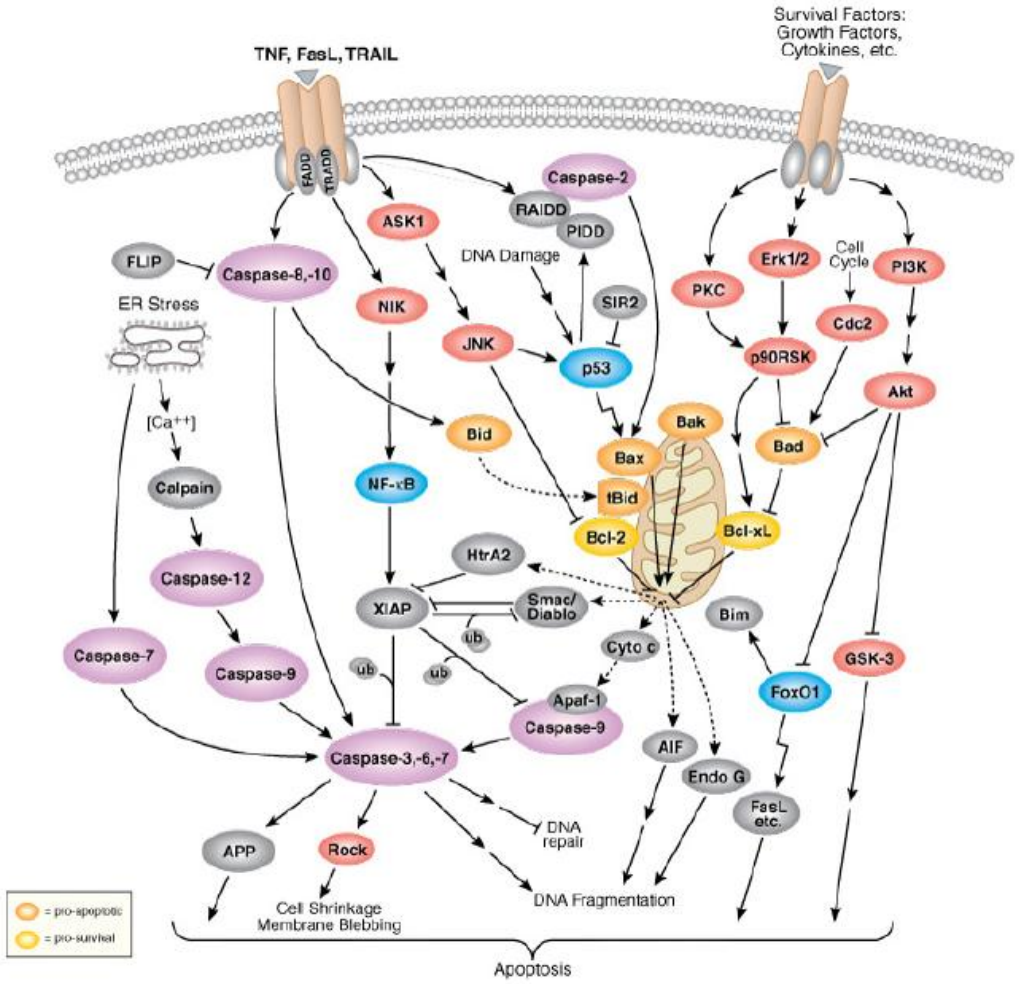


Fig. 3.4.5 *Apoptosis occurs when specific proteases (caspases) digest critical proteins in the cell. The caspases are normally present as inactive procaspases. Two pathways lead to their activation. The death receptor pathway (at the top and left side of the figure) is triggered when ligands bind to death receptors such as CD95/Fas. The mitochondrial pathway is triggered by internal insults such as DNA damage as well as by extracellular signals. In both pathways, procaspases are brought together. They then cleave each other to release active caspase. The binding of ligand (FasL or CD95L) to CD95 brings procaspase 8 molecules together; release of mitochondrial components bring procaspases 9 together. The active caspase 8 and 9 then activate other procaspases such as procaspase 3.*

Figure 6: Apoptosis pathways. From World Cancer Report 2008 [1]

Some mutations however enable cells to evade or avoid apoptotic signals or escape surveillance by the immune system and those cells therefore can keep dividing and proliferating untimely and excessively [1].

Mutated genes that cause cancer are called oncogenes (gain-of-function modification). A mutation of cell cycle suppressor genes (for example p53-gene, RB-gene) can lead to their inactivation and to uncontrolled proliferation (loss-of-function-modification). [4]

1.1.2 Colorectal cancer – Aetiology and Incidence

Colorectal cancer (CRC) accounts for 9,4% of worldwide cancer cases. It is the fourth most common cancer in men and the third most common in women with a total of one million new cases per year.

In terms of prevalence (total percentage of cases in a population) CRC is the second most common cancer worldwide after breast cancer. [5, 6]

The development of CRC is a multistep process initiated by various genetic and molecular changes resulting in signal alterations through affected genes (Figure 7). [7]

About 75% of CRCs are initiated by sporadic somatic mutations. The remaining cases occur due to hereditary syndromes (familial adenomatous polyposis coli (FAP), hereditary nonpolyposis CRC), personal history of adenomatous polyposis coli (APC) and high risk conditions like inflammatory bowel disease and previous diagnoses of cancer (ovary, endometrium, breast, bile duct, pancreas, stomach). [5]

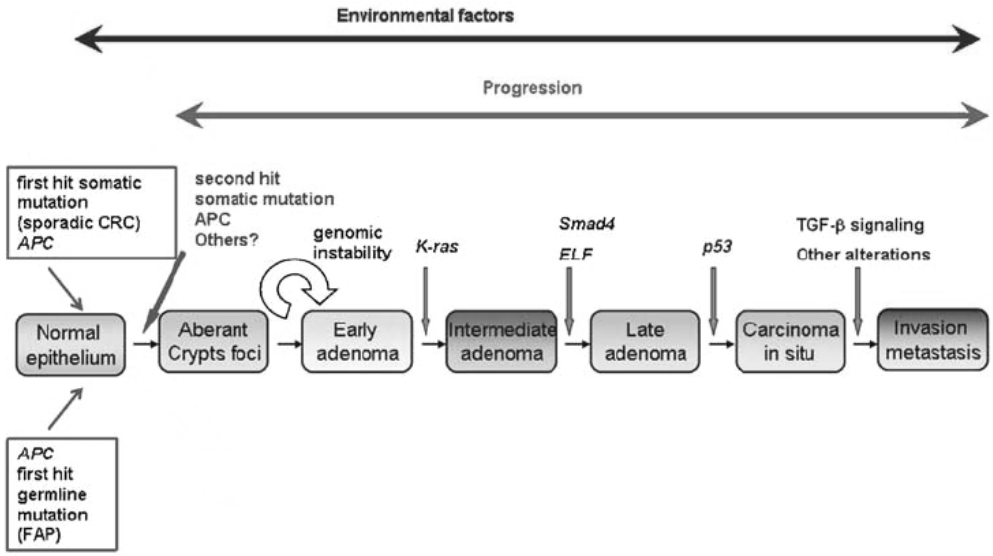


Figure 7: Development of colorectal cancer. Adapted from Mishra et al. (2005) [7]

There is also strong evidence that lifestyle and dietary factors play an important role in the development of CRC. It is most likely that diet highly affects the large intestine which in constant and direct contact with (carcinogenic) food components. [5, 6]

1.1.3 Cancer Chemoprevention

Chemoprevention is the reduction of cancer risk by the use of pharmaceuticals or other agents. [8] The main idea is to continuously attack micro-tumours in tissues during all stages of cancer development (initiation, promotion, progression) through inhibition of angiogenesis, inhibition of cell growth or inactivation of potential carcinogens. [2]

Some chemopreventive substances have already been identified like Vitamin A, E, C and D, Selenium, trace elements, folic acid, non steroidal anti-inflammatory drugs, estrogen receptor modulators or omega-3 fatty acids and polyphenols. [8]

As already mentioned, 30% of tumours develop due to nutritional deficits (Figure 4). Since diet plays an important role in the development of cancer the obvious approach is to use the potential of our daily diet for chemoprevention. [2]

In contrast to chemotherapy with periodic administration of very high doses of chemotherapeutics with severe side-effects this continuous, daily attack of (micro-) tumours ("metronomic therapy", Beliveau and Gringas, 2005) could either prevent micro-tumours from progressing or be used as additional therapy against manifest tumours (Figure 8). [2]

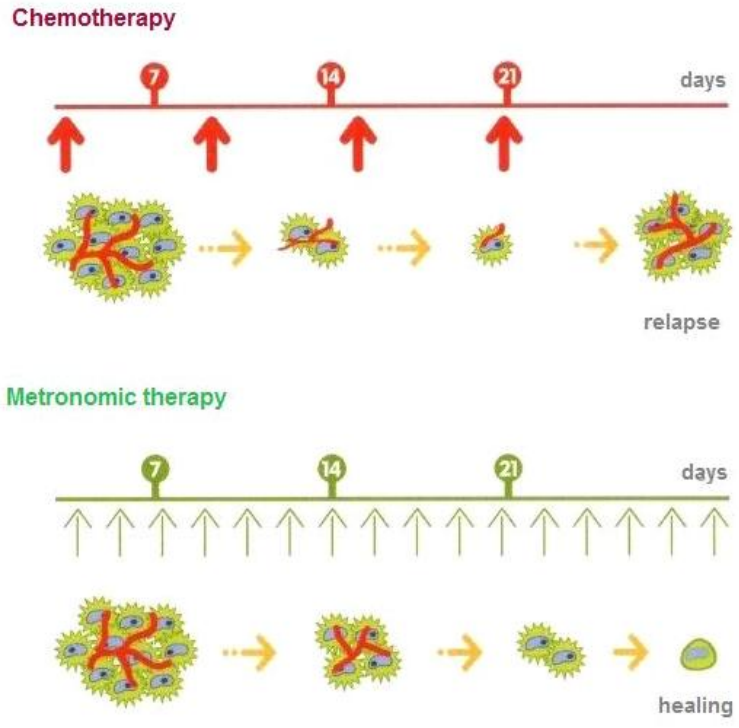


Figure 8: Comparison of chemotherapy and metronomic therapy. Adapted from Beliveau, Gringas (2005) [2]

The spectrum of chemopreventive substances present in our daily diet is vastly diverse. Brassicaceae (cabbage, Brussels sprouts, broccoli…), Alliaceae (garlic, onion…), soja, curcuma, green tea, various berries, tomatoes, citrus fruits, wine

and chocolate bear anticancer potential although the substances present in those aliments are manifold. [2]

This work concentrates on the chemopreventive potential of a specific category of polyphenols present in our daily diet.

1.2 Polyphenols

Polyphenols are prevalent in the plant kingdom. They are the most abundant secondary plant metabolites and consist of a complex mixture of different substances. [9]

Plants use those secondary plant metabolites mainly as a protection against oxidation, external aggressions (viruses, bacteria, funghi) and putrescence.

Polyphenols can be classified as (a) phenolic acids or (b) flavonoids. [10]

Those molecules are highly antioxidant, even superior to vitamin C or E. [9]

Flavonoids are built of three rings. The A-ring is an aromatic compound of either the phloroglucinol type (meta-trihydroxylated) or the resorcinol type (meta-dihydroxylated). It is condensed to the heterocyclic C-ring which can have a pyran, pyrilium or γ-pyrone structure. The C-ring carries a phenyl constituent at C-2 (flavonoids), C3 (isoflavonoids) or C-4 (neoflavonoids). [11]

Flavonoids can be divided into 8 groups, depending on the oxidation status of ring B (Figure 9).

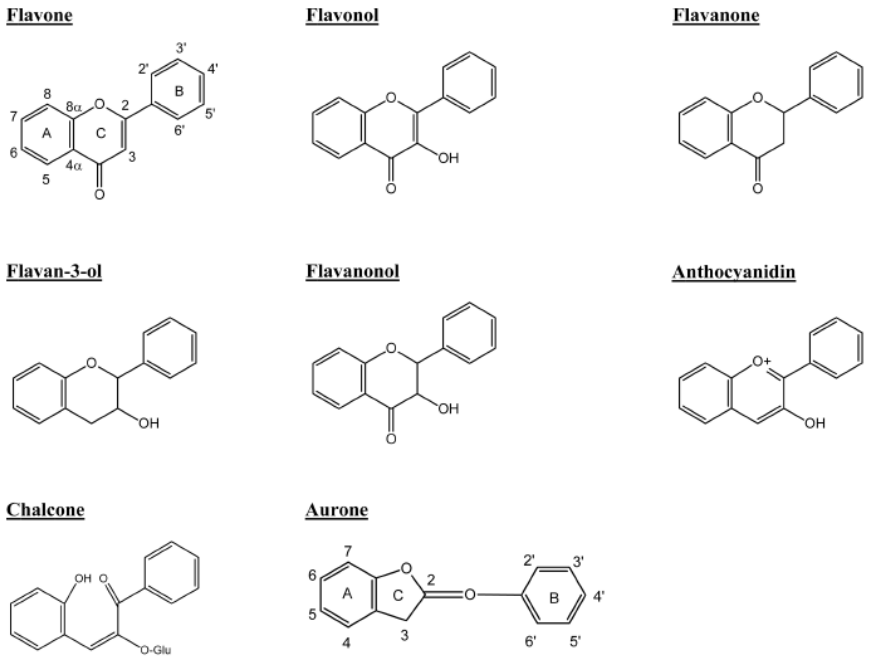
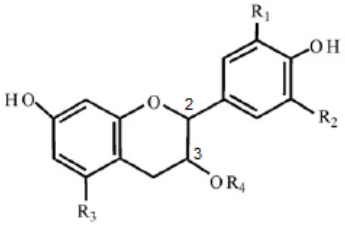


Figure 9: Flavonoid subclasses - the 2-phenylbenzopyrans [11]

1.2.1 Flavan-3-ols

As mentioned above there exists a phloroglucinol and a resorcinol type of flavan-3-ols. (epi)Fisetinidol, and (epi)robinetinidol are examples for the resorcinol type, (epi)afzelechin, (epi)gallocatechin and (epi)catechin are of the phloroglucinol type (Figure 10). [12]



flavan -3-ol	R_1	R_2	R_3	R_4
(epi)afzelechin	H	H	OH	H
(epi)catechin	OH	H	OH	H
galloylated (epi)catechin	OH	H	OH	galloyl
(epi)gallocatechin	OH	OH	OH	H
(epi)fisetinidol	OH	H	H	H
(epi)robinetinidol	OH	OH	H	H

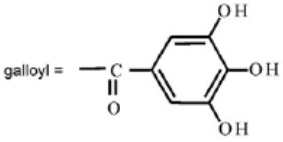


Figure 10: Structures of some flavan-3-ol monomers
There are two chiral centers C2 and C3 therefore four isomers exist. For catechin that would be (+)catechin, (-)catechin, (+)epicatechin and (-)epicatechin. Adapted from Monagas et al. (2010) [12-14]

The most abundant monomers are catechin and epicatechin, they appear either as monomers, dimers, oligomers with a mean degree of polymerisation of three to ten or polymers with a mean degree of polymerisation of more than ten monomers (Figure 11, Figure 12). [11, 12, 15]

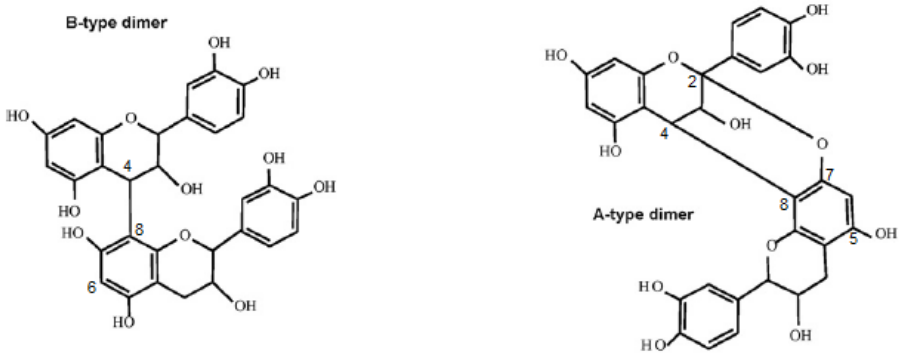


Figure 11: The B-type flavan-3-ol dimers are characterized by a C4-C8 linkage (or less frequently C4-C6) between monomers. A-type dimers have an additional ether bond between C2 and the hydroxy group at C5 or C7 of the other monomer. Adapted from Monagas et al. (2010) [12]

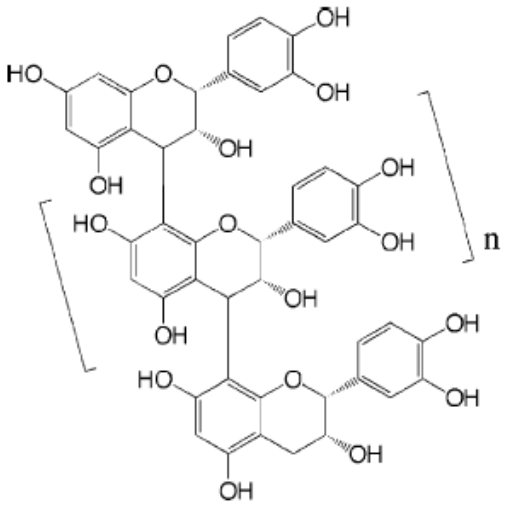


Figure 12: Structure of flavan-3-ol oligomers (n=3-8) and polymers (n >8) [15]

The dimers, oligomers and polymers of flavan-3-ols are also called procyanidins, flavans, flavolans, polyflavans, catechins, macromolecular polyphenolic substances, polymeric proanthocyanidins, oligomeric proanthocyanidins, procyanidins or condensed tannins.

The term “tannin” is related to their astringent effects and must not be confounded with other plant polyphenols like the class of hydrolysable or complex tannins like gallotannins and ellagitannins. [11, 14]

The astringent property and the ability to precipitate proteins are estimated to play an important role in the defence of plants against pathogens and microbes. [11]

The focus of this work lies on oligomeric procyanidins, further referred to as OPCs, consisting of three to ten monomer subunits.

OPCs have already shown several beneficial health effects. They have antimicrobial, antiviral (*Herpes Simplex Virus*), antidiabetic and cardioprotective properties mostly acting through inhibition of enzymes. [16]

Procyanidins affect cell growth and angiogenesis as well as they reduce the carcinogenic potential of radicals via their antioxidant properties. [2]

1.2.2 Anthocyanidins

Anthocyanidins are the oxonium salts of flavanols (Figure 13). [17]

Their glycosides, the anthocyanins, are a large group of water-soluble plant pigments. They are commonly associated with the red or blue colour of flowers and fruits. [18]

In higher plants, the distribution of anthocyanins is up to 50% for cyanidin, and up to 12% for delphinidin, malvidin and pelargonidin. [19]

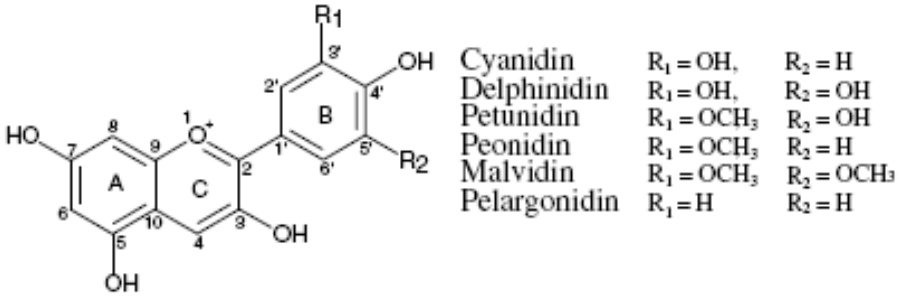


Figure 13: Structural formula of anthocyanidins [19]

At low pH-levels anthocyanins exist as red flavylium salts; in neutral medium they form purple substances; in slightly alkaline medium they appear blue. [17]

The main families that contain pigmented fruits are *Vitaceae* (for example grapes) and *Rosaceae* which include apple, pear, cherry, plum, peach and sloe, blackberry and raspberry, strawberry and quince. Other families with high amounts of anthocyanin-pigmented fruits are *Ericaceae* that include blueberry and cranberry or *Caprifoliaceae* for elderberry. [20]

Anthocyanins have already proven to be highly antioxidant and therefore possess a radical scavenging capacity. [9-11, 18, 21]

Effects on atherosclerosis, inflammation, diabetes, coronary and neuronal diseases as well as antimicrobial and antiulcerative activities have been reported. [22-28]

The effects of anthocyanins on cancerous diseases *in vitro* and *in vivo* have been reported and therefore they were used to compare their effects to those of OPCs. [19, 29-32]

Anthocyanins inhibit angiogenesis and interfere with the synthesis of DNA. They can therefore stop cell growth which leads to apoptosis. [2]

1.3 Concept and Aims

After choosing raw material to work with, the aims consisted in extracting polyphenols and purifying and separating a fraction enriched in OPCs and as minor aim a fraction enriched in anthocyanins.

Fractions were quantified and tested on colon carcinoma cell lines to assess the induction of apoptosis.

1.3.1 Choice of Raw Material

Many fruits, beverages and vegetables as well as cereals or cacao-products contain OPCs. [33, 34]

Some fruits rich in OPCs are listed below (Table 1).

Table 1: OPC contents of several fruits
Monomers, dimers, trimers are listed separately, tetra- to hexamers (4-6mers), hepta- to decamers (7-10mers) as well as OPCs of higher polymerisation grade (>10mers) are pooled together; PA=Proanthocyandins. Adapted from Gu et al. (2004) [34]

No.	Food	Monomers	Dimers	Trimers	4–6 mers	7–10 mers	>10 mers	Total PAs
		mg/100 g (fresh weight foods) mg/L (beverages)						
	Fruits							
1a	Blueberries, cultivated highbush	4.0 ± 1.5	7.2 ± 1.8	5.4 ± 1.2	19.6 ± 3.4	14.5 ± 2.0	129.0 ± 47.3	179.8 ± 50.8
1b	Blueberries, lowbush	3.4 ± 0.5	9.0 ± 0.5	6.8 ± 0.4	25.7 ± 1.2	27.8 ± 1.3	260.4 ± 11.7	331.9 ± 14.0
2	Cranberries	7.3 ± 1.5	25.9 ± 6.1	18.9 ± 3.4	70.3 ± 13.1	62.9 ± 14.7	233.5 ± 49.1	418.8 ± 75.3
3	Blackberries	3.7 ± 2.2	6.7 ± 2.9	3.6 ± 1.9	7.3 ± 5.0	4.2 ± 4.5	1.5 ± 0.0	27.0 ± 17.5
4	Marion berries	0.9 ± 0.0	3.4 ± 0.1	2.4 ± 0.0	2.2 ± 0.0	ND	ND	8.9 ± 0.1
5	Choke berries	5.2 ± 0.2	12.5 ± 0.4	10.3 ± 0.3	40.3 ± 0.8	52.9 ± 3.1	542.6 ± 42.9	663.7 ± 47.7
6	Raspberries	4.4 ± 3.4	11.5 ± 9.8	5.7 ± 5.5	7.7 ± 5.2	0.9 ± 2.2	ND	30.2 ± 23.4
7	Strawberries	4.2 ± 0.7	6.5 ± 1.3	6.5 ± 1.2	28.1 ± 6.5	23.9 ± 3.5	75.8 ± 13.4	145.0 ± 24.9
8	Blackcurrants	0.9 ± 0.2	2.9 ± 0.4	3.0 ± 0.3	10.6 ± 1.7	9.9 ± 1.4	122.4 ± 28.0	147.8 ± 33.0
9	Cherries	4.2 ± 1.1	2.8 ± 0.7	2.8 ± 0.5	6.5 ± 0.8	1.9 ± 0.1	ND	8.2 ± 03.2
10a	Green grapes	1.0 ± 0.1	2.3 ± 0.0	1.9 ± 0.1	8.4 ± 0.3	9.2 ± 0.6	58.9 ± 14.2	81.5 ± 15.0
10b	Red grapes	0.8 ± 0.2	2.0 ± 0.3	1.5 ± 0.2	6.1 ± 0.9	6.2 ± 1.1	44.6 ± 9.9	61.0 ± 12.3
10c	Grape seed (dry)	660.3 ± 8.3	417.3 ± 4.8	290.2 ± 4.5	664.0 ± 8.2	400.3 ± 31.3	1100.1 ± 86.3	3532.3 ± 105.8
11a	Apple, red delicious, with peel	9.6 ± 0.9	13.8 ± 0.6	9.3 ± 0.4	30.2 ± 1.2	25.4 ± 1.2	37.6 ± 2.6	125.8 ± 6.8
11b	Apple, red delicious without peel	6.8 ± 0.9	11.3 ± 1.6	7.2 ± 1.0	24.3 ± 3.4	20.3 ± 3.1	28.7 ± 7.1	98.7 ± 17.0
11c	Apple, golden delicious, with peel	4.7 ± 0.2	10.2 ± 0.2	6.3 ± 0.1	22.8 ± 0.6	19.5 ± 0.7	27.7 ± 2.9	91.1 ± 4.7
11d	Apple, golden delicious, without peel	4.1 ± 0.1	9.4 ± 0.5	5.8 ± 0.5	21.2 ± 2.6	17.5 ± 3.2	22.4 ± 5.7	80.4 ± 12.4
11e	Apple, granny smith	7.5 ± 1.0	15.0 ± 2.3	9.1 ± 1.5	32.9 ± 5.9	30.1 ± 6.1	46.3 ± 9.5	141.0 ± 26.1
11f	Apple, gala	5.9 ± 0.4	9.5 ± 0.3	6.2 ± 0.2	21.3 ± 1.5	18.7 ± 1.4	30.7 ± 5.6	92.4 ± 8.4
11g	Apple, fuji	6.5 ± 1.7	9.9 ± 2.6	6.1 ± 1.4	19.1 ± 4.3	13.8 ± 2.8	14.2 ± 3.1	69.6 ± 15.8

For extraction and enrichment of OPCs, apple and quince were chosen. Quince is not listed in table one, but is rich in OPCs as well. [15, 28, 35]

The choice of apple and quince was made according to the availability and easy supply of raw material. It was decided to use readily available by-products of the cider and juice industry more specifically the pressing residues, also called pomace. [36]

Several million tons of pomace are produced annually worldwide and the disposal of this agro-industrial waste causes serious economical (high transportation costs) and environmental problems as well as bearing health hazards. [36, 37]

Normally pomace is used to feed cattle but there are more and more attempts of value addition. Therefore it is used as microbial substrate for fermentation and lately for the extraction of bioactive molecules. [37]

Pomace consists of 25-30% of the whole fruits, mainly of peel, seeds, stems, core and exhausted tissue and is rich in proteins, vitamins A and C, minerals, dietary fibres and polyphenols. [36]

It has been documented that the amount of polyphenols is generally higher in the peels than in the flesh of fruits. [9, 21, 38]

Additionally the concentration of flavan-3-ols is higher in cider apples than in table apples. [9, 38-40]

As a conclusion pomace could be a very useful and inexpensive material for the extraction of functional food factors.

1.3.2 Extraction and Enrichment

The common procedure for extracting polphenols from fruits is at first a solvent extraction with ethanol, methanol or acetone or mixtures thereof with water. [41]

These extracts are always mixtures of various phenolic substances therefore additional steps for purification have to be taken. [42]

OPCs are sensitive to oxygen, light, acid and alkaline. Protection against oxygen can be achieved by adding ascorbic acid to the extraction solvent as an antioxidant. However antioxidants can interfere with quantification assays as the Folin-Assay and likewise with cell cultures. [43]

Purification steps include liquid-liquid extractions to remove lipoids and unwanted phenolics. [41, 42]

Another step for enrichment and removal of unwanted substances like polyphenols of low molecular weight (phenolic acids, monomers, dimers) sugars, and highly polymerised procyanidins is commonly performed by solid phase extraction (SPE, for example gel filtration chromatography) taking advantage of

size-exclusion and absorptive effects. Polyphenols of low molecular weight and sugars are eluted with demineralised water. Afterwards, with an increasing amount of organic solvent, the elution of OPCs is performed. High molecular weight polymers of procyanidins remain on the stationary phase. [42-45]

1.3.3 Analysis and Quantification of Fractions

Analysis: For the analysis of OPCs reversed phase HPLC (RP-HPLC) is commonly applied. Fractions rich in procyanidins result in a typical unresolved hump in chromatograms (Figure 14). [43]

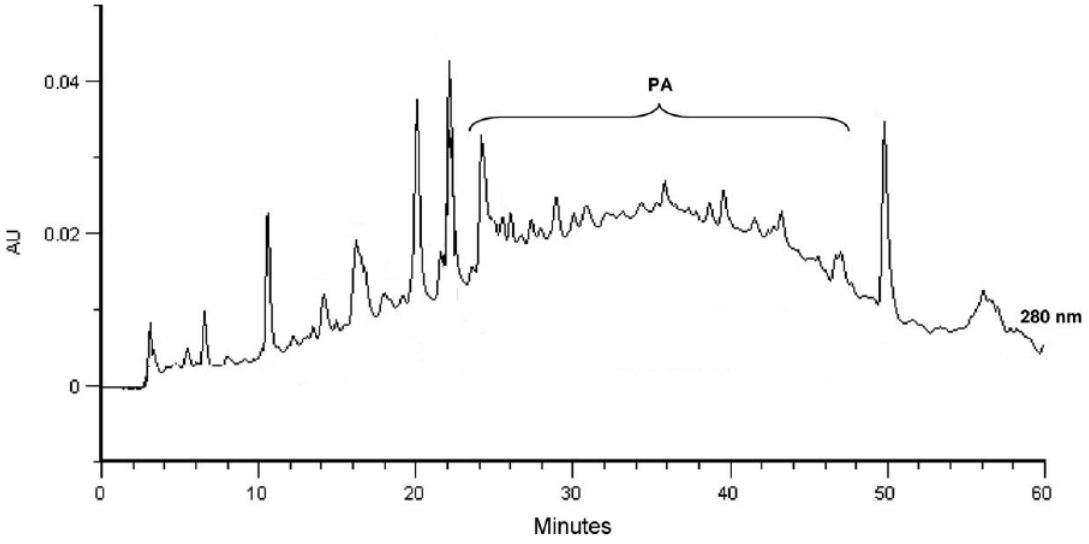


Figure 14: Typical HPLC-chromatogram for procyanidins, recorded at 280nm. Adapted from Goncalves et al. (2005) [46]

Identification can be performed by coupling RP-HPLC with an UV-DAD detector. Flavan-3-ols show absorption spectra with two maxima, one at 200-220nm and another maxima at 275-285nm (Figure 15), Anthocyanins show maximum absorption at 275-285nm and at 520-545nm. [39, 43]

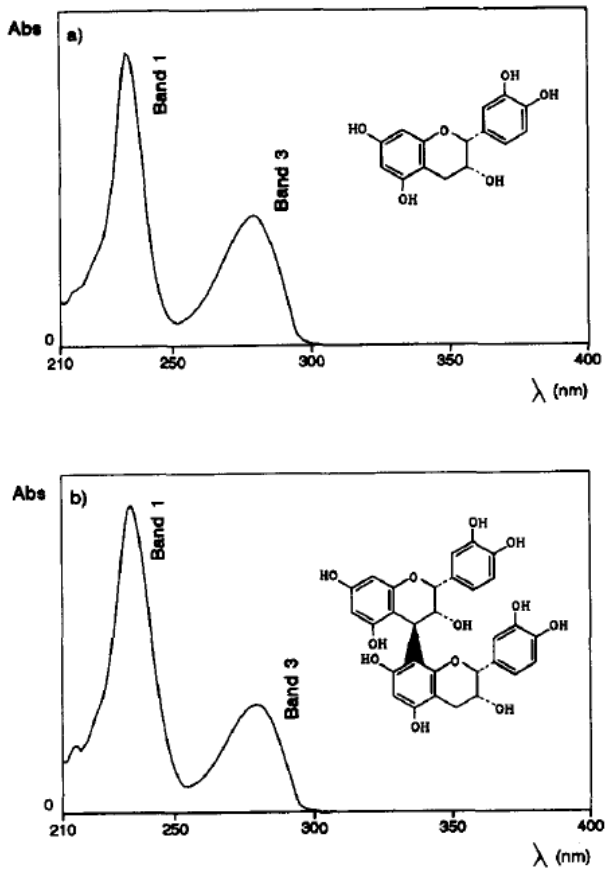


Figure 15: a) UV absorption maxima of (-)catechin, b)absorption maxima of catechin-dimer. Adapted from Bartolomé et al. (1996) [47]

Those methods are only suitable to identify the general phenolic structures of substances but it is not possible to draw conclusions about the degree of polymerisation that way because the UV absorption spectra do not differ much (Figure 15). [43, 47]

Quantification: Quantification is commonly done by reactions that result in a UV-absorbing coloured product.

