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Analysis of vegetable oils and generated by-products: Application of green strategies for extraction and isolation of their bioactive constituents

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To my parents, Fotis and Kaiti

to my brother, Nemos

and to my beloved Theodora

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Abbreviations

2-D NMR: 2 Dimensions Nuclear Magnetic Resonance
ACE: Annular Centrifugal Extraction
AcN: Acetonitrile
AlmS: Almond Seed
AlmSO: Almond Seed Oil
AlmSP: Almond Seed Paste
BSA: Bovine Serum Albumin
$CDCL_3$: Deuterated chloroform
CO_2: Carbon dioxide
COSY NMR: Correlation Spectroscopy Nuclear Magnetic Resonance
CPC: Centrifugal Partition Chromatography
CPE: Centrifugal Partition Extraction
DCM: dichloromethane
ddH_2O: double-distilled water
DMAB: Dimethyl Aminobenzaldehyde
DMSO: Dimethylsulfoxide
DPPH: 1,1-diphenyl-2-picrylhydrazyl
EC: Elemental Composition
EFSA: European Food Safety Authority
ESI: Electrospray ionization
EtOAc: Ethyl acetate
EtOH: Ethanol
EVOO: Extra Virgin Olive Oil
FAO: Food Agriculture Organization of the United States
FnnS: Fennel Seed
FnnSO: Fennel Seed Oil
FnnSP: Fennel Seed Paste
GC-MS: Gas Chromatography-Mass Spectrometry
GrpS: Grape Seed
GrpSO: Grape Seed Oil
GrpSP: Grape Seed Paste
H_2O: Water
H_2O_2: hydrogen peroxide
H_2SO_4: Sulfuric acid
HMBC NMR: Heteronuclear Multiple Bond Correlation Nuclear Magnetic Resonance
HmpS: Hemp Seed
HmpSO: Hemp Seed Oil
HmpSP: Hemp Seed Paste
HPLC: High Performance Liquid Chromatography
HPLC-DAD: High Performance Liquid Chromatography-Diode-Array Detector
HPTLC: High Performance Thin Layer Chromatography
HRMS/MS: High Resolution Mass Spectrometry/Mass Spectrometry
HSQC NMR: Heteronuclear Single Quantum Correlation Nuclear Magnetic Resonance
HT: Hydroxytyrosol
IC100: maximal inhibitory concentration
IC50: half maximal inhibitory concentration
LC-MS: Liquid Chromatography-Mass Spectrometry
LDL cholesterol: Low Density Lipoprotein cholesterol
L-DOPA: levodopa/ L-3,4-dihydroxyphenylalanine
MeOH: Methanol
MFLA: Monoaldehydic Form of Ligstroside Aglycone
MFOA: Monoaldehydic Form of Oleuropein Aglycone
MMP2: matrix metalloproteinase-2/ gelatinase A
MUFA: Monounsaturated Fatty Acids
n-Hept: Heptane
n-Hex: Hexane
NMR: Nuclear Magnetic Resonance
OLEA: Oleacein
OLEO: Oleocanthal
OO: Olive Oil
PBS: Phosphate Buffered Saline

PmgS: Pomegranate Seed

PmgSO: Pomegranate Seed Oil

PmgSP: Pomegranate Seed Paste

PmpS: Pumpkin Seed

PmpSO: Pumpkin Seed Oil

PmpSP: Pumpkin Seed Paste

Prep: Preparative

PUFA: Polyunsaturated Fatty Acids

RDBeq: Ring and Double Bonds Equivalence

RP-HPLC: Reverse Phase High Performance Liquid Chromatography

SFA: Saturated Fatty Acids

SFC: Supercritical Fluid Chromatography

SFE: Supercritical Fluid Extraction

SnfS: Sunflower Seed

SnfSO: Sunflower Seed Oil

SnfSP: Sunflower Seed Paste

SO: Sesame Oil

TLC: Thin Layer Chromatography

TPC: Total Phenolic Content

TPF: Total Phenolic Fraction

Tris HCl: Tris Hydrochloride

UAE: Ultrasound Accelerated Extraction

UPLC-HRMS: Ultra Performance Liquid Chromatography-High Resolution Mass Spectrometry

UV: Ultraviolet

VO: Vegetable Oil

VSO: Vegetables seed oil

VSP: Vegetables seed paste

XIC: Extraction Ion Chromatogram

Thesis summary

Vegetable oils (VOs) are regarded as an integral section of human diet and many early manuscripts refer to their multiple uses in daily life; from food to cosmetic purposes. They represent the main pool of fatty acids in human nutrition and besides their use is associated with food flavoring due to their aroma and their special organoleptic and sensory properties. According to studies, the average consumption of fat reaches the amount of 25 kg per person per year, with the 80% being covered from VOs. Moreover, they play important role in human well-being and enhancement of life quality, due to their dermo-cosmetic privileges. In parallel, the market needs for natural and edible based products in pharmaceutical and dermo-cosmetic sector is emerging year by the year. Taking into consideration all the above, the reasons that VOs are under the scientific focus is clearly evident.

Chemical composition of VOs is characterized mainly by glycerides, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The plethora of health beneficial effects provided from the different fatty acids of VO are wildly known. The most famous properties of MUFA are the cardiovascular diseases prevention through different mechanisms. In parallel, VOs are source of many PUFAs such as linoleic that among other properties is considered as an important anti-atherogenic and anti-thrombotic agent and it cannot be synthetized by the human organism. Furthermore, a great majority of VOs contain an essential fraction of mainly phenol-based chemical structures with interesting biological activities, colloquially called bio-phenols. These compounds represent a very small part of the total oil weight being around 2-5%. Many studies have proved that VOs bio-phenols are responsible for a series of human health beneficial activities. As a consequence, scientific and industrial interest were centered on the investigation of VO chemical composition and biological properties for the development of pharmaceutical and cosmetic products.

Following the demand of the market for natural-based products, it is very important to note that the conception is not only to use starting materials coming from natural sources, but additionally to handle and treat them with green methodologies. The last years, plenty of eco-friendly techniques have been developed and have been optimized for applications on field of natural products. Non-toxic solvents, low energy and less time consuming extraction and separation procedures and use of recycle/reusable by-products are few of the criteria that were taking into account in order to transfer procedures from laboratory to pilot and industrial level. These characteristics can be fulfilled only from state of the art techniques with green environmental

character, like liquid-liquid solid support free chromatography and extraction, supercritical fluids and food grade processes in combination with the use of edible or non-toxic reagents.

After extraction and separation, another vital step is the biological evaluation of natural product extracts and pure compounds. Due to the increasing ecological awareness of the last years, a huge endeavor is given to supersede the *in vivo* models by *in vitro* experiments.

The research axis of the current thesis, was based on the exploration of nine different cold pressed VOs. The thesis is divided into two different parts according to the significance and subsequent production and/or consumption of the studied VO. Two of them, sesame seed oil and olive oil, are considered as worldwide commercial oils and were discussed in the first part of the thesis. At the seconds part, seven oils of limited production were studied. These seed oils come from plants: (*Foeniculum vulgare*) fennel, grape (*Vitis vinifera*), pumpkin Cucurbita pepo), sunflower (*Helianthus annuus*), almond (*Prunus dulcis*), pomegranate (*Punica granatum*) and cannabis-hemp (*Cannabis sativa*) and are globally produced in low quantities.

Based on the most recent data of Food Agriculture Organization of the United states (FAO), sesame and olive oil worldwide production reached 937,000 tonnes and 3,039,000 tonnes, respectively. Both of them, are straightly correlated with the food habits of plenty African, Asian and European civilizations. Their high nutritional properties and the wide medical uses were the stimulus for their further speculation. Numerous studies emphasize the importance of these two oils bioactive constituents on human health. These findings trigger the need to develop methodologies for the recovery of bioactive compounds, known as bio-phenolic fraction, from sesame and olive oil in sufficient amounts, in order to get feasible their biological investigation in deep. To the best of our knowledge, until now there is no scientific reference in pilot scale extraction of bio-phenolic fraction neither for olive oil, nor for sesame oil.

Aim of this thesis first part, is the development of extraction methodologies for the recovery of phenolic fraction from the two oils, employing pioneering liquid-liquid centrifugal techniques and the further fractionation and purification of bioactive solutes, mainly with liquid-liquid countercurrent solid support free chromatography, in quantities that reach up to gram scale.

Precisely, regarding sesame oil, two synchronous extraction liquid-liquid centrifugal methods, Annular Centrifugal Extraction (ACE) and Centrifugal Partition Extraction (CPE), were compared in lab scale, with the intention to obtain its bio-phenolic fraction that is rich in two lignans, sesamin and sesamolin. For the fractionation of extract and the isolation of sesamin and sesamolin in pure form, over 95%, preparative Centrifugal Partition Chromatography (CPC) was applied. In parallel four minor lignans, samin, sesamol, sesaminol and episesaminol were isolated in high purity. All

the minor and major lignans were finally evaluated in relation to their dermo-cosmetic properties against tyrosinase, elastase, collagenase and hyaluronidase enzymes.

Moreover, this study was amplified with the development of a ground-breaking methodology for the recovery of sesame oil extract in pilot scale, in order to meet the *in vivo* experiment needs. Combining liquid-liquid centrifugal extraction of sesame oil with DMSO and food grade adsorption resins, discussed in the current manuscript for the first time, the recovery of major lignans in high quantities was succeed.

For olive oil, several samples were analyzed and the richest in high added value molecules like oleocanthal (OLEO), oleacein (OLEA), monoaldehydic form of oleuropein aglycone (MFOA), monoaldehydic form of ligstroside aglycone (MFLA) and hydroxytyrosol (HT) were selected for extraction. For the first time a pilot scale extraction process of EVOO was took part on liquid-liquid centrifugal extractor. The starting experiments were employed on lab scale instrumentation and scaled up on pilot level providing an enriched bio-phenolic extract of hundreds grams. Prep-CPC analysis of EVOO extract led to the production of enriched fractions with the compounds of interest. Moreover, OLEO, OLEA, MFOA, MFLA and HT were purified on gram scale from CPC fractions with prep-HPLC-DAD. During the purification process of the target compounds, four minor solutes were isolated, three of which are new natural products (EDA lactone, (1*R*, 8*E*)-1-ethoxy-ligstroside aglycon and (1*S*, 8*E*)-1-ethoxy-ligstroside aglycon) and one more which is isolated for the first time from EVOO ((9*E*, 11*E*)-13-oxotrideca-9,11-diencoic acid).

The second part of the thesis discusses the seven limited production cold pressed seed oils and their corresponding by-products. A totally, green pipeline was selected for all the steps of the employed treatment. With the purpose of decreasing at minimum the ecological fingerprint, seed oils were eluted straightly through food grade resins and their extracts were obtained, while for their by-products, experiments with Ultrasound Accelerated Extraction (UAE) and Supercritical Fluid Extraction (SFE) were conducted. Only non-toxic extraction solvents, ethanol and water, were used for the UAE, while SFE extraction was accomplished with CO_2 and ethanol as co-solvent. Crude seed oils' fatty acids were identified after GC-MS analysis, while all the extracts were evaluated for their antioxidant activity and their total phenolic content (TPC). Finally, dermo-cosmetic activities against collagenase, elastase, and tyrosinase enzymes were determined.

The two most interesting seed oils and their by-products extracts (grape and fennel), in terms of enzymatic inhibition and their chemical profile, were further investigated. Grape seed oil resin extract and grape seed paste ultrasound assisted extract were fractionated with CPC. Concerning grape seed paste extract CPC fractions, were also evaluated against tyrosinase, collagenase and elastase enzymes.

Fennel seed oil extract was found rich in anethole, a bioactive compound of high importance for the food industry. After one step CPC fractionation of extract, pure anethole was isolated in high amount, while in parallel a fraction enriched in isomeric forms of 1-(4-methoxyphenyl) propane-1,2-diol was eluted. Supercritical Fluid Chromatography (SFC) was chosen for the complete separation of isomeric forms. Moreover, other minor compounds were identified after prep-TLC treatment of CPC fractions. High interest had also the by-product extract of fennel seeds. After CPC analysis a fraction enriched in syringin was obtained that with the support of prep-HPLC syringin was purified.

To finalize, the current thesis was based on an effort to contribute in the existing knowledge for the ecological extraction and chromatographic treatment of edible vegetable oils as pool of bioactive compounds with health beneficial effects. Novel extraction and separation methodologies were developed for the recovery of high added value compounds, from extensive use oils, sesame and olive oil, with the implementation of state of the art green techniques. Compounds of high scientific interest were isolated in grams, while minor solutes were also isolated and reported for the first time. Additionally, a holistic green strategy for the screening of extracts from seven limited production seed oils (fennel, grape, pumpkin, sunflower, almond, pomegranate and cannabis-hemp seed oils) their respective by-products was designed. Innovative mindsets were proposed for the treatment of the starting materials with minimum environmental fingerprint. The final objective was the dermo-cosmetic evaluation with enzymatic assays of the produced extracts and the further chemical investigation of the most promising of them.

Part I: Worldwide commercial oils

Chapter 1: Recovery of sesamin, sesamolin, and minor lignans from sesame oil using solid support-free liquid–liquid extraction and chromatography techniques and evaluation of their enzymatic inhibition properties. A pilot scale approach for sesame oil extraction.

Abstract

In this study an integrated process for the recovery of sesamin and sesamolin, two high added-value lignans of sesame oil (SO) was developed, using synchronous extraction and chromatography techniques. The extraction of SO phenolic content was studied using two different extraction techniques: Annular centrifugal extraction (ACE) and centrifugal partition extraction (CPE). The derived data of each experiment were compared in terms of revealing the yields, time and solvents consumption showing that CPE is the most effective technique, concerning the solvent consumption. The isolation of lignans was achieved using centrifugal partition chromatography (CPC) both on semi-preparative and preparative scale. The biphasic system used for this purpose was consisted of the solvents: n-Hex/EtOAc/EtOH/H2O in proportion 2:3:3:2 (v/v/v/v) and direct recovery of the two major lignans sesamin and sesamolin was achieved. In parallel the CPC analysis resulted in the isolation of four minor lignans of sesame oil i.e. samin, sesamol, sesaminol and episesaminol. Structure elucidation of isolated lignans was based on HRMS/MS and NMR experiments. High performance liquid chromatography (HPLC) was employed for quantitative analysis of the obtained extracts to determine the purity of the isolated compounds as well. The results of this study demonstrated that sesamin and sesamolin were recovered in purity higher than 95%, verifying the effectiveness of the purposed separation methodology. Finally, due to the general application of sesame oil in cosmetic industry, all the pure compounds were evaluated for their tyrosinase, elastase, collagenase and hyaluronidase inhibition activity. The study was enforced with the design of a pilot scale extraction of SO, in order to obtain high amounts of extract for *in vivo* experiments. For the first time, a pilot scale ACE was applied and DMSO was used as extraction solvent with notable results. Furthermore, two different workstreams were followed in order to obtain the final SO extract. Both were started with pilot scale ACE extraction of SO. As solvent, diluted DMSO (DMSO/H_2O 9:1 v/v) was used. The first workstream, continued with liquid-liquid extraction of the enriched DMSO extract with EtOAc. For the second one, a pioneering methodology was applied. The enriched DMSO extract was loaded in XAD-7 resin. The results of two production methods were correlated concerning the qualitative and quantitative characteristics. Finally, 816 g of SO extract were obtained with a rapid two-step process.

Introduction

SO is a product of high importance, obtained from the seeds of *Sesamum indicum* (Pedaliaceae) and is directly linked to the traditional nutrition of Asian and African people for more than 5.000 years [1,2]. According to Food and Agriculture Organization of the United Nations (FAO) in 2014 the global production of SO exceeded the amount of one and a half million tone to meet market needs [3]. These numbers have attracted scientific interest, leading to the study of the chemical content and bioactivity of SO secondary metabolites [4,5]. Many scientific researches have proved that phenolic compounds of SO have numerous biological activities. Especially sesamin and sesamolin (Figure 1), the two major lignans of SO extract, have been tested *in vitro*, *in vivo* and in clinical studies for numerous activities.

Particularly, *in vivo* experiments have proved the hypocholesterolemic activity of sesamin [6] whereas a clinical study demonstrated positive results against total and LDL-cholesterol on humans, probably synergistic with vitamin E [7]. In addition, this molecule promotes the reduction of fat ratio on human body obviating atherosclerosis and corpulence [8], while experiments on gerbils and mice have demonstrated the neuroprotective role of sesamin against cerebral ischemia [8,9]. Another important activity of this lignan is the anti-inflammatory, via the inhibition of delta 5-desaturase [10], an enzyme which is connected with the pro-inflammatory mediators [11]. When the organism lacks out of glucose, ketone bodies are used to cover the energy demands. Sesamin is able to increase the ketone bodies concentration [12]. This furanofuran lignan also decreases the metabolism of γ-tocopherol and as a result, elevates the concentration of tocopherol [13]. The antioxidant activity of sesamin and its protective role against damages of alcohol and carbon tetrachloride on liver have been proved as well [6].

Sesamolin, the second major lignan of SO, has also a significant number of biological activities. It induces apoptosis of human lymphoid leukemia Molt 4B cells, inhibits the growth of those cells [14] and prevents from mutagenic activity of H_2O_2 [15]. Sesamolin has also free radical scavenging activity and provides protection against neuronal hypoxia [16].

Due to the high pharmacological interest of sesamin and sesamolin, there are several works regarding the isolation and purification of these lignans from *Sesamum indicum* materials like sesame seeds, sesame meal and SO. [17–22]. In literature, silica gel column is referred as separation technique of SO extract, which is mostly applied for laboratory purposes [22]. Moreover, crystallization method is carried out, providing mixture of lignans [18]. Another study is based on semi-preparative HPLC, which has limited capacity of sample treatment [17]. Considering counter-current chromatography, two studies are reported, whereas one of them provide a mixture of sesamin and sesamolin and the other one is characterized by reduced yield of

pure compounds [19,21]. Only in one study CPC technique is applied, but as raw material sesame seed meal was used, while the procedure was more time consuming and the results gave lower recovery and lower purity [20]. However, there is not a previous work for producing these two lignans in high amounts and purity using a low cost and fast methodology. Following this need, our research was targeted to develop a novel approach, which could meet the above-mentioned parameters.

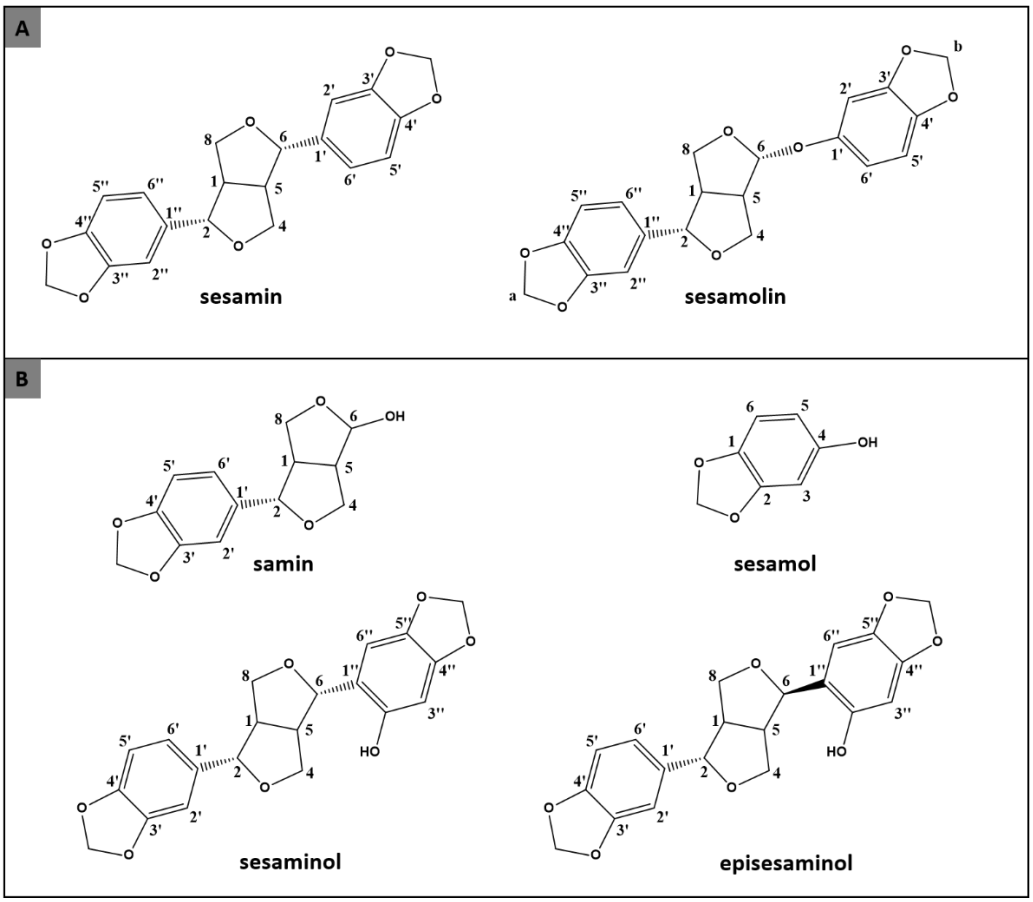


Figure 1: Chemical structures of major (A: sesamin and sesamolin) and minor (B: samin, sesamol, sesaminol and episesaminol) lignans isolated from sesame oil.

The following experimental procedure is based on liquid-liquid techniques. CPE is a solid support-free liquid-liquid extraction technique which is based on the immiscible nature of two phases and the partition of compounds in the formed biphasic system [23–25]. This technique is mainly used for rapid fractionations of mixtures, pH-zone refining separations as well as for the extraction of liquid nature raw materials such as edible oils [25,26]. Low experimental time and solvent consumption rendered it suitable for analytical, preparative, pilot and industrial scale as well [27,28]. Two common extraction methods of CPE are co-current elution and multi-dual mode. At the first method, the biphasic system passes through the column and is separated out of the apparatus. At the second method, column is fed with the stationary phase and the mobile phase

passes through the first. The kinetic nature of two phases can be changed by the rotation of valve ascending/descending, which sets the inlet of solvents in column. Another extraction technique that was used in this study was ACE. This technique can rapidly separates the biphasic systems and is characterized by high mass transfer efficiency per time unit [29]. The centrifugal force is used firstly to mix the two phases and then to separate them [30].

CPC is governed by the same principles of CPE with a difference in the number and the volume of column cells [28]. CPE and CPC columns are metallic cylinders with a number of cells that are proportionally related with theoretical plates. Chromatography column cells are higher in number but smaller in volume than extraction column cells [31]. The one phase (stationary phase) is immobilized by strong centrifugal forces into the column, while the other phase (mobile phase) is pumped through the column, separating thus the mixture compounds, on the basis of their partition coefficient (K_D) [32,33]. The nature of this technique gives the ability of obtainment the maximum amount of the extract and handling high amounts of sample [34,35]. Also, this technique permits the use of various polarity solvent systems which result in widening the chromatographic performances [36]. Another advantage of CPC is the ability of alternation the mobile phase to stationary during a run, accelerating the recovery of compounds [37].

The aim of this study was the development of an effective and capable for scaling up process for the treatment of sesame oil and isolation of sesamin and sesamolin in high purity. The primary step was the extraction of phenolic compounds from SO. Two extraction techniques were compared in order to choose the most advantageous concerning experimental time and extract productivity. The second step was the isolation of sesamin and sesamolin in semi preparative and preparative scale. The last part of the study was the quantification of the obtained lignans using HPLC-DAD. Also, NMR experiments were used for the identification of the targeted molecules. TLC analysis and HPLC experiments were conducted for the qualification of SO extracts. In parallel sesamin, sesamolin and minor compounds isolated from SO were evaluated with enzymatic assays (tyrosinase, elastase, collagenase and hyaluronidase) for their inhibition activity.