Table 2: Comparison of common quantification assays [43, 48, 49]

Method	detection and reference substance	remarks	influence on colour yield
Vanillin Assay	flavan-3-ols, reference catechin	not specific for oligomers, left-over monomers in extracts react as well	reference standard, nature of acid, water content, time, temperature, interfering substances (ascorbic acid, chlorophyll)
Folin-Ciocalteau Assay	total polyphenols, reference gallic acid	not specific for flavan-3-ols, all phenolic substances react	time, temperature, pH, sunlight
Acid butanol assay	condensed procyanidins, reference appropriate procyanidin	reaction of intermediate units, terminal units do not react	structural features such as oxygenation pattern and position of the interflavan bond

As shown in table two, there is no absolutely reliable method to quantify OPCs. The problems of quantification are related to the various structures and the complex mixtures present in extracts. [43, 48]

1.3.4 Proapoptotic Potential

Measurement was done by incubating cell cultures with different concentrations of OPC fractions and analysing the apoptotic status of cells by flow cytometry.

Cell line SW480 is derived from a colon carcinoma stage B according to the Dukes staging system (tumours breached extra-rectal tissues) from a 50 year old patient. [50]

SW620 cells are human metastatic colon carcinoma-derived cells from a lymphatic ganglion of the same patient (stage C, tumour has metastasised to lymph nodes). [50]

In contrast to SW480 cells the SW620 cell line is TRAIL-resistant. [51]

TRAIL (Tumour necrosis factor-related apoptosis-inducing ligand) is a signalling molecule inducing apoptosis through TRAIL receptors (DR4, DR5) (Figure 6). [4]

Flow cytrometry is a technique to characterize cells by phenotype and function. The principle consists of passing single fluorescence marked cells in a fluid stream through a beam of light of a specific wavelength. Scattered and emitted light is collected and transformed into electrical signals. [52]

Light scattering patterns of cells change as a result of morphological changes appearing during apoptosis and necrosis. [53]

Cells can be therefore divided into living cells, cells undergoing different stages of apoptosis, and dead cells. [54]

Extracts will be compared with a commercially available sample to find out if the methods of enrichment and the effects on cell cultures are matchable.

2 Material

2.1 Apple and Quince Pomace

The plant material for the extraction was obtained from the “Ferme Rolli”, a farm which produces vinegar, oil and honey, but also apple and quince juice and cider. The “Ferme Rolli” processes quince (*Cydonia oblonga*) and different varieties of juice and cider apples (*Malus pumila)* (Figure 16).

Figure 16: *Malus pumila* (left side) and *cydonia oblonga* (right side) [55]

Pomace was deep-frozen immediately after pressing of the fruits to avoid enzymatic degradation. It was shrink-wrapped in plastic and stored in the dark to avoid exposure to oxygen and light, because this would also cause degradation of substances.

2.2 Chemicals

All chemicals and substances are listed in the appendix.

2.3 Analytical Apparatus

RP-HPLC was performed on a Varian Star instrument (Varian Incorporation, Palo Alto, CA) with a Nucleodur 100-5 C18ec column (4,6x250mm) (Macherey-Nagel, Düren, GE). RP-HPLC instrument was coupled with an UV- detector. UV-Spectra were measured by a Varian ProStar 330 Photodiode Array detector (PDA). Postrun processing (peak and UV spectra analysis) was done with Polyview2000 software (Varian Incorporation, Palo Alto, CA).

Spectrophotometric measurements were conducted on a VersaMax tunable microplate reader and results were processed with SoftMax Pro Software (Molecular Devices, Sunnyvale CA).

Assessment of apoptosis was done by using a Guava Nexin kit and a Guava PCA system (Guava Technologies, Hayward, CA).

3 Methods

3.1 Extraction of pomace

Apple and quince pomace were defrosted as quickly as possible and divided into portions of about 1kg for extraction (Table 3).

Table 3: Weights of extracted pomace

apple pomace		**quince pomace**	
Portion (no)	weight (g)	Portion (no)	weight (g)
1	959,31	1	875,01
2	950,52	2	1000,26
3	956,65	3	997,89
4	974,37	4	995,52
5	945,15	5	944,34
6	1007		
total (g)	5793	total (g)	4813,02

Samples were extracted with acetone and demineralised water (60:40). The solvent had to be supernatant to the plant material by about 15 cm.

Agitation was performed by magnetic stirring. The mixture was extracted at ambient temperature (20°) and thoroughly protected from light by the use of non-transparent open-top vessels covered with aluminium foil (Figure 17).

Figure 17: Extraction of pomace

Plant Material was extracted for 24 hours under agitation. Then pomace was filtered by aspiration to obtain a clear liquid (Figure 18).

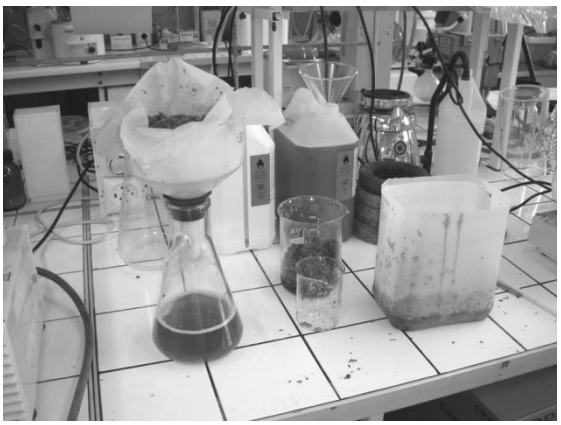


Figure 18: Filtration of pomace

Freshly mixed aqueous acetone was added to the plant material immediately to rerun extraction.

Extraction was performed three times on three consecutive days to extract substances thoroughly.

The aqueous acetone fractions from all three days were united and reduced under pressure to remove acetone and the bigger part of water. The crude extracts of apple and quince had a brown colour and a caramel-like consistence. They were frozen until further progression.

3.2 Purification of Crude Extract

Purification was done by liquid-liquid extraction with two different solvents.

First two to four times with cyclohexane to remove lipophilic substances like chlorophyll, fatty acids and lipids. [41, 42]

The second extraction was effected with ethyl acetate until the organic upper phase was colourless. This was done to remove monomers and to some extent dimers. [56]

The cyclohexane and ethyl acetate phases were put under an extraction hood in open vessels and left to dry.

Solutions thereof were prepared to test on cell cultures (see 3.6.2.1).

The purified aqueous extracts (PFR aq, CFR aq[1]) were stored at 4°C until sample preparation.

[1] PFR/CFR aq means pomme/coing ferme rolli aqueux [fr.] for apple/quince ferme rolli aqueous extract

3.3 Solid Phase Extraction

Solid phase extraction with Sephadex LH-20 gel was executed for further purification and enrichment of OPCs. Several columns were done for each fruit, and fractions were analysed by RP-HPLC to identify fractions rich in OPCs.

3.3.1 Column Preparation

Columns were prepared by soaking Sephadex LH-20 in a mixture of acetone and demineralised water (60:40) and filling it into a column (diameter 2,5 cm) to a height of approx. 25 cm.

The column was then conditioned with pure water until acetone was washed out completely.

3.3.2 Sample Preparation

The aqueous extract was evaporated exhaustively to remove as much water as possible (Table 4). This was necessary to calculate recovery (see 4.4.3). Samples were never completely dry, presumably because of free sugars left in the extract.

Table 4: Sample weights for aple and quince columns

column	PFR aq (g)	CFR aq (g)
1	15,6	23,47
2	15,43	22,95
3	15,07	25,88
4	22	26,64
5	21,77	26,28
6	23,09	26,32
7	22,29	24,28
8	20,94	24,3
9	21,92	25,33
10		24,89
total (g)	**178,11**	**250,34**

Nine columns for apple-extract and ten columns for quince extract were done.

After the first three columns for the apple extract it was decided to increase sample weight. For quince it was necessary to (i) increase weight even more than for apple samples and (ii) do an additional column to increase fraction yield.

3.3.3 Elution

Samples were put on the column and fractions were gained by gradient elution (Table 5, Figure 19).

Figure 19: Column with apple sample

Table 5: Elution gradient for Sephadex LH-20 columns

fraction name	composition MeOH + H_2O(ml)
0%MeOH	0 + 100
10%MeOH	10 + 90
20%MeOH	20 + 80
30%MeOH	30 + 70
40%MeOH	40 + 60
50%MeOH	50 + 50
60%MeOH	60 + 40
70%MeOH	70 + 30
80%MeOH	80 + 20
90%MeOH	90 + 10
100%MeOH	100 + 0
a-w	Acetone: H_20= 60:40

Columns were eluted with fraction a-w until they seemed clean by visual judgement. Nevertheless Sephadex kept a yellowish colour (due to highly

polymerized proanthocyanidins) that could not be removed even through exhaustive washing.

Change of fractions was done when the whole volume of 100ml of mobile phase of one fraction was on the column and the surface of the liquid was on the same level as the Sephadex-gel.

Fractions of 100ml were collected and evaporated to dryness under reduced pressure.

After elution columns were emptied and Sephadex was washed thoroughly with acetone: water (60:40), then with pure water until no more scent of acetone was detectable. After the washing, columns were refilled.

Refilling had to be done after each sample, because the Sephadex –gel sank about 5 cm when adding the extract. This resulted in a denser packing and therefore in a minor flow rate.

The sinking of the Sephadex- gel might be due to the fact that the gel expands more in water than in methanol. Another reason could be the weight of the samples.

Generally flow rates increased with the concentration of methanol in the mobile phase and were higher for apple samples than for quince samples.

However elution was generally very slow and fraction yield low.

3.4 Analysis by reversed phase -HPLC (RP-HPLC)

An adapted gradient elution was performed at a flow rate of 1ml/min (Table 6). [57] Solvent A was water (HPLC-grade) with 0,1% TFA, solvent B was methanol (HPLC-grade).

Table 6: Elution gradient for RP-HPLC. Adapted from Shoji et al. (2004) [57]

time (min)	%A	%B
Initial	90	10
10	90	10
50	50	50
65	50	50
70	0	100
80	0	100
85	90	10

Samples were solubilised using a mixture of water and methanol (1:1) both HPLC-grade and manually injected (Figure 20). Previous to injection, all samples were filtered through a 0,45 µm polystyrene filter.

Figure 20: RP-HPLC apparatus, manual injection

UV spectra were recorded from 190 to 700nm and displayed at 280nm which is the main absorption wavelength for OPCs (see 1.3.3).

Fractions containing only OPCs according to the UV-spectra were united and called PFR OPC and CFR OPC. The enriched extracts were lyophilised previous to quantification.

3.5 Quantification of Enriched Extracts

The enriched fractions were quantified by Vanillin- and Folin-Assay and compared to commercially available samples of enriched polyphenols.

3.5.1 Vanillin Assay

The main principle of the vanillin assay is the condensation reaction of the aldehyde vanillin with a specific position on the flavan-3-ol molecule. The reaction is performed in acidified solution. [58]

The result is a red- coloured adduct that can be measured at 500nm (Figure 21).

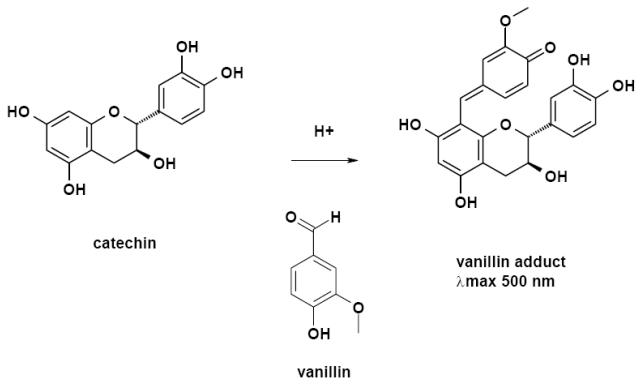


Figure 21: Reaction of vanillin with meta-substituted A-ring of catechin [48]

The vanillin assay is sensitive and specific for flavan-3-ols. Vanillin only reacts with free terminal units of the procyanidins.

For calibration (+)catechin was used.

Reaction time and temperature were fixed at 20 minutes and 30°C.

The assay is sensitive to water so MeOH of HPLC-grade was used (Table 7). [49]

Table 7: stock-solutions for vanillin assay

Solution	concentration	storage
Vanillin	1%(m/v)	0-5°C
Sulfuric acid (H2SO4, 97-95%)	10%(v/v)	0-5°C
Catechin (for Calibration curve)	700µg/ml	0-5°C
	600µg/ml	0-5°C
	500µg/ml	0-5°C
	400µg/ml	0-5°C
	300µg/ml	0-5°C
	200µg/ml	0-5°C
	100µg/ml	0-5°C
PFR OPC (OPC-enriched apple extract)	0,5mg/ml	light protection
CFR OPC (OPC-enriched quince extract)	0,5mg/ml	light protection
PFR aq	5mg/ml	light protection
CFR aq	5mg/ml	light protection

The aqueous extracts of apple and quince (PFR aq, CFR aq) were also measured to calculate the enrichment of flavan-3-ols.

Solutions were pipetted into 96- well plates (Table 8).

Table 8: Pipette schedule for Vanillin assay;
sv=sample-vanillin, c=control, bv=blank vanillin, o=blank

name	H2SO4 10%	sample	vanillin 1%	methanol	total/well
sv	125µl	50µl	125µl	-	300µl
c	125µl	50µl	-	125µl	300µl
bv	125µl	-	125µl	50µl	300µl
o	125µl	-	-	175µl	300µl

Plates were wrapped up in aluminium foil immediately after adding vanillin-solution and left at 30°C under slight agitation.

Absorption was measured after 20 minutes (Figure 22).

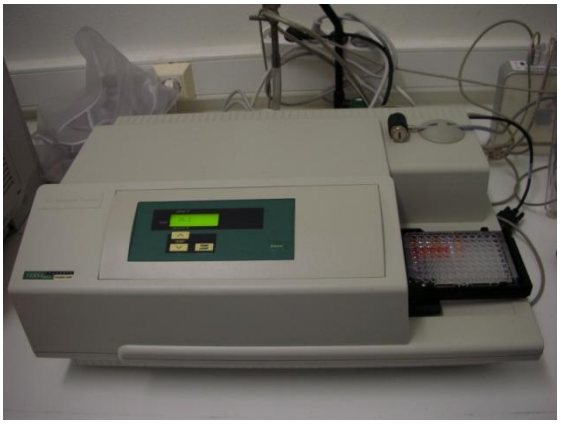


Figure 22: Spectrophotometer with 96-well plate. In some wells the red adduct of the vanillin-reaction can be seen

Catechin for calibration was measured once and all samples were done in triplicate.

Absorptions were calculated as follows:

$$A=(A_{sv}-A_{bv})-(A_c-A_o)$$

The quantity of total flavan-3-ols is calculated according to the calibration curve and expressed as catechin- aequivalents per gram of dry sample. [49, 59]

3.5.2 Folin Assay

The Folin assay is a means for quantification of total phenolic content. [60, 61]

It is not specific for procyanidins, but for all phenolic structures in a sample.

The main principle is the reaction of a phenolic structure with the Folin-Ciocalteau reagent. It results in a blue coloured adduct which absorbs at 650nm or 725nm, depending on the method used. There are various temperature, time and wavelength data for this assay. [40, 59, 60, 62]

An adapted version of the Folin-Ciolteau assay was performed (Table 9).

Table 9: stock-solutions and concentration for adapted Folin assay

Solution	concentration	storage
Folin-Ciocalteau reagent	2M	0-5°C, light protection
Sodium carbonate solution (in demin. Water)	20%(v/v)	
Gallic acid (for Calibration curve)	100µg/ml	-20°C, light protection
	80µg/ml	-20°C, light protection
	60µg/ml	-20°C, light protection
	40µg/ml	-20°C, light protection
	20µg/ml	-20°C, light protection
PFR OPC (OPC-enriched apple extract)	50µg/ml	light protection
CFR OPC (OPC-enriched quince extract)	50µg/ml	light protection

Gallic acid was used as standard for the calibration curve.

The reactions were carried out in a 96-well plate (Table 10). Dry Samples were dissolved in demineralised water. After adding Folin reagent, the plate was agitated vigorously to avoid bubbles. Those would interfere with measurement. The plate remained 30 minutes under ambient temperature (20°C). Then measurement was done immediately at 650nm.

Table 10: Pipette schedule for Folin assay; s=sample, c=control, b=blank

	Na_2CO_3	Sample	Folin	Water	total/well
s	30µl	30µl	10µl	130µl	200µl
c	-	30µl	-	170µl	200µl
b	30µl	-	10µl	160µl	200µl

Calibration curve and samples were measured in duplicate.

Absorption was calculated as follows:

$$A_s - A_b - A_c$$

The total phenolic content is expressed as gallic acid equivalents per gram of sample.

3.6 Proapoptotic Potential

Cell culture maintenance and measurement of proapoptotic potential of extracts were executed by Carole Minker.

3.6.1 Cell Culture

For measurement of proapoptotic potential of the OPC-enriched extracts we used two different cell lines, SW480 and SW620.

Cell lines were graciously offered by the IRCAD (Institut de Recherche sur les Cancers de l'Appareil Digestif[2]).

3.6.1.1 Maintenance of Cells

Cells were cultured in polystyrene flasks of 75 cm^2 (Corning), tips for micropipettes were all sterile. Media and other solutions were purchased from PAN Biotech GmbH.

Table 11: Solutions for cell culture

solution	details
MEM Eagle Alpha Modification	Deoxyribonucleosides, without L-glutamine, with $NaHCO_3$ 2,2g/L, sterile
Foetal Bovine Serum	origin South America, sterile
Trypsin 0,05 %/EDTA 0,02 %	without Ca2+ and Mg2+
Penicillin/Streptomycin	10000 Units Penicillin/ml, 20mg Streptomycin/ml
L-Glutamine	200mM, sterile

The media for cell culture was MEM Eagle Alpha supplemented with 10% of decomplemented foetal bovine serum, 1% of L-Glutamine and 1% of penicillin/streptomycin (Table 11).

[2] =Institute of research of cancers of the digestive tract

Cell culture flasks were placed in an incubator at 37°C and 5% CO_2.

3.6.1.2 Passaging

Cells were divided once a week by removing the media by aspiration and detaching them with 2ml of a mixture of trypsin/EDTA.

After being detached, cells were transferred into 10ml of medium with 20% serum in order to deactivate trypsin and centrifuged at 800 rpm for seven minutes. The supernatant was discarded and cells were resuspended in 30ml media.

Cells were counted using a hemocytometer counting cell. New flasks were prepared with one million cells per flask, media was added and flasks were incubated. Media was changed every two to three days.

Cells were used for tests between passage 93 and 102 for SW620 cell line, and between passages 12 to 22 for SW480 cell line.

3.6.2 Testing of Apple and Quince Samples

3.6.2.1 Preparation of Plates

Tests were executed in 96 well plates for cell culture, made of polystyrene and equally sterile (Costar). Trypinised cells were diluted in medium (10% serum) to obtain a concentration of one million cells per ml.

An amount of 5000 cells per well was established, filling up the wells to a volume of 200 µl with medium.

Celastrol (10mM in DMSO) was used as positive control for proapoptotic measurement with a final concentration of 50µM/well.

The OPC enriched extracts were dissolved in EtOH and H_2O (1:1, both HPLC grade). Five concentrations of apple and quince OPCs, the aqueous extract, and the cyclohexane and ethyl acetate extracts were tested on the cells. Additionally a fraction of anthocyanins and a commercially available enriched OPC fraction (AP) were tested.

For each sample 1µl of the solution of an initital concentration was added to the cell suspension leading to defined concentrations per well (Table 12).

Table 12: Sample concentrations for tests on cell cultures

sample	initial concentration(mg/ml)	final concentration/well (µg/ml)
PFR aq	10	50
CFR aq	10	50
PFR cyclo	10	50
CFR cyclo	10	50
PFR AcOEt	10	50
CFR AcOEt	10	50
PFR anthocyanes	10	50
PFR OPC	25	125
PFR OPC	20	100
PFR OPC	15	75
PFR OPC	10	50
PFR OPC	5	25
PFR OPC	2	10
CFR OPC	25	125
CFR OPC	20	100
CFR OPC	15	75
CFR OPC	10	50
CFR OPC	5	25
CFR OPC	2	10
AP OPC	25	125
AP OPC	20	100
AP OPC	15	75
AP OPC	10	50
AP OPC	5	25
AP OPC	2	10

After pipetting celastrol and sample-solutions into the wells the plates were incubated at 37°C and 5% CO_2 for 24 or 48 hours.

3.6.2.2 Reading of plates

Plates were measured by flow cytometry. Parameters for flow cytometry were set according to the partition of the wells with untreated cells (basal apoptosis) and celastrol-treated cells (over 90% of apoptosis).

Before reading cells were marked with Guava Nexin Reagent.

The reagent combines two methods of staining cells.

Annexin V is a calcium-dependent phospholipid which can specifically bind phosphatidylserine that is exposed on the outer surface of the cell membrane in early stages of apoptosis. [63, 64]

Annexin V is coupled with an fluorochrome and after binding fluorescence can be measured by flow cytometry. [65]

The second staining reagent is 7-AAD (7-Aminoactinomycin D), a DNA intercalating fluorescent dye. [66]

It only permeates into cells when structural integrity of cell membranes is lost during late stages of apoptosis. [65]

With this reagent it is therefore possible to label apoptotic cells and to discriminate between early apoptotic and late apoptotic/dead cells. [54]

After the 24/48 hours of incubation 20µl of Guava Nexin reagent were added to each sample in the 96-well plate and readings were taken.

Results were expressed as percentage of cells in apoptotic state.

3.7 Thin Layer chromatography

Thin layer chromatography (TLC) of the aqueous apple extract and of a butanolic extract (liquid-liquid extraction of the aqueous extract) was done to separate monomers up to hexamers.

The separation is thought to be useful for testing those substances separately on cell culture to work out which ones are the most powerful and/or if there are synergies between certain groups.

Four publicized TLC-systems for OPCs were essayed to find adequate conditions (Table 13).

Table 13: TLC-data [46, 56, 67, 68]

publication	stationary phase	mobile phase	reference	revelation
Goncalves et al. (2005)	Kieselgel 60 F_{254}	toluene: acetone: acetic acid (3:7,5:1)	catechin, epicatechin	10% vanillin in sulfuric acid
Glavnik et al. (2009)	microcrystalline cellulose	n-propanol: H2O: acetic acid (4:2:1)	catechin, epicatechin	10% vanillin in sulfuric acid
Merghem et al. (2004)	Kieselgel 60 F_{254}	toluene: acetone: formic acid (6:6:1)	catechin, epicatechin	10% vanillin in sulfuric acid
Svedström et al. (2002)	Kieselgel 60 F_{254}	ethyl acetate: formic acid: acetic acid: H2O (75:3:2:20), upper phase	catechin, epicatechin	10% vanillin in sulfuric acid

Svedström et al. gave information on $R_{(f)}$ values of substances (Table 14).

$R_{(f)}$ is calculated as follows:

$$R(f) = \frac{S}{M}$$

S= migration distance of substance

M=migration distance of mobile phase (solvent front)

Table 14: publicized R(f) values for mono- to hexamers [68]

publication	polymerisation	R(f) values
Svedström et al. (2002)	monomers	0.8
	dimers	0.71, 0.62
	trimers	0.45, 0,49
	tetramers	0.28
	pentamers	0.11
	hexamers	0.06

The flavan-3-ol monomers catechin and epicatechin were used as internal standards. Other reference substances were not available.

Various quantities of extracts were deposited on the plates with 20µl-micropipettes depending on the intensity of absorption under UV light (wavelength 254nm).

TLC chambers were saturated with the mobile phase for two hours previous to migration.

After migration the plates were examined under UV light (wavelength 254 nm).

Then plates were treated with 10% vanillin in sulphuric acid to colour migrated substances. After revelation plates were examined in daylight.

4 Results

4.1 Column Fraction Yield

Fraction weights for two columns are given as an example (Table 15, Table 16). A complete list is added in the appendix.

Table 15: Fraction weights for apple (Columns no 4 and 5)

Column	*Fraction (%MeOH)*	*weight(g)*
4	0	
4	10	
4	20	
4	30	
4	40	
4	50	0.0154
4	60	0.0226
4	70	0.0276
4	80	0.0399
4	90	0.0356
4	100	0.0326
4	a-w	0.0914
5	0	
5	10	
5	20	
5	30	
5	40	
5	50	0.0112
5	60	0.0182
5	70	0.0279
5	80	0.0377
5	90	0.0388
5	100	0.0319
5	a-w	0.1157

Table 16: Fraction weights for quince (columns no 1 and 2)

Column	*Fraction(%MeOH)*	*weight(g)*
1	0	
1	10	
1	20	
1	30	
1	40	0.0256
1	50	0.0175
1	60	0.0176
1	70	0.0211
1	80	0.0197
1	90	0.0199
1	100	0.012
1	a-w	0.0794
2	0	
2	10	
2	20	0.0688
2	30	0.0207
2	40	0.0172
2	50	0.0116
2	60	0.0164
2	70	0.0155
2	80	0.013
2	90	0.0383
2	100	0.019
2	a-w	0.0917

4.2 RP-HPLC Chromatograms

The following figures show representative RP-HPLC chromatograms.

In general fractions 0 to 20% MeOH were united during elution and fractions 0-30% were only injected for one column for they were not likely to contain OPCs. Fractions 40-60% were injected to detect anthocyanins. Fractions 70-100% and fractions a-w were injected to detect OPCs. When OPCs were identified by UV-spectra fractions were injected in replicate to confirm that all columns worked likewise and contained OPCs.

Corresponding peaks to the published UV-spectra (see 4.3) are indicated by arrows in the RP-HPLC-chromatograms.

The full range of recorded chromatograms is listed in the appendix.

4.2.1 Apple

For the apple extract the first fraction containing OPCs was 70% MeOH (Figure 23).

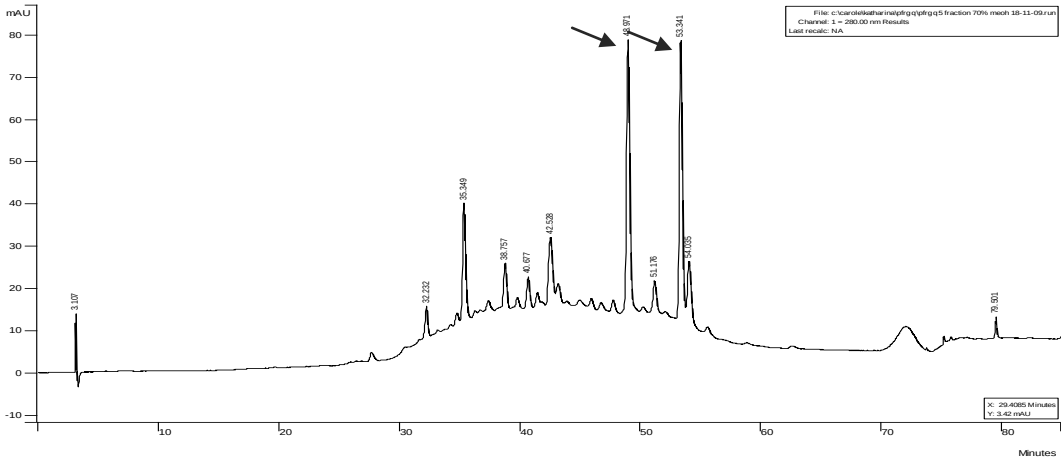


Figure 23: Chromatogram of fraction 70% MeOH, column No. 5

The following figures are representative for fractions 80% MeOH, 90% MeOH, 100% MeOH and fractions a-w. All fractions show the for OPCs typical unresolved hump (Figure 24, Figure 25, Figure 26, Figure 27).

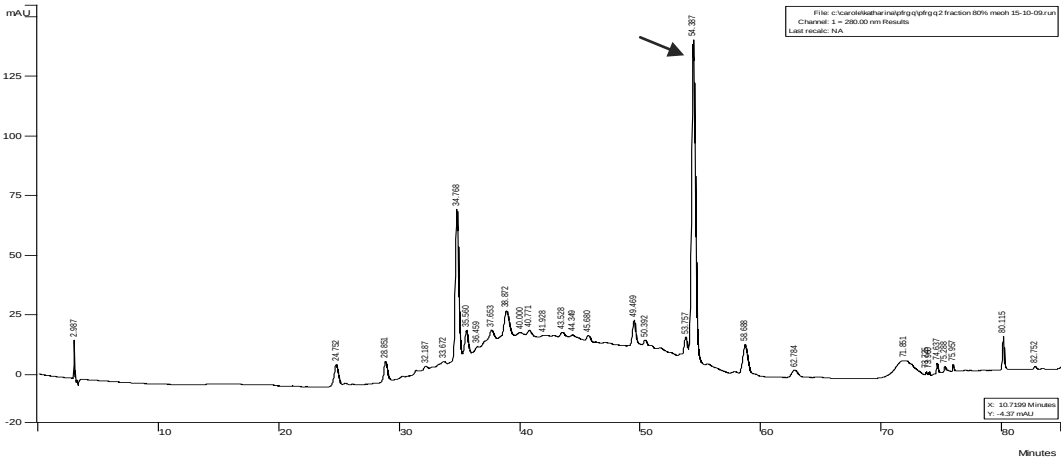


Figure 24: Chromatogram of fraction 80% MeOH, column No. 2

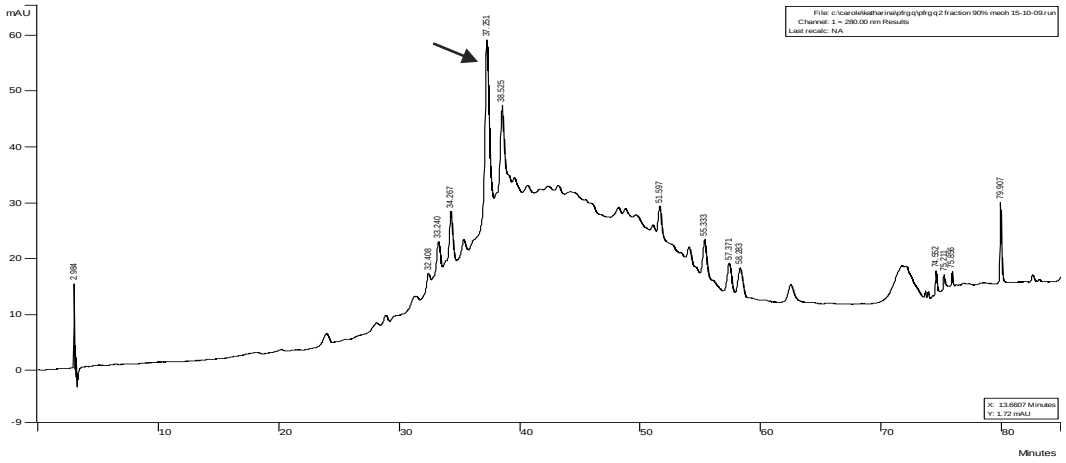


Figure 25: Chromatogram of fraction 90% MeOH, column No. 2

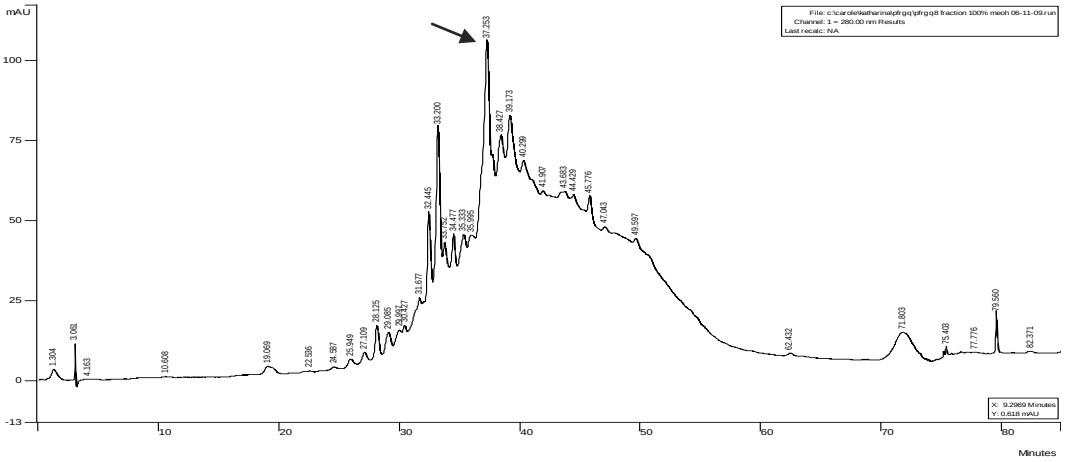


Figure 26: Chromatogram of fraction 100% MeOH, column No. 8

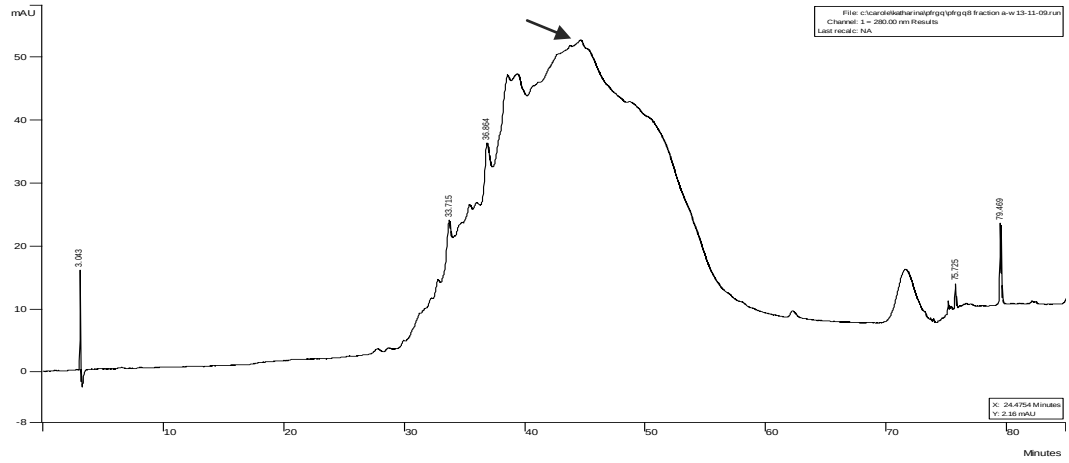


Figure 27: Chromatogram of fraction a-w, column No. 8

4.2.2 Quince

For quince extract, OPCs were detected from fraction 70% MeOH to fraction a-w (Figure 28, Figure 29, Figure 30, Figure 31, Figure 32).

Chromatograms of all fractions again show the unresolved hump, typical for OPCs.

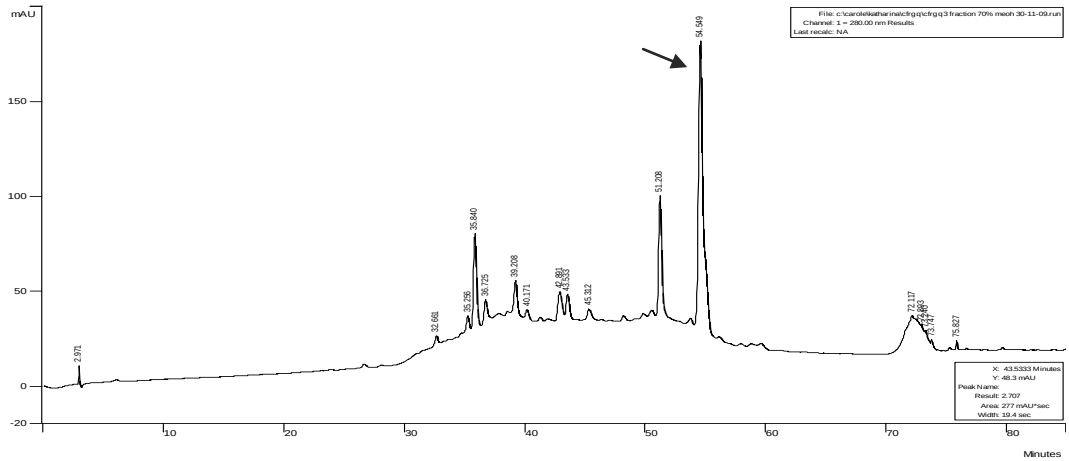


Figure 28: Chromatogram of fraction 70% MeOH, column no. 3

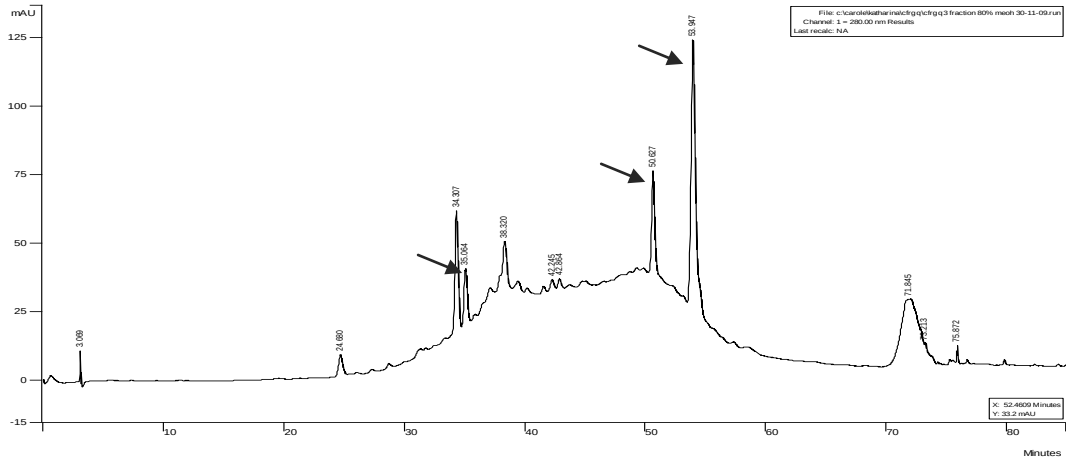


Figure 29: Chromatogram of fraction 80% MeOH, column no. 3

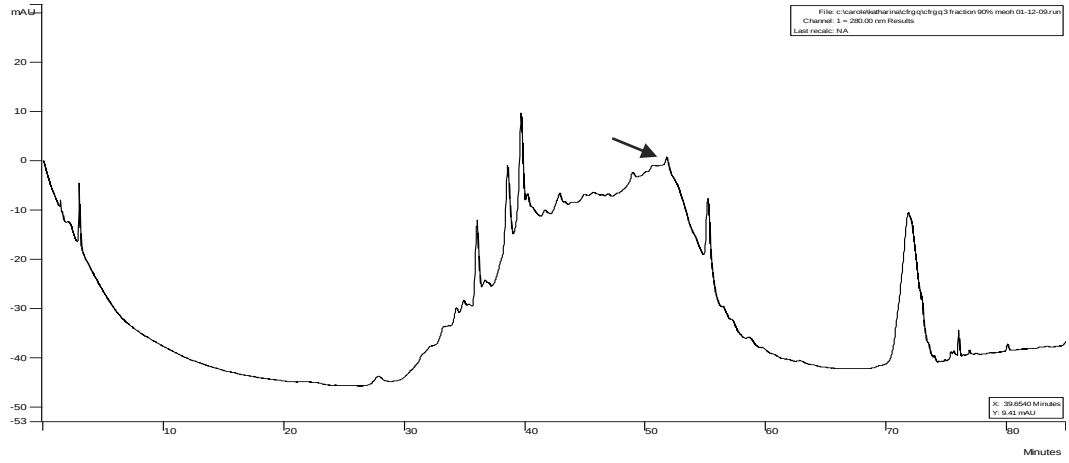


Figure 30: Chromatogram of fraction 90% MeOH, column no. 3

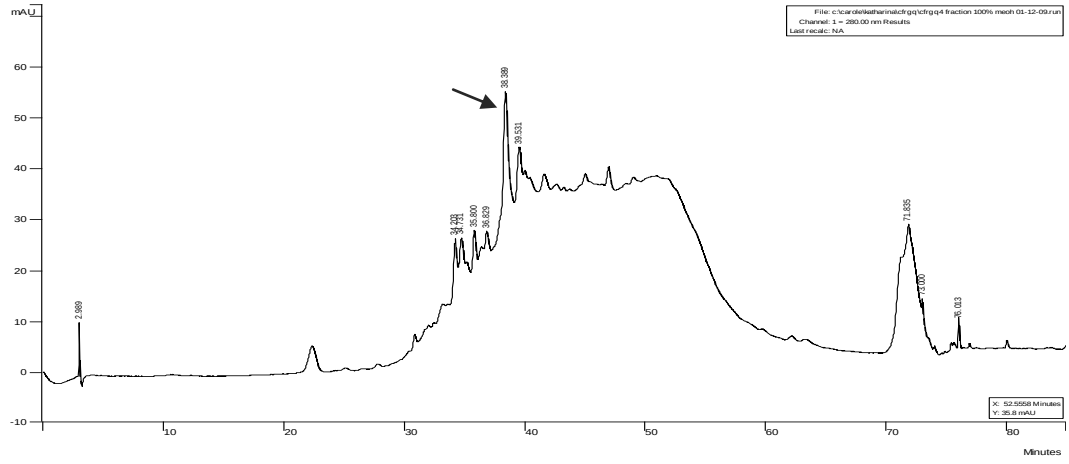


Figure 31: Chromatogram of fraction 100% MeOH, column no. 4

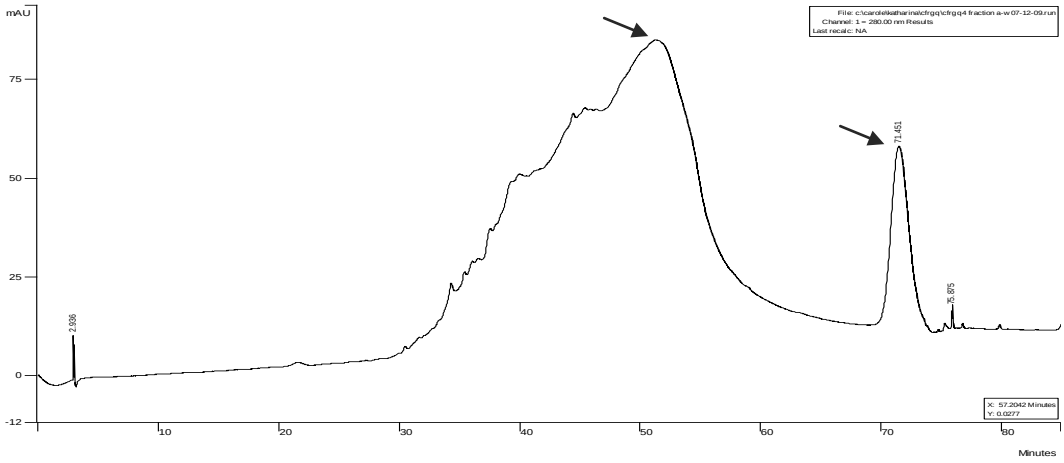


Figure 32: Chromatogram of fraction a-w, column no. 4

4.2.3 Comparison of Chosen Fractions PFR-CFR

The upper chromatograms show quince fractions, the lower apple fractions.

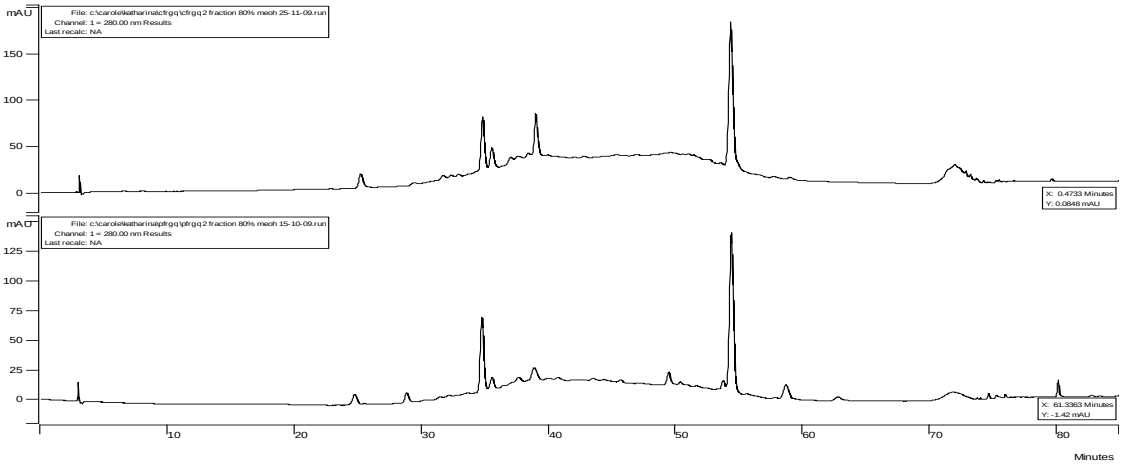


Figure 33: Comparison of fractions 80% MeOH

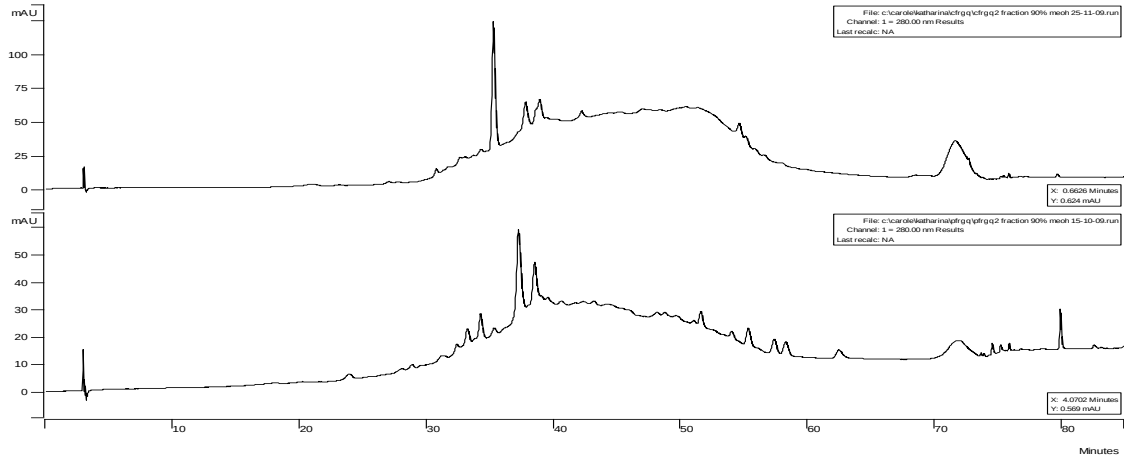


Figure 34: Comparison of fractions 90% MeOH

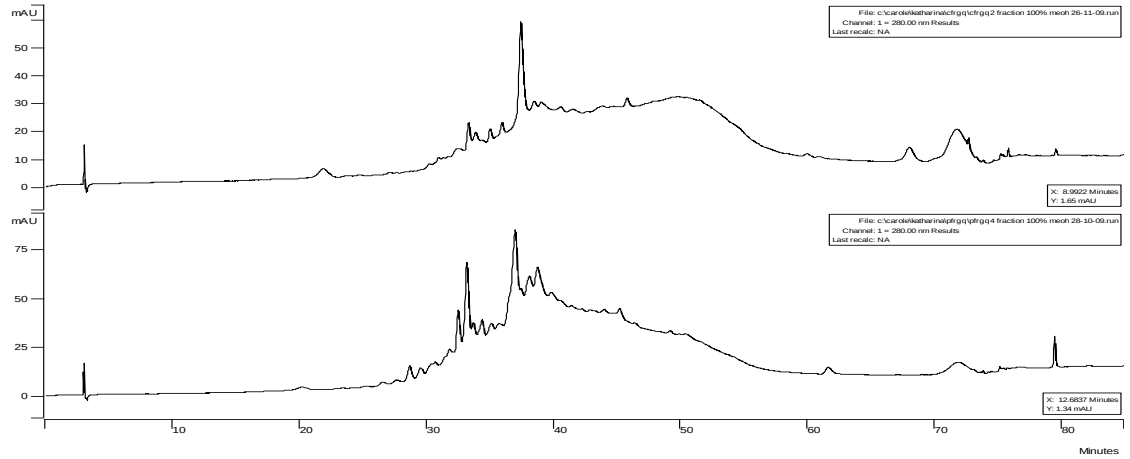


Figure 35: Comparison of fractions 100% MeOH

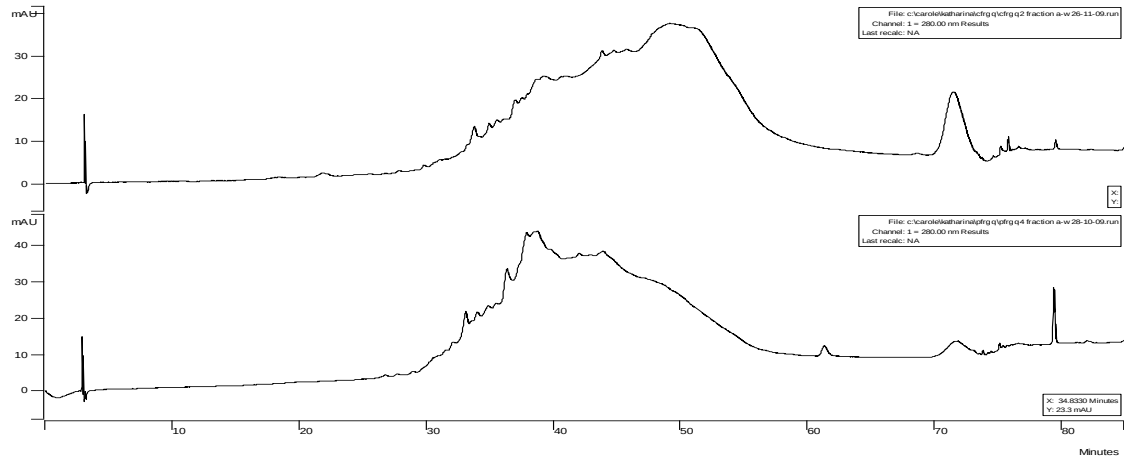


Figure 36: Comparison of fractions a-w

The accordance of apple and quince fractions containing OPCs is confirmed by the apparent similarity of the corresponding chromatograms (Figure 33, Figure 34, Figure 35, Figure 36).

4.3 UV-Spectra

4.3.1 Apple

UV-spectra of fractions 70 and 80% MeOH showed other substances than OPCs (Figure 37, Figure 38, Figure 39).

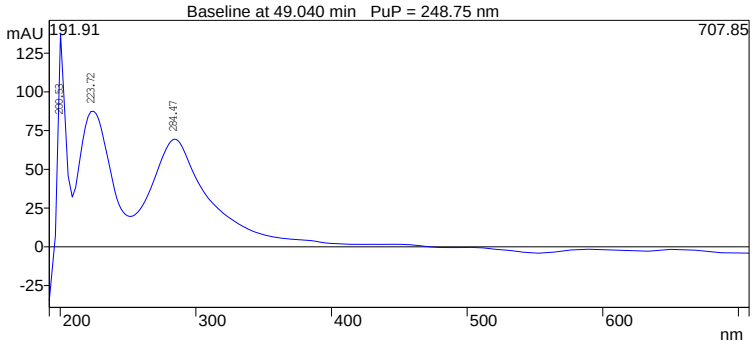


Figure 37: UV-spectrum of non-OPCs in fractions 70% MeOH, column no. 5

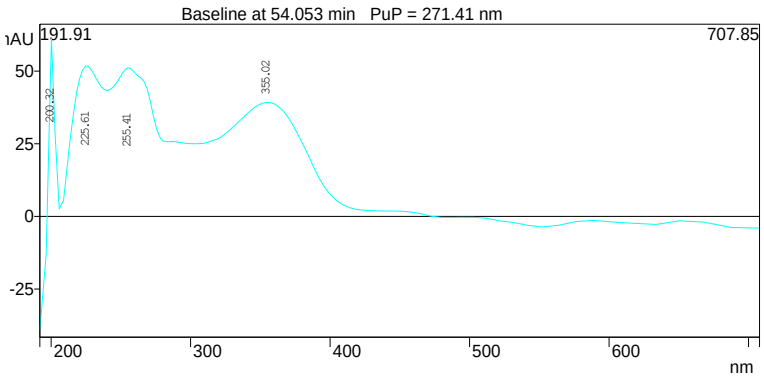


Figure 38: UV-spectrum of non-OPCs in fractions 70% MeOH, column no. 5

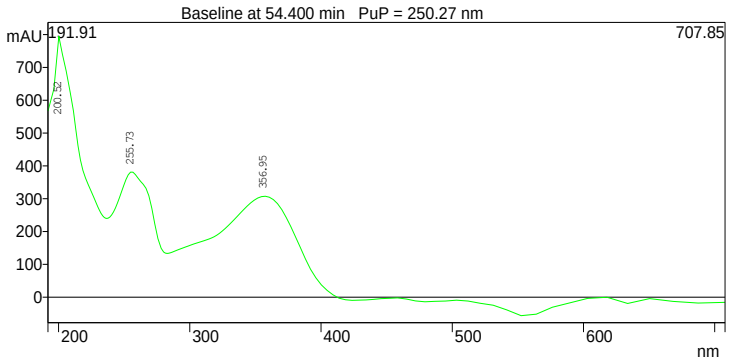


Figure 39: UV-spectrum of non-OPCs in fractions 80% MeOH, column 2

The UV-spectra for fractions 90% MeOH to a-w showed only OPCs. Examples of UV-spectra are given below. (Figure 40, Figure 41, Figure 42).

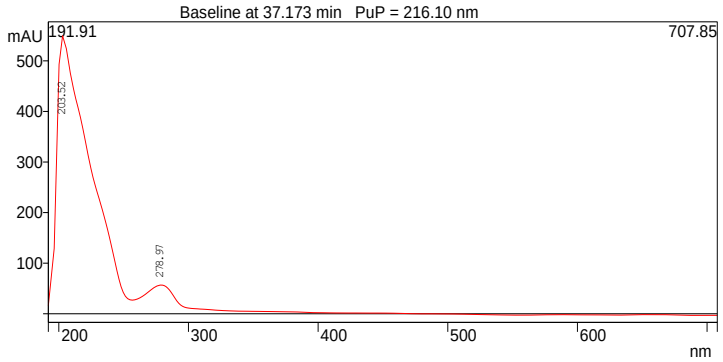


Figure 40: UV-spectrum of OPCs fraction 90% MeOH, column 2

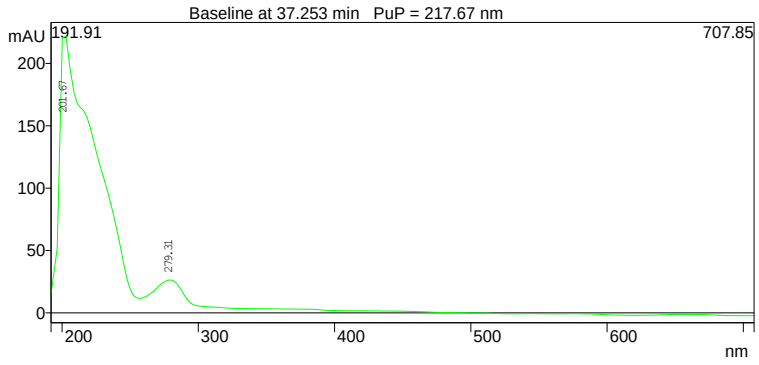


Figure 41: UV-spectrum fraction 100% MeOH, column 8

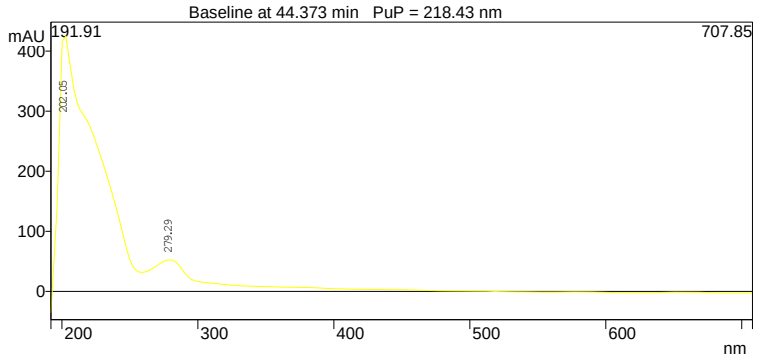


Figure 42: UV-spectrum fraction a-w, column 8

Anthocyanins were not detected in the apple extract.

Fractions 70 and 80% MeOH were not taken into account for the enriched extract.

Fractions 90% MeOH to a-w from column one to nine were united and represented the enriched extract further referred to as “PFR OPC”.

4.3.2 Quince

Likewise to apple in fractions 70 and 80% MeOH other substances than OPCs were found (Figure 43, Figure 44).

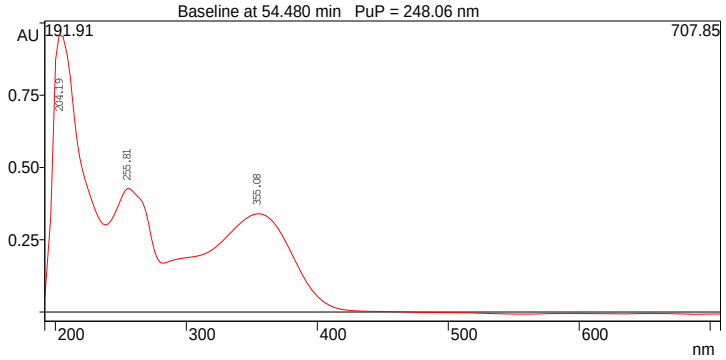


Figure 43: UV-spectrum of non-OPCs, fraction 70% MeOH, column no. 3

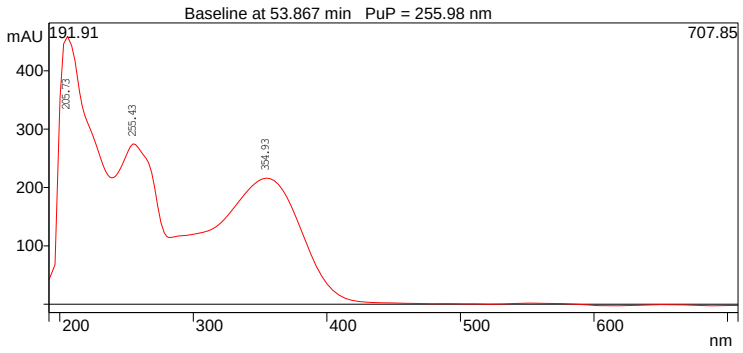


Figure 44: UV-spectrum of non-OPCs, fraction 80% MeOH, column no. 3

Figure 38, Figure 39, Figure 43, Figure 44 are likely to show UV spectra of flavonols like rutin, kaempferol or quercetin. [69]

For some UV-spectra of fraction 80% MeOH it cannot be told for sure whether they belong to OPCs or not (Figure 45, Figure 46). It is possible that they show glycosylised or otherwise substituted OPCs.

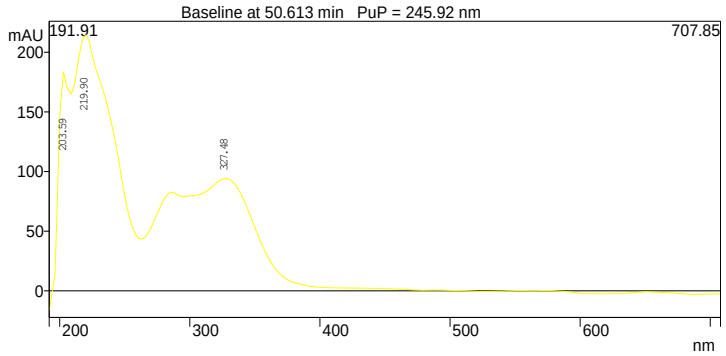


Figure 45: UV-spectra of fraction 80% MeOH, column 3

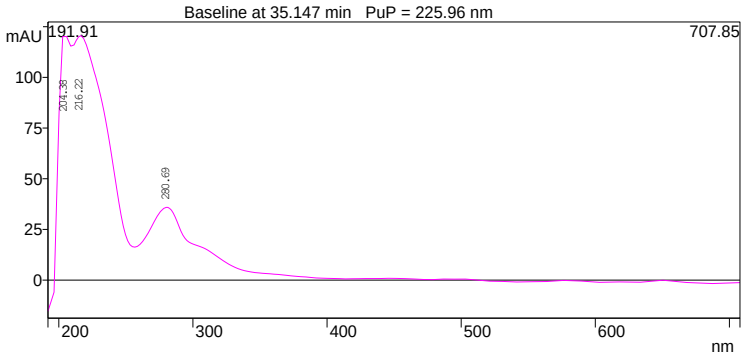


Figure 46: UV-spectra of fraction 80% MeOH, column 3

Fractions 90% MeOH to a-w were free of other constituents than OPCs. A few examples for UV spectra are given below (Figure 47, Figure 48, Figure 49, Figure 50).

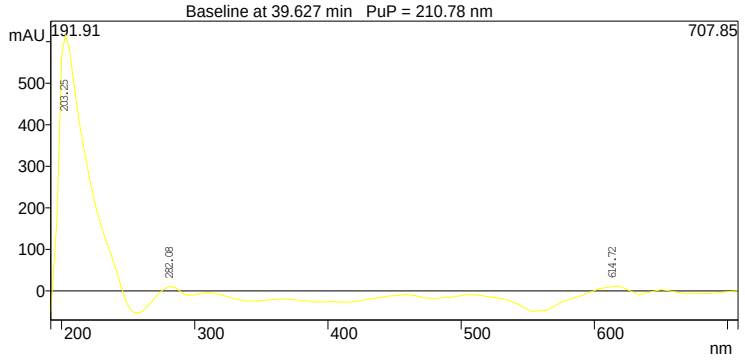


Figure 47: UV-spectrum of OPCs, fraction 90% MeOH, column 3

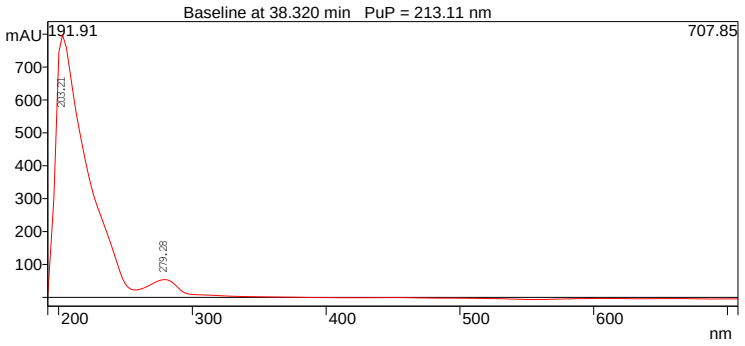


Figure 48: UV-spectrum of OPCs, fraction 100% MeOH, column 4

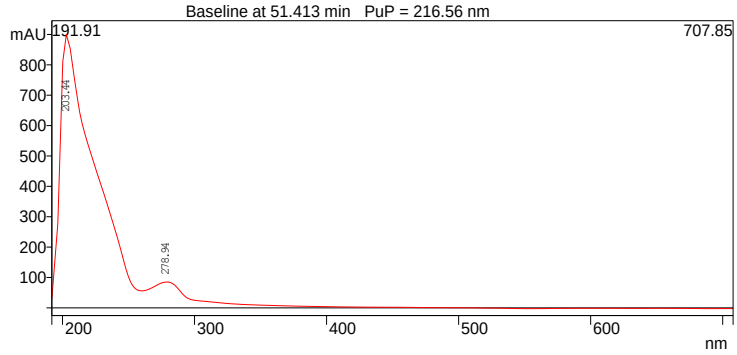


Figure 49: UV-spectrum of OPCs, fraction a-w, column 4

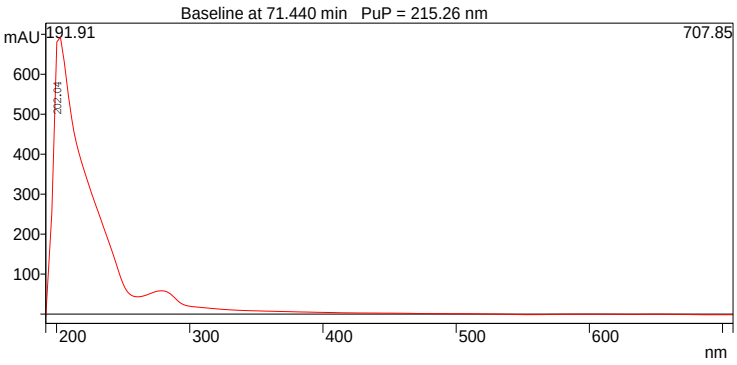


Figure 50: UV-spectrum of OPCs, fraction a-w, column 4

As for apple, no anthocyanins were detected.

Since recovery of quince columns was quite low and UV analysis showed considerable amounts of OPCs, fractions 80% MeOH were added to the enriched extract (fractions 90% MeOH to fractions a-w) to have enough material for further proceeding. The enriched extract is called “CFR OPC”.

4.4 Quantification

4.4.1 Vanillin Assay

Calibration curve:

Absorptions were calculated according to the formula mentioned (3.5.1). Examples for plate pipette schedules and absorption are given below (Table 17, Table 18). All other absorptions and plate pipette schedules for vanillin and Folin-assay are listed in the appendix. In this part only the calculated absorptions and concentrations are given.