In parallel, a pilot scale extraction model of SO was developed to meet the needs of *in vivo* experiments on rabbits. For this purpose, a pilot ACE apparatus was recruited that can handle liters of raw material per minute. Until now, mainly classical liquid-liquid techniques were applied for SO extraction [18,22]. For the first time, a combination of DMSO as extraction solvent of the raw material and the adsorption properties of resin XAD-7 were employed to obtain more than 800 g of SO extract, rapidly from the enriched DMSO extract. Furthermore, a liquid-liquid experiment

with EtOAc as extraction solvent was tested to the enriched DMSO extract. To the best of our knowledge, this was the first attempt to obtain hundreds of grams of SO extract and the first time that ACE instrumentation was employed for the treatment of edible oils.

Materials and Methods

Reagents

The standards of lignans which were used for the quantitative analysis purchased from Sigma-Aldrich (Missouri, USA). Also, all the reagents were purchased from Sigma-Aldrich (Missouri, USA). In detail, for the enzymatic assays mushroom tyrosinase (lyophilized powder, ≥1000 units/mg solid, EC Number: 1.14.18.1), 3,4-dihydroxy-L-phenylalanine, sodium phosphate monobasic, sodium phosphate dibasic, kojic acid, elastase type IV from porcine pancreas (EC Number 254-453-6), N-Succinyl-Ala-Ala-Ala-p-nitroanilide (EC Number 257-823-5), Trizma base reagent grade, elastatinal, collagenase from Clostridium histolyticum (released from physiologically active rat pancreatic islets Type V, ≥1 FALGPA units/mg solid, >125 CDU/mg solid, EC Number: 232-582-9), MMP 2 substrate fluorogenic, chlorexidine, bovine serum albumin, acetic acid glacial, p-(dimethylamino) benzaldehyde, sodium tetraborate, hyaluronidase (released from bovine testes Type I-S, lyophilized powder, 400-1000 units/mg solid, EC Number: 3.2.1.35), hyaluronic acid and tanic acid were purchased also from Sigma-Aldrich (Missouri, USA). The used solvents for the extraction and separation processes were of analytical grade while those used for UPLC-HRMS analysis were of LC-MS grade. All solvents were supplied from Fisher Scientific (Pennsylvania, USA). TLC analysis was performed on Silica gel 60 F_{254} 20cm x 20cm plates purchased from Merck Millipore (Massachusetts, USA). Sesame oil was provided from HAITOGLOU BROS S.A.

Apparatus

The extraction of SO phenolic fraction was performed using two different liquid-liquid techniques: CPE and ACE. CPE experiments were performed on an A-CPC apparatus (Rousselet-Robatel Kromaton, Anonay, France) equipped with a 300 mL capacity extraction column (FCPE300®) while solvents pumped with preparative Lab Alliance Series III P300 pumps (Pennsylvania, USA). ACE laboratory scale experiments were performed using a BXP012 apparatus (Rousselet-Robatel Kromaton, Anonay, France) with 2.2 mL bowl volume. Basic Verderflex pumps (Castleford, United Kingdom) were used for pumping the solvents through the annular extractor. For the pilot line ACE was employed a BXP190 apparatus (Rousselet-Robatel Kromaton, Anonay, France) with 4.2 L bowl volume. Two high throughputs FLUX eccentric worm-drive pumps (FLUX-GERÄTE GmbH, Maulbronn, Germany) were used to feed the extractor.

Semi-preparative and preparative fractionations of SO phenolic fraction were carried out on a FCPC apparatus (Kromaton, Anonay, France) equipped with a 200 mL capacity chromatographic column (FCPC200®) and 1000 mL capacity chromatographic column (FCPC1000®) respectively. Solvents were pumped with preparative Ecom ECP2000 pumps (Prague, Czech Republic). Chromatograms were recorded with a detector UV Flash 14 DAD UV of Ecom (Prague, Czech Republic) and the fractions were collected with a C6-60 Buchi collector (Flawil, Switzerland).

HPLC analysis was performed on a Thermo Finnigan HPLC system (Ontario, Canada) equipped with a SpectraSystem P4000 pump, a SpectraSystem 1000 degasser, a SpectraSystem AS3000 automated injector and an UV SpectraSystem UV6000LP detector. Data acquisition was controlled by the ChromQuest™ 5.0 software (ThermoScientific™).

Nuclear magnetic resonance spectra were registered on 600MHz of Bruker AvanceAVIII-600 spectrometer (Karlsruhe, Germany) and was supported by TopSpin software (Bruker). UPLC-HRMS and HRMS/MS analysis was performed on an AQUITY system (Waters) connected with an LTQ Orbitrap Discovery hybrid mass spectrometer (Thermo Scientific) equipped with an ESI source, in negative and positive mode.

For all the enzymatic assays the reader Infinite 200 PRO series (Tecan, Zürich, Switzerland) was used, supported by software Magellan™ (Tecan, Zürich, Switzerland).

Lab scale liquid-liquid extraction of SOs' lignans

Extraction of lignans using laboratory scale Annular Centrifugal Extractor (BXP012)

Two different experiments were performed using for the extraction the biphasic system SO/Acetonitrile (AcN). In both experiments the rotor speed was set at 3900 rpm and 200 mL of SO were extracted by using 600 mL of acetonitrile. For the first experiment, the extraction was performed on three successive cycles using 200 mL of AcN in each run (total 600 mL of AcN). AcN (upper phase) and SO (lower phase) were pumped through the apparatus at a flow rate of 8 mL/min for each phase (1/1 ratio of the two phases). The total experiment lasted approximately 1 h and 15 minutes (~25 minutes for each cycle). In the second experiment, the flow rate of AcN was increased at 24 mL/min while the flow rate of SO remained stable at 8 mL/min giving thus a ratio of 1/3 SO/AcN into the extraction bowl. The procedure was accomplished on one single run after 25 minutes. Samples were collected from each experiment and were analyzed for quantification of the two targeted lignans via HPLC technique.

Extraction of lignans using FCPE300®

Three extraction runs took place with CPE using multi-dual mode method [26]. CPE column was filled with SO (stationary phase) in descending mode while the flow rate and the rotation were set

at 20 mL/min and 200 rpm, respectively. Then, AcN was pumped in ascending mode at 10 mL/min and 800 rpm in order to equilibrate the biphasic system (SO/AcN) inside the column. Stationary phase retention volume was 200 mL and Sf was calculated at 66.6%. Afterwards, 240 mL of AcN were collected in 12 fractions of 20 mL. Then, the pumping mode switched to descending and 200 mL of untreated SO replaced the extracted SO with a flow rate of 10 mL/min. The above extraction-recovery cycle was repeated three times of 44 min per run. The extraction solvent (AcN) was evaporated under vacuum at 40^{o}C to dryness in order to obtain the SO extract.

Pilot scale extraction of SO

At the first step, SO was extracted with DMSO/H_2O 9:1 v/v using a pilot ACE BXP190. Flow rate of extraction solvent was set at 1 L/min, while the rotation speed of the motor was set at 1000 rpm. When the solvents filled the extraction bowl, SO was fed to the extractor with 1L/min. After 1 hour and 30 minutes 90 L of SO had been treated. Two different approaches were performed for the final obtainment of SO extract. For the first one, 5 L of DMSO extract were diluted in 5 L of H_2O and extracted with 10 L of EtOAc. The final extract of EtOAc was evaporated until dryness and then was dissolved in AcN and was defatted with n-Hex. For the second approach, 5 L of DMSO extract were dissolved in 5 L H_2O and pumped through XAD-7 resin embedded in a column. The recovery of the final SO extract from resin was conducted with EtOH. The other 80 L of DMSO extract were treated with the most efficient method, based on weight and HPLC analysis (discussed in detail in section 3).

Fractionation of SO extract using semi-preparative FCPC200® and preparative FCPC1000® apparatus.

Solvent system selection

Seventeen biphasic solvent systems (Table 1) were created and studied in order to select the appropriate systems for the CPC separation process. All systems were initially tested regarding the solubility of the extract and settling time and then the suitability of biphasic systems was evaluated by TLC and HPLC-DAD. The procedure was as follow: 10 mg of SO extract were weighed into a 10 mL glass tube, 3 mL of each phase of the pre-equilibrated biphasic solvent systems were added to the sample and shaken vigorously. After equilibration of the biphasic system (t<1 min), 1 mL of each layer was evaporated to dryness, the residues were diluted in 1 mL of acetonitrile and analyzed by TLC and HPLC-DAD. The K_D values of the target compounds were expressed as the ratio between the peak area in the stationary phase and the peak area in the mobile phase.

Table 1: Solubility of SO extract and separation of biphasic CPC solvent system.

System	n-Hex	n-Hept	EtOAc	AcN	EtOH	MeOH	H_2O	Solubility of SO extract*	Separation of two phases**
CS1	4	-	1	-	2	-	3	++	+++
CS2	3	-	2	-	2	-	3	++	+++
CS3	2	-	3	-	2	-	3	+++	+++
CS4	1	-	4	-	2	-	3	+++	+++
CS5	4	-	1	-	3	-	2	+++	+++
CS6	3	-	2	-	3	-	2	+++	+++
CS7	2	-	3	-	3	-	2	+++	+++
CS8	1	-	4	-	3	-	2	+++	+++
CS9	-	2	3	-	-	3	2	++	-
CS10	-	2	3	2	-		3	+++	-
CS11	-	2	3	1	-	2	2	++	-
CS12	-	2	3	2	-	1	2	++	-
CS13	-	2	3	1	-	1	3	+++	+++
CS14	-	4	3	-	3	-	2	++	+++
CS15	-	4	3	1	2	-	2	++	+++
CS16	-	3	3	-	3	-	2	++	-
CS17	4	-	2,5	1,5	3	-	2	+++	+++

Semi−preparative CPC analysis

The CPC experiment was carried out in elution extrusion mode by using the biphasic system n-Hex/EtOAc/EtOH/H_2O in proportion 2:3:3:2 (v/v/v/v). Initially the column was filled with the stationary phase (the upper phase) on descending mode at a flow rate of 10 mL/min and setting the rotation speed at 200 rpm. Then, the rotation speed was maximized at 900 rpm and the mobile phase was pumped through the column with a flow rate of 5 mL/min on descending mode. After the system equilibration, the retention volume of the stationary phase was calculated at 105 mL giving a high Sf value of 52.5 %. 110 mg of crude SO extract were dissolved in 10 mL of biphasic system and injected into column. In the elution step 350 mL of mobile phase were passed through the stationary phase at a flow rate of 5 mL/min on descending mode. The experiment was completed by passing 200 mL of the stationary phase on descending mode and extruding the column content. All procedure was monitored by UV detector at 255, 275, 280 and 320 nm while the automatic fraction collector was set to collect fractions every 2 min. The total analysis time was 110 min and finally 55 fractions of 10 mL were collected.

Preparative CPC analysis

The semi-preparative method was scaled−up to preparative column (1000 mL rotor) adjusting all the experimental parameters to the larger scale. After filling the column with the upper stationary phase (500 rpm and 25 mL/min), the rotation speed was increased to 750 rpm and the lower phase of the same system (mobile phase) was pumped at 15 mL/min in descending mode in order to equilibrate the two phases into the column (Sf was calculated at 65 %). Then, 900 mg of the extract were diluted in a mixture of the two phases (ratio 7/3 upper phase/ lower phase) and injected via a 30 mL injection loop. The volume of mobile phase used for the elution step was 1600 mL while the experiment completed by passing 1000 mL of the stationary phase in descending mode (extrusion step). The rotation speed and flow rate were kept stable at 750 rpm and 15 mL/min respectively during the whole experiment. The total analysis time lasted approximately 170 min and finally 130 fractions of 20 mL were collected.

Quantitative analysis of sesamin and sesamolin in crude extracts and CPC fractions

For the quantitative analysis of the two lignans was necessary the construction of standard calibration curves on HPLC-DAD. For the separation, a Supelco Analytical (Sigma-Aldrich) HS C18 column, with dimensions 25 cm x 4,6 mm, 5 μm was used, heated at 40^{o}C. As mobile phase was used in a gradient system consisted of AcN (A) and water (B). The elusion started with 54% of A and reached 79% in 5 minutes. Then, in 5 minutes A reached 83% and during the next 3 minutes was increased to 95%. The gradient continued for 2 minutes with A reaching 100%. In 1 minute the solvent system returned to initial conditions and maintained for 4 minutes. The total running time was 20 minutes and the flow rate was set at 1 mL/min. The injection volume was 10 μL. For sesamin, six concentrations were used: 50, 75, 100, 125, 150 and 175 μg/mL. Also, for sesamolin were used: 25, 50, 75, 100, 125 and 150 μg/mL. As internal standard (IS) was used vanillin in a concentration of 10 μg/mL. For the construction of the calibration curves the ratio area of analyte/IS was used. Linearity was evaluated by coefficient of determination, which were over 0.99 for both analytes (Supplementary Diagrams 1 and 2).

TLC, UHPLC-HRMS/MS and NMR analysis

TLC plates were developed in dichloromethane (DCM). Plates were observed at 254 nm, 366 nm and at visible after treatment with a sulfuric vanillin solution (5% w/v in methanol) – H_2SO_4 (5% v/v in methanol) and heated at 100–120 ˚C for 1 minute.

The phenolic fraction and selected CPC fractions were analyzed using UPLC-HRMS technique. The separation was run in a Fortis C-18 (1.7 μm, 150 x 2.1 mm) column at 40^{O}C. The elution system was consisted of water acidified with 0.1% formic acid (A) and acetonitrile (B) in the

following gradient mode: 0-2min 2% B, 2-18min from 2% to 100% B, 18-20min 100% B, 20-21min from 100% to 2%B and 21-25min 2% B. The flow rate was set at 0.4 ml/min and the injection volume was 10 µl. Ionization was achieved in negative and positive ion mode (ESI+ and ESI-) at 350^{O}C. The mass spectrometric parameters were: sheath gas and aux gas flow rate 40 and 10 units respectively; capillary voltage 30 V and tube lens 100 V for the positive mode and capillary voltage -20V and tube lens -80V for the negative mode. The mass range was adjusted from 113 to 1000 m/z.

NMR samples were dissolved in 600 µL of deuterated chloroform ($CDCL_3$). All the 1H NMR experiments were applied on 600.11 MHz, while 13C NMR spectra were acquired at 150.90 MHz. During all the experiments, temperature was set at 300 K. Spectral width of 1H NMR was set to 14 ppm, offset to 6.5 ppm and scans number to 32. Concerning 2-D NMR experiments, proton spectra were registered according the above-mentioned parameters with 12 scans number for COSY, while carbon spectra width set to 240 ppm, offset to 110 ppm and scans number to 32 and 160 at HSQC and HMBC, respectively. Tyrosinase, elastase, collagenase and hyaluronidase inhibition assays.

Tyrosinase, elastase and collagenase assays were applied following the enzymatic methods described by *Angelis et al.* [38] with some modifications, while the enzymatic assays for the inhibition of hyaluronidase were conducted as described by Kim et al. [39], with some modifications. All the enzymatic assays provide the competitive inhibition activity of the compounds. Three concentrations of pure compounds i.e. 500, 100 and 25 µM (final concentration in the well) were used on the above enzymatic assays. Experiments were performed in triplicates and twice in total while the final DMSO concentrations did not exceed 5% of total volume. The inhibition percentage was calculated by the formula: Inhibition (%) = [((X control – X control's blank) – (X sample – X sample's blank))/(X control – X control's blank)] × 100, where X control is the absorbance or fluoresces of the mixture consisting of buffer, enzyme, sample solvent and substrate and X sample is the absorbance or fluoresces of the mixture of buffer, enzyme, sample or positive control solution and substrate. Blanks contained all the above-mentioned components except the enzyme. Concerning tyrosinase, elastase and collagenase enzymatic assays, the half maximal inhibitory concentration (IC50) of each positive control was used as standard of comparison, while at hyaluronidase enzymatic assay the maximal inhibitory concentration (IC100) was used.

Tyrosinase enzymatic assay: This assay measures the inhibition of the tested samples at the catalytic oxidation of L-DOPA to dopachrome by tyrosinase. Kojic acid (IC50 = 50 μM), was used as positive control. In a 96-well microplate, 80 μL of phosphate buffered saline (PBS) (1/15 M, pH = 6.8), 40 μL of the tested sample (dissolved in the PBS buffer) and 40 μL of mushroom tyrosinase (100 U/mL) (dissolved in PBS buffer) were mixed and incubated in dark for 10 min at room temperature. Afterwards, 40 μL of 2.5 mM L-DOPA (substrate) dissolved in PBS buffer were added and the mixture was incubated for 15 min. The 96-well microplate was measured at 475 nm.

Elastase enzymatic assay: Elastase protocol monitors the release of *p*-nitroaniline from N-succinyl-Ala-Ala-Ala-p-nitroanilide that is stimulated by elastase. Elastatinal (IC50 = 0.5 μg/mL) was used as a positive control. In a 96-well microplate, 70 μL of Trizma buffer (50 mM, pH = 7.5), 10 μL of tested sample (dissolved in Trizma buffer) and 5 μL of elastase (0.45 U/mL) (dissolved in Trizma buffer) were mixed and incubated in dark for 15 min at room temperature. Afterwards, 15 μL of 2 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide (substrate) dissolved in Trizma buffer were added and the mixture was incubated for 30 min at 37^0C. The 96-well microplate was measured at 405 nm.

Collagenase enzymatic assay: Collagenase fragmentates the fluorescence molecule MMP2. The inhibition of the enzyme was measured concerning the reduction of the fluorescent intensity that was produced. Chlorhexidine (IC50 = 50 μM) was used as a positive control. In a 96-well dark microplate, 120 μL of Tris-HCl buffer (50 mM, pH = 7.3), 40 μL of tested sample and 40 μL of collagenase (50 μg/mL) from Clostridium histolyticum (dissolved in Tris-HCl buffer) were incubated for 10 min at 37^0C avoiding light exposure. Afterwards, 40 μL of 50.0 μM MMP2 (substrate) (MCA-Pro-Leu-Ala-Nva-DNP-Dap-Ala-Arg-NH_2) dissolved in Tris-Cl buffer were added and the mixture was incubated in dark for 30 min at 37^0C. The fluorescent intensity of 96-well microplate was measured at an excitation maximum of 320 nm and an emission maximum of 405 nm.

Hyaluronidase enzymatic assay: The inhibition activity of this enzyme was calculated inversely proportional of the production of N-acetyl-D-glucosamine. Tannic acid (IC100= 800 μM) was used as positive control. 100 μL of acetate buffer (0.1M NaCl, pH=3.5), 150 μL of tested sample (dissolved in acetate buffer) and 50 μL of hyaluronidase solution 1% w/v (dissolved in acetate buffer) were added in eppendorfs. Afterwards, 100 μL of bovine serum albumin (BSA) solution 0.2% w/v (dissolved in ddH_2O) were added in each eppendorf and incubated for 20 min at 37^0C.

Then, 50 μL of hyaluronic acid solution 0.5% w/v (dissolved in ddH_2O) were added and incubated for 60 min at 37^0C. 45 μL from each eppendorf were transferred in new eppedorfs containing 10 μL of sodium tetraborate solution 0.8 M (dissolved in ddH_2O) and heated for 3 min at 100^oC and cooled down on ice. In each tube were added 300 μL of dimethylaminobenzaldehyde (DMAB) solution (10% w/v dissolved in 10N HCl and then dissolved 10 times in acetic acid glacial) and incubated for 20 min at 37^0C. Finally, 200 μL from the last eppendorf were transferred in a 96-well microplate and measured at 586 nm.

Results and discussion

Liquid-liquid extraction of phenolic compounds from SO

Several extraction processes of phenolic compounds from SO have been reported previously, both in laboratory and large scale [17,18,21,22,40]. However, the described experimental procedures consume large amount of solvents [18,21], in some cases the sesame oil-solvent ratio is 1:8 [17], and overnight experimental tasks are needed [17,18,22,40]. Also, a lot of the proposed procedures have many steps, like solvent extraction, crystallization and saponification, working in very low (-40oC, 4oC) and high temperatures (70oC), facts leading to long experimental protocols and high energy consumption [17,18,22,40].

In order to avoid all the above-mentioned disadvantages, during the extraction process of SO phenols, two different liquid-liquid techniques were compared. Both ACE and CPE techniques use the centrifugal force in order to achieve a fast mixture and separation of the immiscible liquid phases during the extraction process [28,41]. These two techniques are characterized as green eco-friendly processes due to the low solvent and energy consumption, with industrial applications [28,42,43].

Selection of the suitable method for laboratory scale liquid-liquid extraction

The initial step for the liquid-liquid extraction process was the selection of the most suitable solvent system for the quantitative recovery of bioactive ingredients from SO. Taking advantage of the nonpolar oil nature, SO was used as ingredient of the biphasic system. This fact allowed the treatment of large amount of raw material increasing thus the process efficiency [26]. More specifically, several systems were created and tested by TLC and HPLC (Table 2). The results of this analysis demonstrated that the presence of water as a part of the polar phase (systems ES1-ES8) led to the creation of stable emulsion and thus in unsuitable biphasic systems.

Table 2: Liquid-liquid extraction system that tested on SO.

System	n-Hex	n-Hept	SO	MeOH	EtOH	H2O	AcN	H2O + 5% CH3COOH	H2O + 10% CH3COOH	BuOH	Comments
ES1	3	-	2	-	3	2	-	-	-	-	Creation of emulsion
ES2	3	-	2	-	2,5	2,5	-	-	-	-	Creation of emulsion
ES3	3	-	2	3	-	2	-	-	-	-	Creation of emulsion
ES4	3	-	2	3,5	-	1,5	-	-	-	-	Creation of emulsion
ES5	3	-	2	4	-	1	-	-	-	-	Creation of emulsion
ES6	4	-	1	3	-	2	-	-	-	-	Creation of emulsion
ES7	3	-	2	-	3	-	-	2	-	-	Creation of emulsion
ES8	3	-	2	-	3	-	-	-	2	-	Creation of emulsion
ES9	2	-	1	1	-	-	2	-	-	-	Creation of emulsion
ES10	3	-	2	1	-	-	4	-	-	-	Creation of emulsion
ES11	3	-	2	-	-	-	3	-	-	2	Separation in less than 1 min
ES12	3	-	1	-	-	-	4	-	-	1	Separation in less than 1 min
ES13	-	3	2	-	-	-	3	-	-	2	Separation in less than 1 min
ES14	-	3	1	-	-	-	4	-	-	1	Separation in less than 1 min
ES15	-	-	1	-	-	-	1	-	-	-	Separation in less than 45 sec
ES16	-	-	1	1	-	-	-	-	-	-	Separation in less than 45 sec
ES17	-	-	1	-	1	-	-	-	-	-	Separation in less than 45 sec

On the other hand, the non-aqueous biphasic systems containing mainly acetonitrile, ethanol or methanol (ES11-ES17) resulted in better separation of the two phases. The following HPLC-DAD analysis showed that the addition of butanol in the biphasic systems (ES11-ES14) (Table 3) resulted in an unsatisfactory recovery of the lignans from the feed oil phase.

Table 3: Partition coefficient of sesamin and sesamolin in system ES11, ES12, ES13 and ES14.

Extraction systems	Distribution coefficient of sesamin ($K=C_{up}/C_{low}$)	Distribution coefficient of sesamolin ($K=C_{up}/C_{low}$)
ES11	0,90	0,87
ES12	0,27	0,16
ES13	0,98	1,00
ES14	0,26	0,15

In contrast to these results the direct extraction of SO with acetonitrile, methanol or ethanol (systems ES15, ES16 and ES17 respectively) led to the better recovery of the targeted compounds. These three systems were tested using triple funnel extraction of SO with the corresponding solvent and the recovered upper phases were analyzed by TLC (Figure 2) and HPLC (Table 4).