Table 17: Pipette schedule for vanillin assay calibration with catechin[3]

	1	2	3	4	5	6	7
sv	700µg	600µg	500µg	400µg	300µg	200µg	100µg
b	700µg	600µg	500µg	400µg	300µg	200µg	100µg
bv	BV						
o	O						

Table 18: Absorptions of vanillin assay calibration

	1	2	3	4	5	6	7
sv	1,0494	0,9472	0,8401	0,6963	0,521	0,3527	0,2085
b	0,0414	0,04	0,0384	0,0383	0,0412	0,0387	0,0383
bv	0,0408						
o	0,0374						

The calculations for the calibration curve resulted in the equation y=0,0304x (Table 19, Figure 51).

[3] sv= sample vanillin, b=sample blank, bv: vanillin blank, o=blank

Table 19: Data for vanillin assay calibration

stock-sol. (µg catechin/ml)	Absorbt.	µg/well
0	0	0
100	0,1668	5
200	0,3106	10
300	0,4764	15
400	0,6546	20
500	0,7983	25
600	0,9038	30
700	1,0046	35

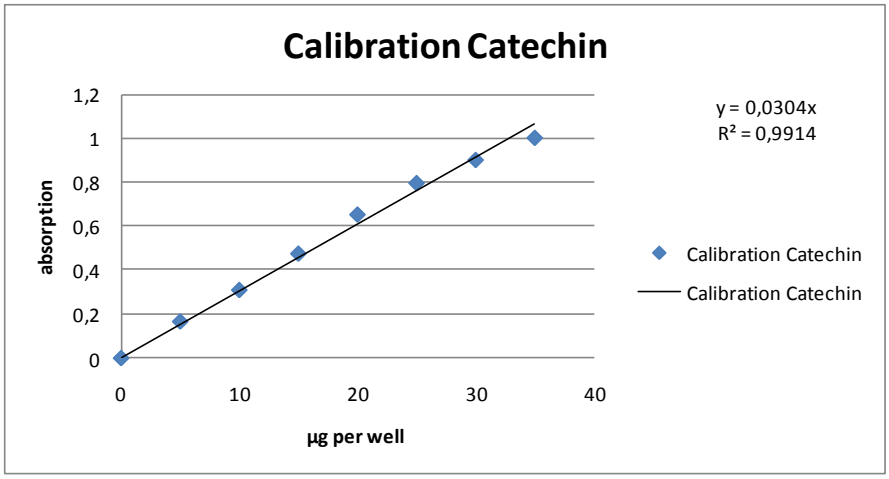


Figure 51: Calibration curve and equation for the vanillin assay

Samples

The content of OPC enriched extracts (PFR/CFR OPC) and aqueous extracts (PFR/CFR aq) were calculated according to the calibration curve equation.

Samples were measured in dupli- or triplicate (Table 20, Table 21).

Table 20: Absorptions and concentrations of enriched extracts. Pipetting 50µl of stock solution into each well results in a content of 25µg per well

stock sol. (µg/ml)	Absorption	Abs. (average)	µg per well (calculated)	µg extract per well	% OPC
PFR OPC					
500	0,6648				
	0,6558	0,6662333	21,916	25	87,664
	0,6781				
CFR OPC					
500	0,5662				
	0,5545	0,55956667	18,407	25	73,628
	0,558				

Table 21: Absorptions and concentrations of aqueous extracts. Taking 50µl of stock solution for each well results in a content of 250µg extract per well

stock sol. (mg/ml)	Absorption	Abs. (average)	µg per well (calculated)	µg extract per well	% OPC
PFR OPC					
5	0,0261				
	0,0263	0,0268333	0,883	250	0,353
	0,0281				
CFR OPC					
5	0,0557				
	0,055	0,0583	1,918	250	0,767
	0,0642				

The content of flavan-3-ols for the enriched extracts was 87,7% for apple extract and 73,6% for quince extract, and for aqueous extracts 0,35% for apple and 0,77% for quince.

Hence apple OPCs were enriched by estimated 248% and quince OPCs by estimated 96%.

Quantification was also done for three commercially available enriched extracts (Cranberry-extract from “Ferco développement”, Oligopin® and from “DRT® (dérivés rétiniques et terpéniques)” and Pineol® from “Berkem®”) to compare results (Table 22). Cranberry extract from Ferco dev. has a content of 18% OPCs (measured by the BL-DMAC method), Oligopin and Pineol have a content of more than 65% (measured by GPC-gel permeation chromatography) and more than 90% (Porter method) OPCs, respectively, according to the internet. [70, 71]

Table 22: Absorptions and concentrations of commercially available extracts

extract	stock (mg/ml)	Absorptions	Abs. average	µg per well (calculated)	µg extract per well	%OPC
CRB	0,5	0,1875	0,1869	6,148	25	24,6
		0,1863				
Pineol	0,5	0,5624	0,5478	18,02	25	72,1
		0,5332				
Oligopin	0,5	0,6399	0,6082	20	25	80
		0,5765				

Quantification by the vanillin-assay showed 24,6% instead of 18% OPCs for the Cranberry extract.

The extract “Pineol” with 72,1% OPCs had a lower concentration than published (>90%).

The extract “Oligopin” with 80% OPCs corresponds to the published data.

4.4.2 Folin Assay

All plate pipette schedules and measured absorptions are added to the annex.

The calibration curve was done with gallic acid as reference. Measurement of calibration was done in triplicate (Table 23). Since there were major variations in absorptions only two of three measured absorptions were taken into account for calculations.

Table 23: Data for Folin assay calibration with gallic acid

Stock sol. (µg/ml)	Absorbtion	Abs. (average)	µg per well
0	0	0	0
20	0,021	0,02015	0,6
	0,0193		
	[0,0183]		
40	0,0733	0,0727	1,2
	0,0721		
	[0,107]		
60	0,1275	0,12555	1,5
	0,1236		
	[0,1712]		
80	0,1873	0,1773	2,4
	0,1673		
	[0,2731]		
100	0,2303	0,2202	3
	0,2101		
	[0,3209]		

Calculated absorptions resulted in an equation of y=0,073x for calibration curve (Table 23, Figure 52).

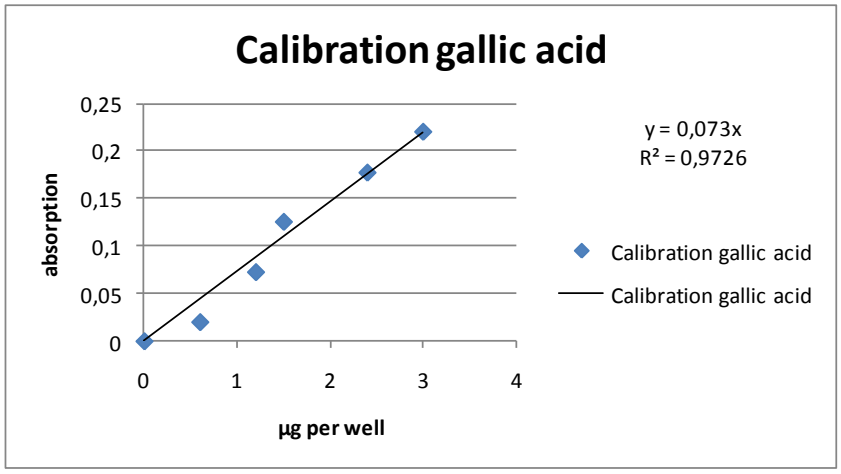


Figure 52: Calibration curve and equation for the Folin assay

Samples

The total polyphenol contents of OPC enriched extracts (PFR/CFR OPC) were calculated according to the calibration curve equation.

All samples were measured in triplicate. Again, variations in absorptions occurred. Values in parentheses were not considered (Table 24).

Table 24: Absorptions and concentrations of enriched extracts. Pipetting 30µl of stock solution into each well results in a content of 1,5µg per well.

stock sol. (µg/ml)	Absorption	Abs. (average)	µg per well (calculated)	µg extract per well	% total polyphenol content
PFR OPC					
50	0,1016	0,1047	1,434	1,5	95,6
	[0,0803]				
	0,1078				
CFR OPC					
50	[0,4426]	0,158	2,164	1,5	144,3
	0,1602				
	0,1558				

The result for apple was 95,6% total polyphenol content and for quince more than 100% total polyphenol content.

Aqueous extracts and commercially available samples were not quantified.

4.4.3 Recovery and Enrichment

Recovery for the applied method for extraction, purification and enrichment was calculated based on the weights of pomace, aqueous and enriched extract (Table 25).

Table 25: Weights and recovery for apple and quince

fruit	pomace (g)	aqueous extract (g)	extract on columns (g)	OPC enriched fraction (g)
apple	5793	499,69	178,11	1,4314
quince	4813,02	600,54	250,34	1,369

pomace/ aqueous extract	aqueous extract/ OPC enriched fr.	pomace/ OPC enriched fr.
8,33%	0,80%	0,07%
12,48%	0,55%	0,07%

4.5 Cell Culture

Effects on apoptosis of for cell lines SW480 and SW620 are presented by dose response curves (Figure 53, Figure 54).

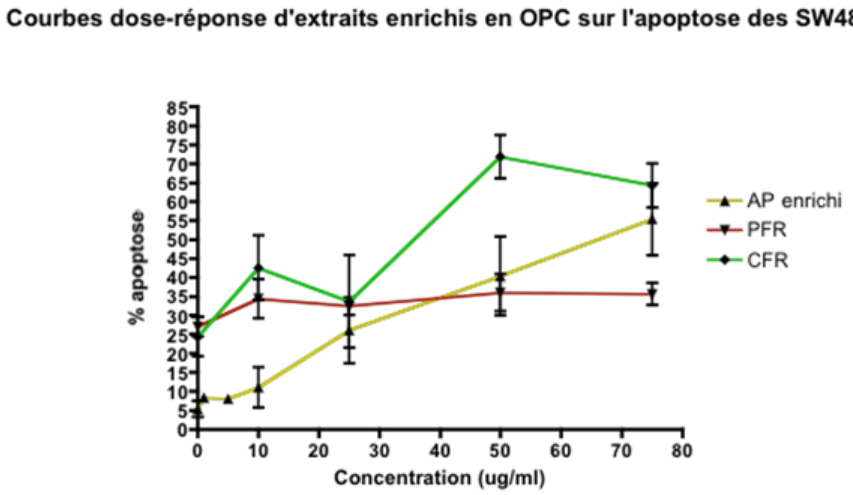


Figure 53: Dose-response curve of OPC enriched extracts on apoptosis of SW480

“AP enrichi” is a commercially available OPC enriched extract. Here it was used as a reference. PFR OPC (red) had no dose dependant effect on the apoptosis of cells. Apoptosis remained at 27-35%.

For CFR OPC (green) and AP enrichi (yellow) a dose dependant effect could be observed. Even if the effect of CFR OPC on cell apoptosis decreased after reaching a maximum of 71% at 50µg/ml the effect at 80µg/ml was 10% higher than for AP.

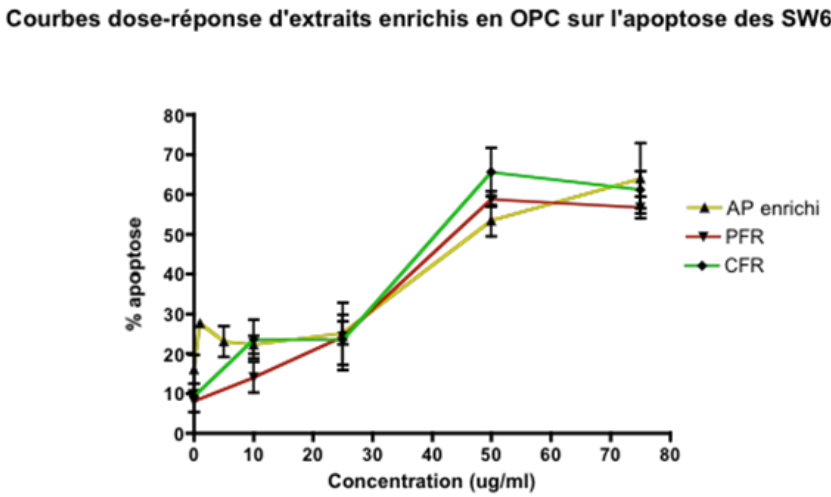


Figure 54: Dose-response curves for OPC enriched extracts on apoptosis of SW620

The three extracts had comparable effects on apoptosis of SW620 cells. A dose dependant increase of apoptosis could be observed. Compared to SW480 the effects of PFR OPC and CFR OPC started at lower levels.

Diagrams were made for both cell lines to compare the effects of OPC extracts to the effects of celastrol induced apoptosis, normal apoptosis, aquaeous extracts, anthocyanins and cyclohexane/ethyl acetate phases from liquid-liquid extraction. The samples mentioned above were tested at a concentration of 50µg/ml (Figure 55, Figure 56).

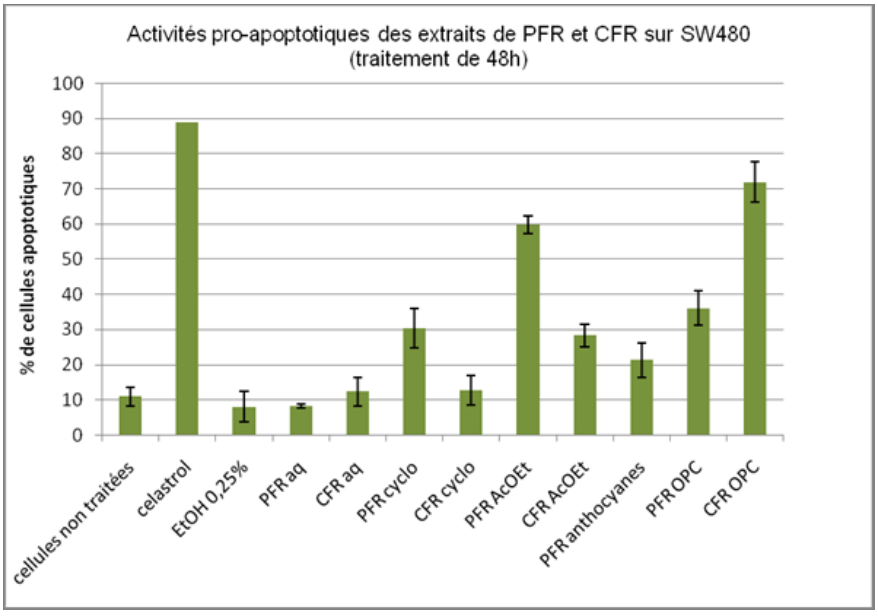


Figure 55: Pro-apoptotic activities of PFR and CFR extracts on SW480 (48h treatment)

SW480 cells had to be treated 48 hours to observe effects. Besides celastrol treated cells (89%) the highest amounts of apoptotic cells were counted for CFR OPC (71%) and the ethyl acetate fraction of PFR (60%), followed by PFR OPC (37%), the cyclohexane fraction of PFR (30%), the ethyl acetate fraction of CFR (28%) and anthocyanins (22%). Untreated cells had an apoptosis rate of 11%.

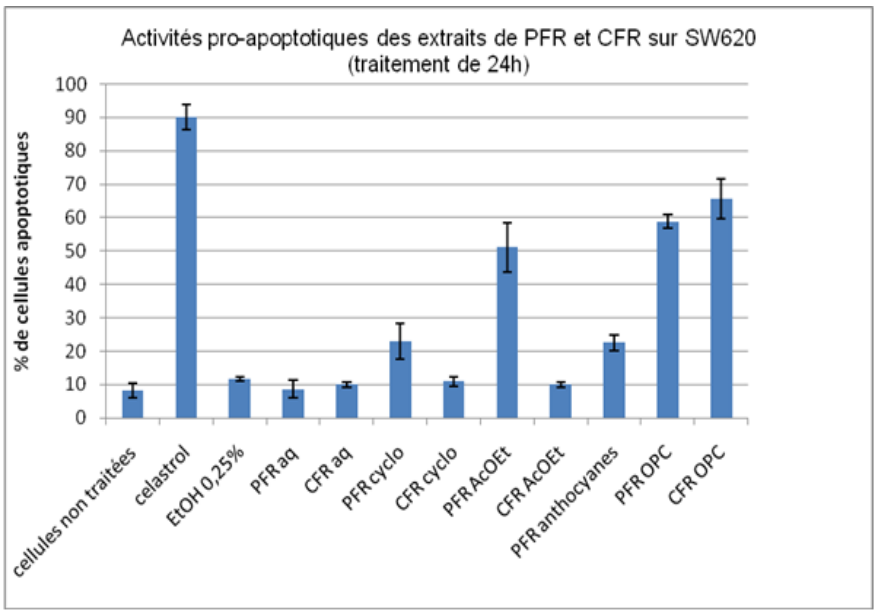


Figure 56: Pro-apoptotic activities of PFR and CFR extracts on SW620 (24h treatment)

Extracts showed effects after treatment of 24h on SW620 cells. Besides celastrol treated cells (90%) the highest amounts of apoptotic cells were counted for CFR OPC (67%), PFR OPC (58%) and the ethyl acetate fraction of PFR (51%) followed by the cyclohexane fraction of PFR (23%) and anthocyanins (22%). Untreated cells had an apoptosis rate of 9%.

4.6 Thin Layer chromatography

Three TLC-systems (Goncalves, Glavnik, Merghem) showed no separation of extract constituents (Figure 57, Figure 58, Figure 59, Figure 60, Figure 61, Figure 62). In one system no migration of the extract spot could be seen (Figure 61, Figure 62).

Figure 57: TLC Goncalves et al (2005), UV 254nm after migration

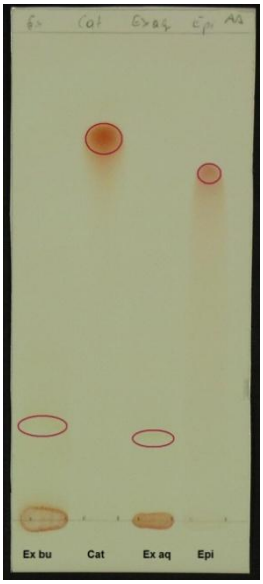


Figure 58: TLC Goncalves et al. (2005), after revelation

Figure 59: TLC Glavnik et al (2009), UV 254nm after migration

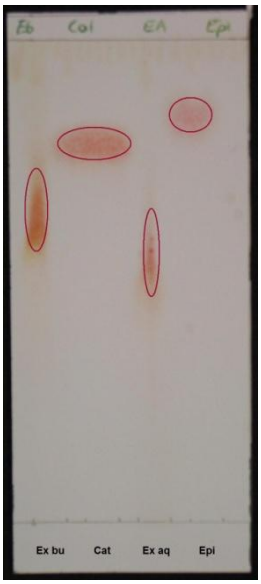


Figure 60: TLC Glavnik et al. (2009), after revelation

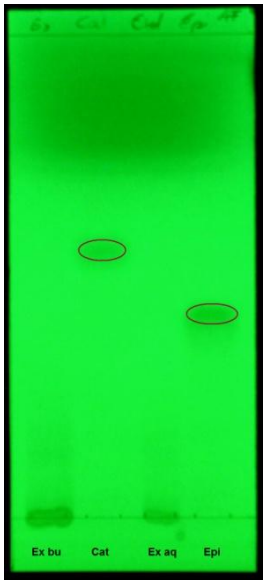


Figure 61: TLC Merghem et al (2004), UV 254nm after migration

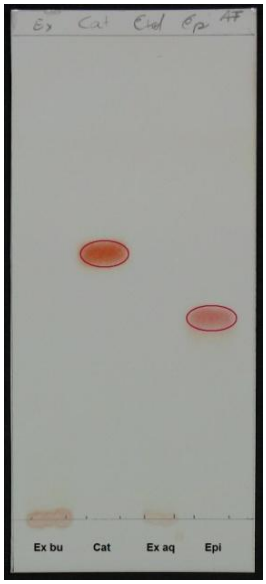


Figure 62: TLC Merghem et al. (2004), after revelation

Figure 63: TLC Svedström et al (2002), UV 254nm after migration

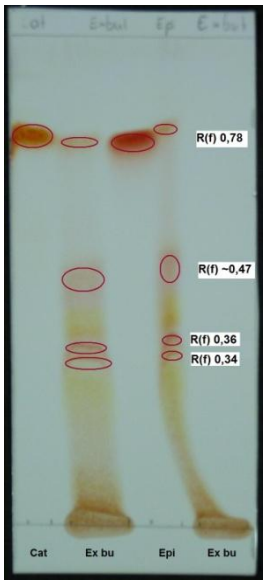


Figure 64: TLC Svedström et al. (2002), after revelation

The TLC-system after Svedström showed a separation of extracts constituents (Figure 63, Figure 64). A comparison with published R(f) values showed monomers, trimers and eventually tetramers (Table 26).

Table 26: R(f) value analysation

polymerisation	R(f) TLC	R(f) publication
monomers	0.78	0.8
dimers	-	0.71, 0.62
trimers	~0.47	0.45, 0,49
tetramers	0.34, 0.36	0.28
pentamers	-	0.11
hexamers	-	0.06

5 Discussion

5.1 Recovery and Enrichment

According to the RP-HPLC chromatograms and the corresponding UV-spectra the method of extraction and purification can be applied for the selected plant material. The comparison of chromatograms of apple and quince showed that columns worked in a reproducible manner.

However the RP-HPLC-DAD method does not give information on the polymerisation degree of flavan-3-ols.

Yet monomers and almost all the dimers should have been exhaustively eliminated from the extract by liquid-liquid extraction and size-exclusion chromatography.

For OPC analysis according to the degree of polymerisation, normal-phase HPLC system published by Svedström et al. or Shoji et al. could be used. [57, 72]

A content of 70mg trimers to decamers per 100g for both apple and quince (0,07%) is in accordance with published data of Gu et al. and Cetkovic et al., so pomace seems to be a potent source for OPCs. [34, 59]

The use of Sephadex is very expensive and the applied method of extraction and enrichment is a very long process. To obtain a reasonable amount of OPC enriched extract many columns are required for recovery per column is very low.

Hence this method should only be used on a laboratory scale and would not be suitable for industrial scale-up.

5.2 Quantification

Data of calibration and samples were homogeneous for the Vanillin assay.

Sun et al. stated that this assay is quite specific to a narrow range of flavanols (monomers and polymers) and to dihydrochalcones with a single bond at the 2,3-position and a free meta-oriented hydroxyl group on the B-ring. Nevertheless variations in water content, temperature and time lead to different colour yields. [49]

According to Broadhurst and Jones the assay is also sensitive to interfering substances (ascorbic acid, chlorophyll, anthocyanins), the concentration of vanillin, and the acid nature and concentration. Changes in those factors can largely influence results. [73]

As published by Scalbert et al. (+)catechin as a reference standard can lead to underestimation of a sample. By contrast, Price et al. published that the standardisation with (+)catechin results in an overestimation of flavan-3-ols. [74, 75]

The overestimation could be seen for the commercial sample CRB with 24,6% OPC instead of 18%. On the other hand, the other commercial sample Pineol showed only 72,1% OPCs instead of >90% as published on the internet so this would account for an underestimation.

Hence data cannot be interpreted as totally precise and the results for Vanillin quantification should be interpreted as approximate values.

The inclusion of quince fractions 80%MeOH in the enriched extract explains the lower result for quince (73,63%) than for apple (87,66%) as in the quince fraction 80%MeOH other substances than flavan-3-ols were identified.

The results of the Folin assay show 144% total polyphenols for quince and considering the heterogeneous absorption data this assay does not seem suitable for enriched extracts. Hümmer et al. as well as Sun et al. also claim the lack of specifity, the varying specificity and poor reproducibility of this assay. [43, 49] According to them the Folin assay is more suitable for crude extracts with lower quantities of polyphenols.

Recently, Prior et al. published a multi-laboratory validation of a colorimetric method for the quantification of OPCs in cranberry extracts. This method uses DMAC (4-dimethylaminocinnamaldehyde) as reagent with a high selectivity for flavan-3-ols and commercially available procyanidin A2 as reference. [76]

As reported by Aubert C., DMAC reacts specifically with meta-diphenols, forming a green carbonium ion. The assay is more likely not to interfere with anthocyanins (for the determination wavelength for the coloured product is at 640nm) or with any other flavanoids or ascorbic acid. [77]

Cunningham et al. and Payne et al. also reported on the DMAC quantification method for OPCs to be adequate for cranberry and chocolate-samples. [78]

According to Aubert, C. more reproducible results could be achieved with the DMAC-method. [77]

Therefore the method could be considered as an alternative to the Vanillin and the Folin assay.

5.3 Proapoptotic Potential

Fini et al. documented the induction of apoptosis by apple polyphenols in colon carcinoma cell lines RKO, SW48 and SW480. A normalisation of the expression of tumour suppressor protein p53 could also be shown. [79]

Alterations of signalling pathways that trigger apoptosis in colon cancer cell line SW620 have been reported by Gossé et al. [80]

As shown by Maldonado-Celis et al. procyanidins trigger the up-regulation of the receptors DR4 and DR5 of TNF-related apoptosis inducing ligand (TRAIL). Binding of TRAIL to those receptors leads to the activation of the extrinsic death receptor pathway (Figure 6). [51, 81]

All those publications are in good accordance with the data received from PFR/CFR treated cells and present likely ways of molecular function of OPCs.

The high potential of quince extract to induce apoptosis is apparent in both cell lines. There is a dose dependant increase of the effect.

The dose-response curves for apple extract show bivalent data for cell lines SW480 and SW620. The effect on SW620 cells corresponds to data received for quince but there is a lack of increase of a proapoptotic effect in SW480 cells for no obvious reasons.

The incubation time of 48 hours for SW480 cells compared to 24h for the other cell line indicated that cells need a longer time to respond to treatment but the response then is alike. A reason for this delayed reaction is a difference in metabolism between the two cell lines.

The antitumor effect of anthocyanins (PFR anthocyanes) that occurred in both cell lines has also been documented by Cooke et al. and Yun et al. [19, 31]

As the ethyl acetate extraction was done to remove monomers and dimers from the aqueous extract the high efficiency of these extracts on the induction of apoptosis could be ascribed to those substances. This would be in accordance with the review of Giovannini et al. [23]

However it is possible that other molecules in the ethyl acetate fraction cause the effect on the cell lines.

5.4 Future Perspectives

Bioavailability and cancer reduction: According to Scalbert et al. and Manach et al. procyanidins consisting of more than three monomer subunits are only poorly intestinally absorbed. [82, 83]

Kahle et al. observed that they are obliged to be degraded microbial but nonetheless higher polymers reach the colon and are likely to exert local effects. [84]

Gossé et al. carried out trials with rats with chemically induced colon carcinogenesis. They were fed with apple procyanidins (=AP enrichi) (0,01% in drinking water) for six weeks and a significant reduction of preneoplastic lesions and aberrant crypt foci could be observed. [80]

Such animal trials would be interesting for the apple and quince extracts as well.

Chemoprevention: treatment or dietary intake: According to Scalbert et al. it has to be distinguished between chemoprevention through normal diet and chemoprevention through pharmacological doses of OPCs that would be far beyond dietary intake, for the effects, pharmacology and pharmacokinetics would be largely different. [24]

Regarding toxicology and safety of polyphenols Shoji et al documented that the apple polyphenol extract AP showed no mutagenicity, acute or sub-chronic toxicity effects at average dietary levels. The extract AP is used as food additive and nutritional supplement. [57]

Chemotherapy: Beliveau and Gringas and Scalbert et al. report synergies of (epi)gallocatechingallat and the flavonoid quercetin with chemotherapeutics and radiotherapy on cancer cell lines. [2, 24]

It would be interesting to determine if those synergies also existed for OPCs.

5.5 Thin Layer Chromatography

The results of the executed TLC-systems show they are not sufficient for effectively separating single substances. More trials and modifications are needed.

An attractive method for separation of procyanidin oligomers was presented by Shibusawa et al. [85] The use of counter current chromatography could be very interesting for separation of extracts by degree of polymerisation. Substances could be tested separately for effects and possible synergies.

5.6 Conclusion

Apple and quince pomace have been shown to be potent, easily available and cheap sources of OPCs.

The applied methods for extraction and enrichment of OPCs are efficient but fastidious. If applied in an industrial scale they would need reconsideration and optimisation. Sanofi Aventis developed a method for the extraction of OPCs from grapes. The medicine containing these OPCs (Endothélon®) is used as peripheral angioprotector and venotonic. The applied method could be used as a model for an extraction procedure.

The proapoptotic effects on human colon cancer cell lines are evident for OPCs from both fruits and further investigations in animal models would be desirable.

Substances that induce programmed cell death in colon cancer cells are very likely to have chemopreventive powers in inflammatory or cancerous diseases of the gastrointestinal tract.

Therefore OPCs present an interesting class of substances which are easily applicable through diet. The prevention of cancer through diet means a constant and modest effort.

Another interesting future approach would be the use of *per os* OPC extracts as adjuvant in chemotherapy or radiotherapy.

However, many more trials and more data on the effects, pharmacology, pharmakocinetics and adverse effects of concentrated OPCs are needed.

Since OPCs have always been part of our diet no secondary effects are expected for the amounts of OPCs averagely ingested through normal diet.

6 Abstract

Cancer chemoprevention is the use of pharmaceuticals or other agents to reduce cancer risk.

As cancer is worldwide becoming a more and more serious problem a lot of effort is taken to find new chemopreventive substances.

Oligomeric procyanidins (OPCs, trimers to decamers) are a subclass of polyphenols that have already proven to have antimicrobial, antiviral, antidiabetic and cardioprotective properties.

This work concentrates on the ability of OPCs from specific sources to induce apoptosis in colorectal cancer cell lines.

OPCs were extracted from apple and quince pomace with a mixture of acetone and demineralised water (60:40). After liquid-liquid extractions with cyclohexane and ethyl acetate, the extracts were fractionated on Sephadex LH-20 columns with a methanol/water elution gradient.

The fractions were analysed by reversed phase HPLC coupled with an UV-VIS detector. Fractions containing major amounts of OPCs were united to the enriched OPC extract.

After quantification (vanillin and Folin assay) the extract was tested on colorectal cancer cell lines SW480 and SW620 to assess the induction of apoptosis.

The OPC enriched quince extract showed high activity in both cell lines (71% and 67% of apoptotic cells in contrast to 11%/9% apoptosis rate of untreated cells).

The effect of the OPC enriched apple extract on the cell line SW480 was relatively low (35%) but higher for the cell line SW620 (58%).

Additionally to the tests on cell lines it was tried to separate monomers to oligomers from the extracts by thin layer chromatography. Isolated substances could be tested on cell cultures for effects and possible synergies.

No satisfying results could be obtained by TLC-separation.

As a conclusion, OPCs have relevant effects on cancer cells and are likely to be of aid in the prevention or the treatment of tumours of the gastrointestinal tract.

7 Zusammenfassung

Die Chemoprävention von Krebs ist die Verwendung von Arznei- oder anderen Mitteln um das Krebsrisiko zu vermindern.

Da Krebs weltweit zu einem immer bedeutenderen Problem wird, bemüht man sich, neue chemopräventive Substanzen zu finden.

Oligomere Procyanidine (OPC, Trimere bis zu Decamere) sind eine Unterklasse der Polyphenole, deren antimikrobielle, antivirale, antidiabetische und kardioprotektive Wirkung schon gezeigt werden konnte.

Diese Arbeit beschäftigt sich mit der Fähigkeit der OPCs aus spezifischen Quellen in kolorektalen Krebszelllinien Apoptose zu induzieren.

OPCs wurden aus den Pressrückständen von Äpfeln und Quitten mit einer Mischung aus Azeton und demineralisiertem Wasser (60:40) extrahiert. Nach flüssig-flüssig Extraktionen mit Cyclohexan und Ethylazetat wurden die Extrakte mittels einer Sephadex LH-20 Säule mit einem Methanol/Wasser Gradienten fraktioniert.

Die Fraktionen wurden mit RP-HPLC, gekoppelt mit einem UV-VIS Detektor, analysiert. Fraktionen die zum Großteil OPCs enthielten wurden zu einem OPC angereicherten Extrakt vereint.

Nach der Gehaltsbestimmung (Vanillin und Folin-Assay), wurde der Extrakt an den kolorektalen Krebszelllinien SW480 und SW 620 getestet um das Ausmaß der Apoptose-Induktion festzustellen.

Der OPC angereicherte Quitten-Extrakt zeige hohe Aktivität in beiden Zelllinien (71% und 67% apoptotische Zellen im Gegensatz zu 11% bzw 9% Apoptoserate bei unbehandelten Zellen).

Der Effekt des OPC angereicherten Apfel-Extraktes auf die Zelllinie SW480 war relativ gering (35%), für die Zelllinie SW620 jedoch höher (58%).

Zusätzlich zu den Zelltests wurde versucht Monomere und Oligomere mittels Dünnschichtchromatographie zu isolieren. Isolierte Substanzen könnten an Zellkulturen getestet werden um ihre Effekte und mögliche Synergien aufzuzeigen. Die DC-Trennung ergab keine zufriedenstellenden Ergebnisse.

Zusammenfassend lässt sich sagen, dass OPCs erhebliche Effekte auf Krebszellen haben und möglicherweise in der Prävention oder der Behandlung von gastrointestinalen Tumoren von Nutzen sein können.

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9 Curriculum Vitae

Name	Katharina Schüller
Day of Birth	January, 26th , 1986
Nationality	Austria
Adress	Fasangartengasse 55 1130 Vienna
Academic Studies	October 2004 to January 2005 Diploma Study History of Art, University of Vienna Since March 2005 Diploma Study Pharmacy, University of Vienna September 2009 to January 2010 Visiting student at the Université de Strasbourg UMR 7200 Pharmacognosie et Molécules naturelles bioactives Diploma thesis: Phytonutriments and Chemoprevention: the role of polyphenols in the prevention of colorectal cancer
Education	General qualification for University entrance June 2004 GRG 13 Wenzgasse 7 1130 Vienna Academic high school with emphasis on languages
Work Experience	July 2006 Internship at Roche Diagnostics (marketing) Engelhorngasse 3 A-1211 Vienna July 2007 Internship at AGES (department of radiation protection) Spargelfeldstraße 191 1220 Vienna July 2008 Internship at St Anna Apotheke, Dr. Peithner KG Meidlinger Hauptstraße 86 1120 Wien

Appendix

Chemicals and substances

Chemicals	Technical information	Company
(+)Catechin	≥98% (HPLC), powder	Sigma-Aldrich
1-Butanol	ACS reagent	VWR
Acetic Acid	>99,7% , ACS reagent	Sigma-Aldrich
Acetone	min 99% v/v, technical grade	VWR
Cyclohexane	technical grade	Carlo Erba Reactifs
Ethanol absolute Normapur	min 99,85% v/v	VWR
Ethanol TechniSolv	min 95,00% v/v	VWR
Ethyl acetate	technical grade	Carlo Erba Reactifs
Folin Ciocalteu phenol reagent	2M	Sigma-Aldrich
Formic Acid	~ 98%	Fluka
Gallic Acid	97%	Sigma-Aldrich
Guava Nexin reagent	Guava Nexin Kit	Millipore
Heptane	no data	SDS
Methanol HPLC	water content <300mg/kg	Carlo Erba Reagents
Methanol TechniSolv	min 99,50% v/v	VWR
Sephadex LH-20	lipophilic	Sigma-Aldrich
Sodium Carbonate	99,95-100,05%, ACS reagent	Sigma-Aldrich
Sulfuric Acid	95-97% v/v	Fluka
TLC plate	Silica gel 60 F254	Merck
Toluol UniSolv	no data	Merck
Trifluoracetic Acid	liquid	Sigma-Aldrich
Vanillin	synthesis grade	Merck
water milliq	Satorius, reverse Osmosis 0,2µm, Carlo Erba Reagents	Amtrol, Carlo Erba Reagents

Fraction yield apple

Col.	Fraction (%MeOH)	weight(g)
1	0	
1	10	
1	20	
1	30	
1	40	
1	50	
1	60	0.0406
1	70	0.0189
1	80	0.0179
1	90	
1	100	
1	a-w	

Col.	Fraction (%MeOH)	weight(g)
4	0	
4	10	
4	20	
4	30	
4	40	
4	50	0.0154
4	60	0.0226
4	70	0.0276
4	80	0.0399
4	90	0.0356
4	100	0.0326
4	a-w	0.0914

Col.	Fraction (%MeOH)	weight(g)
2	0	
2	10	
2	20	
2	30	
2	40	
2	50	0.012
2	60	0.0222
2	70	0.0258
2	80	0.0426
2	90	
2	100	
2	a-w	

Col.	Fraction (%MeOH)	weight(g)
5	0	
5	10	
5	20	
5	30	
5	40	
5	50	0.0112
5	60	0.0182
5	70	0.0279
5	80	0.0377
5	90	0.0388
5	100	0.0319
5	a-w	0.1157

Col.	Fraction (%MeOH)	weight(g)
3	0	
3	10	
3	20	
3	30	
3	40	
3	50	0.0122
3	60	0.0228
3	70	0.0293
3	80	0.0369
3	90	
3	100	
3	a-w	

Col.	Fraction (%MeOH)	weight(g)
6	0	
6	10	
6	20	
6	30	
6	40	
6	50	0.0121
6	60	0.0191
6	70	0.0315
6	80	0.0298
6	90	0.0402
6	100	0.0349
6	a-w	0.096

Col.	Fraction (%MeOH)	weight(g)
7	0	
7	10	
7	20	
7	30	
7	40	
7	50	0.0126
7	60	0.02
7	70	0.0261
7	80	0.0398
7	90	0.0366
7	100	0.0323
7	a-w	0.1053

Col.	Fraction (%MeOH)	weight(g)
8	0	
8	10	
8	20	
8	30	
8	40	
8	50	0.012
8	60	0.0214
8	70	0.0271
8	80	0.0348
8	90	0.0351
8	100	0.0514
8	a-w	0.0926

Col.	Fraction (%MeOH)	weight(g)
9	0	
9	10	
9	20	
9	30	
9	40	
9	50	0.0132
9	60	0.0199
9	70	0.0245
9	80	0.0377
9	90	0.0634
9	100	0.0286
9	a-w	0.1091

Fraction yield quince

Column	Fraction(% MeOH)	weight(g)
1	0	
1	10	
1	20	
1	30	
1	40	0.0256
1	50	0.0175
1	60	0.0176
1	70	0.0211
1	80	0.0197
1	90	0.0199
1	100	0.012
1	a-w	0.0794

Column	Fraction(% MeOH)	weight(g)
2	0	
2	10	
2	20	0.0688
2	30	0.0207
2	40	0.0172
2	50	0.0116
2	60	0.0164
2	70	0.0155
2	80	0.013
2	90	0.0383
2	100	0.019
2	a-w	0.0917

Column	Fraction(% MeOH)	weight(g)
3	0	
3	10	
3	20	
3	30	0.095
3	40	0.0119
3	50	0.0116
3	60	0.0145
3	70	0.0184
3	80	0.0253
3	90	0.0234
3	100	0.0219
3	a-w	0.0976

Column	Fraction(% MeOH)	weight(g)
4	0	
4	10	
4	20	
4	30	0.3984
4	40	0.0899
4	50	0.0155
4	60	0.0149
4	70	0.0334
4	80	0.0312
4	90	0.024
4	100	0.0213
4	a-w	0.1227

Column	Fraction(% MeOH)	weight(g)
5	0	
5	10	
5	20	
5	30	0.0174
5	40	0.0139
5	50	0.0221
5	60	0.0155
5	70	0.0214
5	80	0.022
5	90	0.0252
5	100	0.0215
5	a-w	0.0934

Column	Fraction(% MeOH)	weight(g)
6	0	
6	10	
6	20	
6	30	0.0142
6	40	0.0213
6	50	0.0133
6	60	0.0169
6	70	0.0224
6	80	0.021
6	90	0.0262
6	100	0.0193
6	a-w	0.089

Column	Fraction(% MeOH)	weight(g)
7	0	
7	10	
7	20	
7	30	0.072
7	40	0.0151
7	50	0.0129
7	60	0.0157
7	70	0.0197
7	80	0.0225
7	90	0.0216
7	100	0.0158
7	a-w	0.0855

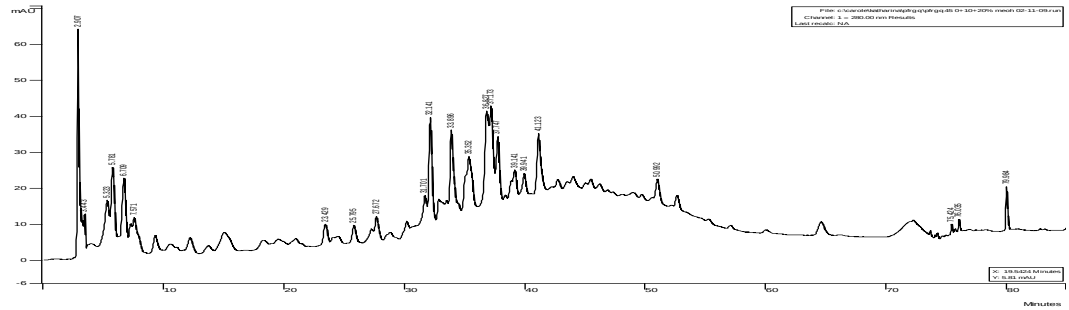
Column	Fraction(% MeOH)	weight(g)
8	0	
8	10	
8	20	
8	30	0.0173
8	40	0.0137
8	50	0.0162
8	60	0.0165
8	70	0.0171
8	80	0.0233
8	90	0.0213
8	100	0.0168
8	a-w	0.0643

Column	Fraction(% MeOH)	weight(g)
9	0	
9	10	
9	20	
9	30	0.0238
9	40	0.0154
9	50	0.0126
9	60	0.0158
9	70	0.0202
9	80	0.0232
9	90	0.0208
9	100	0.0187
9	a-w	0.0654

Chromatograms apple

Fractions 0+10+20% MeOH

C45



Fractions 30% MeOH

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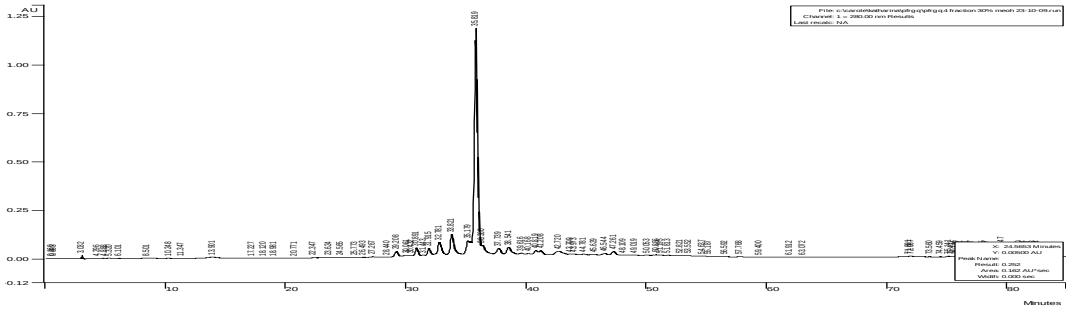

Fractions 40%MeOH

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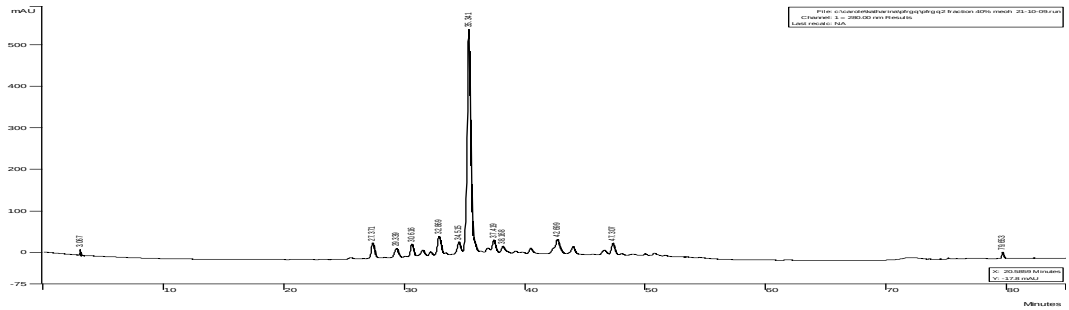

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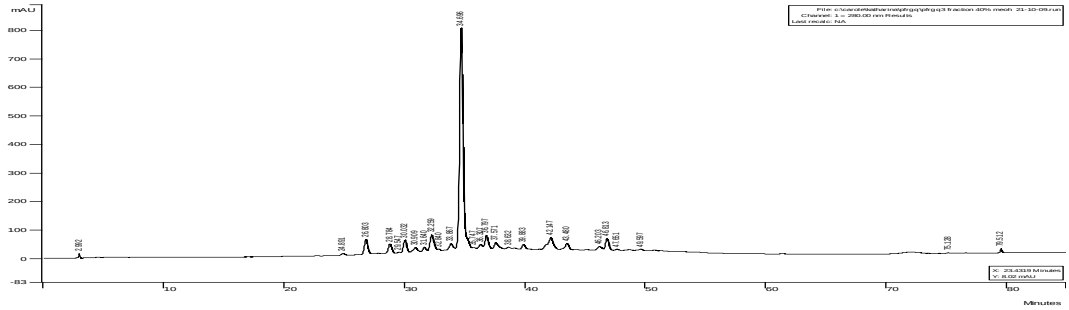

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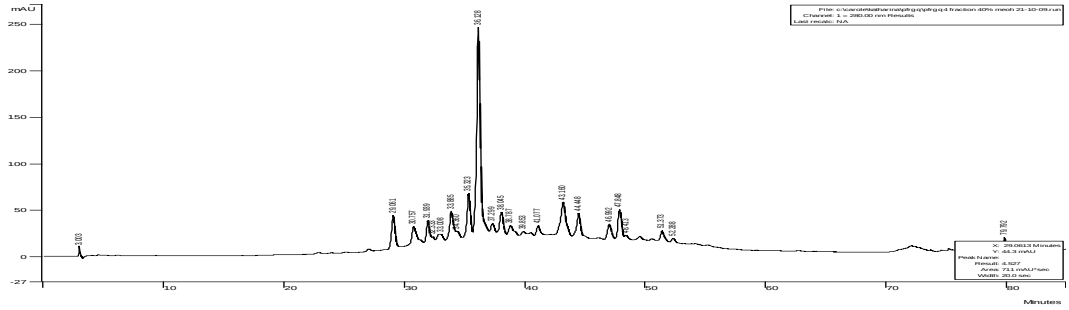
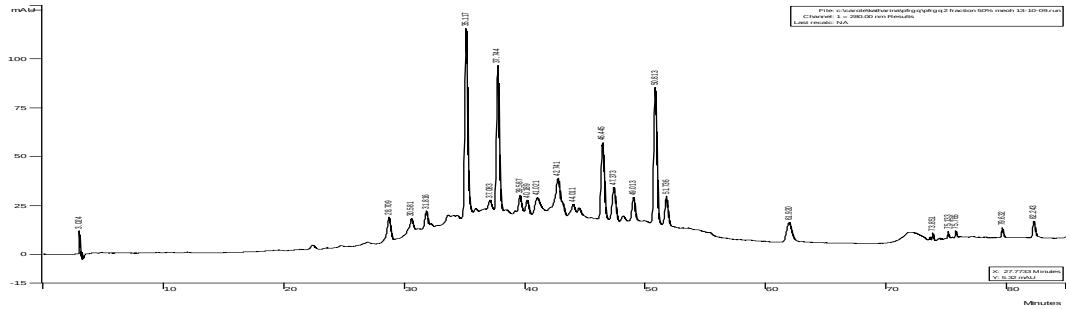


Fractions 50% MeOH

C2



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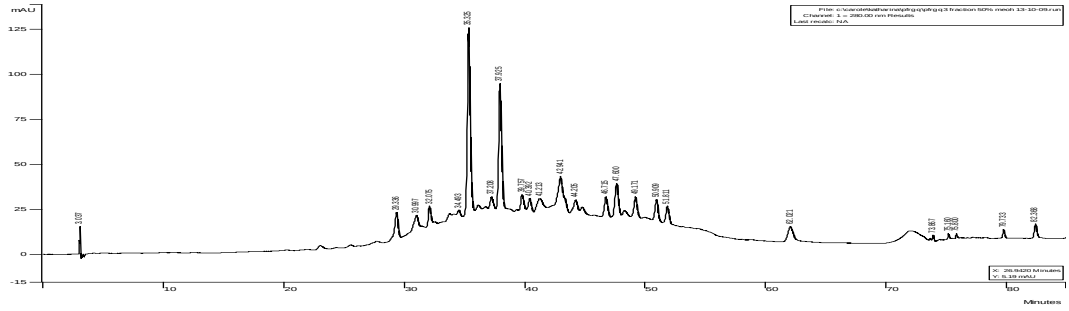

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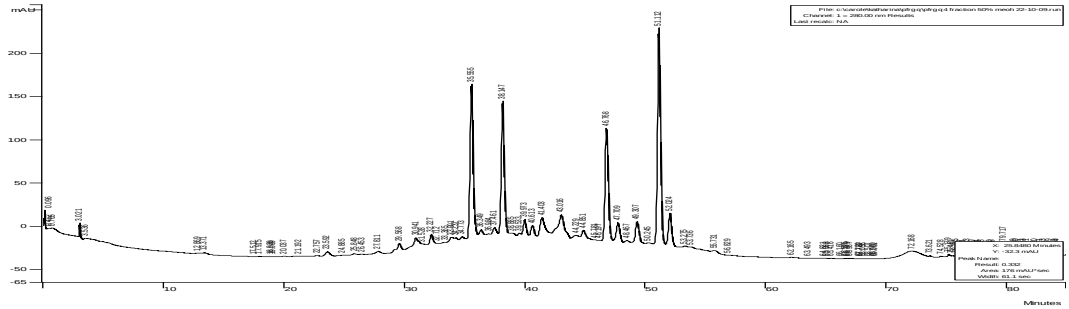

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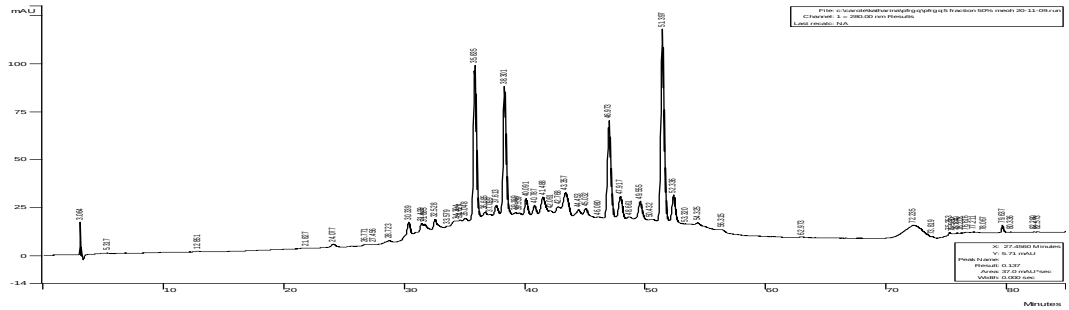

Fractions 60%MeOH

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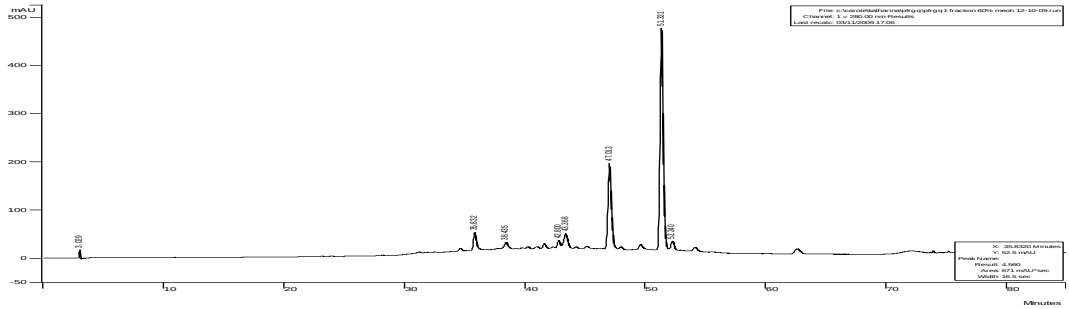

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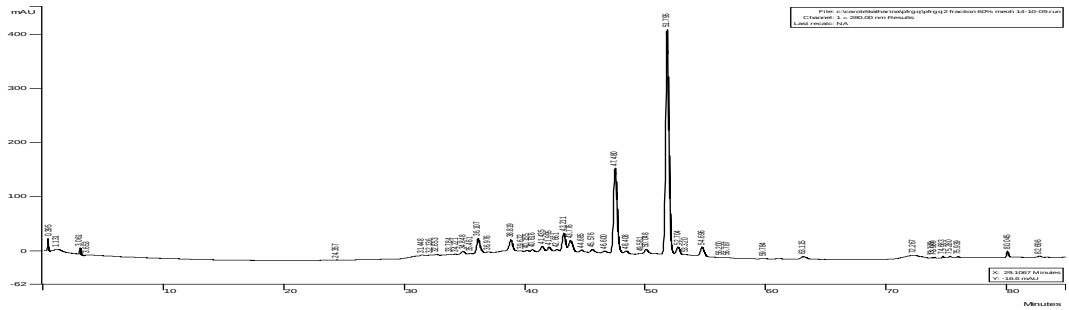

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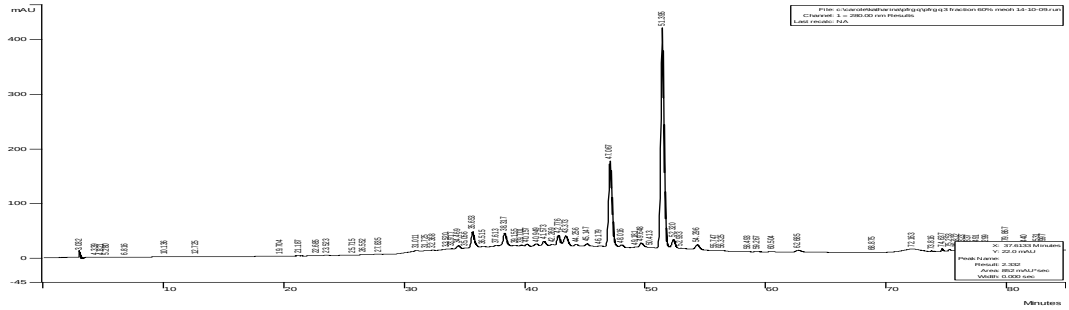

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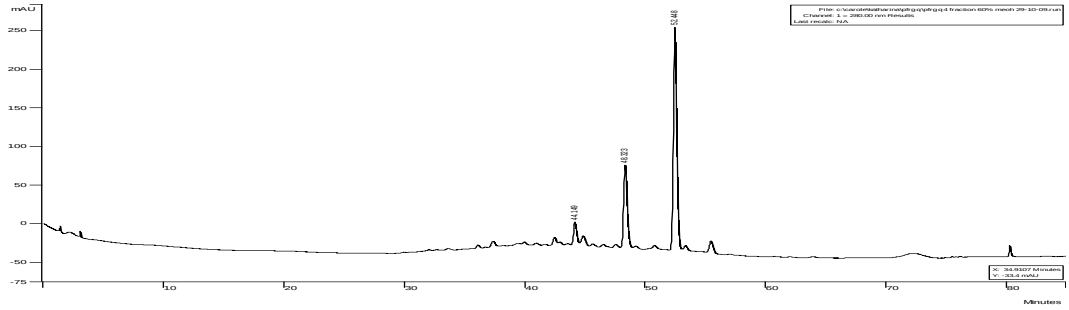

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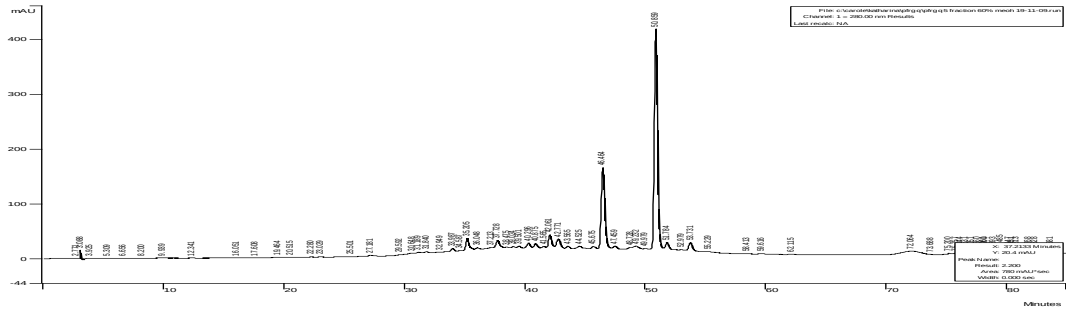

Fractions 70% MeOH

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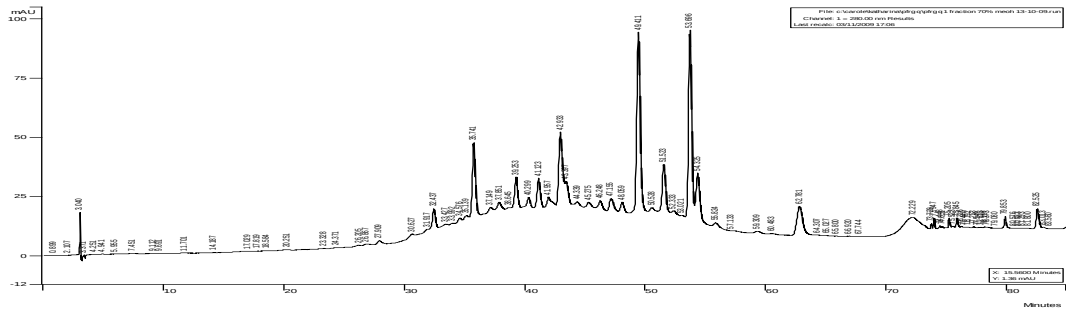

X

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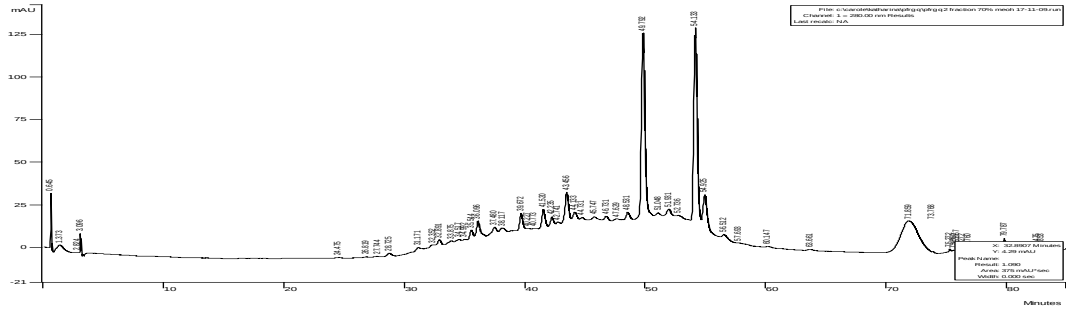

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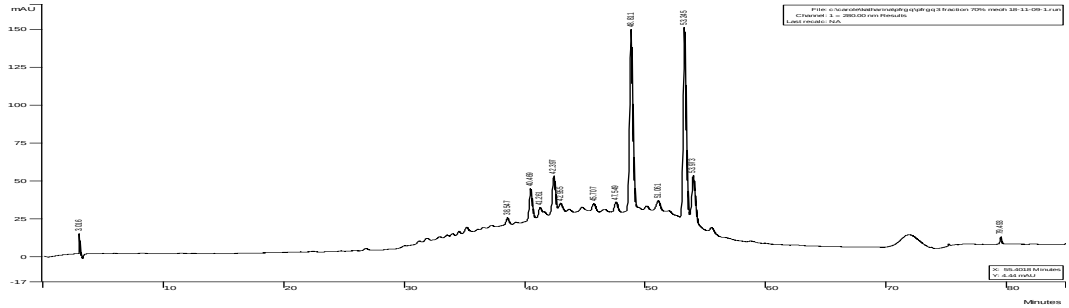

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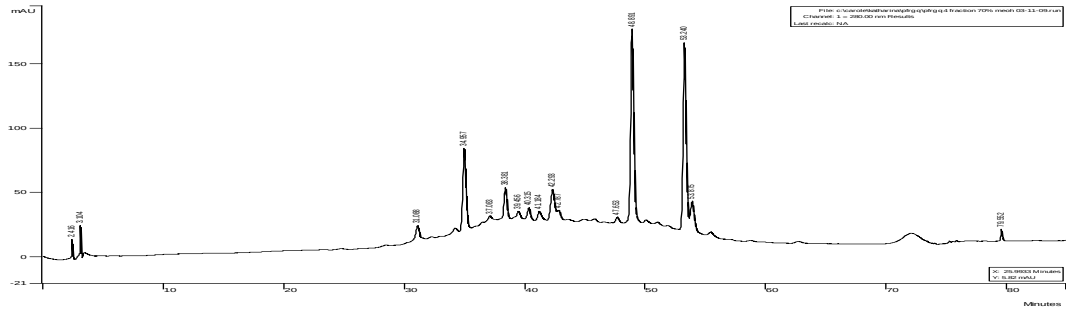

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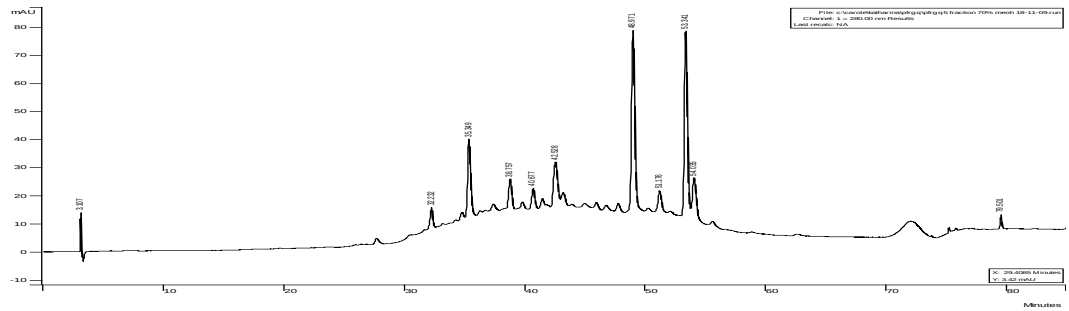

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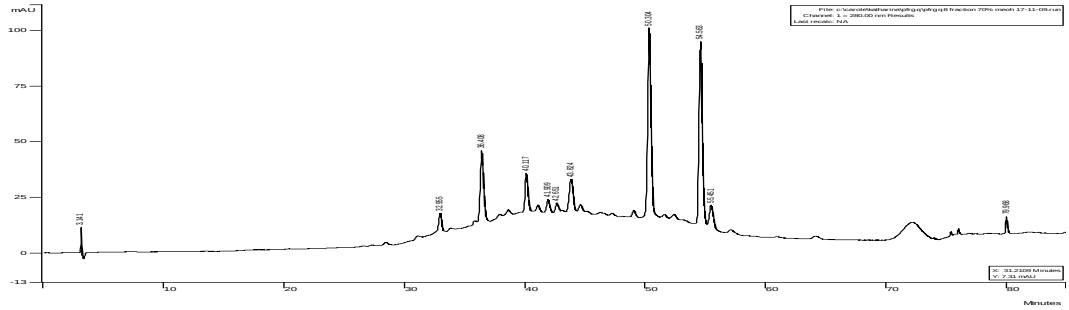
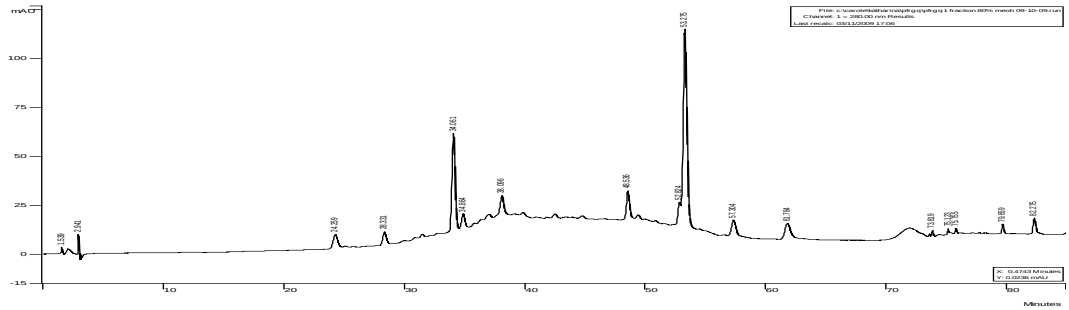