Both techniques demonstrated that system ES15 (extraction with AcN) is the most effective in receiving the lignan fraction and thus was chosen for the liquid-liquid extraction of SO.

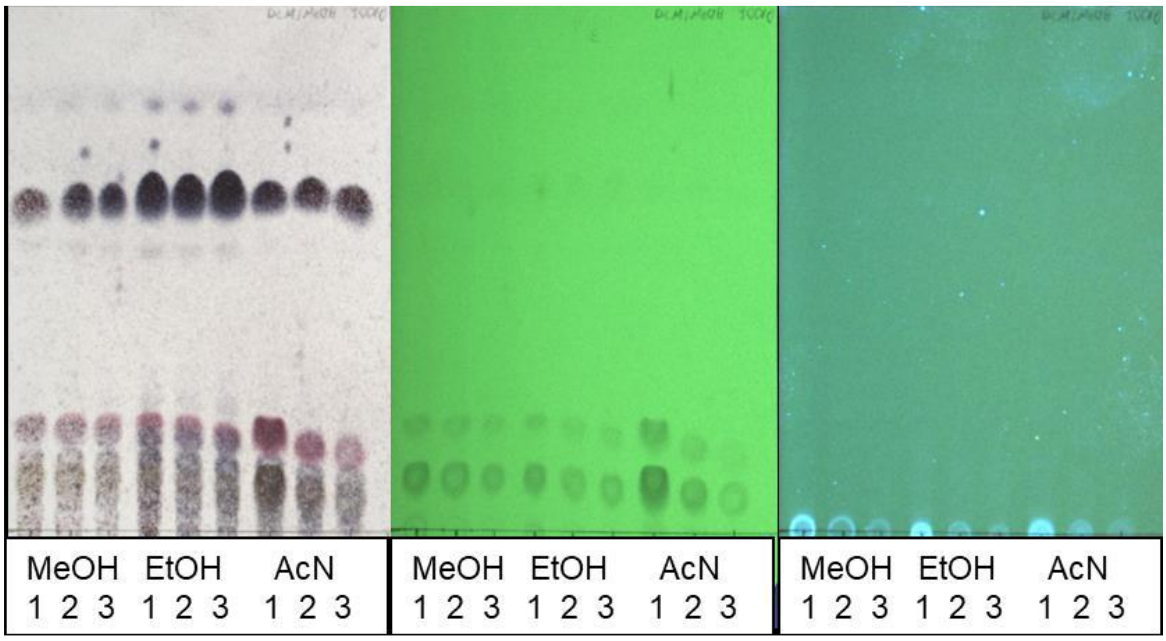


Figure 2: TLC of systems ES15-ES17 in visible sprayed with vanillin solution (left photo), in 254 nm (middle photo) and in 366 nm (right photo).

Table 4: Area on HPLC chromatographs of sesamin and sesamolin in each of triple extraction for solvent systems ES15-ES17.

Extractions	Area of sesamin's peak	Area of sesamolin's peak
ES15 1th	13475090	8538380
ES15 2th	6255833	2946539
ES15 3th	2222615	945313
ES16 1th	6063140	2441181
ES16 2th	4429955	1837144
ES16 3th	3785633	1610077
ES17 1th	2177219	2177219
ES17 2th	1980610	1980610
ES17 3th	1547120	1547120

Liquid-liquid extraction using ACE.

ACE is a liquid-liquid extraction technique with numerous advantages such as high mass transfer coefficient, high inter facial areas, low solvents consumption and flexible phase ratios [44]. In order to find the critical parameters for the analytical scale ACE extraction of SO using acetonitrile it was necessary to standardize the solvents flow rate and rotor speed. After several trials was found that the flow rate of SO should be lower than 14 mL/min and the rotation of the annular rotor at 3800-4050 rpm. Under these conditions the two phases of the biphasic system are mixed and separate rapidly into the extraction bowl eliminating thus the formation of an emulsion that affects the quality of the extraction. The first experiment aimed to check the extraction efficiency using acetonitrile as extraction solvent. For this purpose, 200 mL of SO were extracted with 600 mL of acetonitrile in three successive cycles (200 mL in each cycle). The experiment lasted 75

min and totally 2.02 g of SO extract were obtained. The quantitative HPLC analysis of the obtained extract showed the present of 1.05 g of sesamin and 0.36 g of sesamolin (5.25 mg of sesamin and 1,80 mg of sesamolin per mL of SO) (Table 5). In order to reduce the process time and to increase the efficiency of the method the ACE extraction was repeated. The main difference from the previous experiment was that the flow rate of AcN was increased three times (24 mL/min), replacing the triple extraction. Thus, 200 mL of SO were extracted with 600 mL of AcN in a single run and in total time of 25 min. The procedure resulted in the recovery of 1.68 g of extract which contains 0.84 g of sesamin and 0.29 g of sesamolin (Table 5).

Table 5: Comparison of two extraction techniques used for the treatment of SO in regard to yield, time and solvents consumption

Extraction Technique	SO volume	Extraction Time	Yield g/200mL SO	Solvent consumption	Yield sesamin	Yield sesamolin
ACE	i. 200 mL	i. 75 min	i. 2.02	i. 600 mL AcN	i. 1.05 g	0.36 g
	ii. 200 mL	ii 25 min	ii.1.68	ii. 600 mL AcN	ii. 0.84 g	0.30 g
CPE	a. 200 mL	44 min	1.97	240 mL AcN	1.01 g	0.35 g
	b. 200 mL	44 min	2.01	240 mL AcN	1.03 g	0.36 g
	c. 200 mL	44 min	1.94	240 mL AcN	0.96 g	0.34 g

As a result, it was observed the continuous receiving of lignans but reduced recovery. At these conditions, 4.20 mg of sesamin and 1.50 mg of sesamolin were obtained from each mL of SO. It is important to note that is the first time that ACE technique was applied for the extraction of bioactive compounds, not only from SO, but generally from edible oils.

Liquid-liquid extraction using CPE

The experiment was repeated successfully using multi-dual mode method. SO was used as stationary phase while acetonitrile (mobile phase) was pumped through the SO in ascending mode. After passing approximately one column volume (240 mL) of mobile phase the experiment stopped and the collected fractions (12 fractions of 20 mL) ware analyzed by TLC.

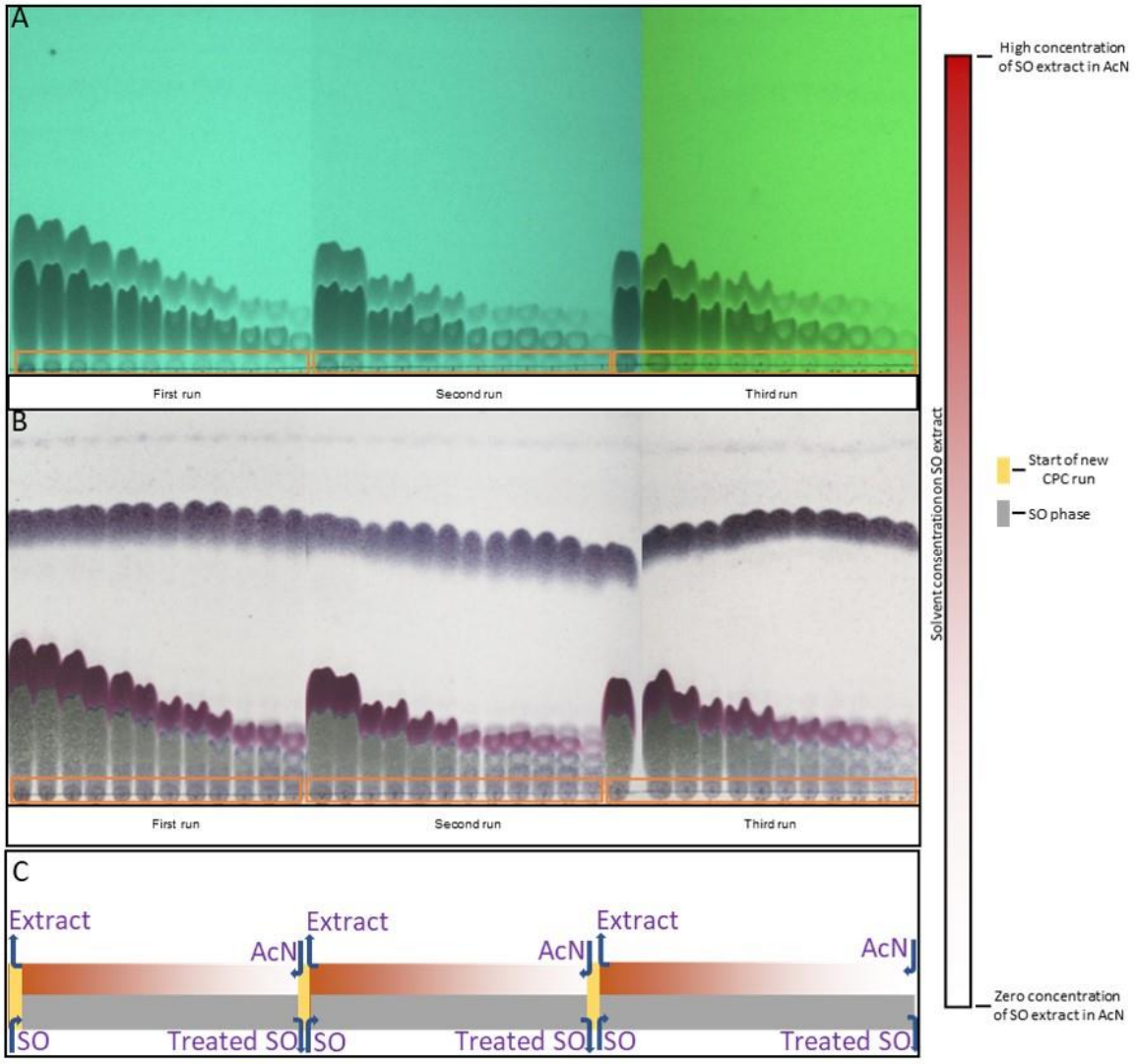


Figure 3: TLC analysis of CPE fractions from three continuous runs in 254 nm (A) and in visible sprayed with vanillin solution (B). Schematic presentation of concentration of extraction solvent in lignans during the extraction process (C).

As it is observed in the TLC analysis of CPE fractions (Figure 3), the first two fractions are fully enriched in SO extract. Thereafter, the next four fractions are highly concentrated, while the following fractions have a decreasing amount of SO extract. It has to be noted that even the appliance of concentrated spots on the TLC plate, the final fraction provides a negligible amount of the extract. The fact that after twelve fractions we obtained the total amount of SO extract, proves the effectiveness of AcN as extraction solvent. Moreover, it is important to underline the repeatability of CPE technique. The above procedure was repeated two more times by replacing each time the treated SO with the fresh one in descending mode (multi-dual mode process). All the repetitions (three runs) provide the exact same phenomenon, a total recovery of lignans' extract after 12 fractions. The fractions of each run were combined, evaporated under vacuum and weighted, yielding 1.97, 2.01 and 1.94 g respectively. The quantitative HPLC analysis of the above extracts reveals that sesamin constituted approximately the 50% (1.01, 1.03 and 0.96 g) of total

extract, while sesamolin was included also in high amount of approximately 18% (Table 5, repetitions a, b and c).

Overall, the first experiment (i) of ACE provided an adequate extract yield, but with high solvent (ratio SO/AcN 1/3) and time consumption. Concerning the second ACE experiment (ii), the experimental time decreased at 1/3, but the solvent consumption remained the same, while the yield decreased for 17%. Although, CPE gave a high amount of extract after reasonable time, while the solvent needs decreased almost 3 times. In detail, after 44 minutes and with an extraction ratio 1/1.2 of SO-AcN, CPE technique is able to obtain the total extract of sesame oil. The above seems to lead at the conclusion that CPE is the best laboratory scale extraction solution, because it is efficient on yield, time and solvent consumption, with high repeatability. It should be highlighted that this technique was applied for first time, concerning the recovery of lignan' fraction from SO. According to UPLC-HRMS analysis of SO extract, sesamin and sesamolin were detected in the SO extract. Sesamin molecular ion was 355.1176 m/z and sesamolin molecular ion was 371.1142 m/z in positive mode. Also, other minor lignans as well as fatty acids were detected (Table 6).

Table 6. HPLC-HRMS (orbitrap) data of sesame oil total liquid-liquid extraction sample.

Rt	Experimental mass	Theoretical mass	Delta (ppm)	Molecular formula	RDB	ESI	Comments
8.76	137.0244	137.0246	1.625	$C_7 H_4 O_3$	5.5	-	sesamol
10.32	249.0767	249.0768	-0.5135	$C_{13}H_{14}O_5$	7.5	-	samin
10.55	371.1126	371.1125	0.2991	$C_{20}H_{18}O_7$	11.5	+	Sesaminol/episesaminol
11.88	371.1142	371.1125	4.5752	$C_{20}H_{18}O_7$	11.5	+	Sesamolin
12.88	355.1176	355.117	0.0859	$C_{20}H_{18}O_6$	11.5	+	sesamin
20.89	279.2325	279.2319	2.1332	$C_{18}H_{32}O_2$	3.5	-	Linoleic acid

Pilot scale ACE extraction

In natural products field the conduction of *in vivo* experiments is a demanding task due to the high amounts of extract required for such experiments. Thus, the aim of this extraction was the obtainment of high amounts of SO extract, in a short period of time. For this reason, a pilot scale ACE BXP190 was selected for the extraction of 90 L SO. BXP190 has the capability to manage flow rates up to 53 L/min with maximum rotor speed at 2900 rpm. In order to avoid the emulsion and to have a factional disperse of the biphasic system (SO-DMSO/H_2O), the apparatus was not used at the manufacture limits. After 1.5 h, 90 L of SO were extracted with 90 L DMSO/H_2O (9:1 v/v). As it is already mentioned, two different procedures were compared in order to use the most efficient. At the first one, 5 L of the extract were diluted in H_2O and extracted with EtOAc yielding 77.64 g. Because the extract was obviously full of lipids, was defatted with final weight of 12.86 g. For the second one, 5 L of extract were diluted in 5 L of H_2O and passed through XAD-7. The resin extract was evaporated and weighted 18.41g. As it is noticed, the second method produced higher amount of final extract and finally the rest 80 L of DMSO extract were treated with resin

XAD-7, providing final yield of 785 g. Both extracts were analyzed with HPLC giving the same profile (Figure 4).

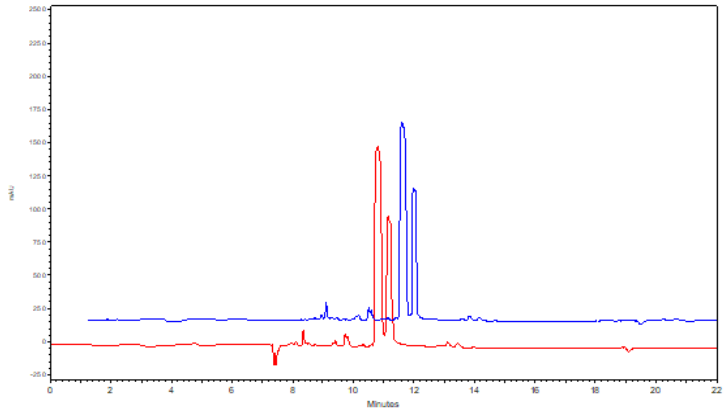


Figure 4: Superimposed HPLC-DAD chromatograms of sesame oil extract from laboratory scale process (red) and sesame oil extract from pilot scale process (blue) at 280 nm.

Separation of sesamin and sesamolin from crude extracts by CPC

Study of the CPC solvent systems

Crucial step in the innovated chromatographic process of CPC was to find the biphasic system needed for the separation (Table 1) and then for the distribution of the target compounds by using TLC analysis. Based on this test, systems CS9, CS10, CS11, CS12 and CS16 were rejected because they do not meet the required specifications (Table 1) while at systems CS1, CS2, CS14 and CS15 were observed unsatisfactory distributions of sesamin and sesamolin in the TLC chromatograms. Seven biphasic systems (CS3-CS8 and CS17) were further investigated using HPLC in order to calculate the partition coefficient values (K_Ds) of the target compounds and thus the suitability of the biphasic systems (based on the values of the separation factor α, which follow the rule K_{D1}/K_{D2}, $K_{D1} \geq K_{D2}$). The results of this analysis are given in Table 2.

The study of K_Ds values and separation factors showed that five of the tested biphasic systems (CS3, CS6, CS7, CS8 and CS17) meet the criteria for a satisfactory separation of the target compounds ($\alpha > 1.5$). Given that, the higher value of α enables better separation of the two compounds and the treatment of higher amount of extract, system CS7 (n-Hex/EtOAc/EtOH/H_2O in proportion 2:3:3:2 v/v/v/v) seems to be the most effective ($\alpha = 2.78$) and thus this system was chosen for the CPC analysis of the SO extract.

Purification of lignans by semi-preparative FCPC200® and the scale-up operation on a preparative FCPC1000®

The capability of the selected method (elution – extrusion) and biphasic system (*n*-Hex/EtOAc/EtOH/H_2O in proportion 2:3:3:2 v/v/v/v) to efficiently isolate the two major lignans

of SO extract was initially tested in semi–preparative column. Except the phenolic part, SO extract contains high amount of nonpolar fatty compounds such us glycerides and fatty acids. Due to this nonpolar nature of the extract the experiment was run in reverse mode by using as stationary phase the upper (nonpolar) phase of the system. This fact stabilizes the fatty compounds at the beginning of the column (due to the close affinity with the nonpolar phase) eliminating thus their co-elution with sesamin and sesamolin. After equilibrating the two phases into the column (Sf = 52.5%),110 mg of crude SO extract were injected via a 10 mL injection loop. The elution step was completed by passing 350 mL of the aqueous mobile phase in descending mode and then the column content was extruded by passing 200 mL of the upper stationary phase also in descending mode. The experiment lasted 115 min while the separation process was monitored by UV at 255, 275, 288 and 320 nm (Figure 5A). All resulting fractions (55 fractions of 10 mL) were analyzed using TLC and fractions with similar chemical composition were put together. The result of this analysis was the recovery of 31.6 mg of sesamin and 14.1 mg of sesamolin both in a purity higher than 95% as this was calculated from the quantitative HPLC analysis.

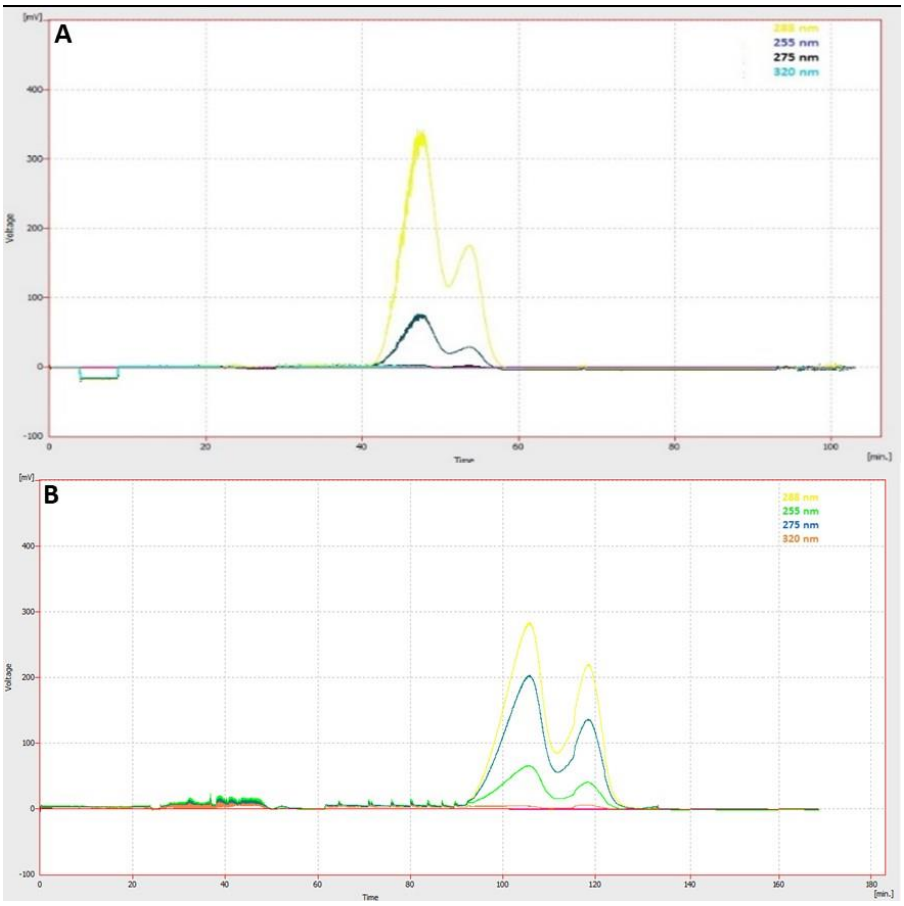


Figure 5: UV Chromatogram (λ=288, 275, 255 and 320 nm) of semi-preparative elution-extrusion CPC (A) in comparison to preparative elution-extrusion CPC (B), indicating the better separation of the two main lignans during the scaling up from semi preparative to preparative mode. Biphasic solvent system: n-Hex/EtOAc/EtOH/H_2O in proportion 2:3:3:2 (v/v/v/v).

The result obtained from semi-preparative analysis being very promising thus separation was scaled-up to preparative CPC mode. The scaling-up from 200 mL column (semi-preparative) to 5-fold larger, 1L CPC column (preparative) can be easily applied paying particular attention on rotational speed and flow-rate, two parameters that effect on stationary phase retention [45]. After equilibrating the two phases into the column, 900 mg of SO extract were injected. The elution step of the experiment was completed after passing 1600 mL of aqueous mobile phase in descending mode and then the column was extruded by pumping 1000 mL of the upper stationary phase also in descending mode. The CPC procedure was monitored with a UV detector and the chromatogram (at 255, 275, 288 and 320 nm), presented in Figure 5B, shows a recovery of the target compounds according to their K_Ds values in the used biphasic system. By setting the rotational speed at 750 rpm and the flow rate of the eluent at 15 mL/min the retention factor of stationary phase (Sf) was approx. 65%, much higher than Sf calculated for the semi−preparative CPC experiment (52.5%). Higher retention of the stationary phase led to higher theoretical plate number and thus in better fractionation of the extract. Indeed, the comparison of the CPC chromatograms obtained from semi-preparative and preparative analysis revealed that the preparative process resulted in better separation of the two major lignans (Figure 5AB).

The preparative CPC process lasted 170 min while fraction collector was set to collect 20 ml fractions (total 130 fractions). All fractions were analyzed by TLC in order to check the quality of the separation. The analysis showed that the lignans were recovered during the elution step of the experiment while the fatty compounds were collected in the last fractions of the experiment during the extrusion of the column content. The fractions containing sesamin (fractions 51 – 64) and sesamolin (fractions 64 – 75) were subjected to quantitative HPLC analysis. The result of this analysis (presented as fractogram in Figure 6) showed that fractions 51-63 and 65-75 contain only sesamin and sesamolin respectively while only one fraction (64) contains a mixture of both compounds. Sesamin fractions were pooled and evaporated to dryness, yielding 276.07 mg, while the combined sesamolin fractions yielded 138.15 mg. The following UPLC-HRMS, NMR and quantitative HPLC analysis of combined fractions showed that both sesamin and sesamolin were isolated in high purity (>95%) and good recovery (61.3% and 87.7 % of the total amount of their

SO extract content, respectively) verifying the efficient separation of these two bioactive compounds by using the proposed preparative elution-extrusion CPC method.

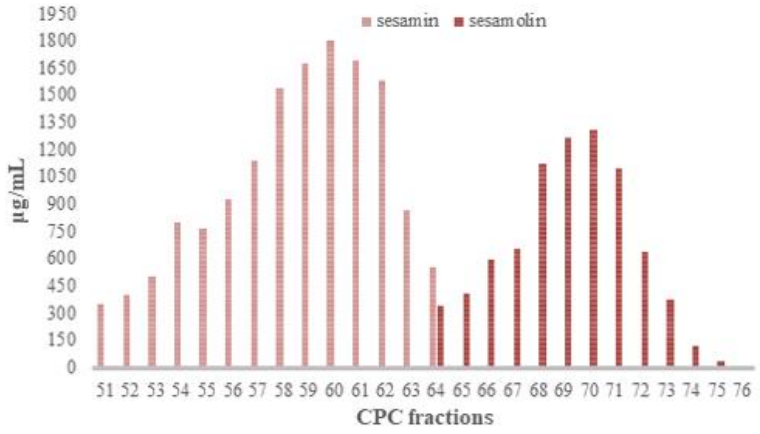


Figure 6: Fractogram obtained from quantification analysis of preparative CPC fractions 51-76.

Except the isolation of the two major lignans, the preparative CPC analysis led to the additional separation of four minor lignans of SO extract i.e. samin, sesamol, sesaminol and episesaminol (Figure 1). In more details, fractions 13-18 (4.3 mg) samin, fractions 22-31 (9.4 mg) sesamol while fractions 35-39 (8.2 mg) and fractions 40-49 (6.6 mg) contained a mixture of sesaminol and episesaminol in ratios of approximately 85/15 and 45/55 respectively. It is important to note that samin and sesamol were recovered in one step separation procedure in high purity as this was determined by ^{1}H-NMR analysis (Figure 7a-11d).

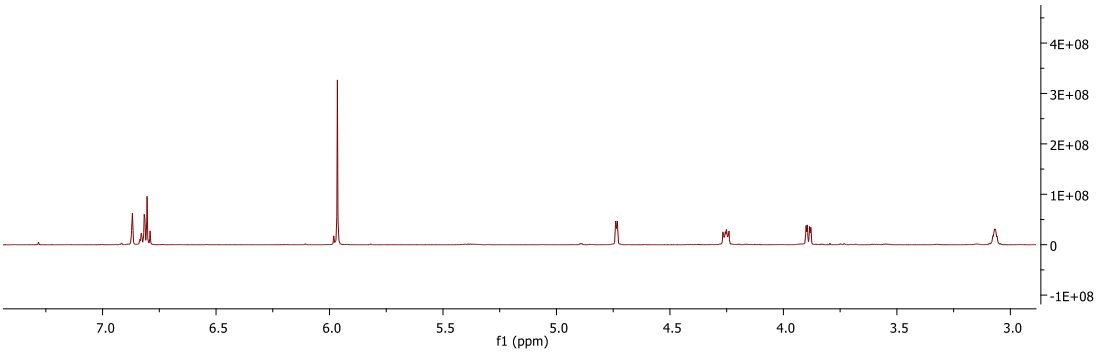


Figure 7: a) ^{1}H NMR spectra of sesamin in $CDCl_3$.

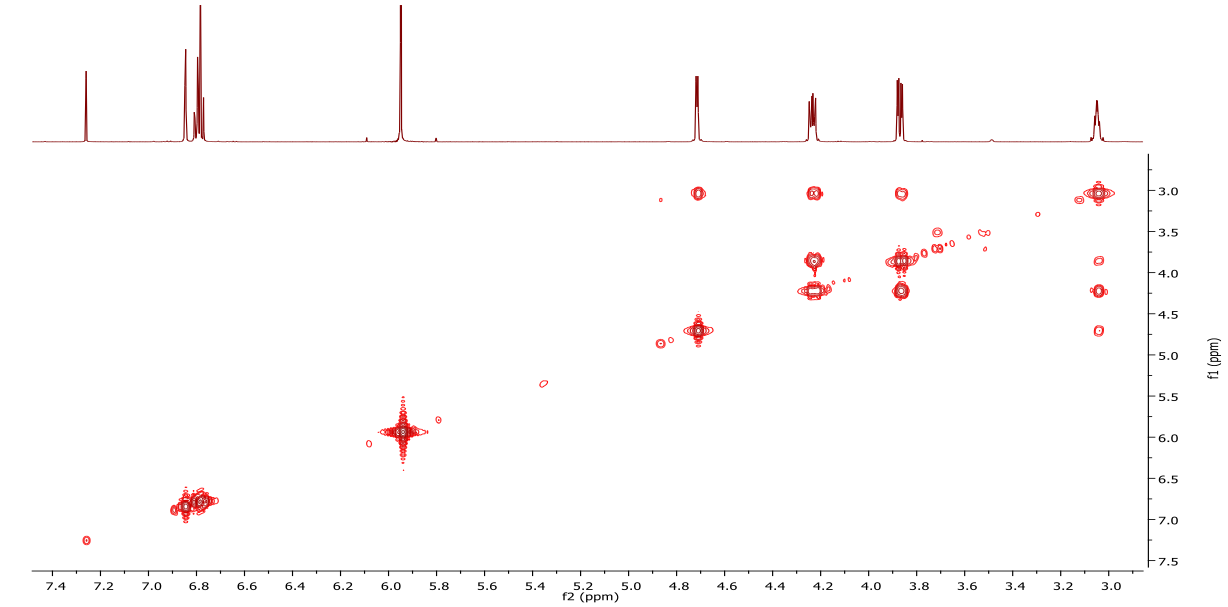


Figure 7: b) 2D COSY NMR spectra of sesamin in $CDCl_3$.

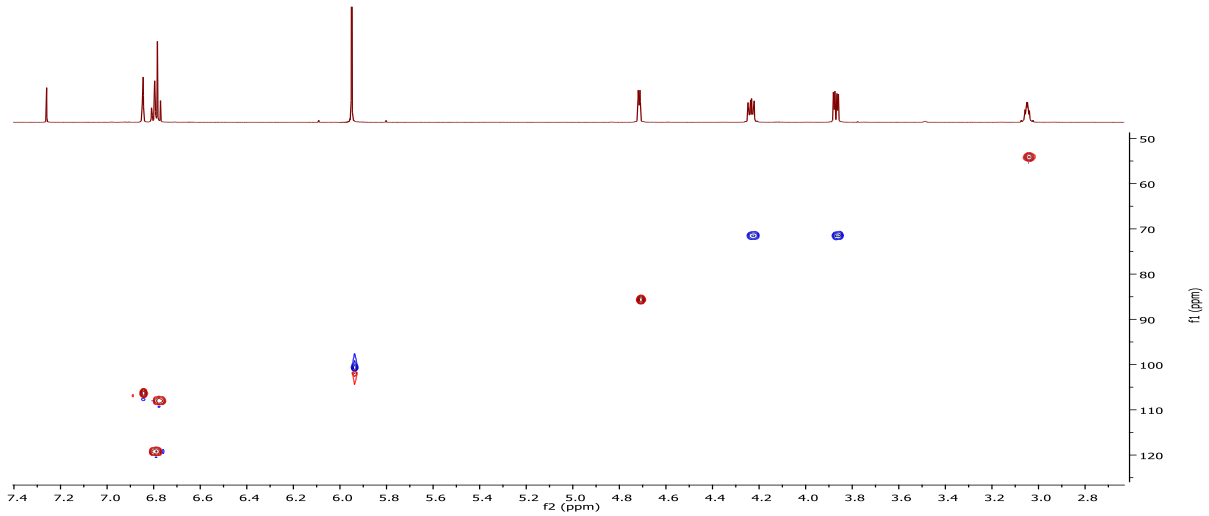


Figure 7: c) 2D HSQC-DEPT NMR spectra of sesamin in $CDCl_3$.

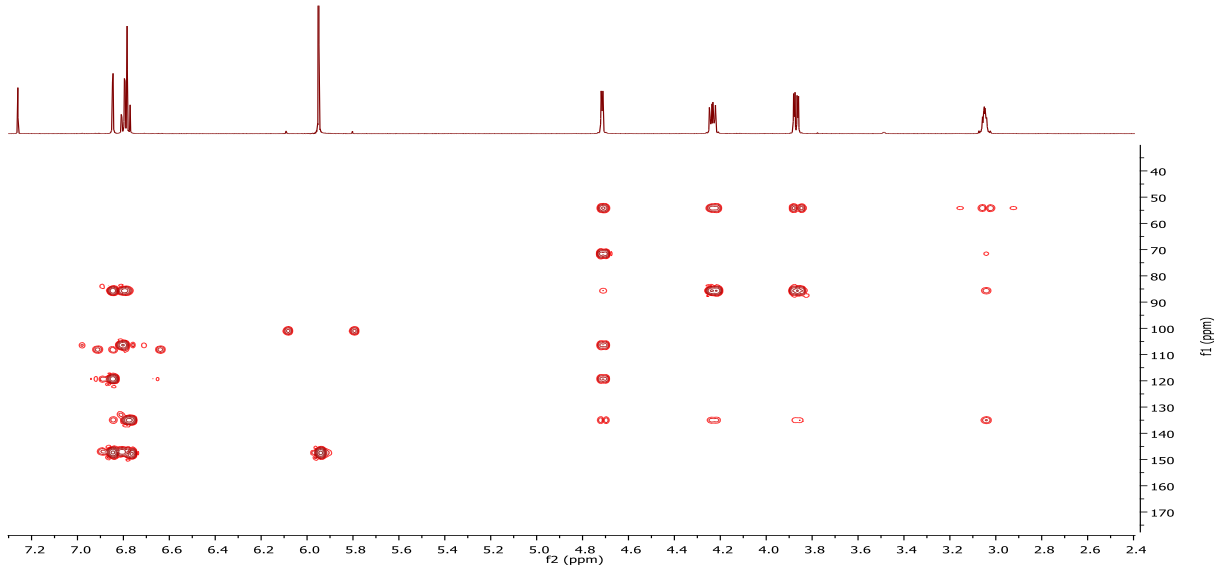


Figure 7: d) 2D HMBC NMR spectra of sesamin in $CDCl_3$.

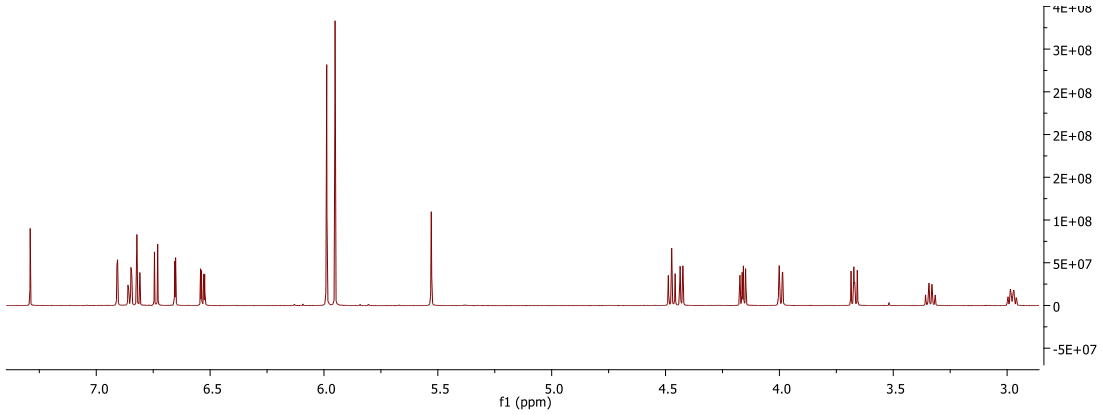


Figure 8: a) ^1H NMR spectra of sesamolin in $CDCl_3$.

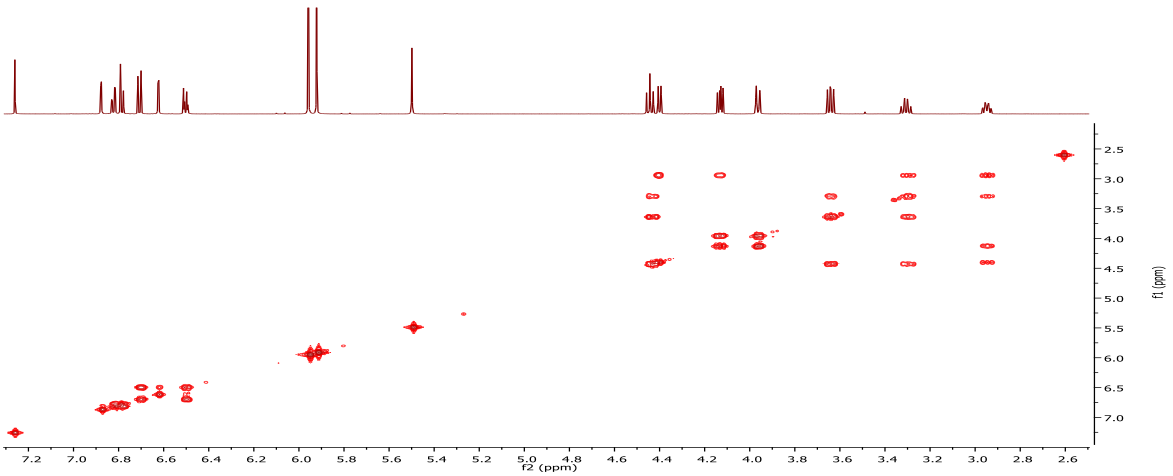


Figure 8: b) 2D COSY NMR spectra of sesamolin in $CDCl_3$.

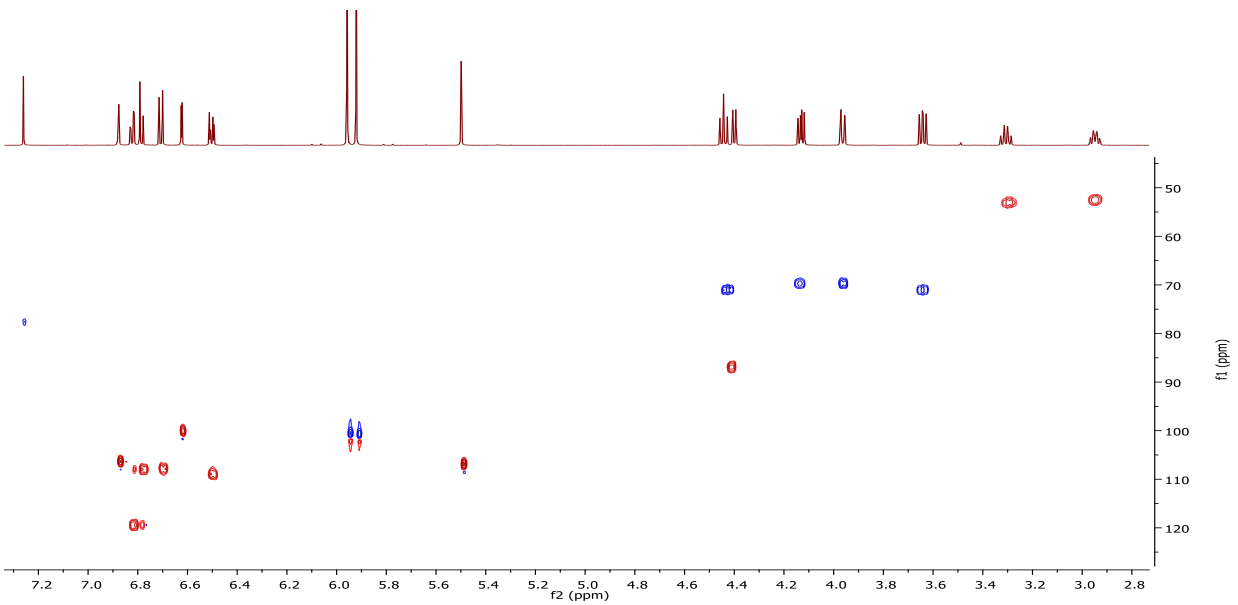


Figure 8: c) 2D HSQC-DEPT NMR spectra of sesamolin in $CDCl_3$.

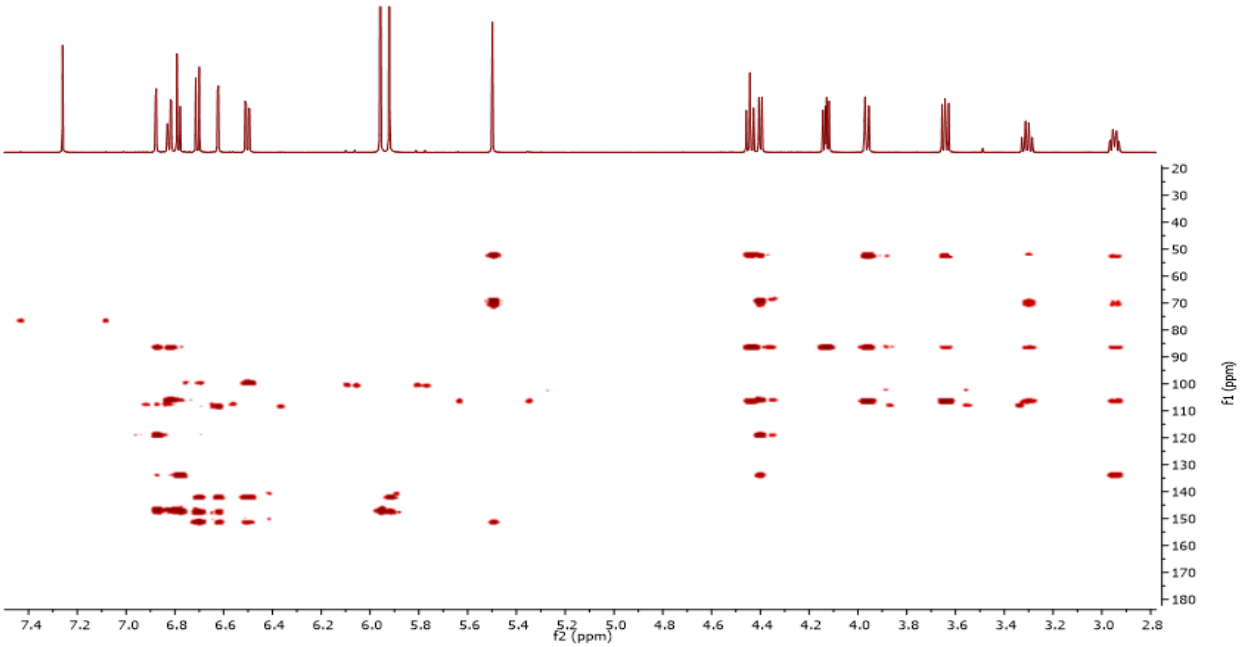


Figure 8: d) 2D HMBC NMR spectra of sesamolin in $CDCl_3$.

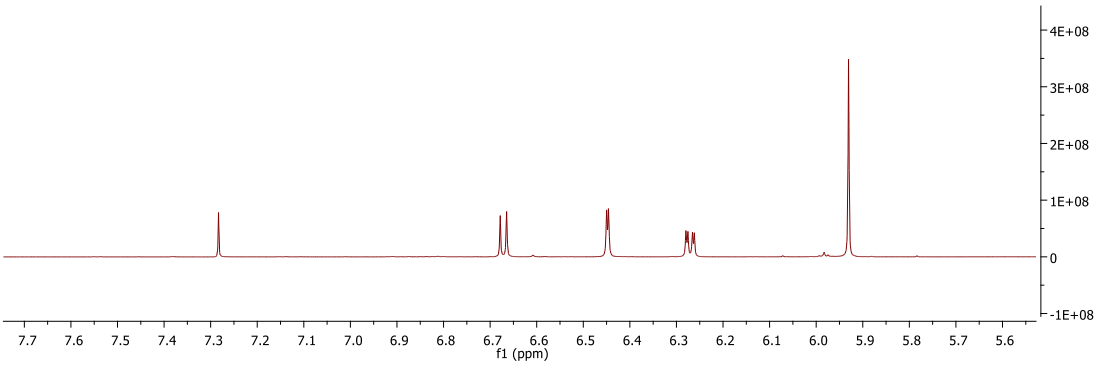


Figure 9: ^1H NMR spectra of sesamol in $CDCl_3$.

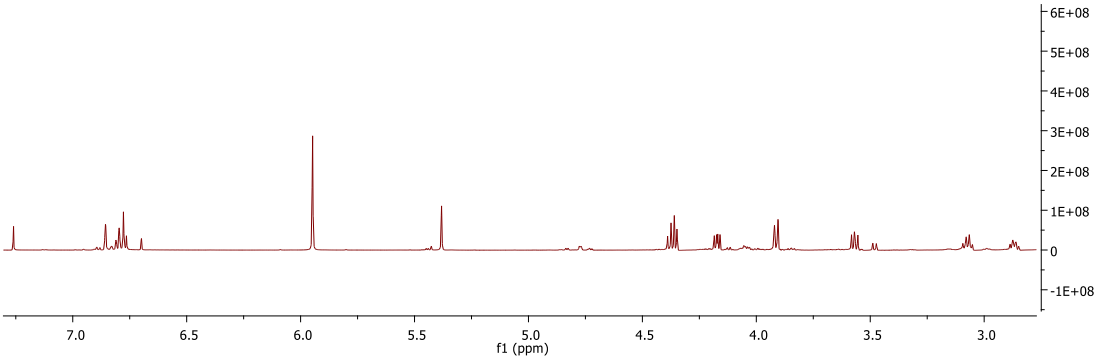


Figure 10: a) ^{1}H NMR spectra of samin in $CDCl_3$.

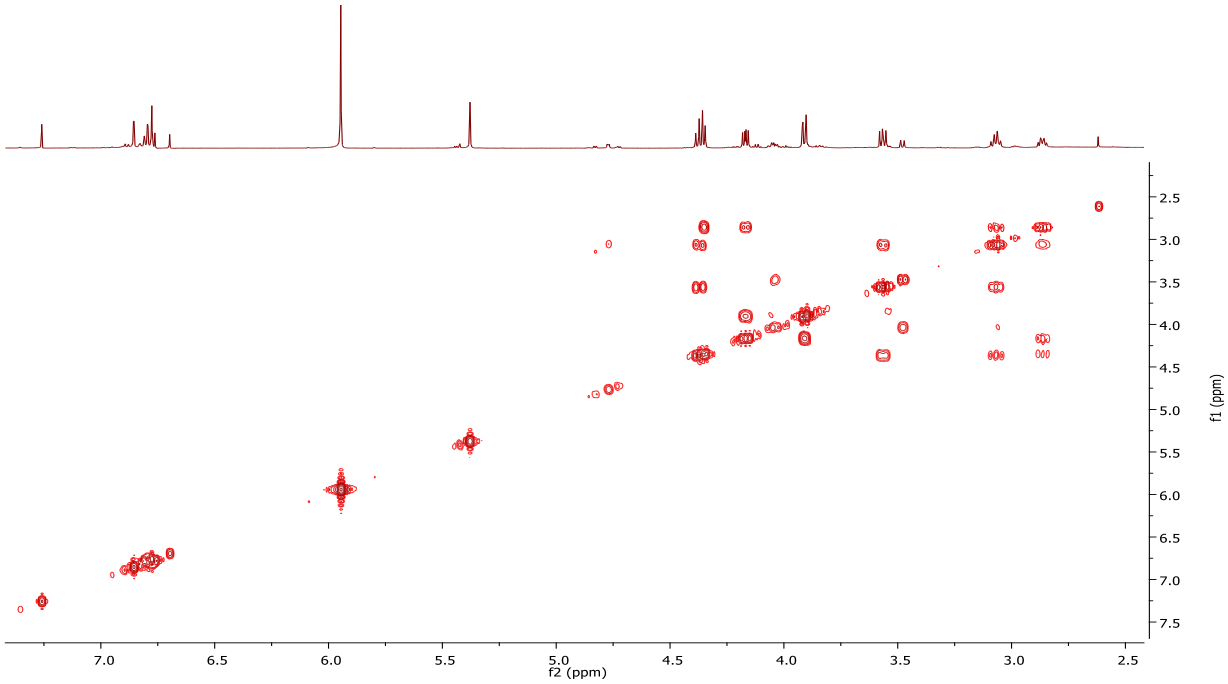


Figure 10: b) 2D COSY NMR spectra of samin in $CDCl_3$.