Fractions 80%MeOH

C1



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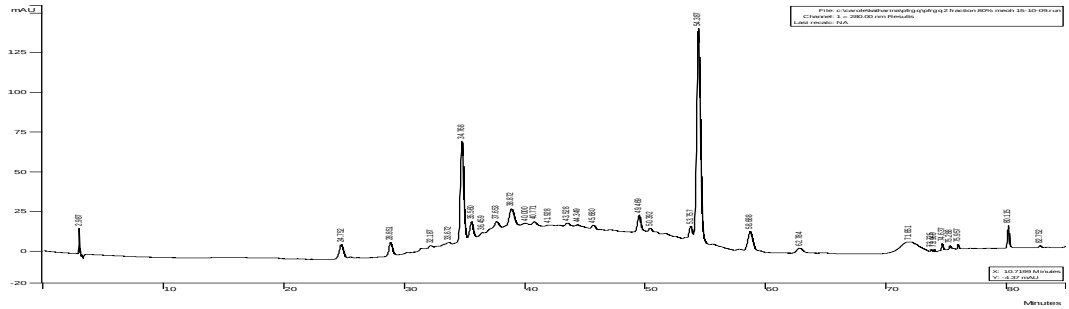

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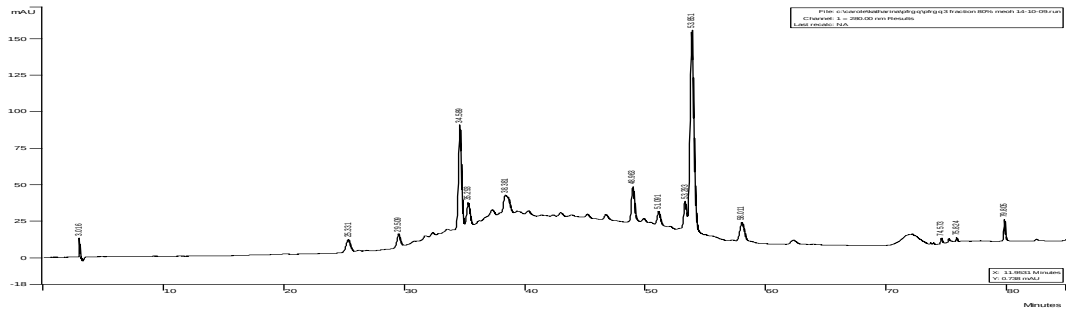

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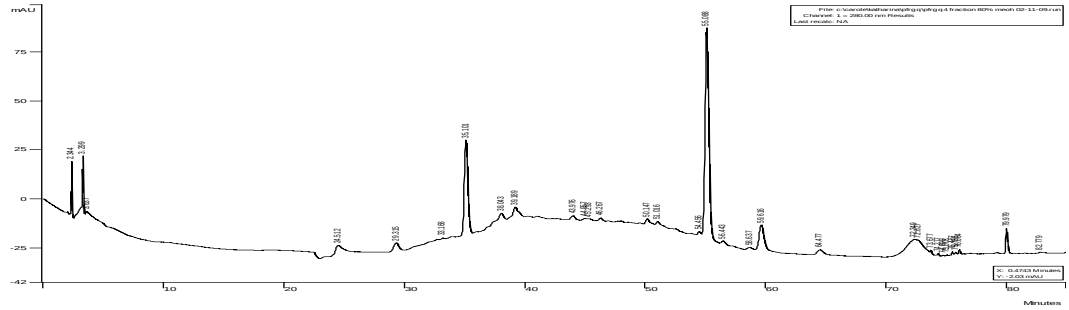

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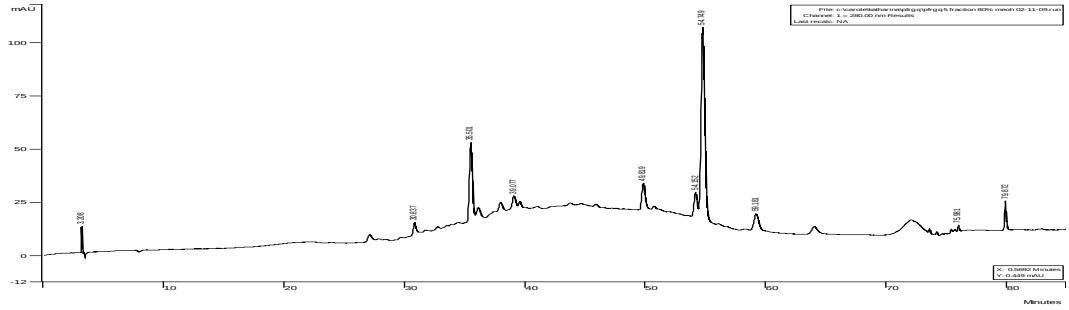

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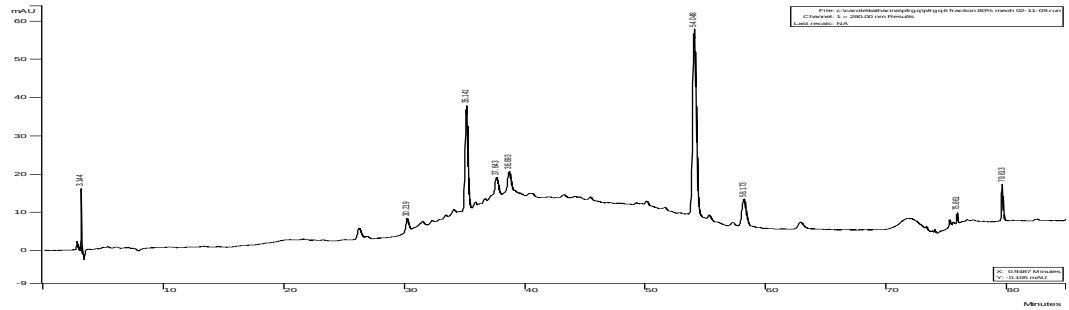

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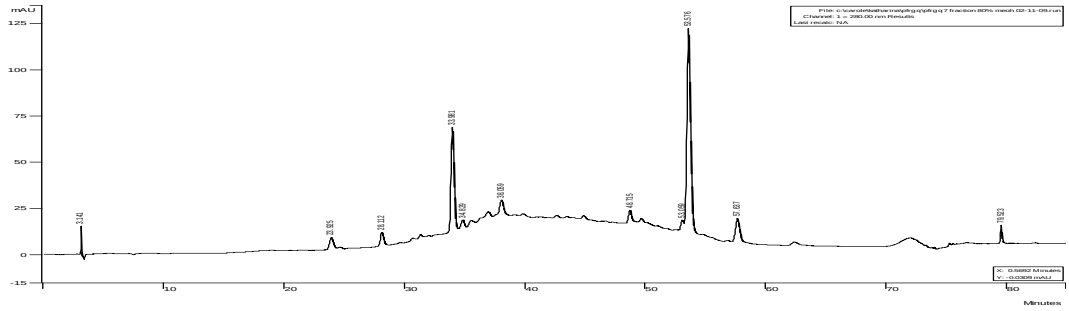

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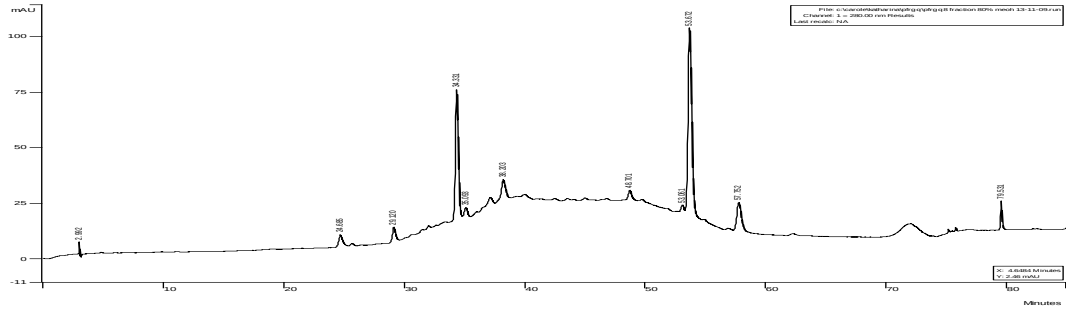
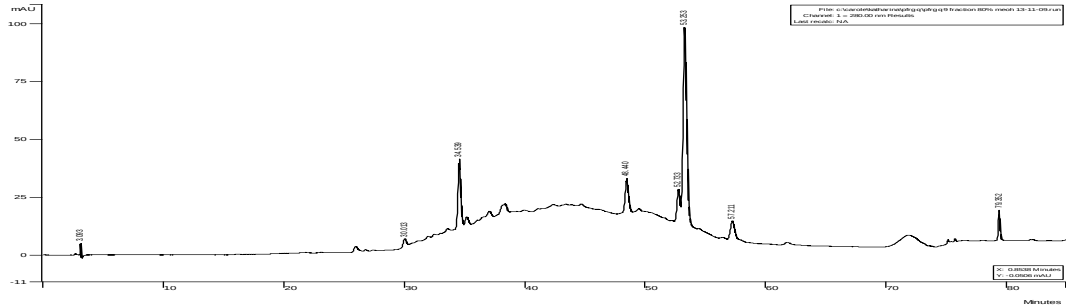
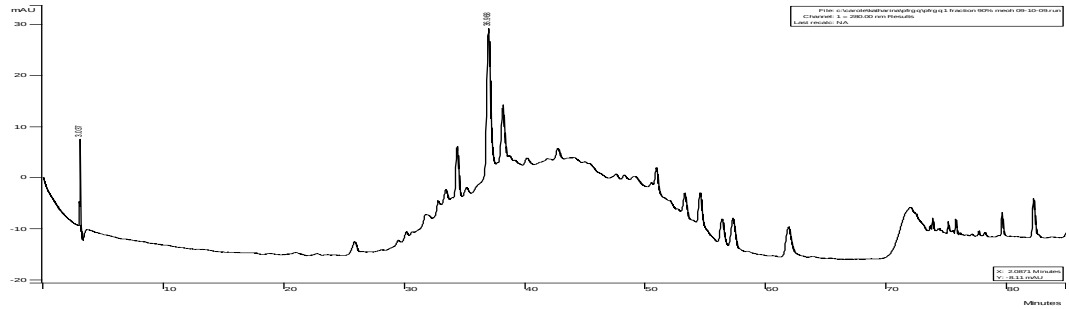


C9

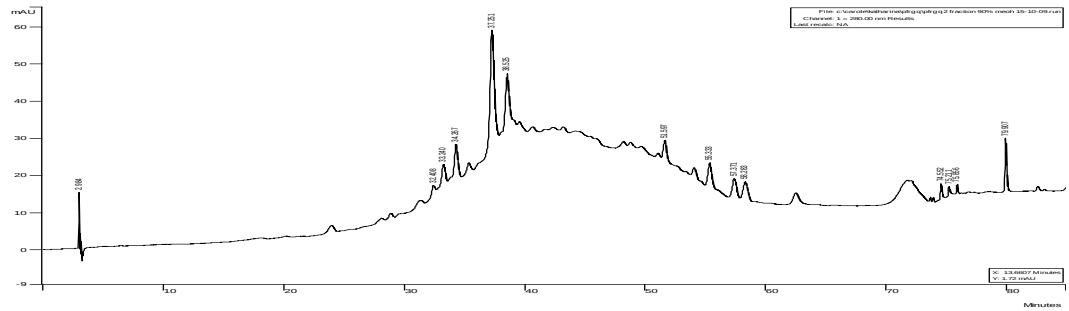


Fractions 90%MeOH

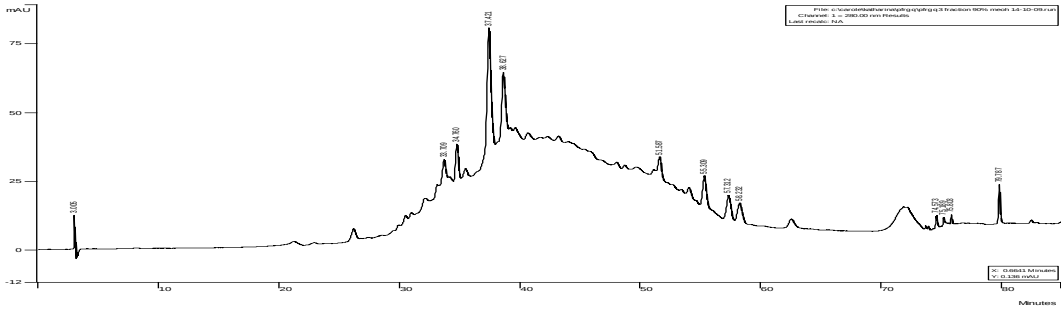
C1



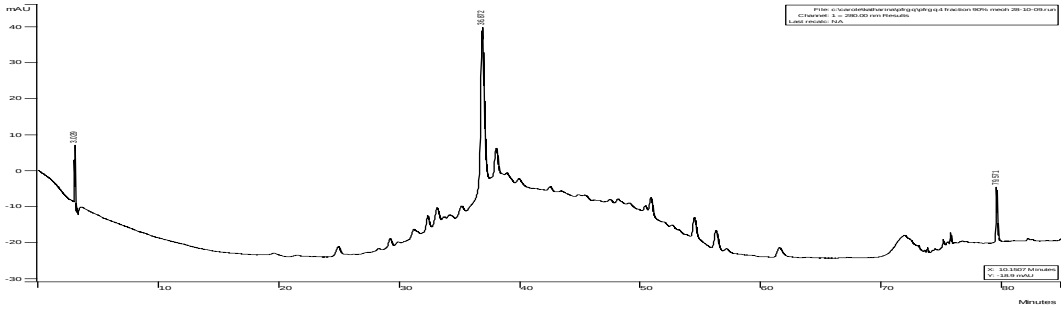
C2



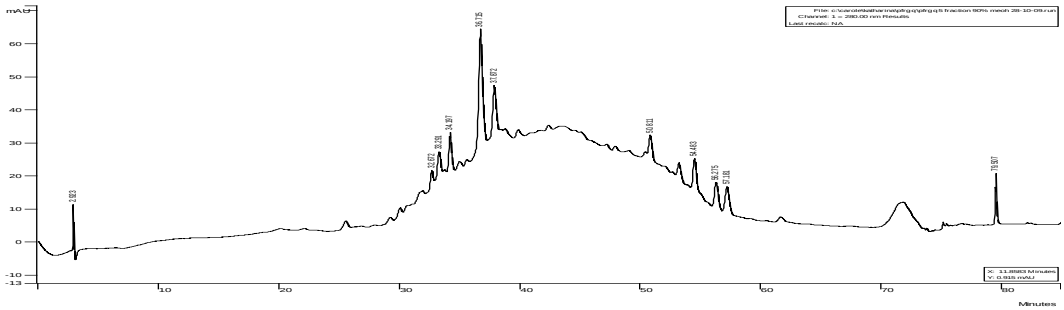
C3



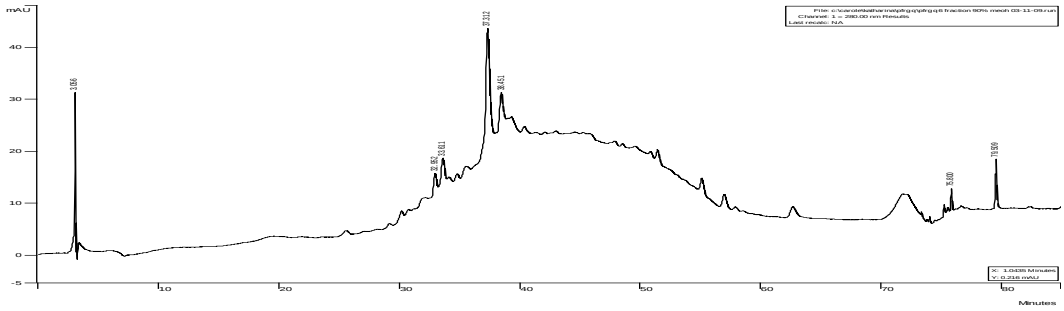
C4



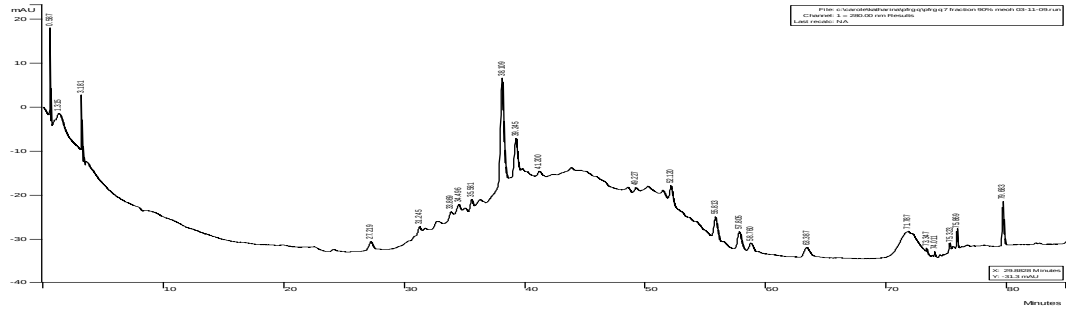
C5



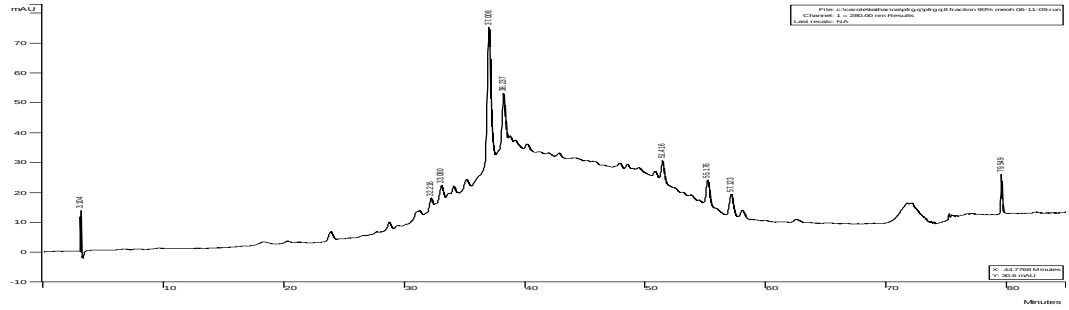
C6



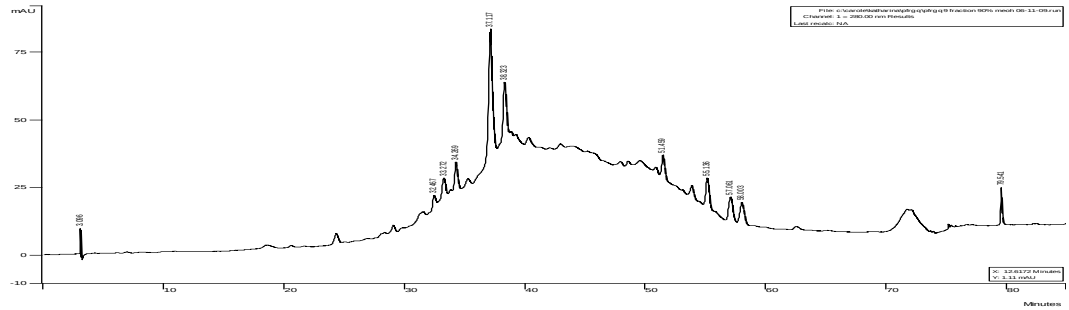
C7



C8

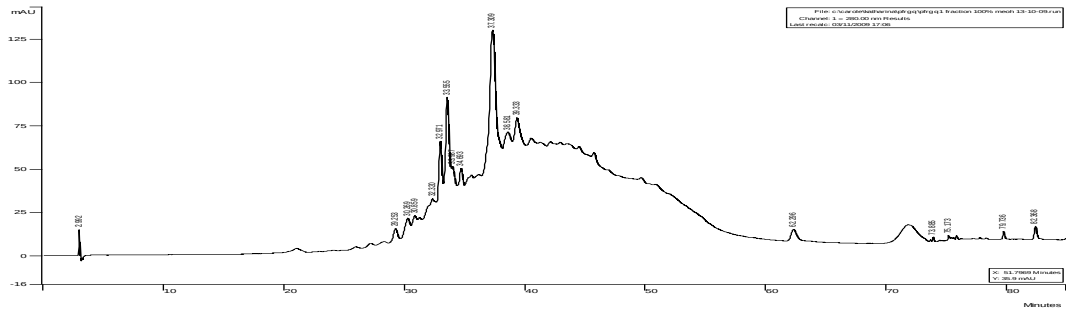


C9

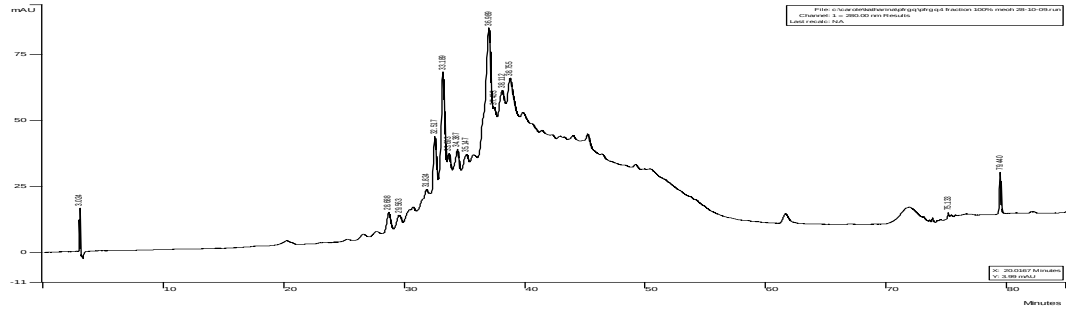


Fractions 100%MeOH

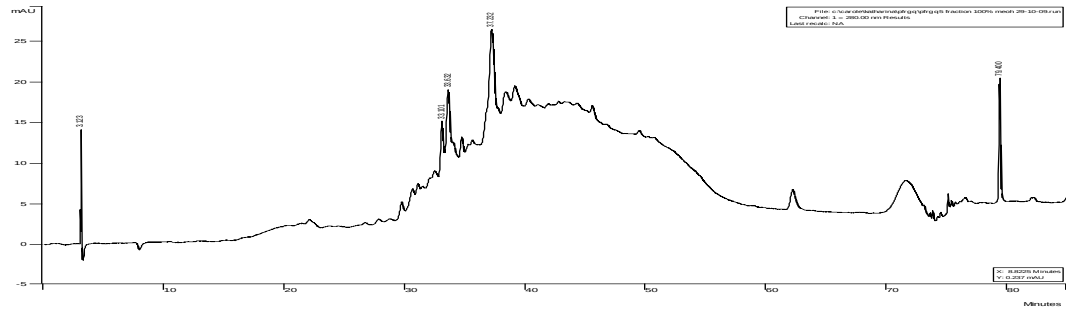
C1



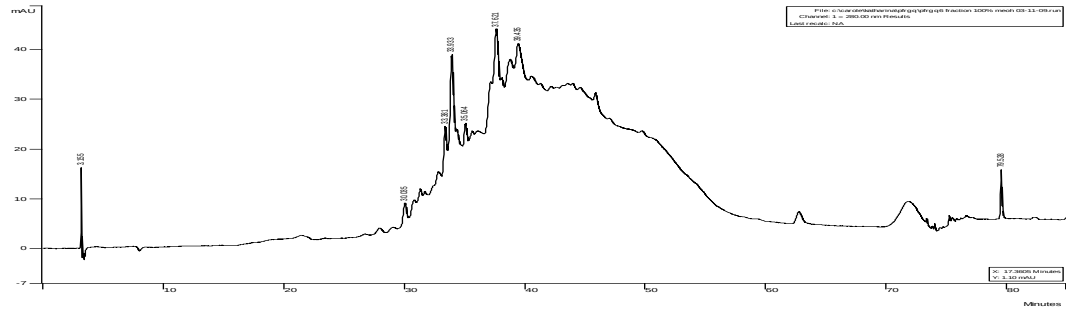
C4



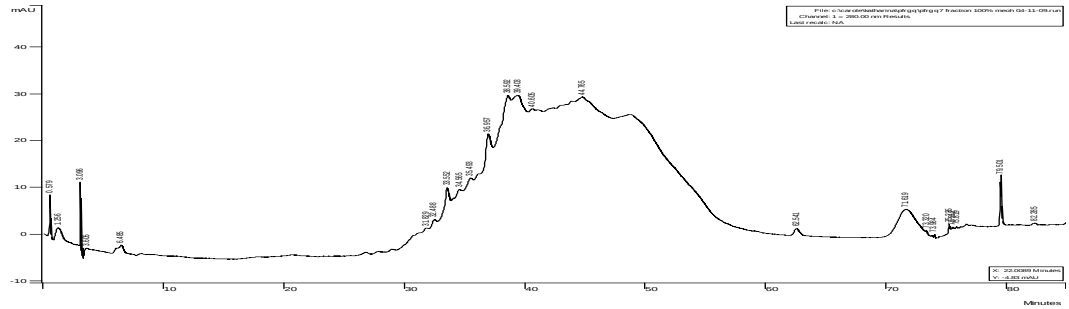
C5



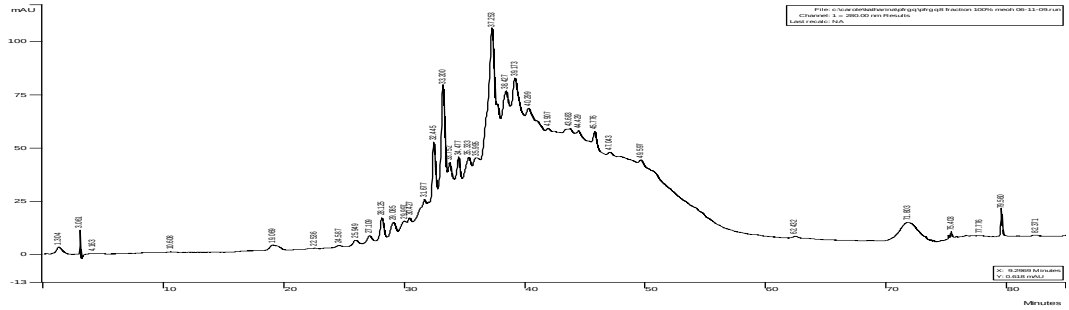
C6



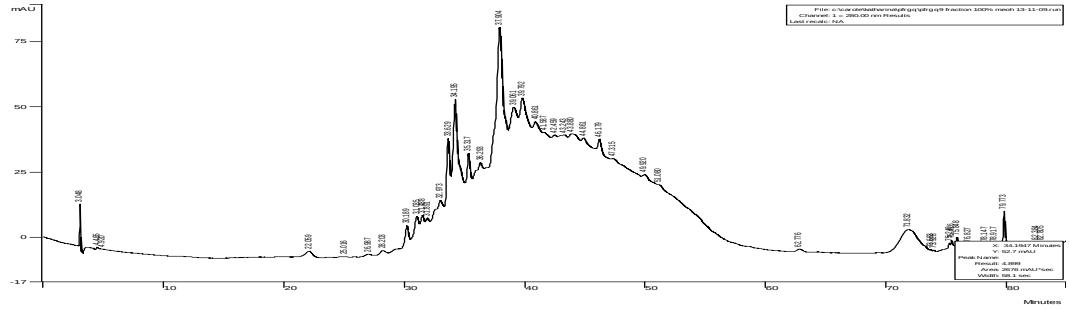
C7



C8

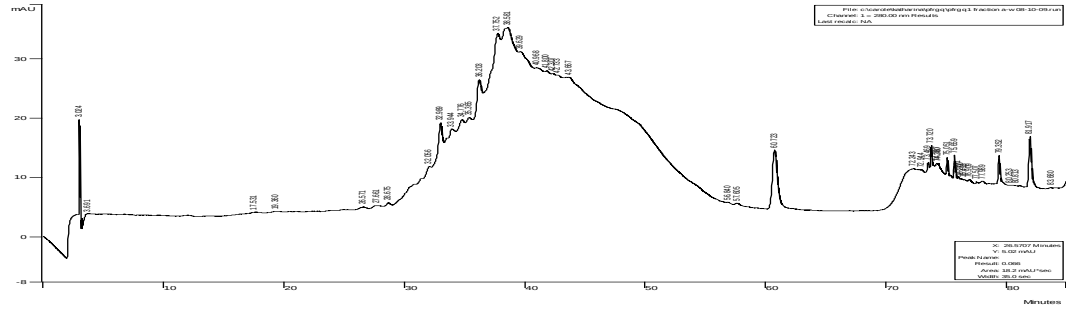


C9

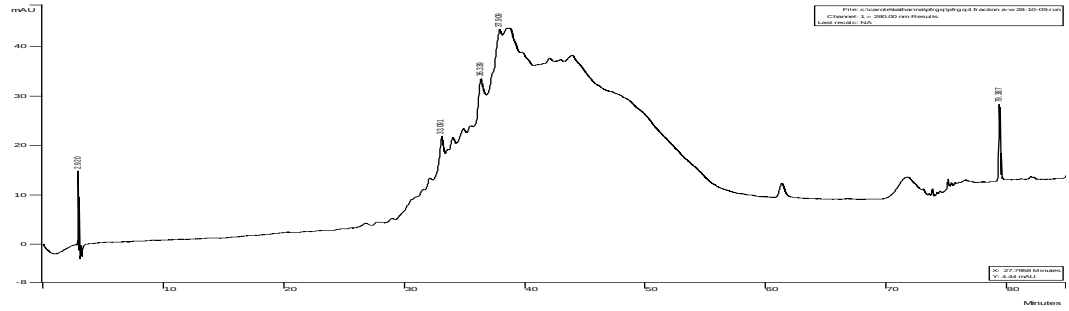


Fractions a-w

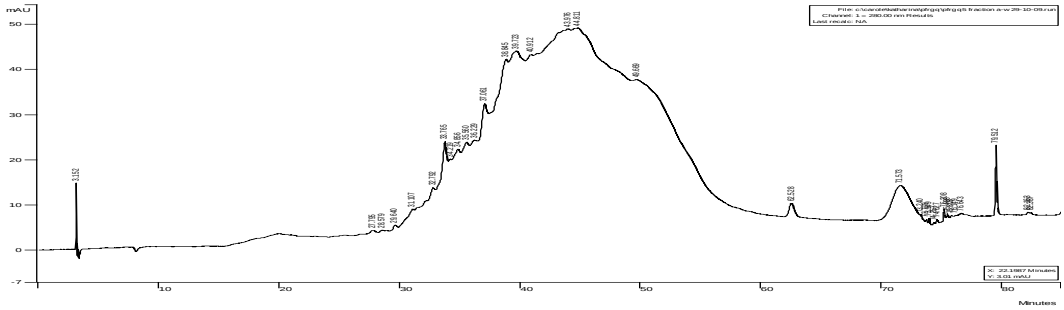
C1



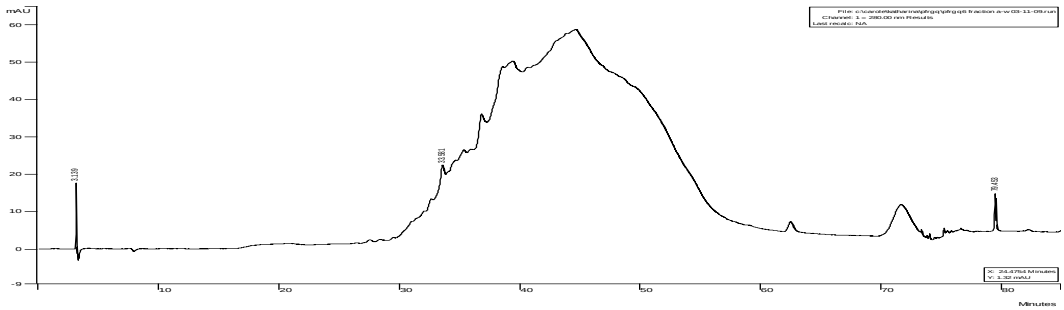
C4



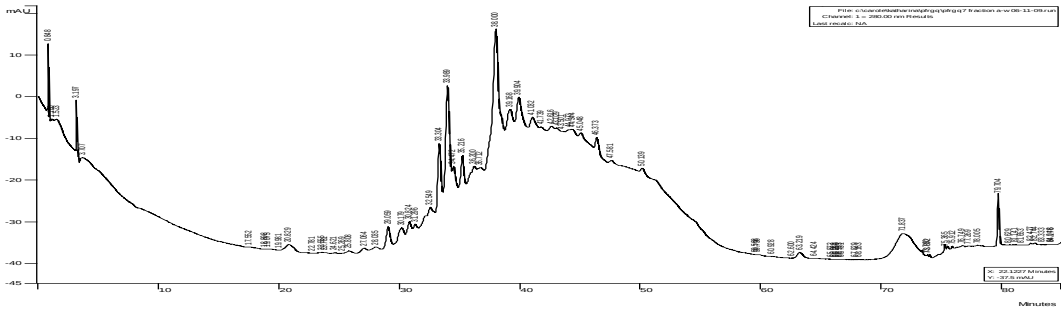
C5



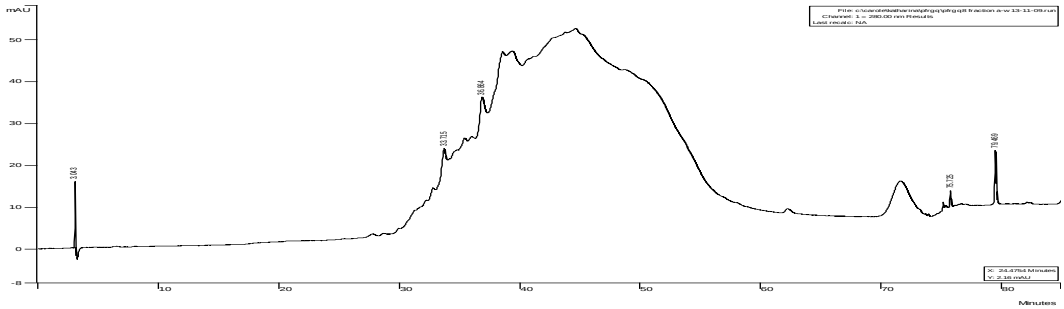
C6



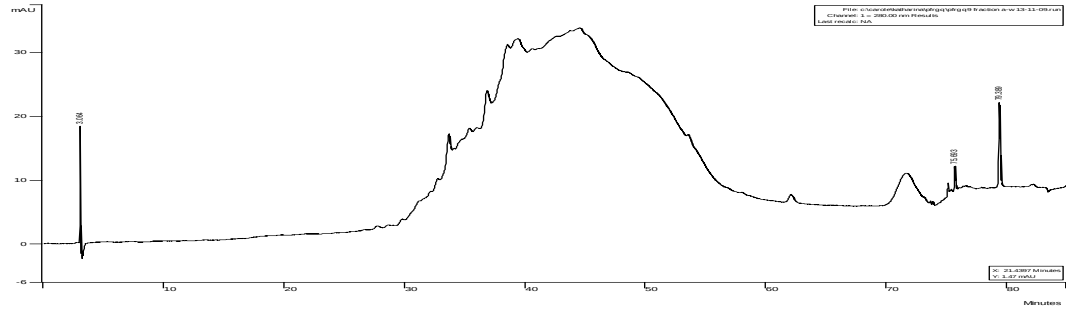
C7



C8



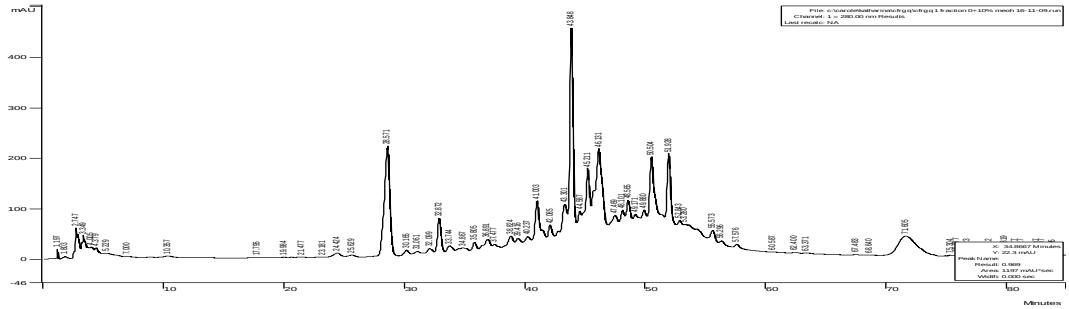
C9



Chromatograms quince

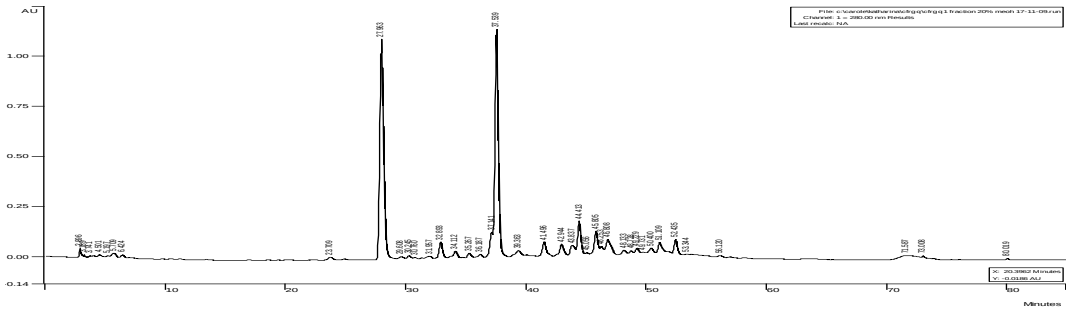
Fractions 0+10MeOH

C1



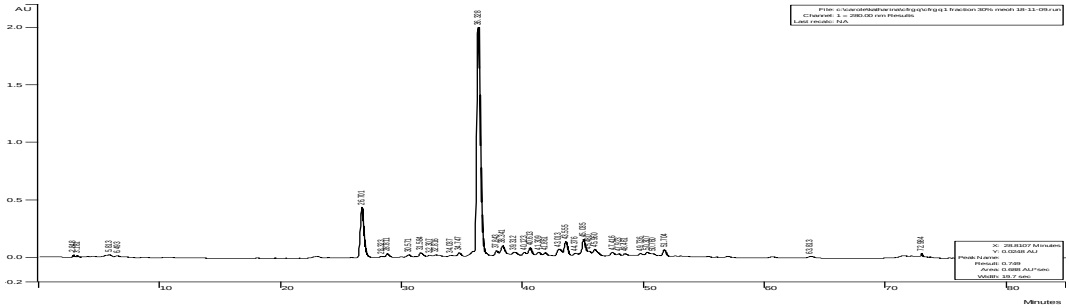
Fractions 20% MeOH

C1

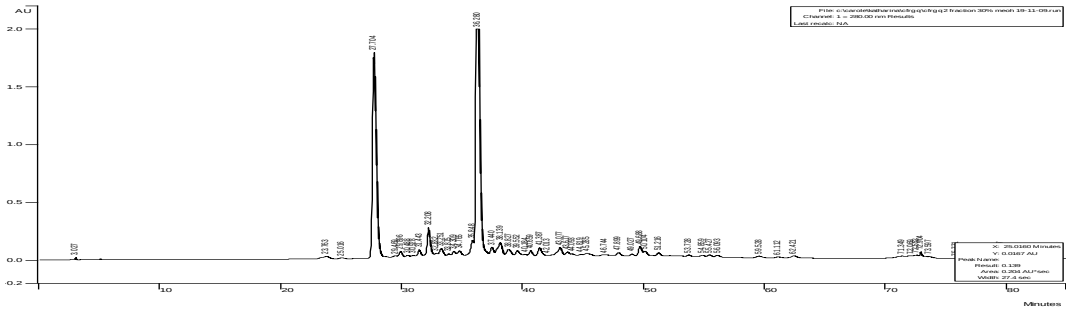


Fractions 30% MeOH

C1

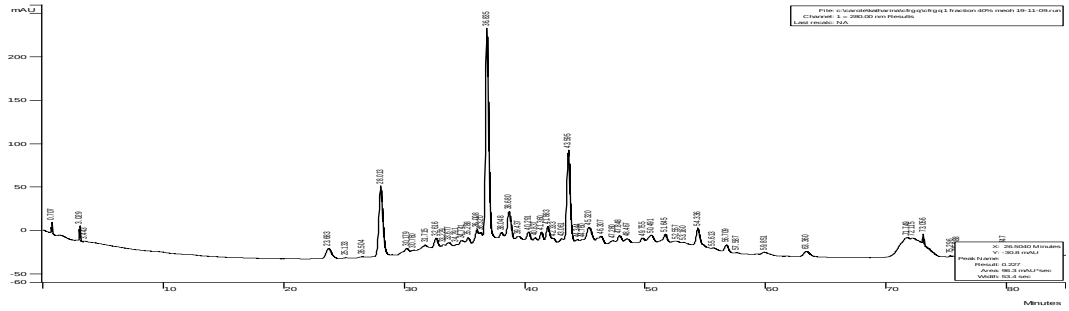


C2

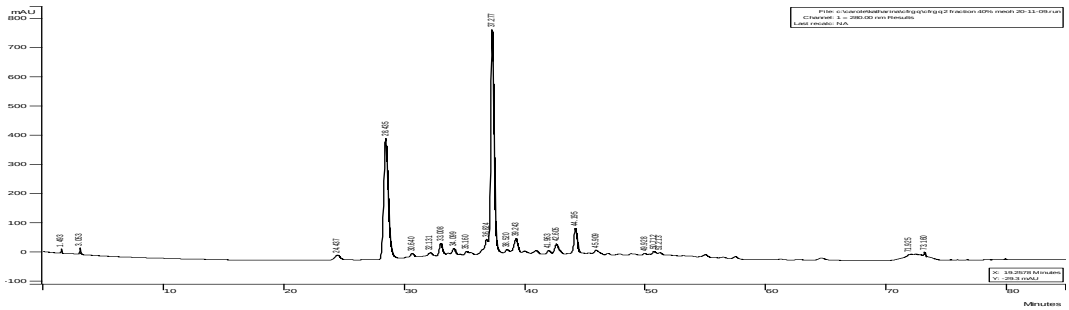


Fractions 40% MeOH

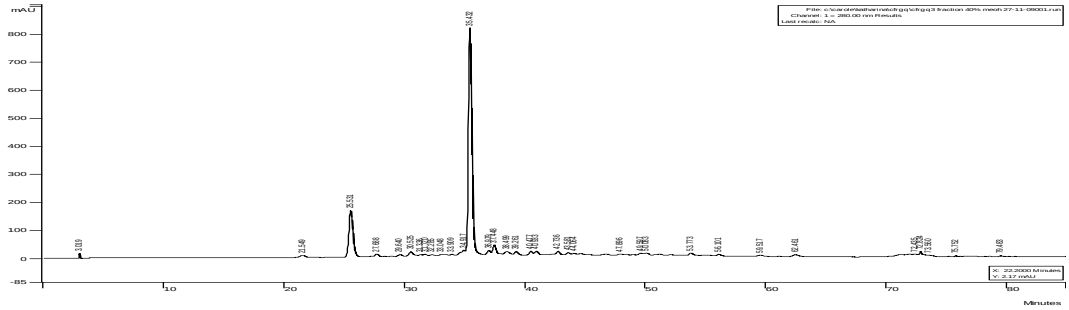
C1



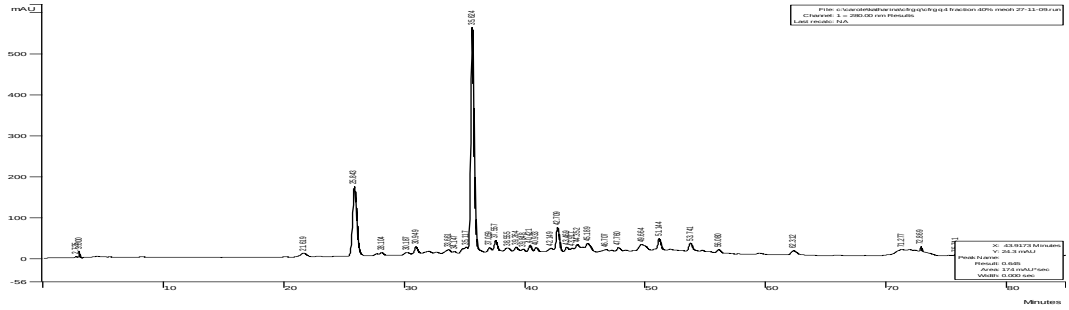
C2



C3

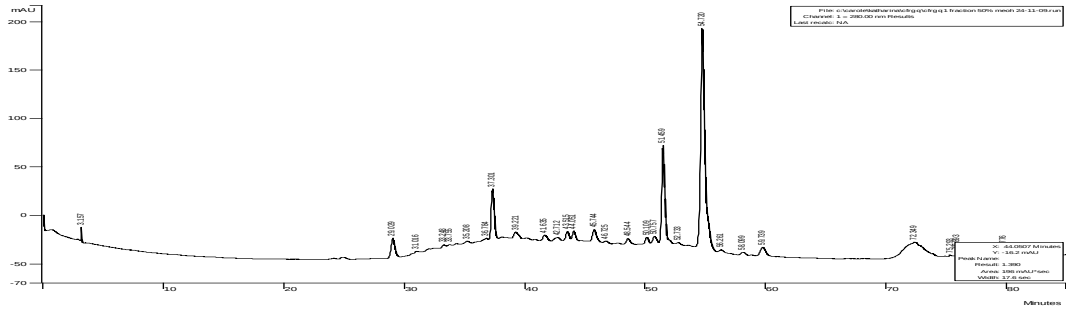


C4

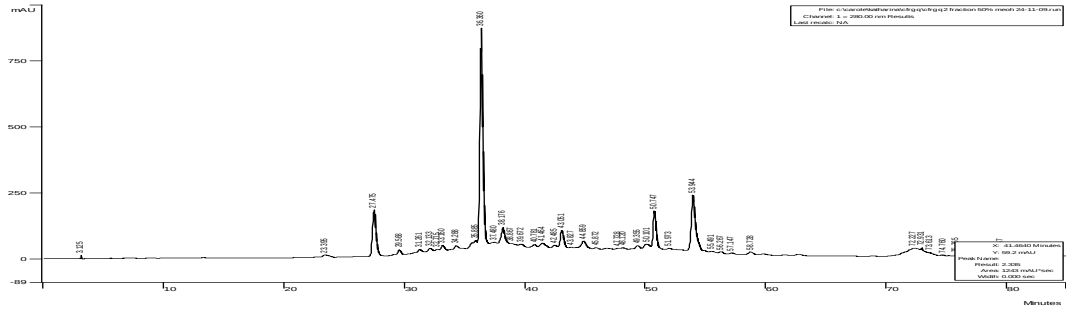


Fractions 50% MeOH

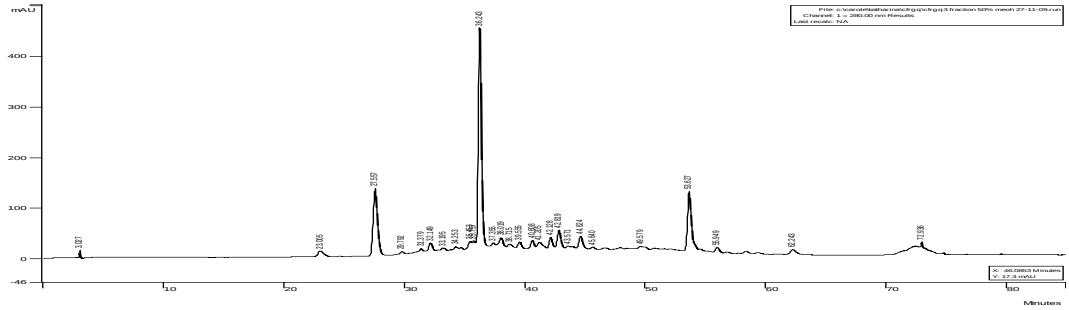
C1



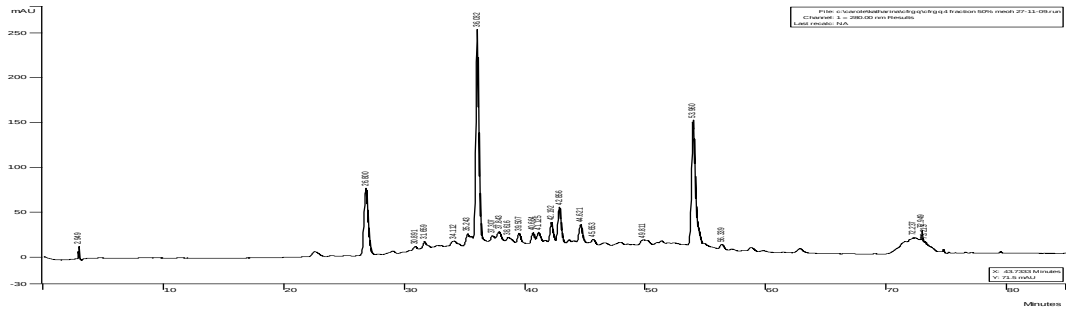
C2



C3



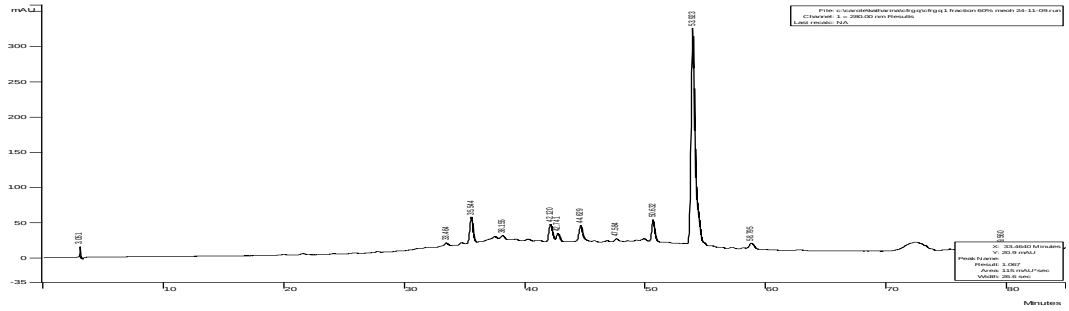
C4



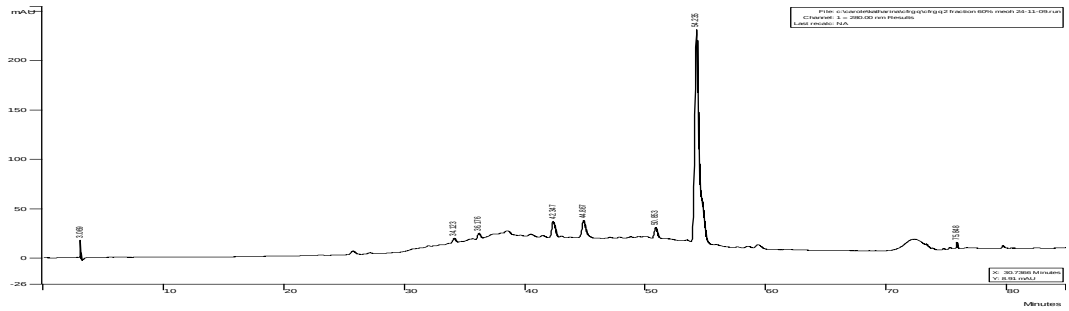
C5

Fractions 60% MeOH

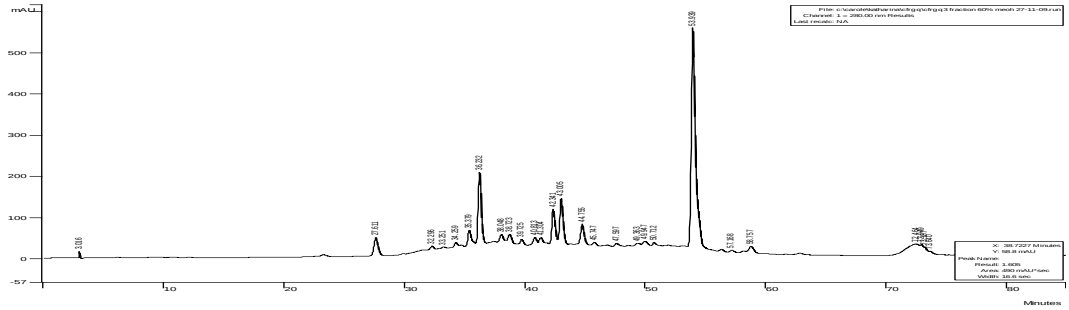
C1



C2

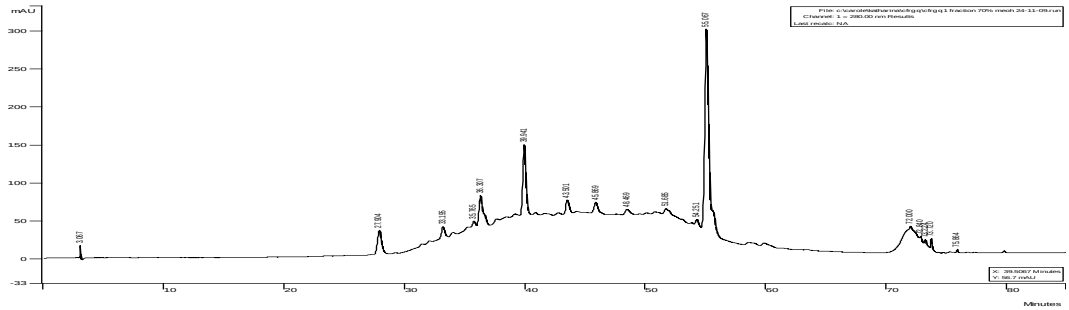


C3

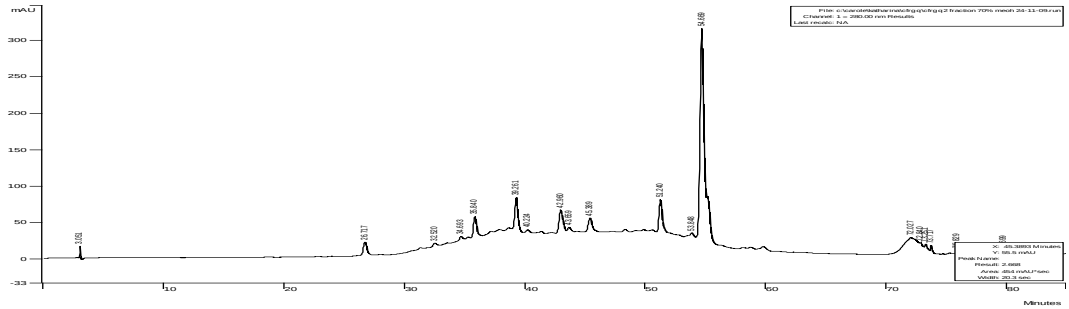


Fractions 70% MeOH

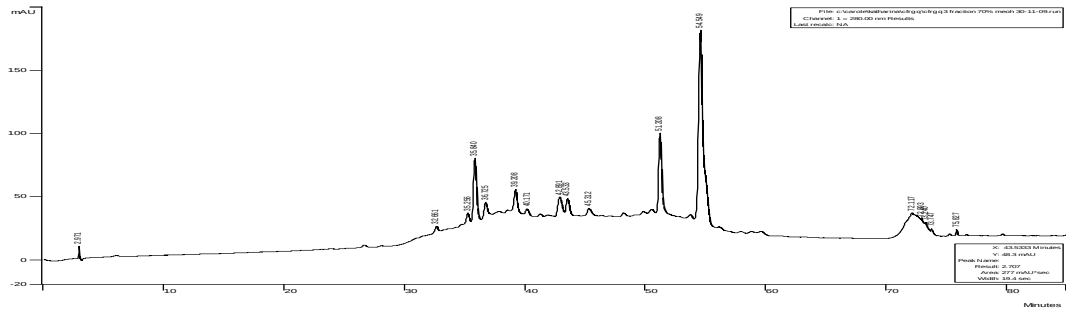
C1



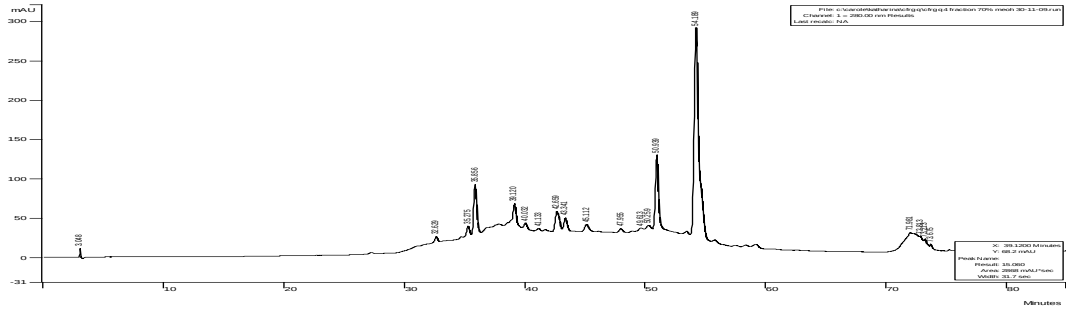
C2



C3

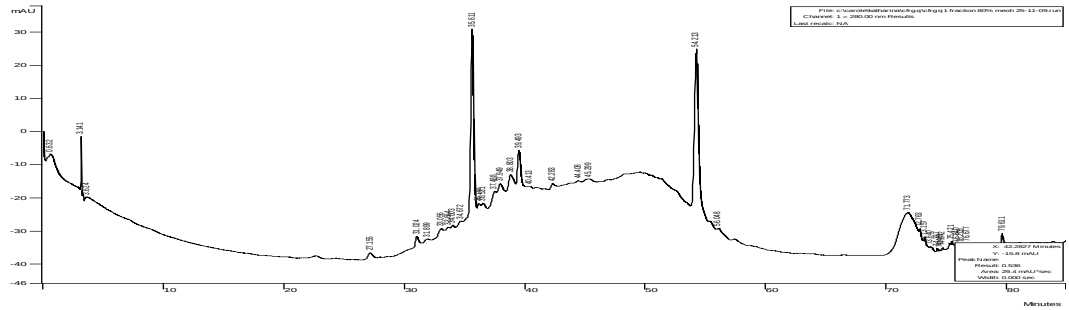


C4

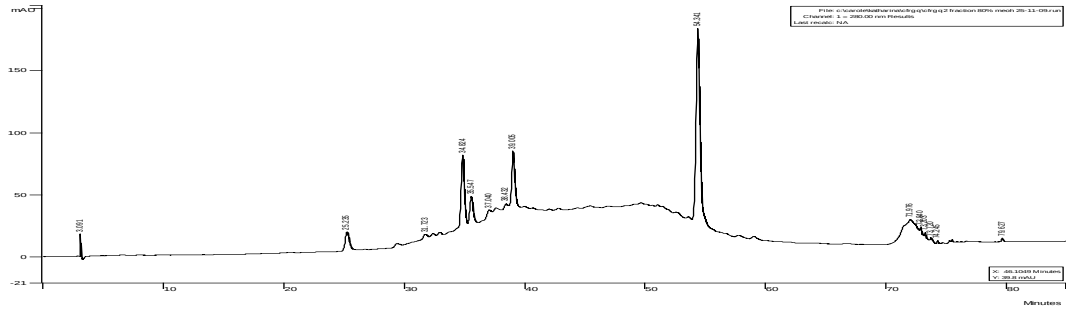


Fractions 80% MeOH

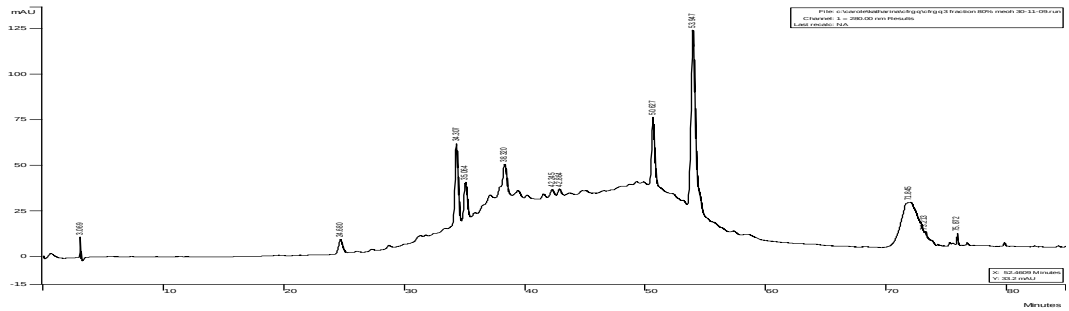
C1



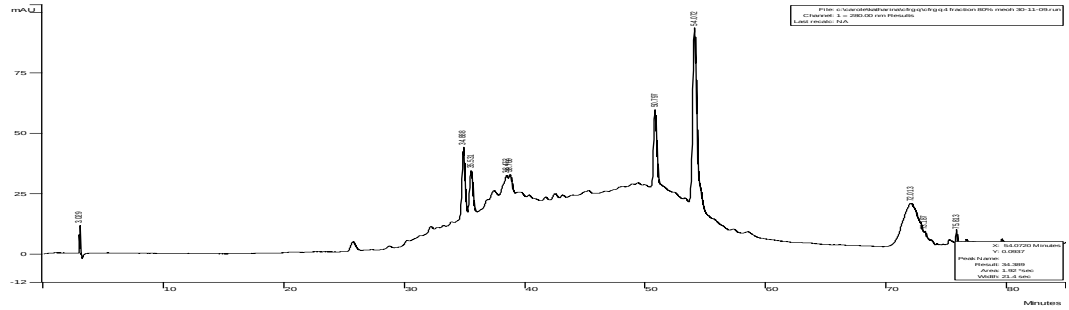
C2



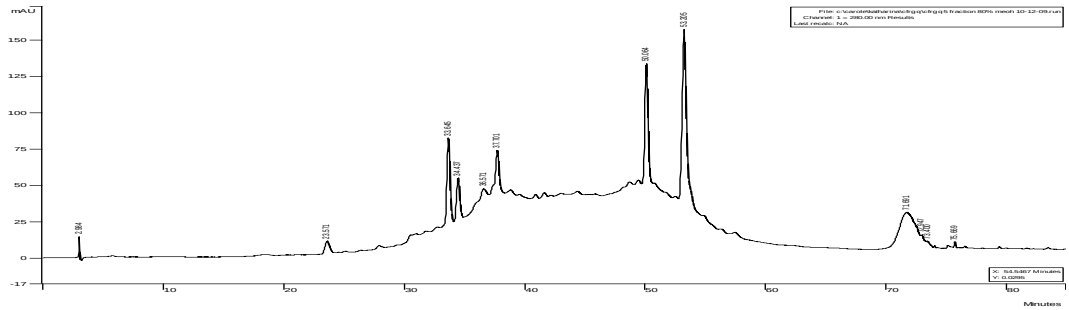
C3



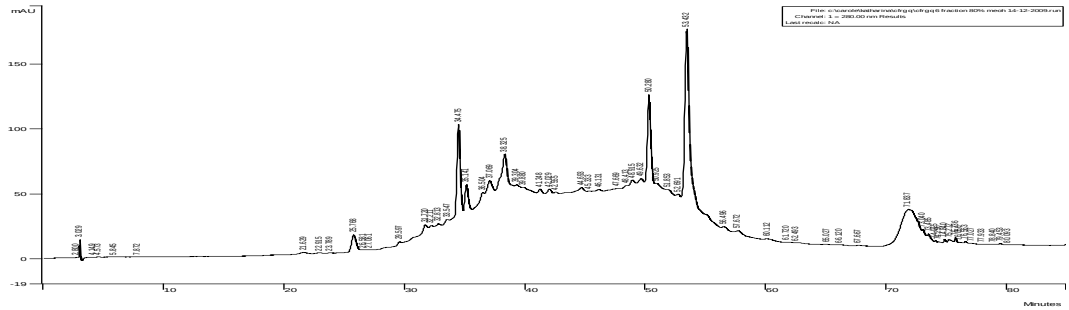
C4



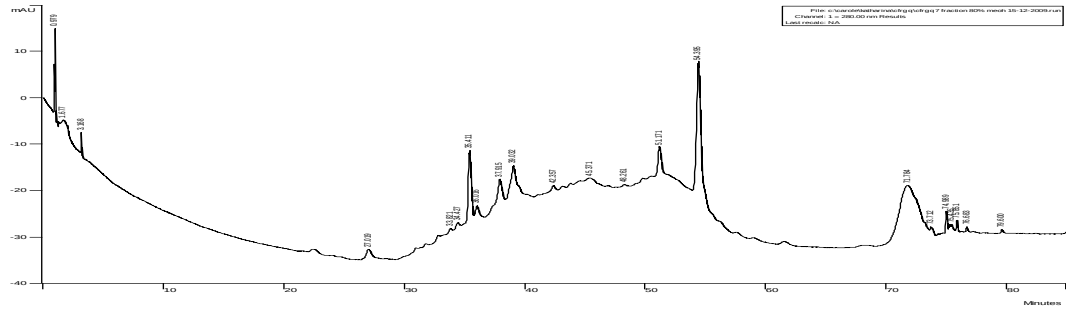
C5



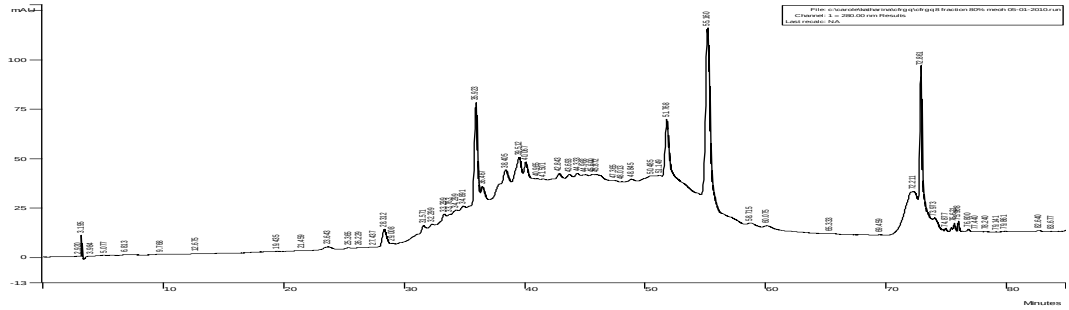
C6



C7

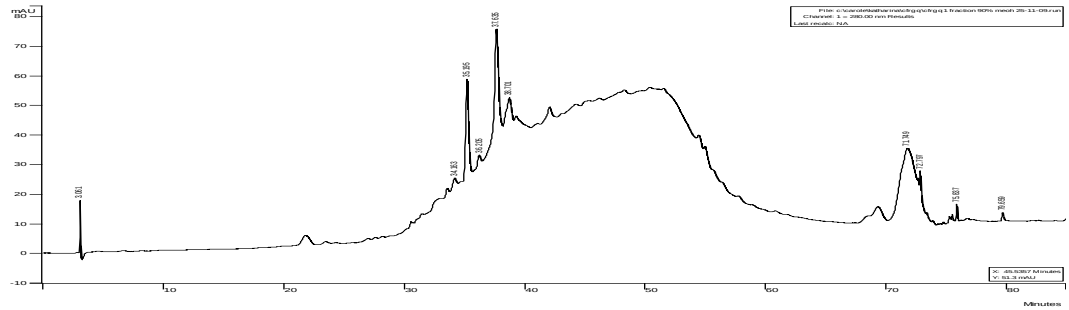


C8

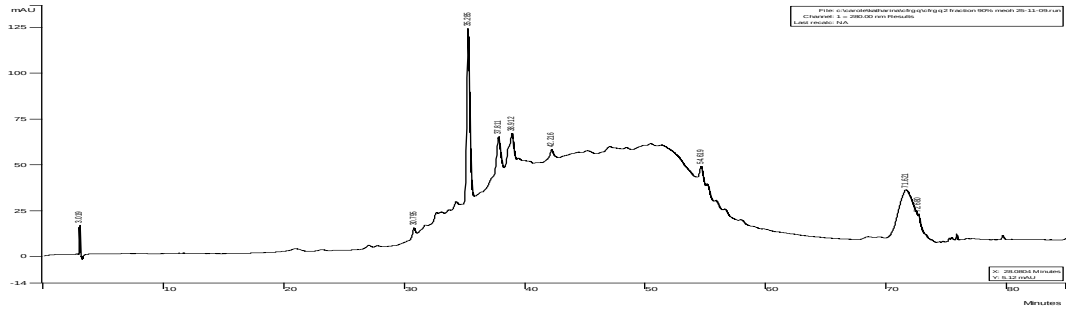


Fractions 90% MeOH

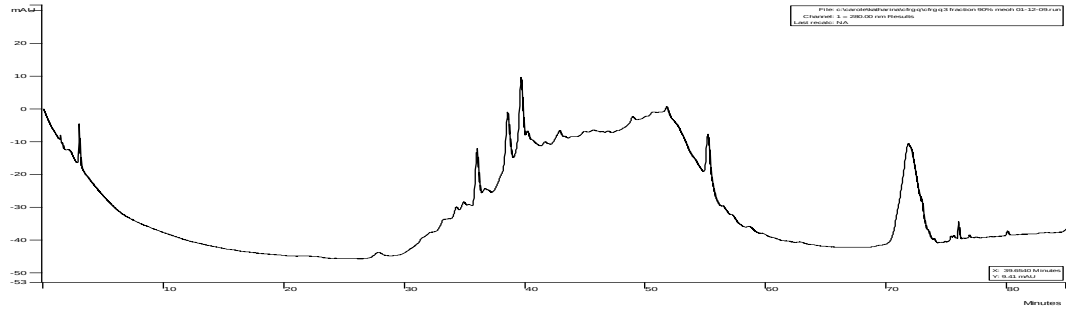
C1



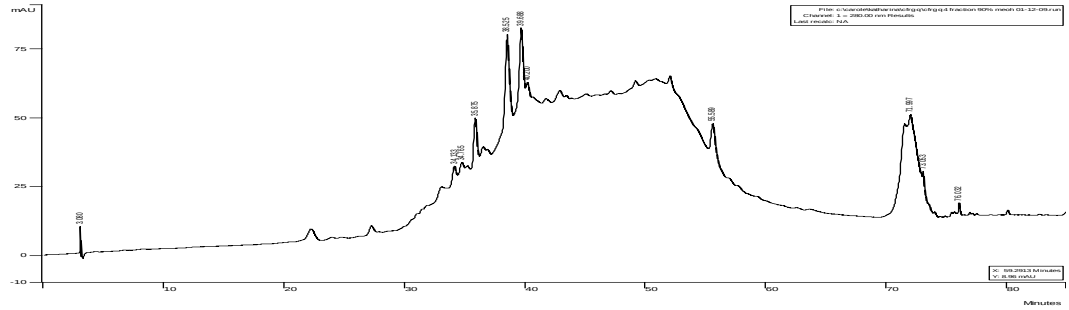
C2



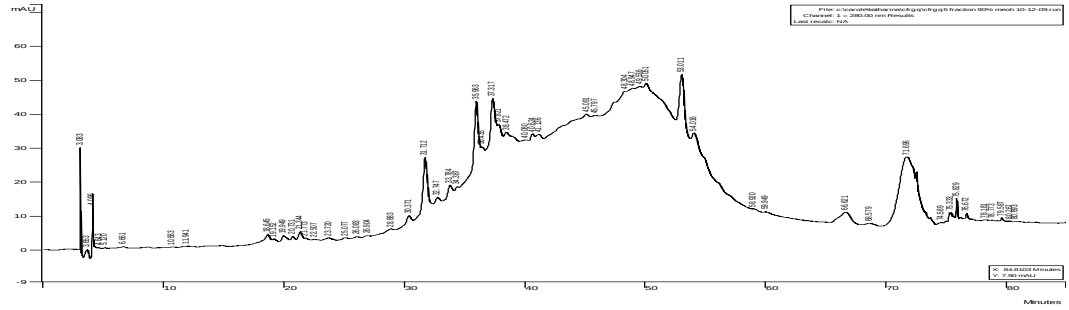
C3



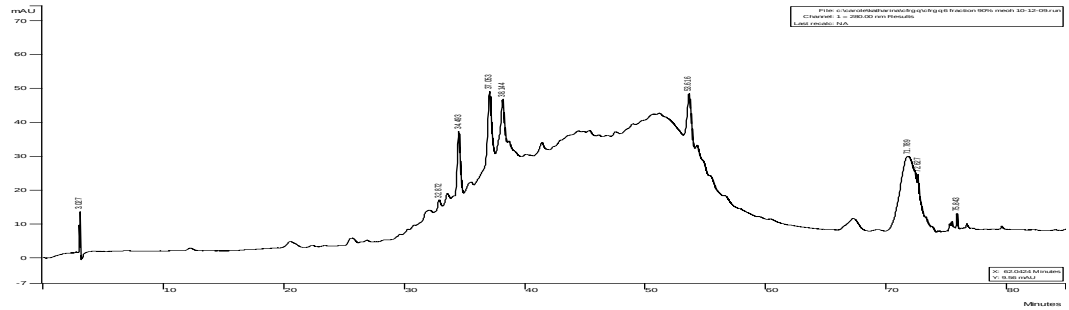
C4



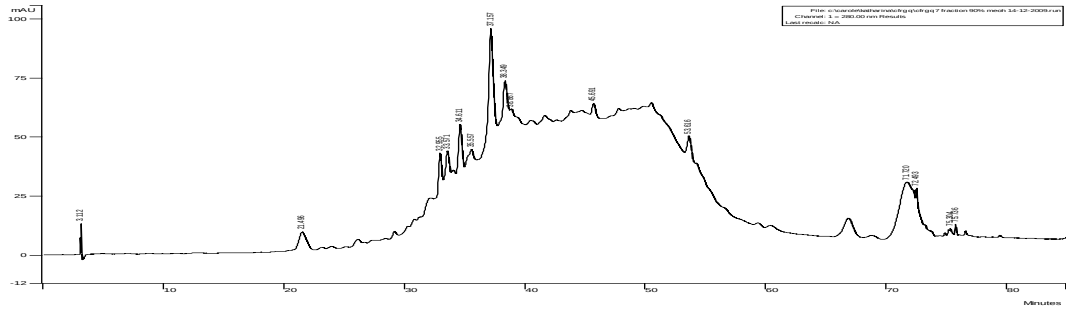
C5



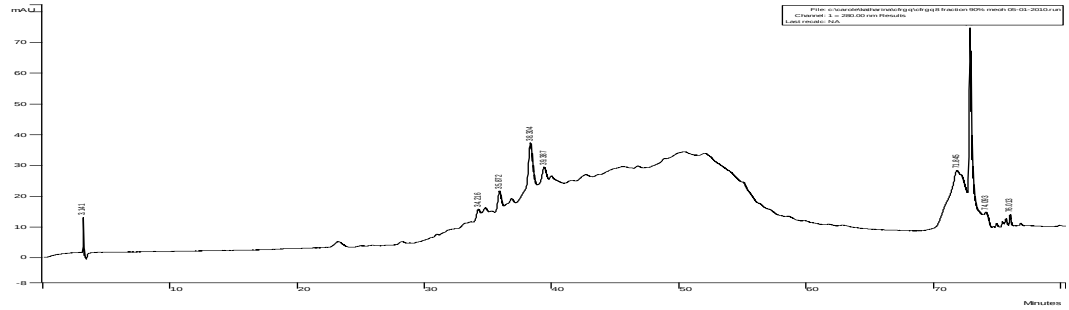
C6



C7

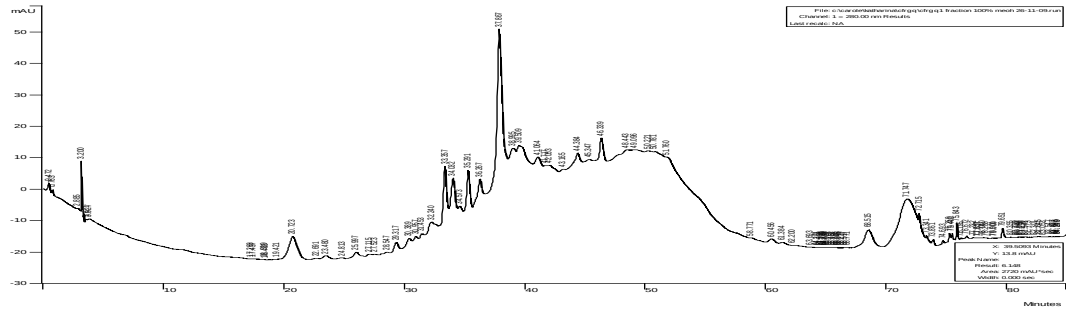


C8

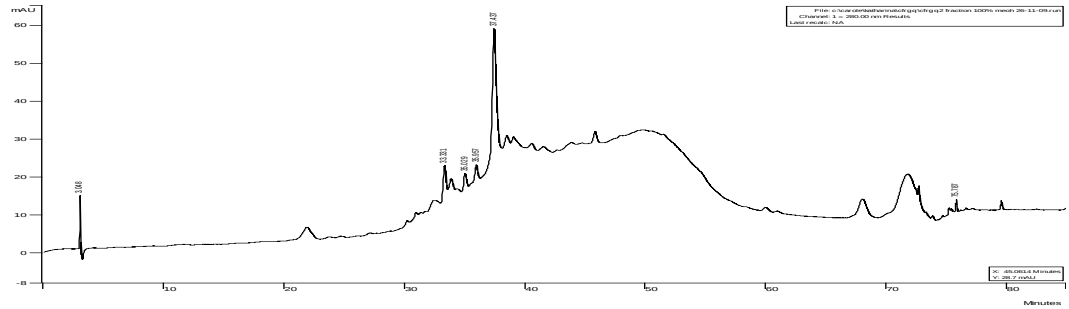


Fractions 100% MeOH

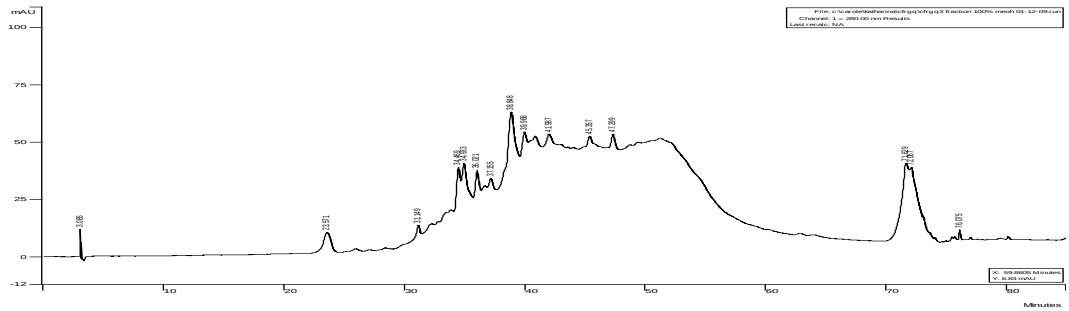
C1



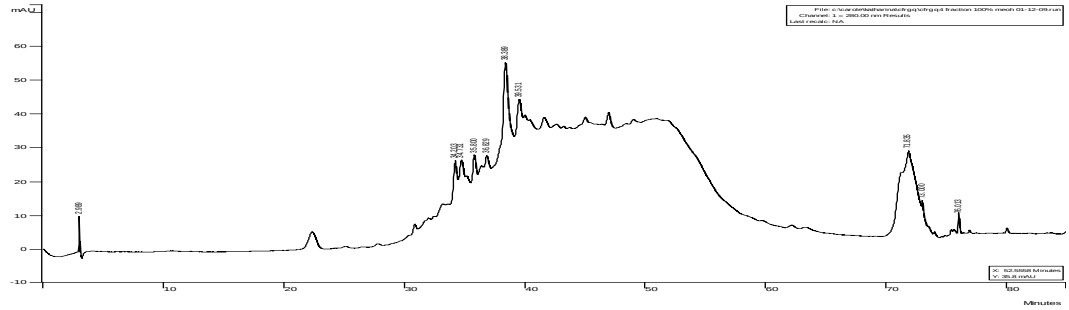
C2



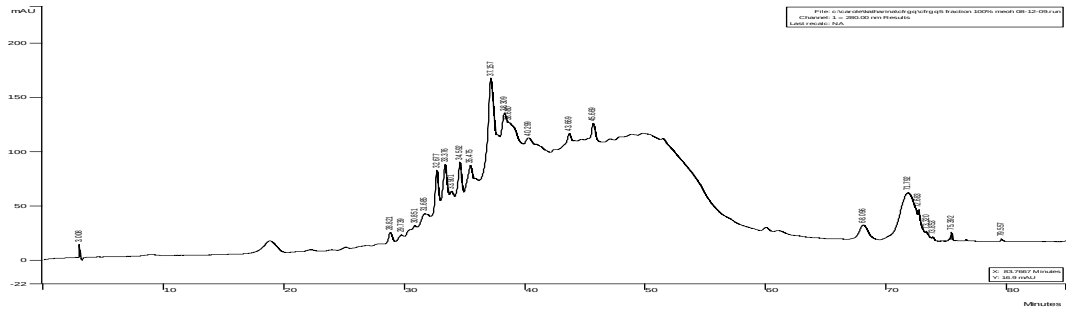
C3



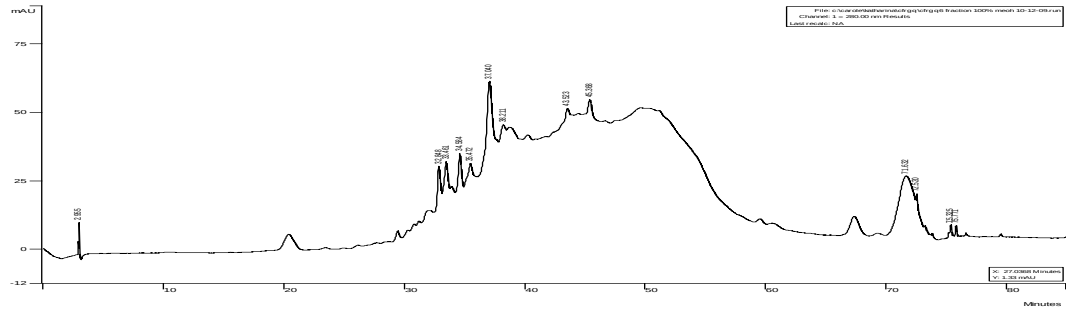
C4



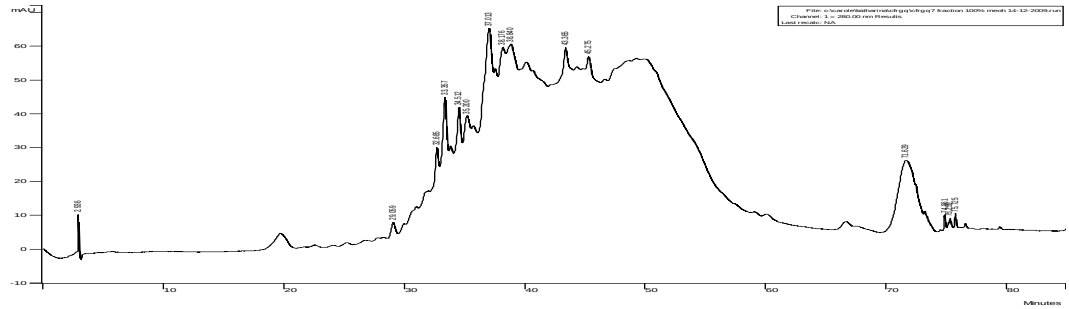
C5



C6

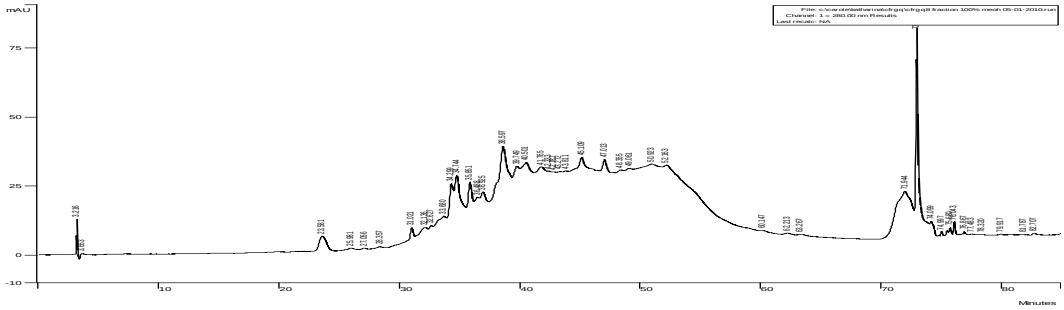


C7



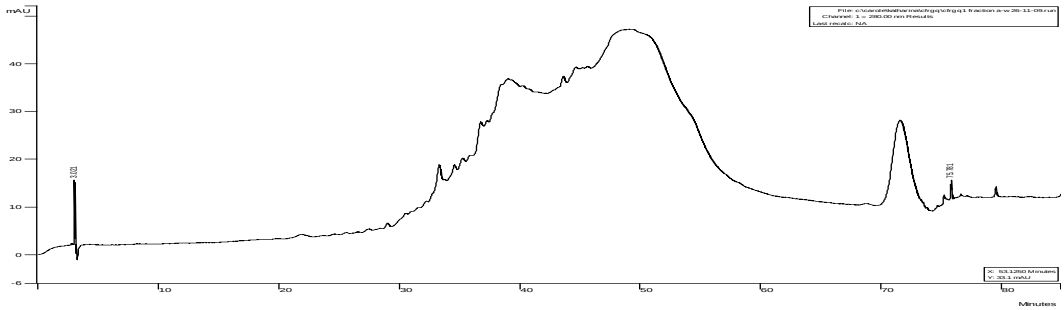
XXX

C8

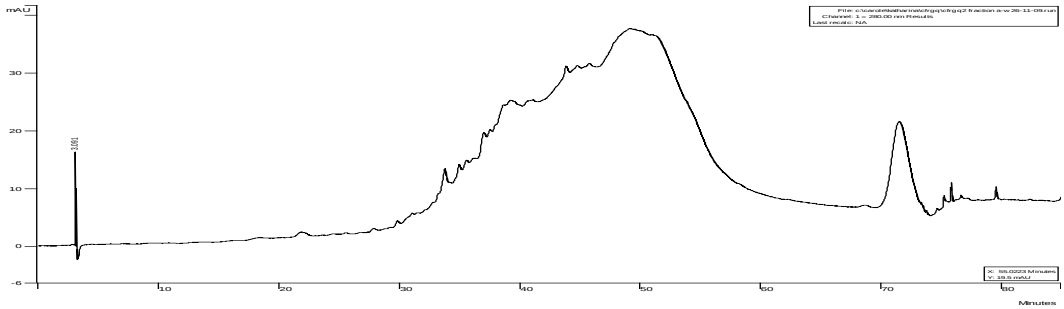