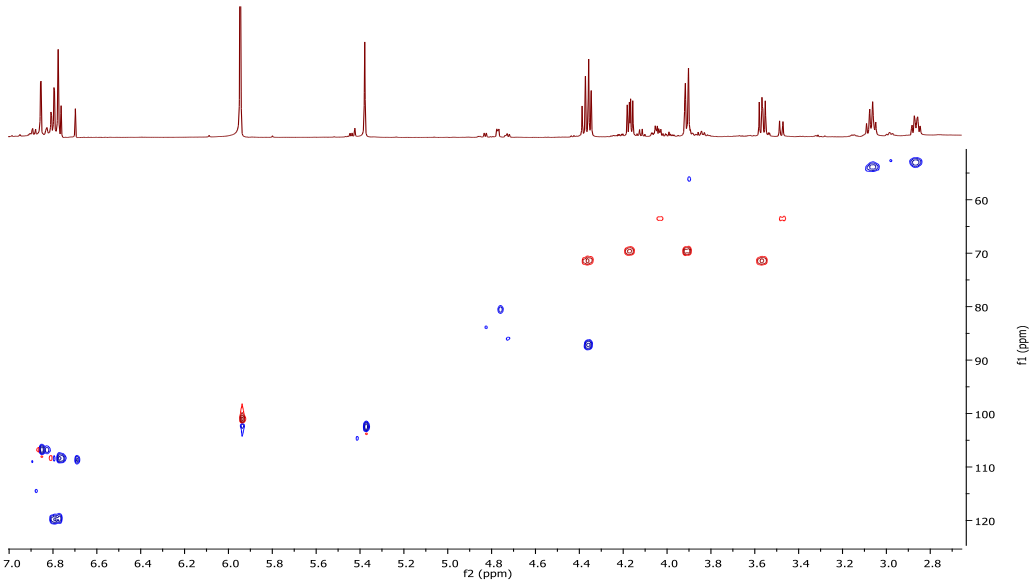


Figure 10: c) 2D HSQC-DEPT NMR spectra of samin in $CDCl_3$.

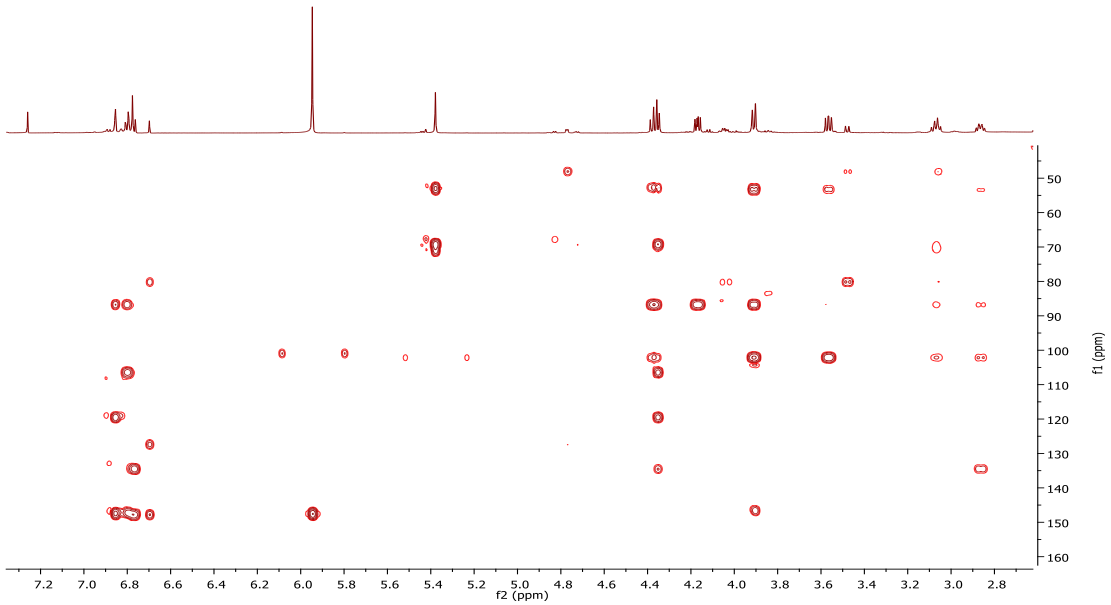


Figure 10: d) 2D HMBC NMR spectra of samin in $CDCl_3$.

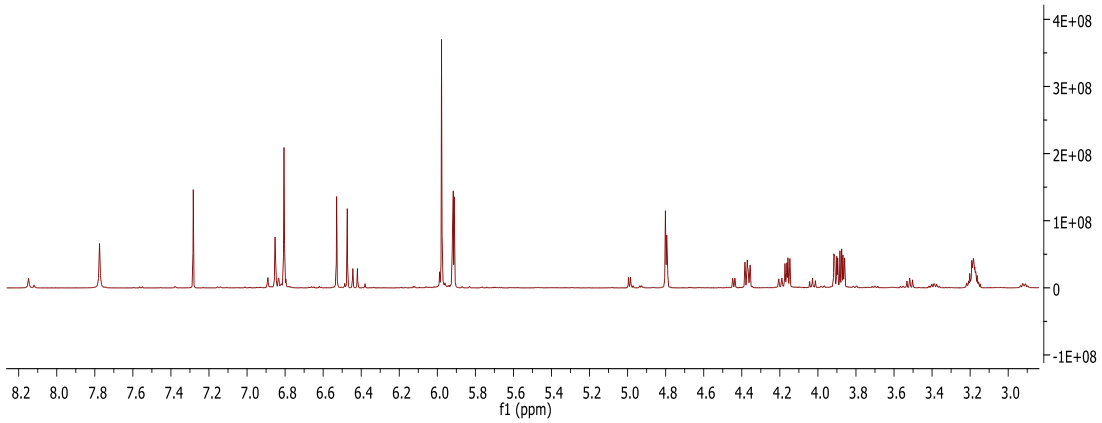


Figure 11: a) ^1H NMR spectra of sesaminol-episesaminol in $CDCl_3$.

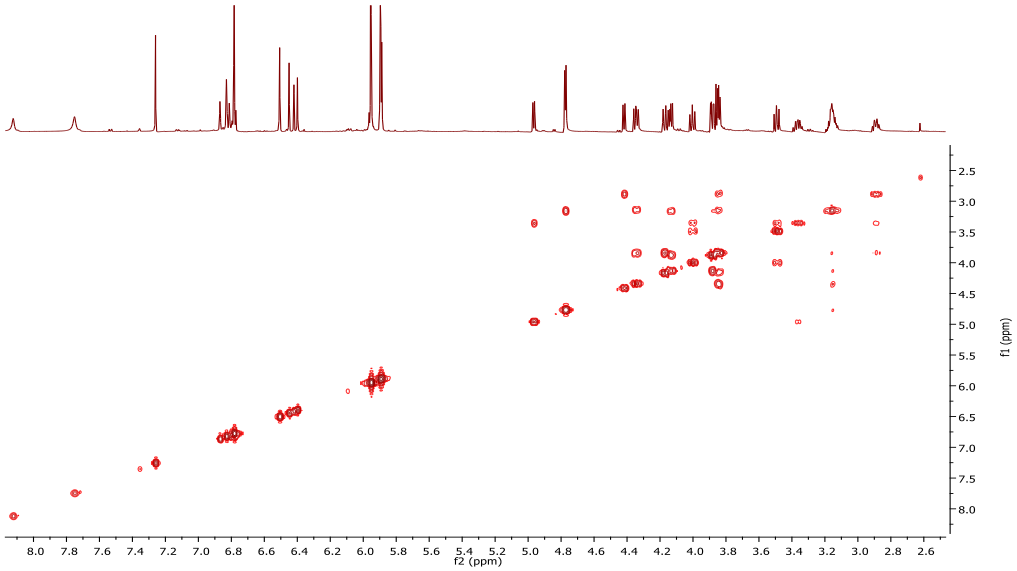


Figure 11: b) 2D COSY NMR spectra of sesaminol-episesaminol in $CDCl_3$.

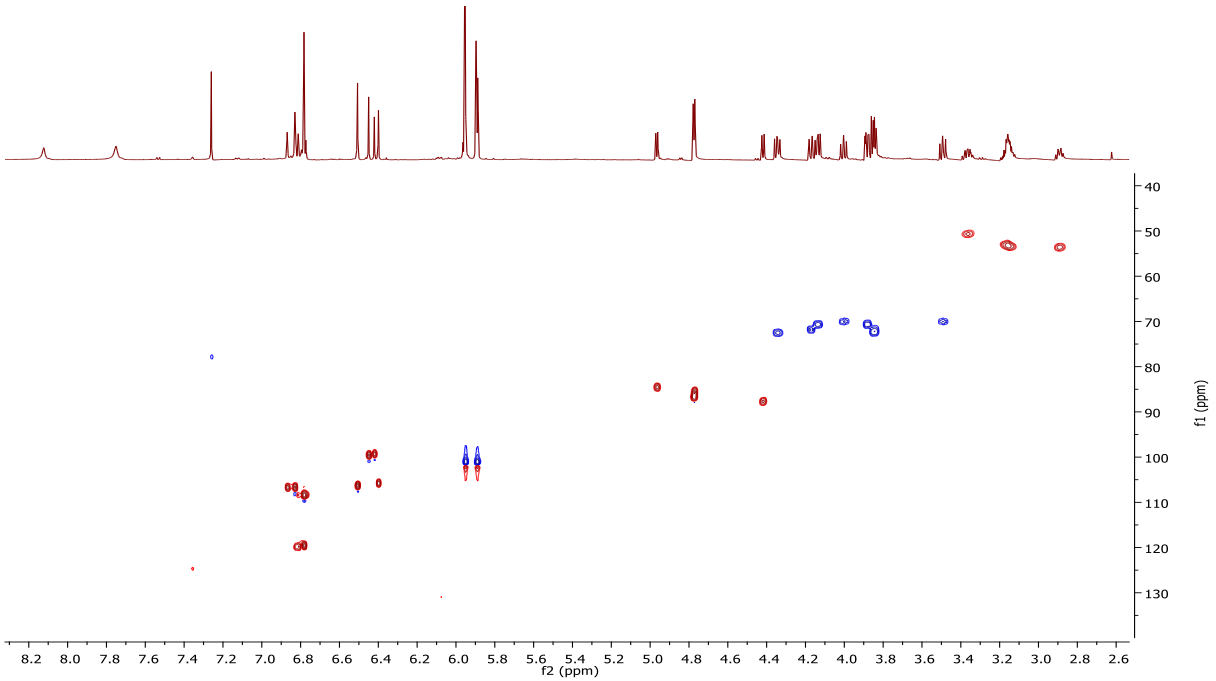


Figure 11: c) 2D HSQC-DEPT NMR spectra of sesaminol-episesaminol in $CDCl_3$.

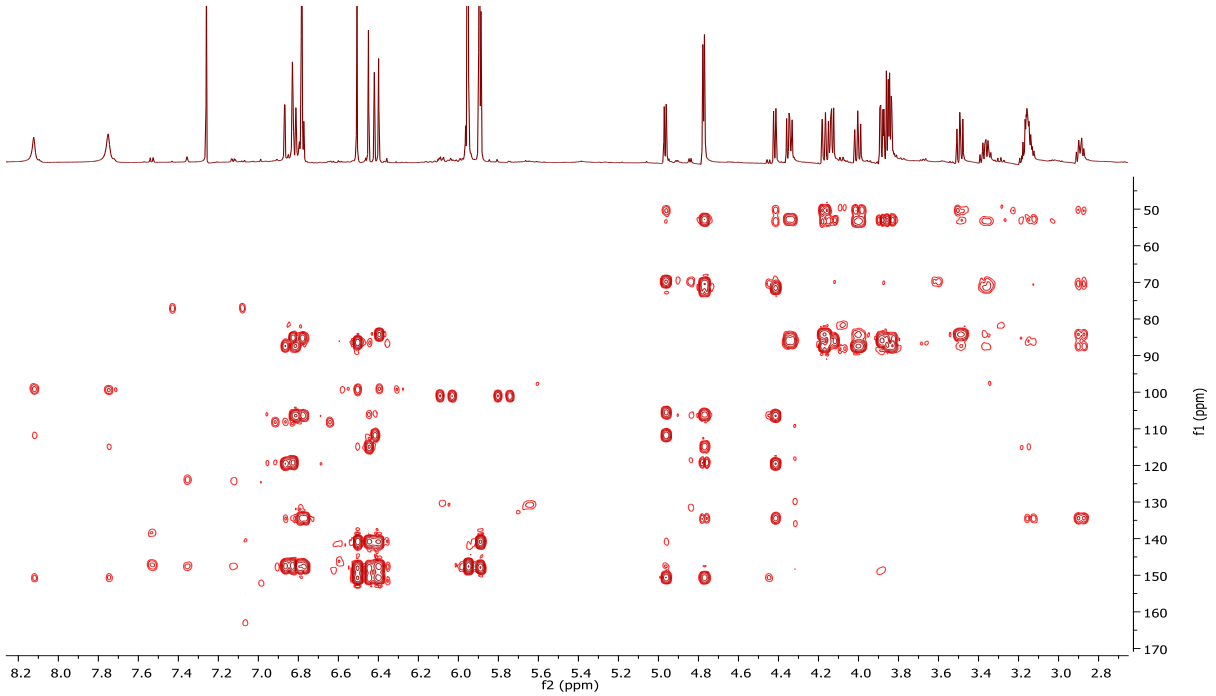


Figure 11: d) 2D HMBC NMR spectra of sesaminol-episesaminol in $CDCl_3$.

The structure elucidation of the isolated compounds was achieved by studying HRMS/MS and NMR (1D and 2D) spectra and verified by comparison the experimental data with the corresponding bibliographic data [40,46–50]. Experimental data of ^{1}H and ^{13}C NMR of the isolated compounds are referred at Table 7.

Table 7. NMR signals of sesamin, sesamolin, sesamol, samin, sesaminol and episesaminol.

Protons	Sesamin	sesamolin	sesamol	samin		sesaminol		Episesaminol	
	^{1}H (ppm)	^{1}H (ppm)	^{1}H (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
1	**3.05**, m	**2.95**, m		**2.86**, m	**53.14**	**3.16**, m	**53.3**	**2,88**, m	**53.6**
2	**4.71**, d J= 4.5 Hz	**4.40**, d J=7.1 Hz		**4.35**, d J=6.8 Hz	**87.34**	**4.77**, m	**85.5**	4.42, d J=7.0 Hz	**87.8**
3			**6.43**, d J=2.3 Hz						
4a	**4.23**, dd J= 9.2, 6.9 Hz	**4.44**, t J=9.0 Hz		**4.38**, t J=9.1 Hz	**71.64**	**4.34**, dd J=9.4, 7.5 Hz	**72.6**	**4.17**, brd J=9.5 Hz	**71.9**
4b	**3.86**, dd J= 9.2, 3.8 Hz	**3.64**, dd J=9.2, 7.4 Hz		**3.57,** dd J=9.1, 7.4 Hz	**71.65**	**3.85**, dd J=9.4, 5.3 Hz	**72.6**	**3.85,** m	**71.9**
5	**3.05**, m	**3.31**, m	**6.25**, dd J=8.4, 2.3 Hz	**3.07**, m	**54.08**	**3.16**, m	**53.3**	**3.36**, m	**50.8**
6	**4.71**, d J=4.5		**6.65**, d J=8.4 Hz	**5.38**, bs	**102.6**	**4.78**, bs	**86.7**	**4.96**, d J=5.0 Hz	**84.6**
8a	**4.23**, dd J= 9.2, 6.9 Hz	**4.13**, dd J=9.2, 5.9 Hz		**4.17**, dd J=9.1, 5.9 Hz	**96.84**	**4.14**, dd J=9.4, 2.4 Hz	**70.6**	**4.00**, d J=9.0 Hz	**70.4**
8b	**3.86**, dd J= 9.2, 3.8 Hz	**3.96**, dd J=9.1, 0.8 Hz		**3.91**, bd J=8.5 Hz	**96.84**	**3.88**, dd J=9.4, 2.4 Hz	**70.6**	**3.49**, d J=9.0 Hz	**70.4**
2′	**6.85**, d J=1.5 Hz	**6.88**, d J=1.7 Hz		**6.86**, d J=1.5 Hz	**106.85**	**6.83**, brs	**106.7**	6.86, brs	**106.8**
5′	**6.77**, d J=8.1 Hz	**6.78**, d J=8.0 Hz		**6.77**, d J=7.9 Hz	**108.44**	**6.78**, brs	**108.4**	**6.78**, d J=8.1 Hz	**108.4**
6′	**6.80**, dd J=8.1 Hz	**6.82**, dd J=8.0, 1.7 Hz		**6.80**, dd J=7.9, 1.5 Hz	**119.91**	**6.78**, brs	**119.5**	**6.81**, brd J=8.1 Hz	**119.9**
2′′	**6.85**, d J=1.5 Hz	**6.62**, d J=2.3 Hz				**6.0**, s	**106.4**	**6.40**, s	**105.9**
5′′	**6.77**, d J=8.1 Hz	**6.71**, d J=8.5 Hz				**6.45**, s	**99.5**	**6.42**, s	**99.4**
6′′	**6.80**, dd J=8.1 Hz	**6.82**, dd J=8.5, 2.3 Hz							
-O-CH_2-O-	**5.95**, s		**5.91**, s	**5.95**, s	**101.15**				
-O-CH_2-O- a		**5.92**, s				**5.95**, s	**101.0**	**5.95**, s	**101.0**
-O-CH_2-O- b		**5.96**, s				**5.89**, s	**101.1**	**5.89**, s	**101.1**
-OH						**7.75,** brs	**150.8**	**8.12**, brs	**150.9**

Tyrosinase, elastase, collagenase and hyaluronidase inhibition activity of SO compounds.

All isolated lignans were evaluated for their tyrosinase, elastase, collagenase and hyaluronidase inhibitory activities. For all the enzymatic assays the IC50 of positive controls was used, with only exception the hyaluronidase assay where for the positive control was used at the IC100 concentration (see experimental part).

The tyrosinase inhibition assay showed that sesamol and sesamolin are able to inhibit the enzyme activity, in contrast to sesamin, samin, sesaminol and episesaminol. In detail, sesamol exhibited an important inhibition activity at 500 μM (52.34%), while no activity was present at doses of 100 μM and 25 μM. Sesamolin presented moderate anti-tyrosinase activity at 500 μM (27.78%) and

weak activity at 100 μM and 25 μM (Figure 12). These results are in agreement with literature data reporting the potent anti-tyrosinase activity of sesamol and sesamolin [51]. The above results demonstrate a potent correlation between the structure of tested compounds and the anti-tyrosinase activity. Although sesamin, sesamolin, sesaminol and episesaminol are structurally related compounds, only sesamolin inhibited the tyrosinase activity, showing that sesamol moiety seems necessary for the enzyme inhibition.

All tasted compounds expressed important activity on collagenase assay. The sesaminol/epi-sesaminol mixture revealed the highest anti-collagenase activity with inhibition values of 91.99% at 500 μM, 71.94% at 100 μM and 40.36% at 25 μM. Sesamin presented moderate anti-collagenase activity with inhibition value of 61.16% at 500 μM, 40.77% at 100 μM and 44.71% at 25 μM. Samin revealed anti-collagenase activity with inhibition values of 65.66% at 500 μM, 40.65% at 100 μM and 40.33% at 25 μM. Sesamolin also presented moderate activity with inhibition value of 54.05% at 500 μM, 36.20% at 100 μM and 47.83% at 25 μM while sesamol, revealed the lowest anti-collagenase activity with inhibition values of 46.19% at 500 μM, 48.20% at 100 μM and 36.57% at 25 μM (Figure 12). To our knowledge, this is the first report connecting SO lignans with collagenase activity.

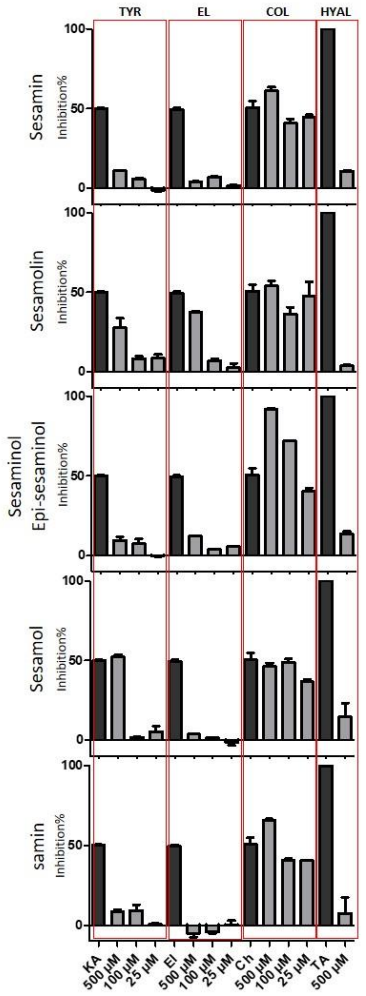


Figure 12: Tyrosinase, elastase, collagenase and hyaluronidase inhibition activity of isolated compounds. Tested concentration for tyrosinase, elastase and collagenase inhibition: 500 µM, 100 µM and 25 µM. Tested concentration for hyaluronidase inhibition: 500 µM.

Regarding the elastase and hyaluronidase inhibition assays, all the tested compounds were found to be non-effective compared to the positive controls. The only exception was sesamolin which present a moderate anti-elastase activity at the highest dose of 500 µM with inhibition value of 37.24% (Figure 12).

Conclusion

This study constitutes a holistic procedure for the swift isolation of sesamin and sesamolin in high purity, using techniques with scale up to pilot and industrial prospects. Two different approaches were used for the extraction of SO lignans, in laboratory scale, based on innovative liquid-liquid techniques, ACE and CPE, to obtain both sesamin and sesamolin in high amounts. Although, lab-scale CPE needs almost the 1/3 of solvent volume that was required from ACE in order to obtain the total extract. The green characteristic is not the only advantage of CPE. Also, this procedure is less time consuming. CPC, as the superior liquid-liquid solid support free technique can treat the

SO extract, giving high recovery of sesamin and sesamolin with purity over 95% with the minimum time consumption. The ability of CPC technique to analyze high portions of sample has as a result the isolation of other minor compounds from the SO extract in high purity. Obtaining compounds in pure form, permitted the realization of enzymatic assays. As a result, significant anti-collagenase activity was observed from all the isolated molecules. Concerning the pilot extraction of SO, a high throughput pilot line ACE was used in combination with adsorption resin XAD-7. For the first time, an innovative strategy was developed, where DMSO was used as the primary extraction solvent and adsorption resins were assisted to obtain rapidly the high amount of extract that is necessary for *in vivo* experiments.

References

[1] H. Zhang, H. Miao, L. Wang, L. Qu, H. Liu, Q. Wang, M. Yue, Genome sequencing of the important oilseed crop Sesamum indicum L., Genome Biol. 14 (2013) 1–9. https://doi.org/10.1186/gb-2013-14-1-401.

[2] J.R. Sarkis, I. Michel, I.C. Tessaro, L.D.F. Marczak, Optimization of phenolics extraction from sesame seed cake, Sep. Purif. Technol. 122 (2014) 506–514. https://doi.org/10.1016/j.seppur.2013.11.036.

[3] Food and Agriculture Organization of the United Nations (FAO), Production Statistics - Crops, Crops Processed - knoema.com, (2017). https://knoema.com/FAOPRDSC2017/production-statistics-crops-crops-processed?tsId=1023090 (accessed March 1, 2017).

[4] C.V. da S. Barbosa, A.S. Silva, C.V.C. de Oliveira, N.M.L. Massa, Y.R.F. de Sousa, W.K.A. da Costa, A.C. Silva, P. Delatorre, R. Carvalho, V. de A. Braga, M. Magnani, Effects of Sesame (Sesamum indicum L.) Supplementation on Creatine Kinase, Lactate Dehydrogenase, Oxidative Stress Markers, and Aerobic Capacity in Semi-Professional Soccer Players, Front. Physiol. 8 (2017) 196. https://doi.org/10.3389/fphys.2017.00196.

[5] K. Dossa, D. Diouf, L. Wang, X. Wei, Y. Zhang, M. Niang, D. Fonceka, J. Yu, M.A. Mmadi, L.W. Yehouessi, B. Liao, X. Zhang, N. Cisse, The Emerging Oilseed Crop Sesamum indicum Enters the "Omics" Era., Front. Plant Sci. 8 (2017) 1154. https://doi.org/10.3389/fpls.2017.01154.

[6] M. Nakai, M. Harada, K. Nakahara, K. Akimoto, H. Shibata, W. Miki, Y. Kiso, Novel Antioxidative Metabolites in Rat Liver with Ingested Sesamin, J. Agric. Food Chem. 51 (2003) 1666–1670. https://doi.org/10.1021/jf0258961.

[7] J.L. Penalvo, A. Hopia, H. Adlercreutz, Effect of sesamin on serum cholesterol and triglycerides levels in LDL receptor-deficient mice, Eur J Nutr. 45 (2006) 439–444. https://doi.org/10.1007/s00394-006-0617-8.

[8] A.A. Dar, N. Arumugam, Lignans of sesame: purification methods, biological activities and biosynthesis--a review, Bioorg Chem. 50 (2013) 1–10. https://doi.org/10.1016/j.bioorg.2013.06.009.

[9] B.-H. Chung, J.J. Lee, J.-D. Kim, D. Jeoung, H. Lee, J. Choe, K.-S. Ha, Y.-G. Kwon, Y.-M. Kim, Angiogenic activity of sesamin through the activation of multiple signal pathways, Biochem Biophys Res Commun. 391 (2010) 254–260. https://doi.org/10.1016/j.bbrc.2009.11.045.

[10] S. Ohnmacht, P. Nava, R. West, R. Parker, J. Atkinson, Inhibition of oxidative metabolism of tocopherols with ω-N- heterocyclic derivatives of vitamin E, October. 141 (2008) 520–529. https://doi.org/10.1016/j.surg.2006.10.010.Use.

[11] M.G. Obukowicz, D.J. Welsch, W.J. Salsgiver, C.L. Martin-Berger, K.S. Chinn, K.L. Duffin, a Raz, P. Needleman, Novel, selective delta6 or delta5 fatty acid desaturase inhibitors as antiinflammatory agents in mice., J. Pharmacol. Exp. Ther. 287 (1998) 157–166.

[12] K.R. Anilakumar, A. Pal, F. Khanum, A.S. Bawa, Nutritional, Medicinal and Industrial Uses of Sesame (Sesamum indicum L.) Seeds -An Overview, Agric. Conspec. Sci. Cus Agric. Conspec. Sci. Kandangath Raghavan ANILAKUMAR. 75 (2010) 159–168.

[13] J.H.Y. Wu, J.M. Hodgson, M.W. Clarke, A.P. Indrawan, A.E. Barden, I.B. Puddey, K.D. Croft, Inhibition of 20-hydroxyeicosatetraenoic acid synthesis using specific plant lignans: In vitro and human studies, Hypertension. 54 (2009) 1151–1158. https://doi.org/10.1161/HYPERTENSIONAHA.109.139352.

[14] Y. Miyahara, H. Hibasami, H. Katsuzaki, K. Imai, T. Komiya, Sesamolin from sesame seed inhibits proliferation by inducing apoptosis in human lymphoid leukemia Molt 4B cells., Int. J. Mol. Med. 7 (2001) 369–371.

[15] R. Grougnet, P. Magiatis, H. Laborie, D. Lazarou, A. Papadopoulos, A.-L. Skaltsounis, Sesamolinol glucoside, disaminyl ether, and other lignans from sesame seeds, J Agric Food Chem. 60 (2012) 108–111. https://doi.org/dx.doi.org/10.1021/jf2040687.
[16] S.-H. Park, S.-N. Ryu, Y. Bu, H. Kim, J.E. Simon, K.-S. Kim, Antioxidant Components as Potential Neuroprotective Agents in Sesame (Sesamum indicumL.), Food Rev. Int. 26 (2010) 103–121. https://doi.org/10.1080/87559120903564464.
[17] A.A. Dar, N.K. Verma, N. Arumugam, An updated method for isolation, purification and characterization of clinically important antioxidant lignans - Sesamin and sesamolin, from sesame oil, Ind. Crops Prod. 64 (2015) 201–208. https://doi.org/10.1016/j.indcrop.2014.10.026.
[18] M. V. Reshma, C. Balachandran, C. Arumughan, A. Sunderasan, D. Sukumaran, S. Thomas, S.S. Saritha, Extraction, separation and characterisation of sesame oil lignan for nutraceutical applications, Food Chem. 120 (2010) 1041–1046. https://doi.org/10.1016/j.foodchem.2009.11.047.
[19] S. Hammann, M. Englert, M. Müller, W. Vetter, Accelerated separation of GC-amenable lipid classes in plant oils by countercurrent chromatography in the co-current mode, Anal. Bioanal. Chem. 407 (2015) 9019–9028. https://doi.org/10.1007/s00216-015-9068-5.
[20] J.S. Jeon, C.L. Park, A.S. Syed, Y.M. Kim, I.J. Cho, C.Y. Kim, Preparative separation of sesamin and sesamolin from defatted sesame meal via centrifugal partition chromatography with consecutive sample injection, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1011 (2016) 108–113. https://doi.org/10.1016/j.jchromb.2015.12.062.
[21] X. Wang, Y. Lin, Y. Geng, F. Li, D. Wang, Preparative Separation and Purification of Sesamin and Sesamolin from Sesame Seeds by High-Speed Counter-Current Chromatography, Cereal Chem. 86 (2009) 23–25. https://doi.org/10.1094/CCHEM-86-1-0023.
[22] J. Lee, E. Choe, Extraction of lignan compounds from roasted sesame oil and their effects on the autoxidation of methyl linoleate, J. Food Sci. 71 (2006) 430–436. https://doi.org/10.1111/j.1750-3841.2006.00137.x.
[23] T. Michel, E. Destandau, V. Pecher, I. Renimel, L. Pasquier, P. André, C. Elfakir, Two-step Centrifugal Partition Chromatography (CPC) fractionation of Butea monosperma (Lam.) biomarkers, Sep. Purif. Technol. 80 (2011) 32–37. https://doi.org/10.1016/j.seppur.2011.04.002.
[24] A. Berthod, T. Maryutina, B. Spivakov, O. Shpigun, I.A. Sutherland, Countercurrent chromatography in analytical chemistry (IUPAC Technical Report), Pure Appl. Chem. 81 (2009) 355–387. https://doi.org/10.1351/PAC-REP-08-06-05.
[25] C. Ungureanu, L. Marchal, A.A. Chirvase, A. Foucault, Centrifugal partition extraction, a new method for direct metabolites recovery from culture broth: Case study of torularhodin recovery from Rhodotorula rubra, Bioresour. Technol. 132 (2013) 406–409. https://doi.org/10.1016/j.biortech.2012.11.105.
[26] A. Angelis, M. Hamzaoui, N. Aligiannis, T. Nikou, D. Michailidis, P. Gerolimatos, A. Termentzi, J. Hubert, M. Halabalaki, J.H. Renault, A.L. Skaltsounis, An integrated process for the recovery of high added-value compounds from olive oil using solid support free liquid-liquid extraction and chromatography techniques, J. Chromatogr. A. 1491 (2017) 126–136. https://doi.org/10.1016/j.chroma.2017.02.046.
[27] G.M. Kumar, I. Neelam, A. Ajitha, V. Uma, M. Rao, CENTRIFUGAL PARTITION CHROMATOGRAPHY: AN OVERVIEW, 4 (2014) 353–360.
[28] M. Hamzaoui, J. Hubert, J. Hadj-Salem, B. Richard, D. Harakat, L. Marchal, A. Foucault, C. Lavaud, J.-H. Renault, Intensified extraction of ionized natural products by ion pair centrifugal partition extraction, J. Chromatogr. A. 1218 (2011) 5254–5262. https://doi.org/10.1016/J.CHROMA.2011.06.018.

[29] X. Jing, P. Ning, H. Cao, Z. Sun, J. Wang, Separation of V(V) and Cr(VI) in leaching solution using annular centrifugal contactors, Chem. Eng. J. 315 (2017) 373–381. https://doi.org/10.1016/j.cej.2017.01.014.
[30] W. Duan, T. Sun, J. Wang, Separation of Nd3+ and Fe3+ by non-equilibrium solvent extraction using an annular centrifugal contactor, Sep. Purif. Technol. 146 (2015) 108–113. https://doi.org/10.1016/j.seppur.2015.03.044.
[31] J. Goll, G. Audo, M. Minceva, Comparison of twin-cell centrifugal partition chromatographic columns with different cell volume, J. Chromatogr. A. 1406 (2015) 129–135. https://doi.org/10.1016/j.chroma.2015.05.077.
[32] C. Roullier, M. Chollet-Krugler, A. Bernard, J. Boustie, Multiple dual-mode centrifugal partition chromatography as an efficient method for the purification of a mycosporine from a crude methanolic extract of Lichina pygmaea, J Chromatogr B Anal. Technol Biomed Life Sci. 877 (2009) 2067–2073. https://doi.org/10.1016/j.jchromb.2009.05.040.
[33] Z. Ning, C. Wang, Y. Liu, Z. Song, X. Ma, D. Liang, Z. Liu, A. Lu, Integrating Strategies of Herbal Metabolomics, Network Pharmacology, and Experiment Validation to Investigate Frankincense Processing Effects, Front. Pharmacol. 9 (2018) 1–18. https://doi.org/10.3389/fphar.2018.01482.
[34] J.-S. Jeon, C.Y. Kim, Preparative separation and purification of flavonoids and stilbenoids from Parthenocissus tricuspidata stems by dual-mode centrifugal partition chromatography, Sep. Purif. Technol. 105 (2013) 1–7. https://doi.org/10.1016/j.seppur.2012.11.010.
[35] A. Agalou, M. Thrapsianiotis, A. Angelis, A. Papakyriakou, A.-L. Skaltsounis, N. Aligiannis, D. Beis, Identification of Novel Melanin Synthesis Inhibitors From Crataegus pycnoloba Using an in Vivo Zebrafish Phenotypic Assay, Front. Pharmacol. 9 (2018) 265. https://doi.org/10.3389/fphar.2018.00265.
[36] A. Toribio, L. Boudesocque, B. Richard, J.-M. Nuzillard, J.-H. Renault, Preparative isolation of glucosinolates from various edible plants by strong ion-exchange centrifugal partition chromatography, Sep. Purif. Technol. 83 (2011) 15–22. https://doi.org/10.1016/j.seppur.2011.07.001.
[37] I.A. Sutherland, Recent progress on the industrial scale-up of counter-current chromatography, J Chromatogr A. 1151 (2007) 6–13. https://doi.org/10.1016/j.chroma.2007.01.143.
[38] A. Angelis, J. Hubert, N. Aligiannis, R. Michalea, A. Abedini, J.M. Nuzillard, S.C. Gangloff, A.L. Skaltsounis, J.H. Renault, Bio-guided isolation of methanol-soluble metabolites of common spruce (Picea abies) bark by-products and investigation of their dermo-cosmetic properties, Molecules. 21 (2016) 1–15. https://doi.org/10.3390/molecules21111586.
[39] J.S. Kim, Y.C. Seo, R.H. No, H.Y. Lee, Improved cosmetic activity by optimizing the Lithospermum erythrorhizon extraction process, Cytotechnology. 67 (2013) 51–65. https://doi.org/10.1007/s10616-013-9657-0.
[40] M. Dachtler, F.H.M. Van De Put, F. V. Stijn, C.M. Beindorff, J. Fritsche, On-line LC-NMR-MS characterization of sesame oil extracts and assessment of their antioxidant activity, Eur. J. Lipid Sci. Technol. 105 (2003) 488–496. https://doi.org/10.1002/ejlt.200300835.
[41] J.-Q. Xu, W.-H. Duan, X.-Z. Zhou, J.-Z. Zhou, Extraction of phenol in wastewater with annular centrifugal contactors, J. Hazard. Mater. 131 (2006) 98–102. https://doi.org/10.1016/J.JHAZMAT.2005.09.011.
[42] W. Duan, C. Song, Q. Wu, X. Zhou, J. Zhou, Development and Performance of a New Annular Centrifugal Contactor for Semi-Industrial Scale, Sep. Sci. Technol. 40 (2005) 1871–1883. https://doi.org/10.1081/SS-200064531.
[43] D.H. Meikrantz, L.L. Macaluso, W.D. Flim, C.J. Heald, G. Mendoza, S.B. Meikrantz, A New Annular Centrifugal Contactor for Pharmaceutical Processes, Chem. Eng. Commun.

189 (2002) 1629–1639. https://doi.org/10.1080/00986440214582.
[44] T.V. Tamhane, J.B. Joshi, R.N. Patil, Performance of annular centrifugal extractors: CFD simulation of flow pattern, axial mixing and extraction with chemical reaction, Chem. Eng. Sci. 110 (2014) 134–143. https://doi.org/10.1016/J.CES.2013.08.016.
[45] N. Fumat, A. Berthod, K. Faure, Effect of operating parameters on a centrifugal partition chromatography separation, J. Chromatogr. A. 1474 (2016) 47–58. https://doi.org/10.1016/j.chroma.2016.10.014.
[46] S. Yamauchi, N. Yamamoto, Y. Kinoshita, Stereoselective Synthesis of the Optically Active Samin Type of Lignan from L-Glutamic Acid, Biosci. Biotechnol. Biochem. 64 (2000) 878–881. https://doi.org/10.1271/bbb.64.878.
[47] P.C. Kuo, M.C. Lin, G.F. Chen, T.J. Yiu, J.T.C. Tzen, Identification of methanol-soluble compounds in sesame and evaluation of antioxidant potential of its lignans, J. Agric. Food Chem. 59 (2011) 3214–3219. https://doi.org/10.1021/jf104311g.
[48] W.J. Liu, Y.J. Chen, D.N. Chen, Y.P. Wu, Y.J. Gao, J. Li, W.J. Zhong, L. Jiang, A new pair of enantiomeric lignans from the fruits of Morinda citrifolia and their absolute configuration, Nat. Prod. Res. 32 (2018) 933–938. https://doi.org/10.1080/14786419.2017.1371163.
[49] S. Xia, L. Gan, K. Wang, Z. Li, D. Ma, Copper-Catalyzed Hydroxylation of (Hetero)aryl Halides under Mild Conditions, J. Am. Chem. Soc. 138 (2016) 13493–13496. https://doi.org/10.1021/jacs.6b08114.
[50] L. Liu, Z. Li, C. Chen, H. Li, L. Xu, Z. Yu, Efficient dealkylation of aryl alkyl ethers catalyzed by Cu2O, Tetrahedron. 74 (2018) 2447–2453. https://doi.org/10.1016/j.tet.2018.03.070.
[51] M. Srisayam, N. Weerapreeyakul, K. Kanokmedhakul, Inhibition of two stages of melanin synthesis by sesamol, sesamin and sesamolin, Asian Pac. J. Trop. Biomed. 7 (2017) 886–895. https://doi.org/10.1016/j.apjtb.2017.09.013.

Chapter 2: Pilot continuous centrifugal liquid-liquid extraction of extra virgin olive oil biophenols and gram-scale recovery of pure oleocanthal, oleacein, MFOA, MFLA and hydroxytyrosol.

Abstract

This study provides an efficient pilot-scale liquid-liquid extraction process for the recovery of total phenolic fraction (TPF) from extra virgin olive oil (EVOO) and the further purification of the major TPF constituents (oleocanthal, oleacein, monoaldehydic form of oleuropein aglycone, monoaldehydic form of ligstroside aglycone and hydroxytyrosol) using successively centrifugal partition chromatography and reversed phase high pressure liquid chromatography. Special attention was paid for the selection of the appropriate raw material, which should be rich in the targeted high-value compounds. For the extraction of the selected EVOOs, a green technique based on liquid-liquid partitioning using the biphasic system *n*-Hept/EVOO/EtOH/H_2O 3:2:3:2 v/v was carried out, first on pioneering laboratory-scale annular centrifugal extractor (ACE) and then on pilot device. 240 L from three different EVOOs (3x80 L of each EVOO) were extracted yielding 285 g of TPF in approximately 2 hours. The second step of this procedure was the purification of the target compounds. A preparative centrifugal partition chromatography (CPC) process was firstly applied for the initial TPF fractionation using a step gradient elution extrusion method with a series of 4 biphasic systems composed of n-Hept/EtOAc/EtOH/H_2O in ratios 4:1:3:2, 3:2:3:2, 2:3:3:2 and 1:4:3:2 (v/v/v/v). Preparative reversed phase high performance liquid chromatography (prep-RP-HPLC) was then carried out to polish the target compounds from the enriched CPC fractions resulting in purity higher than 97%. In parallel, the above purification analysis led to the isolation of four minor TPF constituents of which three are new natural products (EDA lactone, (1*R*, 8*E*)-1-ethoxy-ligstroside aglycon and (1*S*, 8*E*)-1-ethoxy-ligstroside aglycon) and one [(9*E*, 11*E*)-13-oxotrideca-9,11-diencoic acid] is described for the first time as EVOO constituent.

Introduction

Olive oil (OO), since ancient time has a highly economic importance, especially for the Mediterranean area [1]. Nowadays, OO has expanded all over the world, from USA and Chile to Australia, Japan and China [2–4]. OO and especially extra virgin olive oil (EVOO) is straightly correlated with the Mediterranean diet and the providing health benefits [5,6]. Most of the beneficial characteristics of EVOO are attributed to minor compounds, named biophenols which compose the total phenolic fraction (TPF) [7]. Hydroxytyrosol (HT) is the most known phenylethanoid of EVOO phenolic fraction. Other significant compounds of TPF are the secoiridoid derivatives oleacein (OLEA), oleocanthal (OLEO), monoaldehydic form of oleuropein aglycone (MFOA) and monoaldehydic form of ligstroside aglycone (MFLA) (Figure 1) [8]. These molecules have been widely investigated, providing significant bioactivities. It is worth noting that the last years, the scientific interest has focused on HT and its derivatives as the most important non-nutritive components of olive oil, due to EFSA (European Food & Safety Authority) health claim about their protecting effect on cardiovascular system [9].

Specifically, numerous biological activities were reported for HT, such as strong antioxidant and anti-inflammatory properties [10][11]. Even more, are the studies investigating anti-cancer activity, neuroprotective effects, potency against Alzheimer's disease and anti-inflammatory properties of OLEO [12–14]. Additionally OLEA, has shown significant bioactivities such as anti-oxidant, anti-inflammatory, anti-proliferative, anti-microbial and anti-cancer properties [14–17]. To the best of our knowledge, there are only few studies for the activity of MFOA, focus mainly on its antioxidant properties, while for the bioactivity of MFLA there is no significant information in the literature [18,19]. It is noteworthy, that there are limited reports related to the biological activity of the above mentioned secoiridoids in animal models. One critical reason is the required quantities and purity levels in such studies, thus hindering significantly their investigation in animal models and their comprehensive biological and pharmacological evaluation. [20–22].

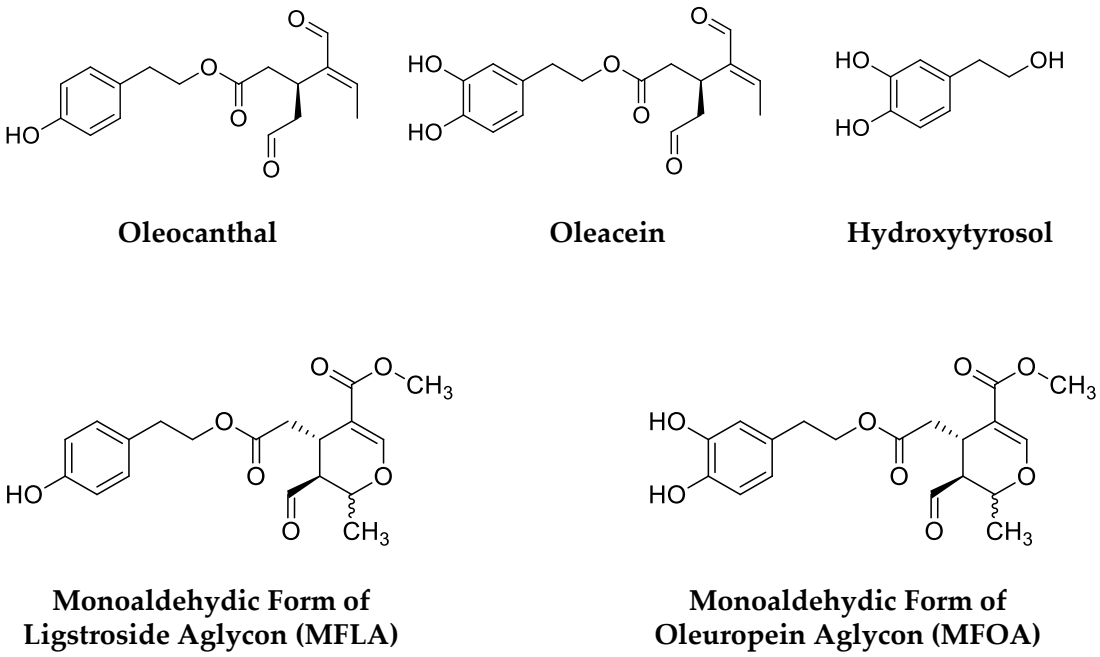


Figure 1: Structures of the main phenolic compounds of olive oil.

In the current study, an integrated process combining continuous liquid-liquid extraction and chromatographic techniques with scale-up capabilities was developed for the isolation of OO phenolic compounds in high yield. The liquid-liquid extraction step was carried out using liquid-liquid centrifugal extraction devices, which allows the development of continuous processes. Two types of equipment were used: a single stage annular centrifugal extractor (ACE) using two co-axial cylinders in order to ensure phase mixing [23] for the lab scale feasibility step and then a single stage centrifugal extractor using a rotating agitator disc in order to mix the two phases for the pilot-scale extraction. Both apparatuses are based on continuous counter-current liquid-liquid extraction principle, the only difference of them being allocated at the phase mixing mechanism. They provide high mass transfer efficiency as well as excellent phase decantation, thus leading to high throughputs in compact contactors [24]. The rapid start-up and shut-down of experimental procedure makes it also very useful for the industrial sector [25]. Centrifugal extraction has already been used in many different industrial fields, including pharmaceutical or food industry [26].