Fractions aw

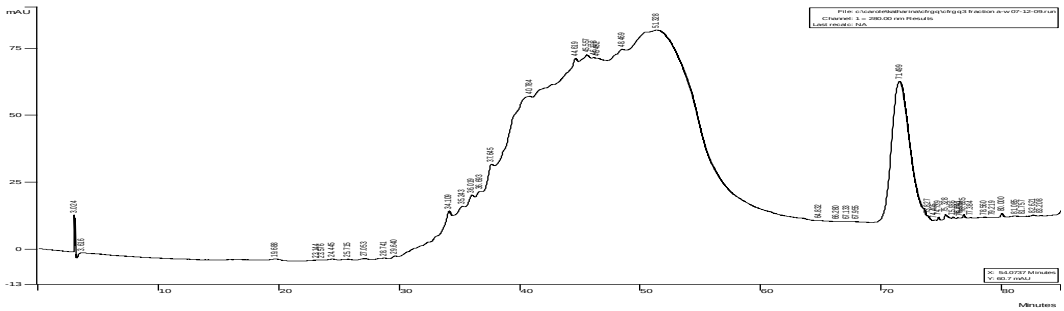
C1



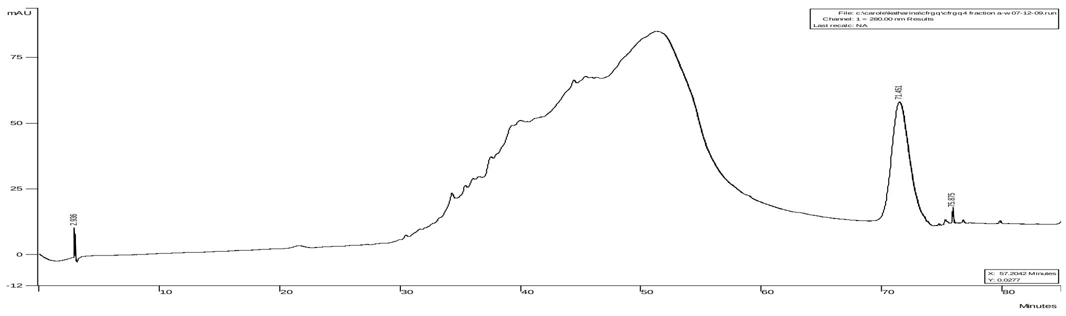
C2



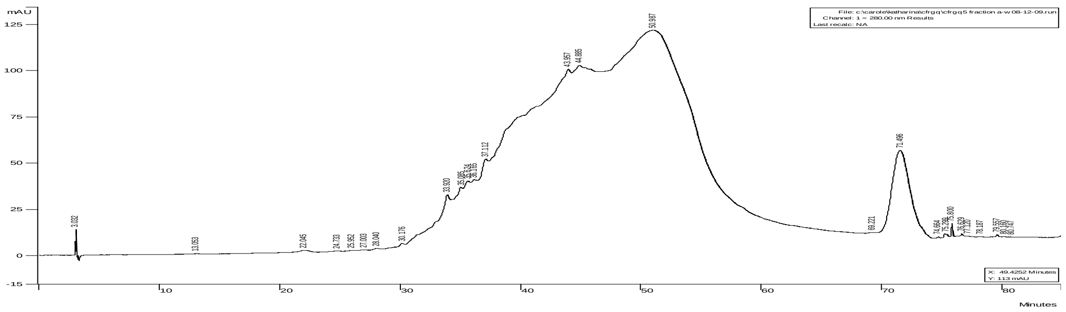
C3



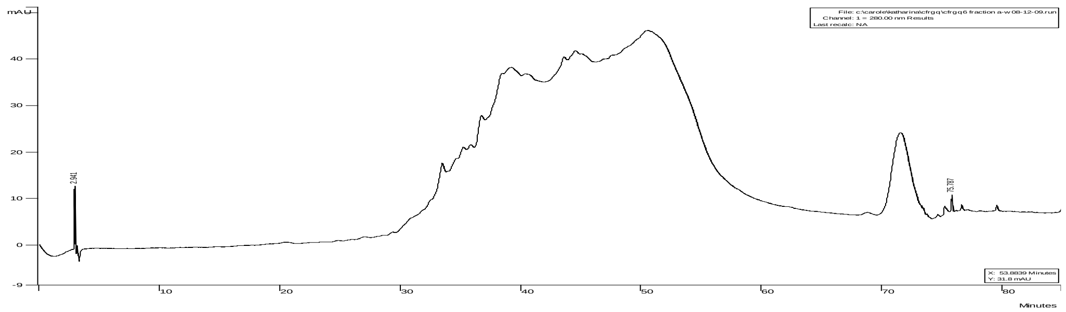
C4


File: c:\carole\katharina\cfrgq\cfrgq4 fraction a-w 07-12-09.run
Channel: 1 = 280.00 nm Results
Last recalc: NA
mAU
75
50
25
0
-12
10
20
30
40
50
60
70
80
Minutes
71.451
X: 57.2042 Minutes
Y: 0.0277

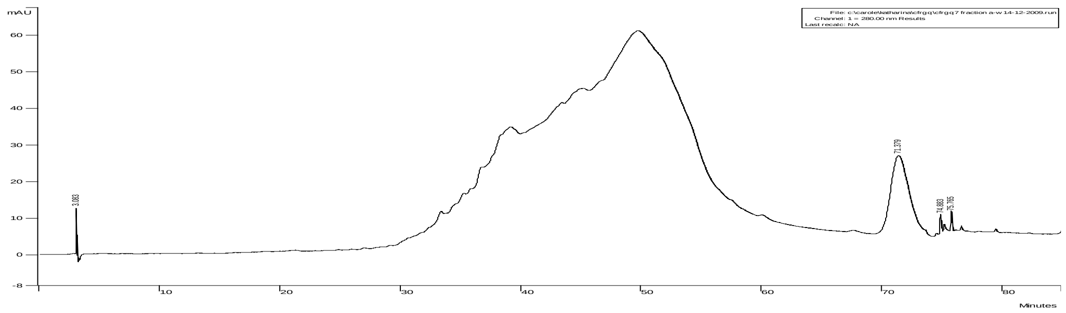
C5


File: c:\carole\katharina\cfrgq\cfrgq5 fraction a-w 08-12-09.run
Channel: 1 = 280.00 nm Results
Last recalc: NA
mAU
125
100
75
50
25
0
-15
10
20
30
40
50
60
70
80
Minutes
50.987
71.496
X: 49.4252 Minutes
Y: 113 mAU

C6


File: c:\carole\katharina\cfrgq\cfrgq6 fraction a-w 08-12-09.run
Channel: 1 = 280.00 nm Results
Last recalc: NA
mAU
40
30
20
10
0
-9
10
20
30
40
50
60
70
80
Minutes
X: 53.8839 Minutes
Y: 31.8 mAU

C7


File: c:\carole\katharina\cfrgq\cfrgq7 fraction a-w 14-12-2009.run
Channel: 1 = 280.00 nm Results
Last recalc: NA
mAU
60
50
40
30
20
10
0
-8
10
20
30
40
50
60
70
80
Minutes
71.379

Pippette schedules and absorptions Vanillin

Calibration	1	2	3	4	5	6	7
sv	700µg	600µg	500µg	400µg	300µg	200µg	100µg
b	700µg	600µg	500µg	400µg	300µg	200µg	100µg
bv	B						
o	O						

Calibration	1	2	3	4	5	6	7
sv	1,0494	0,9472	0,8401	0,6963	0,521	0,3527	0,2085
b	0,0414	0,04	0,0384	0,0383	0,0412	0,0387	0,0383
bv	0,0408						
o	0,0374						

OPC	1	2	3	4	5	6	7
sv	PFR OPC 0,5	PFR OPC 0,5	PFR OPC 0,5	CFR OPC 0,5	CFR OPC 0,5	CFR OPC 0,5	
b	PFR OPC 0,5	PFR OPC 0,5	PFR OPC 0,5	CFR OPC 0,5	CFR OPC 0,5	CFR OPC 0,5	
sv							
b							
bv	B	B	B	B	B	B	
o	O	O	O	O	O	O	

OPC	1	2	3	4	5	6	7
sv	0,746	0,7347	0,7567	0,634	0,6189	0,6248	
b	0,077	0,0747	0,0737	0,0636	0,0602	0,0619	
sv							
b							
bv	0,0426	0,0426	0,0427	0,0426	0,0426	0,0427	
o	0,0384	0,0384	0,0378	0,0384	0,0384	0,0378	

aq	1	2	3	4	5	6	7
sv	PFR aq 5	PFR aq 5	PFR aq 5	CFR aq 5	CFR aq 5	CFR aq 5	
b	PFR aq 5	PFR aq 5	PFR aq 5	CFR aq 5	CFR aq 5	CFR aq 5	
sv							
b							
bv	B	B	B	B	B	B	
o	O	O	O	O	O	O	

aq	1	2	3	4	5	6	7
sv	0,0729	0,073	0,073	0,1031	0,1029	0,1084	
b	0,0426	0,0425	0,0428	0,0432	0,0437	0,0421	
sv							
b							
bv	0,0426	0,0426	0,04	0,0426	0,0426	0,04	
o	0,0384	0,0384	0,0379	0,0384	0,0384	0,0379	

commercial	1	2	3	4	5	6	7
sv	CRB 0,5	CRB 0,5	Pin 0,5	Pin 0,5	Oli 0,5	Oli 0,5	
b	CRB 0,5	CRB 0,5	Pin 0,5	Pin 0,5	Oli 0,5	Oli 0,5	
sv							
b							
bv	B	B	B	B	B	B	
o	O	O	O	O	O	O	

commercial	1	2	3	4	5	6	7
sv	0,3232	0,3223	0,6168	0,588	0,696	0,6379	
b	0,1315	0,1318	0,0502	0,0506	0,0519	0,0572	
sv							
b							
bv	0,0426	0,0426	0,0426	0,0426	0,0426	0,0426	
o	0,0384	0,0384	0,0384	0,0384	0,0384	0,0384	

Pipette schedules and absorptions Folin

Calibration	1	2	3	4	5	6	7
s	AG 100	AG 100	AG 100	AG 80	AG 80	AG 80	
c	AG 100	AG 100	AG 100	AG 80	AG 80	AG 80	
s	AG 60	AG 60	AG 60	AG 40	AG 40	AG 40	
c	AG 60	AG 60	AG 60	AG 40	AG 40	AG 40	
s	AG 20	AG 20	AG 20				
c	AG 20	AG 20	AG 20				
b	b						

Calibration	1	2	3	4	5	6	7
s	0,3908	0,3022	0,2822	0,2576	0,2391	0,3451	
c	0,0333	0,0353	0,0355	0,0337	0,0352	0,0354	
s	0,2431	0,1991	0,1952	0,1452	0,1435	0,1791	
c	0,0353	0,035	0,035	0,0353	0,0348	0,0355	
s	0,091	0,09	0,0909				
c	0,0334	0,0351	0,035				
b	0,0366						

OPC	1	2	3	4	5	6	7
s	PFR OPC	PFR OPC	PFR OPC	CFR OPC	CFR OPC	CFR OPC	
c	PFR OPC	PFR OPC	PFR OPC	CFR OPC	CFR OPC	CFR OPC	
b	b						

OPC	1	2	3	4	5	6	7
s	0,176	0,1561	0,1836	0,517	0,2358	0,2343	
c	0,0346	0,036	0,036	0,0346	0,0358	0,0387	
b	0,0398						