Secondly, centrifugal partition chromatography (CPC) [27,28] was used for the fractionation of TPF in order to obtain enriched fractions in compounds of interest [29] CPC is a liquid-liquid solid support free chromatography based on solutes partitioning at least between two immiscible phases in a thermodynamic equilibrium [30]. A CPC column consists of a series of partition cells linked in cascade by ducts in cascade and arranged in a centrifuge (one axis, two high pressure rotary seals). The stationary phase is maintained inside the column by the centrifugal force acceleration generated by column rotation (50 to 800 times the gravity, depending on the apparatus), while the

mobile phase is pumped through it. CPC technique has numerous advantages mainly due to the absence of chromatographic solid support. It is characterized by total sample recovery (irreversible adsorption on solid support are avoided), high repeatability, low solvent consumption and high capacity [8][31]. Additionally, it is considered as gentle and versatile technique leading to high selectivity, as a huge range of solvent combinations are available and reported in literature. [32,33][29][34][35]. Moreover, the industrial potential of CPC was demonstrated using a scale up methodology based on both column capacity and mass transfer efficiency as invariants parameters [36]. Using continuous centrifugal extraction and CPC at-line offers advantages concerning the amounts and the required quality for *in vivo* experiments and human studies. It has to be highlighted that both of the above techniques, continuous centrifugal liquid-liquid extraction such as ACE and CPC, could be considered as green methodologies. Indeed, they are characterized by a low solvent consumption when the intensification step is correctly carried out. Moreover, the short experimental duration minimizes the energy expenditure and the liquid-liquid nature of the techniques provides the ability to recycle the used solvents reducing thus drastically the generated wastes. All these characteristics have a positive impact on the environmental fingerprint of these liquid-liquid separation techniques [37,38].

Three EVOO from different geographical areas of Greece were selected, based on HPLC analysis, for their high abundance in the compounds of interest. TPF extraction was conducted with ACE in the selected OO samples, in both lab- and pilot-scale. Afterwards, the fractionation of the corresponding TPFs was carried out using CPC in step gradient elution mode. Selected fractions, enriched in the target compounds, were finally purified with preparative reversed phase HPLC (prep-RP-HPLC), reaching purity higher than 97% (HPLC based). Thus, the combination of two liquid-liquid support-free techniques (i.e. ACE and CPC) with prep-RP-HPLC also based on the partition mode for the polishing step allowed the extraction of TPF and the isolation of OLEO, OLEA, MFOA, MFLA and HT in considerably high yields. It should be also underlined that due to the high TPF availability and consequently the high quantity of CPC fractions, four minor constituents, never been reported before in olive oil were also isolated and structurally identified by NMR and HRMS analysis.

Materials and Methods

Reagents

All the reagents were purchased from Sigma-Aldrich (Missouri, USA). The solvents used for the extraction and separation processes were of analytical grade while those used for UPLC-HRMS analysis were of LC-MS grade. All solvents were supplied from Fisher Scientific (Pennsylvania,

USA). TLC analysis was performed on Silica gel 60 F254 20cm x 20cm plates purchased from Merck Millipore (Massachusetts, USA). The standard compounds (purity > 98%) used for the HPLC qualitative and quantitative analysis were purchased from Chembiotin (Greece) (Hydroxytyrosol, Tyrosol standards) and Pharmagnose S.A. (Inofyta, Greece) (Oleocanthal, oleacein, MFOA and MFLA standards).

Apparatus

The analytical scale extractions were performed on a laboratory scale BXP012 apparatus (Rousselet-Robatel Kromaton, Annonay, France) with 2.2 mL bowl volume and internal diameter of 12 mm. The two phases of the biphasic system were pumped into the annular extractor using two Basic Verderflex pumps (Castleford, United Kingdom) connected to the respective inlets for heavy and light phase. The large-scale extractions of OO biophenols were performed using the pilot scale ACE BXP190 (mono-stage unit) (Rousselet-Robatel Kromaton, Annonay, France). The apparatus consists of a casing and a bowl driven by a motor directly coupled to the extractor pendular shaft. The bowl has an internal diameter of 190 mm with a useful volume of 4.2 liters. The two liquid phases are mixed in the annular space of the extractor and are separated in the weir system which consists of a stable light phase weir of 90 mm and an interchangeable heavy phase weir of 100 mm. The motor is fed by a variable frequency drive to achieve variable speed operation with the maximum acceptable speed of 2900 rpm. Both heavy and light phases were pumped through the extractor with two ATEX FLUX eccentric worm-drive pumps with FBM 4000 Ex Motor and flow rate up to 75 L/min (FLUX-GERÄTE GmbH, Maulbronn, Germany) connected to the two solvent inlets of the apparatus.

The TPF fractionations were performed on a FCPC1000® apparatus (Kromaton, Anonay, France) equipped with rotor made of 45 circular partition disks engraved with 32 partition cells (555 µL per cell). The total column volume capacity is 955 mL. The partition cells are interconnected by capillary ducts, the latter representing a volume of 156 mL (dead volume). Rotation speed could be adjusted from 200 to 1000 rpm, producing a stable centrifugal force field in the partition cells up to 161 g at 1000 rpm. The solvents were pumped through two preparative ECP2000 pumps (Ecom, Prague, Czech Republic), and the effluent was monitored online by a Flash 14 UV-DAD detector (Ecom, Prague, Czech Republic). Fractions were collected by a C6-60 collector (Büchi, Flawil, Switzerland). The LC system (pumps and UV detector) was controlled by Clarity^{TM} 5.4 (DataApex, Prague, Czech Republic).

The purification of target compounds from enriched CPC fractions was performed on a preparative HPLC chain consisted of two Lab Alliance preparative 36 Pumps (State College, USA), a UV

Flash 06S DAD 800 Detector of ECOM (Prague, Czech Republic) and injection valve Rheodyne 7755-027 equipped with 1 mL loop (Target Analysis, Thessaloniki, Greece). The separation was run on a Supelco supelcosil™ LC-18, 25 cm x 21.2 mm, 5μm (Pennsylvania, USA) preparative HPLC column while the fractions were collected manually based on UV chromatograms. The HPLC system was controlled by Clarity™ 5.4 (DataApex, Prague, Czech Republic).

HPLC qualitative and quantitative analysis was performed on a Thermo Finnigan HPLC system (Ontario, Canada) equipped with a SpectraSystem 1000 degasser, a SpectraSystem P4000 pump, a SpectraSystem AS3000 automated injector and an UV SpectraSystem UV6000LP detector. Data acquisition was monitored by the ChromQuest™ 5.0 software (ThermoScientific™).

The pure compounds were analyzed by UPLC-ESI-HRMS using an Acquity H-Class UPLC system (Waters Corp., Milford, USA), equipped with a quaternary pump, an autosampler, an online vacuum degasser, and a temperature-controlled column and sample compartment and hyphenated to a hybrid LTQ-Orbitrap Discovery XL (Thermo Scientific, Brehmen, Germany) mass spectrometer with an electrospray ionization (ESI) source. Xcalibur 2.0.7 (Thermo Scientific) software was used for data acquisition and processing.

NMR spectra (^{1}H, ^{13}C, COSY, NOESY, HSQC-DEPT, HMBC) were recorded on a Bruker Avance III 600 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), equipped with a 5-mm PABBI 1H/D-BB inverse detection probe with a z-gradient. ^{1}H and ^{13}C NMR spectra were acquired at 600.15 MHz and 150.91 MHz, respectively.

HPLC-DAD analysis for olive oil selection

A HPLC-DAD method was employed according to the COI / T.20 / Doc No 29 method (International Olive Council (IOC) 2009) for the screening of in-house olive oil library [39]. For sample preparation, 2.0 g of olive oil and 1 mL of the internal standard solution (syringic acid) were mixed and vortexed for 30 s. Then, 5 mL of the methanol/water (80:20 v/v) ware added and vortexed for 1 min more. The mixture was placed in an ultrasonic bath for 15 min at room temperature and then centrifuged for 25 min. An aliquot of the supernatant phase was forwarded for HPLC analysis after filtration. HPLC quantification was achieved on a reversed-phase Spherisorb Discovery HS C18 column (250 × 4.6 mm, 5μm, Supelco) using a mobile phase consisted of 0.2% aqueous orthophosphoric acid (A) and methanol/acetonitrile (50:50 v/v) (B). The applied gradient elution was as follows: 0 min, 96% A and 4% B; 40 min, 50% A and 50% B; 45 min, 40% A and 60% B; 60 min, 0% A and 100% B; 70 min, 0% A and 100% B; 72 min, 96% A and 4% B; 82 min, 96% A and 4% B. A flow rate of 1.0 mL/min was used and the injection volume was set to 20μl. Chromatograms were monitored at 280 nm.

In total 10 EVOO samples were analyzed and the levels of HT, OLEA, OLEO, MFOA and MFLA were determined (Figure 2). The same analysis method was used to determine the levels of the target compounds in TPFs obtained from the pilot-scale extraction as well as the purity of the isolated compounds. The purity of the OLEO, OLEA, MFOA, MFLA and HT was also supported by UPLC-ESI-HRMS/MS and NMR analysis.

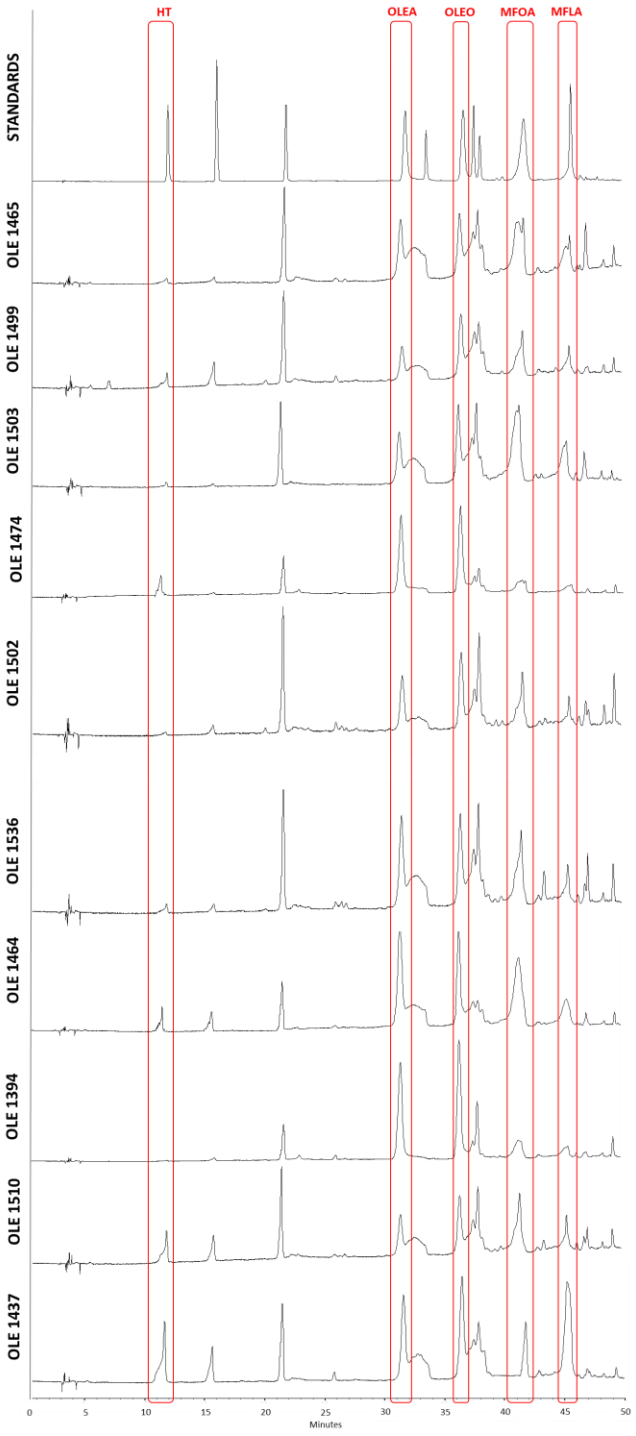


Figure 2: HPLC chromatograms of standard compounds, and 10 EVOO at 280 nm.

Liquid-liquid extraction of EVOO

The continuous centrifugal liquid-liquid extraction processes were started with parallel pumping of both feed oil phase (light) and extraction phase (heavy) into the extractor bowl. The biphasic system was mixed (at the mixing zone) and then was separated (by centrifugal forces) to treated oil phase and enriched aqueous phase. The biphasic system used for the extractions previously described by *Angelis et al* [8], was the following: n-Hept/EVOO/EtOH/H_2O in ratio 3:2:3:2 (v/v/v/v/v). The extraction procedure was initially developed in a laboratory scale extractor (BXP012) using *EVOO-I* as study material and then the method was transferred in pilot-scale extractor (BXP190) where all three chosen OOs were treated.

Extraction of TPF using the laboratory scale annular centrifugal extractor BXP012

The feed oil phase was prepared by mixing 80 mL of *EVOO-I* and 120 mL of *n*-heptane while the extraction phase was prepared by mixing 120 mL ethanol and 80 mL water. The rotation speed of laboratory scale ACE BXP012 was set at 3950 rpm and the flow rate at 8 mL/min for both phases (Table 1). Subsequently, the biophenol-enriched aqueous phase was evaporated under vacuum. The experiment was repeated two more times. The repeatability of the process was evaluated by comparing the yields standard deviation, as well as using TLC and RP-HPLC-DAD analysis of the three obtained TPFs.

Extraction of TPF using pilot scale extractor BXP190

The pilot-scale extraction of OO TPF was performed using a mono-stage pilot scale ACE BXP190. The first step of the procedure was the separately preparation of the two phases of solvent system: 200 L of the feed oil phase composed of *n*-Hept/EVOO (in ratio 3:2 v/v) and 200 L of the aqueous extraction phase composed of EtOH/H_2O (in ratio 3:2 v/v). The extraction procedure started by pumping the heavy phase at a flow rate of 10 L/min and at the rotation speed of 1050 rpm. When the heavy phase came out from the extractor, its flow rate decreased at 5 L/min and the light phase was pumped with the same flow (5 L/min), while the rotation speed was maintained at 1050 rpm (Table 1). The extraction procedure lasted 40 min and finally 200 L of treated oil phase and the same volume of enriched in biophenols aqueous phase were separately collected into two different barrels.

The next step of the procedure was the evaporation of the collected extract and raffinate in order to recover the biophenolic fraction as well as the treated olive oil, respectively. The solvents (n-heptane and the mixture of EtOH/H_2O) obtained from the evaporation were recycled and reused in further extraction processes. The evaporation was performed in a QVF thermal evaporation system (200 L evaporation vessel) under 15-100 mbar vacuum at 39^oC, while the condensation of

the produced solvents vapors was facilitated using coolant (glycol) at -7°C. The TPF obtained after processing of 80 L EVOO was 85.8 g (*EVOO-I –> TPF-I*).

The procedure was repeated for the extraction of *EVOO-II* and *EVOO-III*. Each extraction was performed by using the recycled solvents. The TPFs obtained after extraction of 80 L of *EVOO-II* and 80 L of *EVOO-III* were 109.4 g (*TPF-II*) and 89.5 g (*TPF-III*), respectively.

Table 1: Experimental parameters of EVOO extractions and the corresponding yields, using analytical and pilot scale ACE.

Biphasic system	*n*-Hept/EVOO/ethanol/water 3:2:3:2 (v/v/v/v)			
Aparatus	BXP012 (Analytical scale)		BXP190** (Pilot scale)	
EVOO	*EVOO-I*	*EVOO-I*	*EVOO-II*	*EVOO-III*
Flow rate of feed oil phase	8 mL/min	5 L/h	5 L/h	5 L/h
Flow rate of extraction phase	8 mL/min	5 L/h	5 L/h	5 L/h
Volume of treated EVOO	80 mL	80 L	80 L	80 L
Volume of feed oil phase	200 mL	200 L	200 L	200 L
Volume of extraction phase	200 mL	200 L	200 L	200 L
Rotation speed	3950 rpm	1050	1050	1050
Experimental Time	20 min	~40 mir	~40 min	~40 min
Yield of TPFs	**83.3 mg* (±0.36)**	**85,8 g**	**109.4g**	**89.5g**

** The average of three repetitions (standard variation)*
***The heavy phase weir used for both three pilot analysis was of 100 mm.*

Isolation procedure

Preparative CPC fractionation of TPFs using step-gradient elution extrusion method.

Preparative CPC fractionations of the produced TPFs (*TPF-I*, *TPF-II* and *TPF-III*) were achieved by following a previously described method with slight modifications [8]. All experiments were run in a step gradient elution extrusion mode using the series of four biphasic systems (composed of the solvents n-Hept/EtOAc/EtOH/H_2O in ratio 4/1/3/2 (S1), 3/2/3/2 S2), 2/3/3/2 (S3) and 2/4/3/2 (S4), v/v/v/v) and the same experimental parameters. The aqueous phase of S1 was used as stationary phase while the gradient elution was achieved by pumping successively the upper phases (mobile phases, MP) of the four systems in ascending mode (500 mL of MP1, 1100 mL of MP2, 1400 mL of MP3 and 1000 mL of MP4). The experiments were ended by extruding the column content using the lower phase of S4 as mobile phase in descending mode. The injected sample mass of each TPF was 10 g, while rotation speed and flow rate were remained constant during all experiment at 900 rpm and 15 mL/min, respectively. All collected fractions (190 fractions of 25 mL) were initially off-line analyzed by TLC and combined based on their chemical profile similarity (Figure 3).

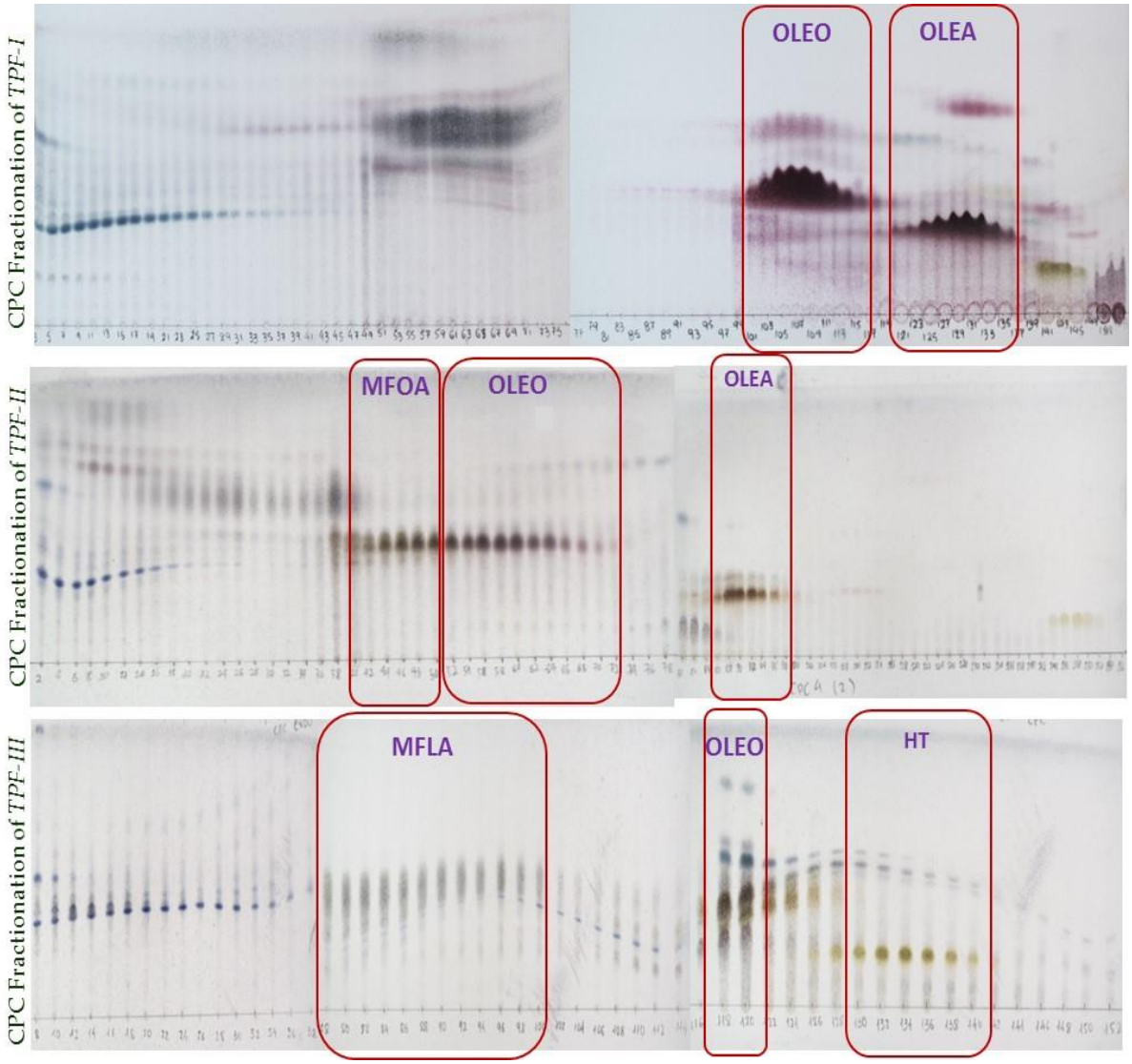


Figure 3: TLC chromatograms of CPC fractions obtained from the analysis of TPF I (upper-high levels of OLEO and OLEA), TPF II (middle- high levels of MFOA, OLEO and OLEA) and TPF III (lower- high levels of MFLA, OLEO and HT).

Preparative HPLC purification

Different preparative HPLC methods were developed for each target compound using H_2O (solvent A)-CH_3CN (solvent B) gradients at flow rate of 15 mL/min. The gradient profiles of methods A-E were as described below. Method A (for OLEO enriched fractions): 0 min – 80% A, 20 min- 70% A, 25 min– 10% A and 30 – 80% A; Method B (for OLEA enriched fractions): 0 min – 80% A, 20 min- 70% A, 25 min– 70% A, 30 min– 10% A and 35 – 80% A; Method C (for MFLA enriched fractions): 0 min – 80% A, 10 min – 70% A, 22 min- 50% A, 25 min- 50% A, 30 min– 10% A and 35 min – 80% A; Method D (for MFOA enriched fractions): 0 min – 800% A, 20 min – 70% A, 25 min- 50% A, 30 min– 10% A and 35 min – 80% A; and Method E (for HT enriched fractions): 0 min – 100% A, 5 min – 99% A, 10 min- 95% A, 20 min- 50% A, 25 min–

10% A and 30 min – 100% A. All the samples were dissolved in CH_3CN in order to reach a concentration of 150 mg/mL and were injected with 1 mL volume capacity loop.

TLC, UHPLC-HRMS/MS and NMR analysis

TLC analysis was performed on normal phase plates. The chromatograms were development with a mixture of dichloromethane (CH_2Cl_2) and methanol (MeOH) in ratio CH_2Cl_2/MeOH 95:5 v/v and observed at 254 nm, 366 nm and at visible after treatment with a sulfuric vanillin solution (5% w/v in methanol) – H_2SO_4 (5% v/v in methanol) and heated at 100–120OC for 1 minute.

For UHPLC-HRMS/MS analysis all the samples were diluted in MeOH/H2O 1:1 v/v and injected (10μL) on a Fortis C-18 (1.7 μm, 150 x 2.1 mm) column at 40oC. The flow rate was set at 400 μL/min while acidified water with 0.1% formic acid (A) and CH3CN (B) were used as elution system, in the following gradient mode: 0-3 min 95% A, 3-21 min from 95% to 5% A, 21-23 min 5% A, 23-24 min from 5% to 95% B and 24-30 min 95% A. Ionization was achieved in negative ion mode (ESI-) at 350°C. The mass spectrometric parameters were: capillary voltage -20V and tube lens -80V for the negative mode; sheath gas and aux gas flow rate 40 and 10 units, respectively. The mass range was 113 - 1000 m/z.

NMR samples were dissolved in 600 μL of deuterated chloroform ($CDCl_3$) and experiments were recorded using standard Bruker microprograms. Chemical shifts (δ) are expressed in ppm, while coupling constants (J) in Hz. The multiplicity of vertices is expressed as s (singlet), brs (broad singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets) and m (multiplet).

Results and Discussion

EVOOs selection

In a previous study of our research group the TPFs of 130 EVOO's - constituting our in-house library of EVOOs - from all over Greece, were analyzed [22]. As it was expected, the results showed a significant difference in the yields of both TPF and its major components *i.e.* hydroxytyrosol, tyrosol, oleacein and oleocanthal, depending on the olive variety, geographical origin, production procedure and cultivation practice. Thus, 10 EVOO samples with high levels of phenolic compounds were re-collected during the 2018-2019 harvesting period and were re-analyzed. All samples showed different levels of the target compounds after HPLC-DAD analysis (Figure 2). The best candidate of EVOOs for the designed pilot-scale isolation workflow were OLE_1474 obtained from Lakonia-Peloponnese (code name *EVOO-I*), OLE_1464 from Heraklion-Crete (code name *EVOO-II*) and OLE_1437 from Lasithi-Crete (code name *EVOO-III*). The three EVOOs contained high levels of two or more target compounds. More precisely, *EVOO-I* showed high levels for OLEA and OLEO, *EVOO-II* high levels for OLEO, OLEA and MFOA,

while the *EVOO-III* was rich in HT, OLEO and MFLA. Therefore, the three EVOOs were re-procured in higher quantities for the following steps of this study.

Continuous liquid-liquid centrifugal extraction of selected EVOOs

Two different extraction methods were previously developed [8] on a laboratory-scale Centrifugal Partition Extractor equipped with 231 partition cells for a column volume of 303 mL: a sequential strategy consisted of several "extraction-recovery" cycles and a continuous strategy based on stationary phase co-current elution. In both cases, EVOO was used as mobile phase diluted in food grade n-heptane (feed mobile phase) and the required biphasic system was obtained by adding ethanol and water as polar solvents [8]. Considering its efficacy, the same biphasic system was used in the present work. Therefore, the liquid-liquid extraction feasibility using the biphasic system n-Hept/EVOO/EtOH/H_2O in ratio 3:2:3:2 (v/v/v/v/v) was firstly evaluated on a lab-scale single stage ACE with a bowl diameter of 12 mm and a bowl volume of 2.2 mL (BXP012). ACE technique have been previously used for the lab-scale extraction of lignans from sesame oil by *Michailidis et al* [40] with significant results and the current study reports the use of this technique for EVOO biophenols extraction for the first time. Considering the density ratio between the two phases of 1.22 (feed oil phase density = 704,4 kg/m^3, extraction phase density = 853.8 kg/m^3), and the quite low viscosity of the EVOO-rich upper phase, the rotation speed was set at 3950 rpm corresponding to a centrifuge acceleration of 1046 g (up to 6700 g can be reached with this equipment). The flow rate was fixed at 8 mL/min for each phase, corresponding to 50% of the maximum flow rate for this lab-scale extractor and an average residence time of 8 s for each phase. The 80 mL of *EVOO-I* were successfully extracted in 20 min, providing 83.23 mg of TPF, (done in triplicate, standard deviation: 0.36), without the presence of persistent emulsion at the outlet of the extractor, corresponding to a productivity of 113 g/(h.L) of extractor capacity. It is important to note that in the recently developed CPE extraction methods [8], the productivity was calculated at 8.9 g/(h.L) of CPE column using the co-current CPE process, even though the analyzed olive oil was slightly more concentrated in TPF (1.3 g TPF/L of olive oil) compared to *EVOO-I* (1.04 g TPF / L of olive oil). This first results highlighted the efficiency of the single stage continuous liquid-liquid centrifugal extraction process for TPF recovery from EVOO.

The scale-up was directly achieved on a pilot-scale single stage liquid-liquid centrifugal contactor with the following bowl dimensions: 190 mm and 4.2 L for diameter and capacity, respectively. The phase mixing take place in the annular space and the separation occurs in the weir system (light phase weir of 90 mm and heavy phase weir of 100 mm). The centrifuge acceleration was chosen as scale change invariant in order to provide nearly the same mixing state and so mass

transfer of polyphenolic compounds [41]. Considering the diameter of the bowl, the rotation speed was set at 1000 rpm, reaching approximately 1060 g. The flow rate of each phase was set at 5 L/min, corresponding to a residence time of 25 s in order to allow an efficient dispersion-coalescence of the dispersed phase together with a complete mass transfer of the compounds of interest. The above flow rates resulted in high quality of both separated phases by allowing more time to achieve efficient separation. Nevertheless, this parameter should be further optimized for an industrial application in order to increase productivity.

80 L of each EVOO (*EVOO-I*, *EVOO-II* and *EVOO-III*) were extracted, tending to recycle the solvents between the successive extractions. The three final TPFs i.e. *TPF-I* from *EVOO-I*, *TPF-II* from *EVOO-II* and *TPF-III* from *EVOO-III* were evaporated to dryness yielding 85.9, 109.4 and 89.5 g respectively (Table 1). The corresponding productivities were 30.6 g/(h.L), 39.7 g/(h.L) and 31.9 g/(h.L).

The liquid-liquid extraction process developed here being only governed by single stage phase transfer phenomena, we can assume that the productivity decrease (113 g/(h.L) *vs* 34 g/(h.L), ratio 3.3) observed between the lab-scale and the pilot-scale, respectively is mainly due to the selected flow rate on the pilot extractor that increased the residence time (8 s *vs* 25 s, ratio 3.1).

Each TPF obtained after the continuous liquid-liquid extraction step was analyzed by HPLC in order to qualitatively visualize their chemical profiles (Figure 4). As expected, *TPF-I* was rich in OLEO and OLEA, *TPF-II* in MFOA, OLEO and OLEA and *TPF-III* in MFLA, OLEO and HT.

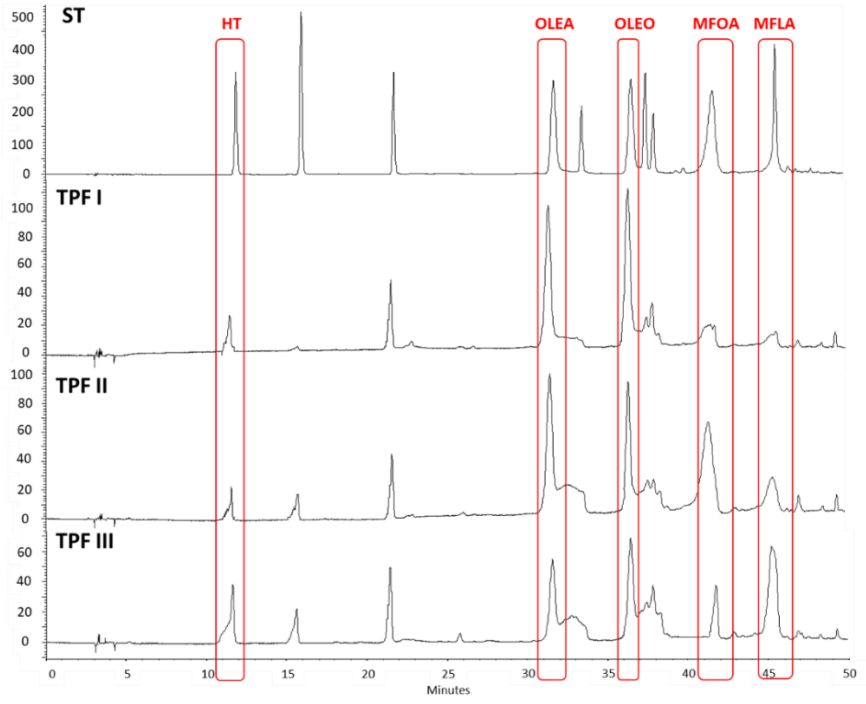


Figure 4: HPLC chromatograms of standard compounds, *TPF-I* (rich in OLEA and OLEO), *TPF-II* (rich in OLEA, OLEO and MFOA) and *TPF-III* (rich in HT, OLEO and MFLA) at 280 nm.

CPC fractionation of TPFs and purification of target compounds

10 g of each TPF were fractionated by CPC using a previously described step-gradient elution extrusion method [8]. The different biphasic solvent systems were all composed of n-heptane, ethyl acetate, ethanol and water (4/1/2/3, 3/2/2/3, 2/3/2/3 and 1/4/2/3 v/v/v/v for S1, S2, S3, S4, respectively). Previous work demonstrated that the compositions of the aqueous phases of these systems are very close, making it possible to use the gradient mode without dramatic disturbing of the hydrodynamic equilibrium during the process [8]. The fractions were pooled based on their chemical profile similarities, after TLC off-line analysis (Figure 3).

The CPC analysis of *TPF-I* resulted in the recovery of 1135.3 mg of the enriched OLEO fraction and 1009.1 mg of the enriched OLEA fraction as well as in the recovery of 143.5 mg, 102.3 mg and 38.2 mg of fractions enriched in HT, MFLA and MFOA, respectively. The same analysis of *TPF-II* led to the recovery of 1045.2 mg, 983.6 mg, 845.8 mg, 112.6 mg and 93.2 mg of CPC fractions enriched in OLEO, OLEA, MFOA, HT and MFLA, respectively. The CPC analysis of *TPF-III* resulted enriched fractions in 1348.5 mg, 845.3 mg, 372.9 mg, 148.1 mg and 83.2 mg of MFLA, OLEO, HT, OLEA and MFOA, respectively. Finally, certain amounts of enriched fractions in MFLA (1544 mg) , MFOA (967.2 mg) , OLEO (3025.8 mg), OLEA (2140.8 mg) and HT (629 mg) were recovered from the three CPC analysis (Table 2). The purity of the target compounds in the enriched CPC fractions was determined based on HPLC and NMR analysis and found to be more than 80%.

Table 2: The yields of the enriched in the target compounds CPC fractious recovered from the CPC fractionation of the three TPF extracts obtained after continuous liquid-liquid extraction of the three selected EVOO.

Analyzed TPF	Enriched CPC fractions				
	MFLA	MFOA	OLEO	OLEA	HT
TPF-I	102.3 mg	38.2 mg	1135.3 mg	1009.1 mg	143.5 mg
TPF-II	93.2 mg	845.8 mg	1045.2 mg	983.6 mg	112.6 mg
TPF-III	1348.5 mg	83.2 mg	845.3 mg	148.1 mg	372.9 mg
Total	1544 mg	967.2 mg	3025.8 mg	2140.8 mg	629 mg

In order to succeed high purity of the target compounds, all the enriched CPC fractions were purified with prep-RP-HPLC (Figure 5). Aliquots of 150 mg/mL were injected via a 1 mL injection loop, while the chromatographic separations were run using different elution method for each target compound (as described at experimental part).

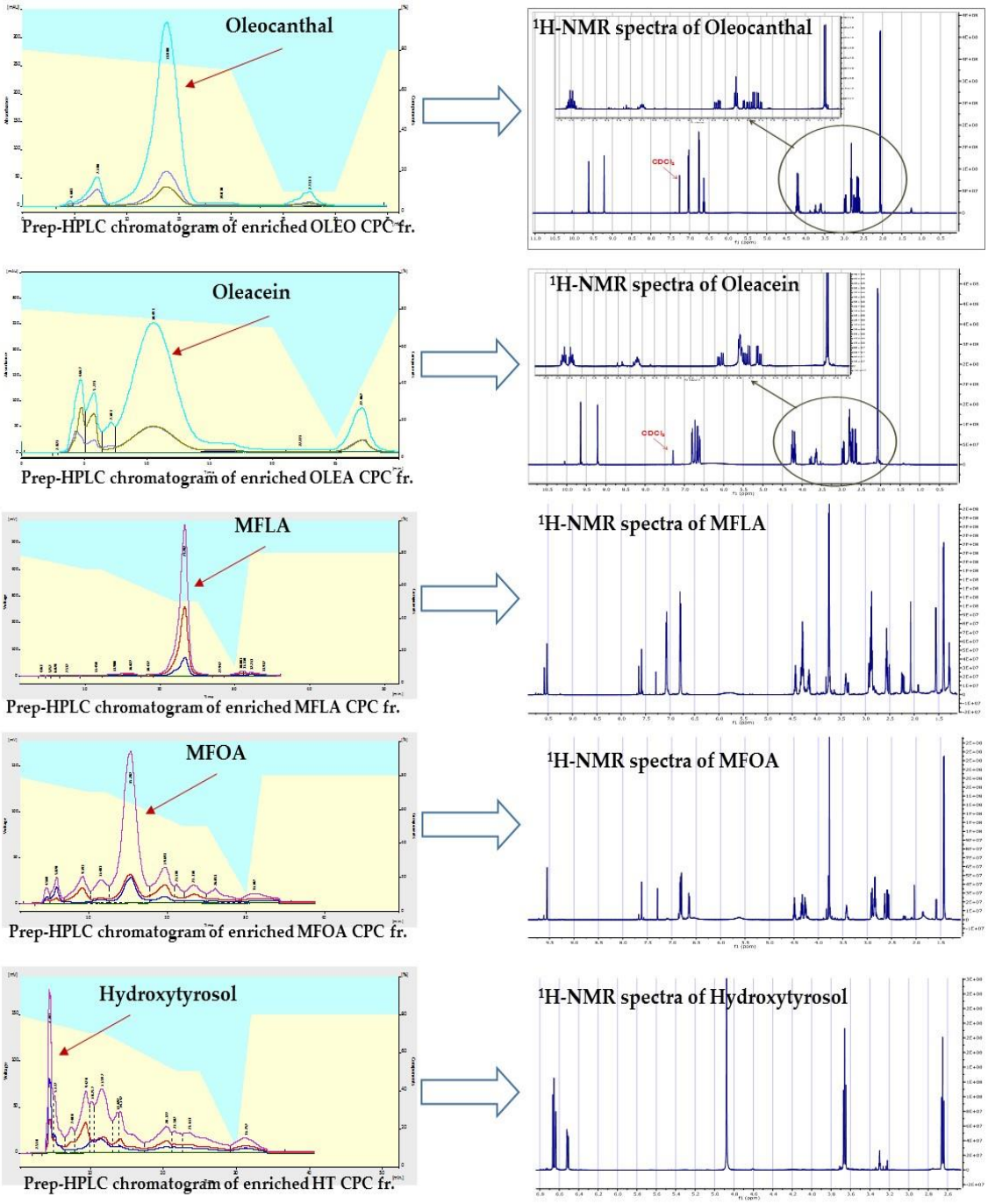


Figure 5: Preparative HPLC chromatograms and 1H-NMR spectra of purified major EVOO compounds.

This purification step led to the isolation of 1030.4 mg of MFLA (96% purity), 683.4 mg of MFOA (97% purity), 2020.6 mg of OLEO (98% purity), 1463.7 mg of OLEA (98% purity) and 407.4 mg of HT (97% purity) (Figure 6). The purity of the isolated compounds was determined by quantitative HPLC analysis using standard compounds for calibration curves plotting. The applied procedure, including the CPC fractionation of the EVOO extract and followed by the prep-RP-HPLC polishing step of the enriched fractions, is an efficient method for the gram-scale recovery of purified TPF high-value compounds.

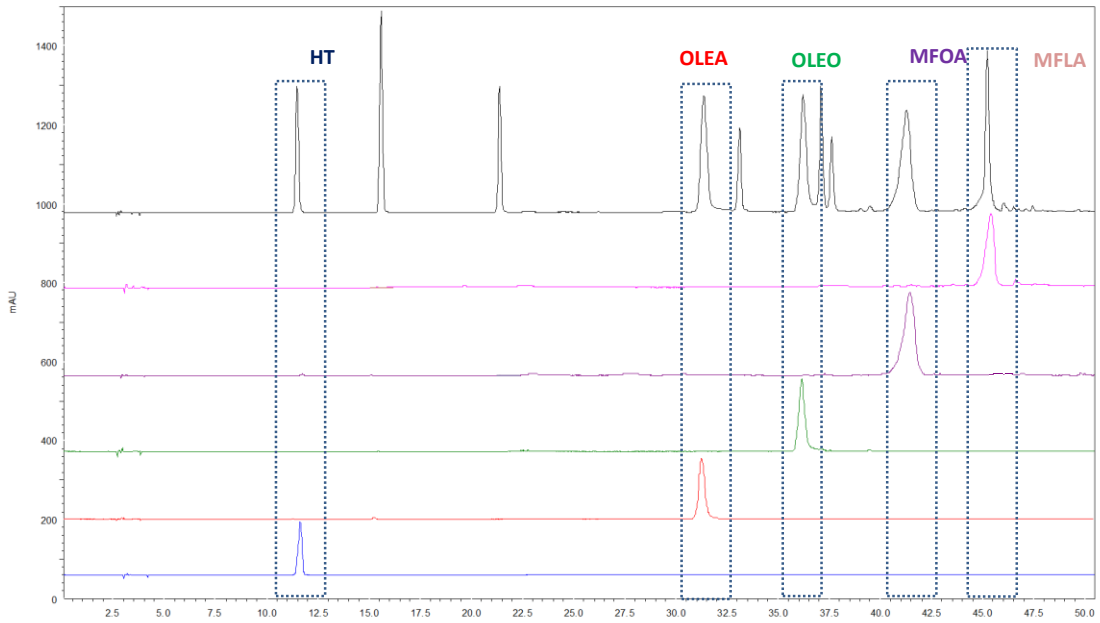
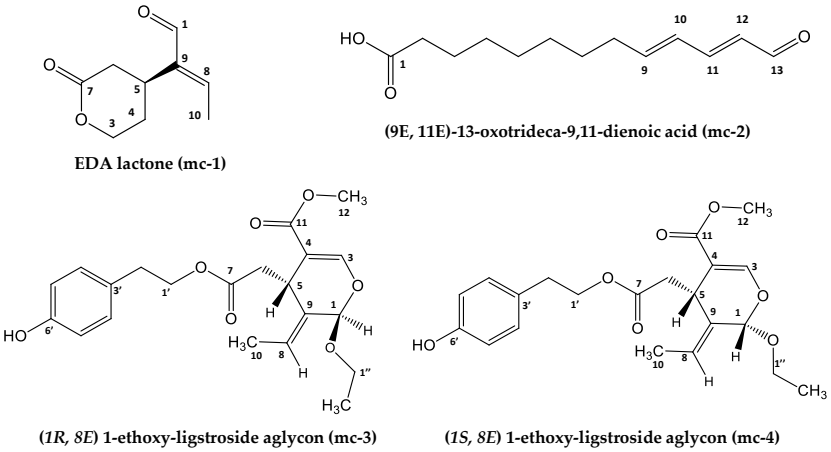


Figure 6: HPLC chromatograms of purified major EVOO compounds.

Isolation and structure elucidation of new EVOO metabolites

The high complexity of TPF in combination with the alteration of OO phenolic composition depending on olive variety and differentiation on harvesting period or cultivation area, make this raw material a continuous source of original natural products. To date, over than 100 metabolites have been reported as EVOO constituents apart from lipids [42] [43] [8,44]. Nevertheless, the use of solid support-free liquid-liquid techniques for the upstream steps of the fractionation process (*i.e.* continuous liquid-liquid centrifuge extraction and CPC) prevented irreversible adsorption phenomena and chemical degradation and/or artifact formation. This extraction-fractionation process combined with the pilot-scale strategy (80 L of each EVOO were used as starting material) and the prep-RP-HPLC allowed the purification, apart from the major metabolites, of four minor compounds (Figure 7).



EDA lactone (mc-1)

(9E, 11E)-13-oxotrideca-9,11-dienoic acid (mc-2)

(*1R, 8E*) 1-ethoxy-ligstroside aglycon (mc-3)

(*1S, 8E*) 1-ethoxy-ligstroside aglycon (mc-4)

Figure 7: Structures of isolated minor compounds.

In more detail, the purification process of OLEO resulted in the isolation of one more minor secoiridoid (18 mg) which was identified as the lactonic form of EDA (**mc-1**: Fig. 7). The structure of **mc-1** was elucidated by means of 1D and 2D NMR experiments as well as HRMS analysis (Figure 8a-8e). The ESI (-)-HRMS spectrum of this compound showed a pseudomolecular ion $[M-H]^-$ at *m/z* 167.0718 with a suggested EC of $C_9H_{11}O_3$ and RDBeq of 4 (Figure 8e). The ^1H NMR spectrum showed the presence of a total of ten peaks of which three correspond to the protons of an 1-Oxobut-2-en-2-yl moiety [a doublet at δ 9.36 (J = 1.4 Hz) (H-10), a quartet at δ 6.68 (J = 7.2 Hz) (H-8) and a doublet at δ 2.02 (3H, J = 7.2 Hz) (H-10)] and seven peaks correspond to the protons of a δ-valerolactonic moiety [two peaks at δ 4.57 and 4.31 (H-3a/3b), one multiplet at δ 3.51 (H-5), two doublet of duplet at δ 2.96 and 2.43 (H-6a/6b) and two multiplets at δ 2.14 and 1.88 (H-4a/4b)]. Further study of 2D-NMR spectra (COSY, HSQC-DEPT, HMBC) verified the structure of the isolated compound. The complete ^1H and ^{13}C NMR data of **mc-1** are presented in table 3 while 1D and 2D spectra are shown in Figures 8a-8d. Due to the formation of δ-valerolactonic ring through the reaction of aldehydic group at C-3 with the carboxyl group at C-7 of EDA, the asymmetric carbon C-5 of **mc-1** maintains the same stereochemical configuration as in EDA skeleton i.e 5S- configuration. To the best of our knowledge, there are not previous references regarding this metabolite and thus is characterized as new natural product with the given name EDA lactone.

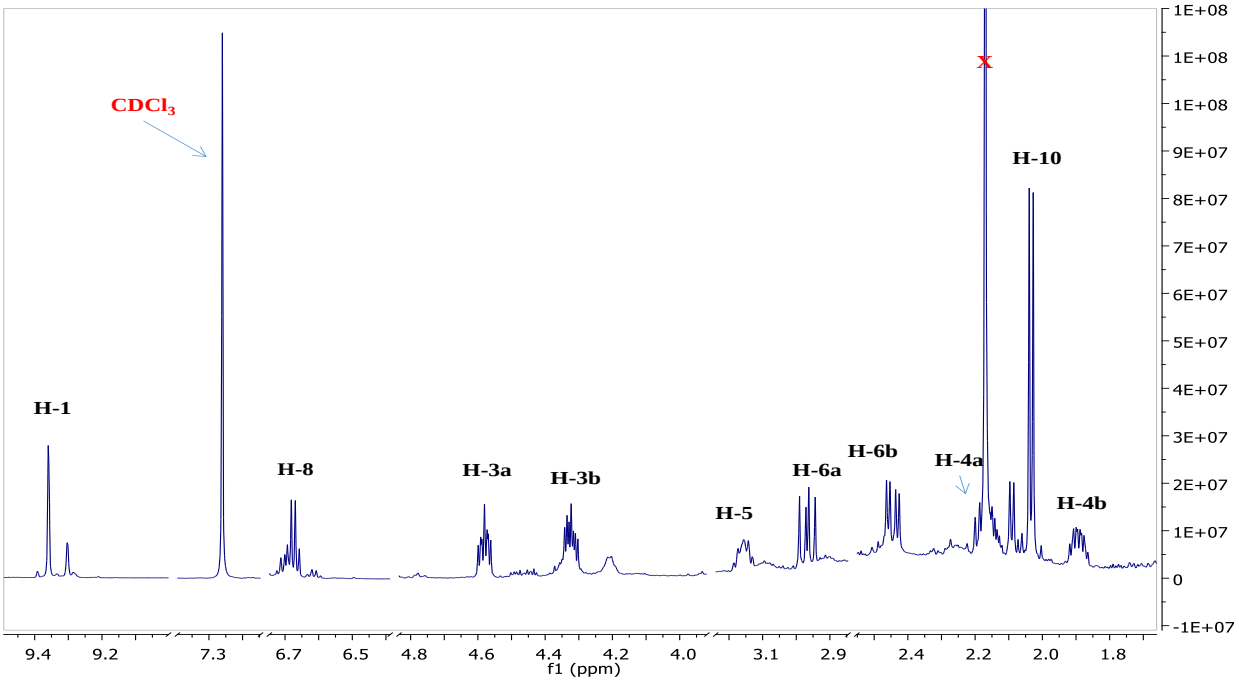


Figure 8a: 1H-NMR spectrum of EDA lactone.

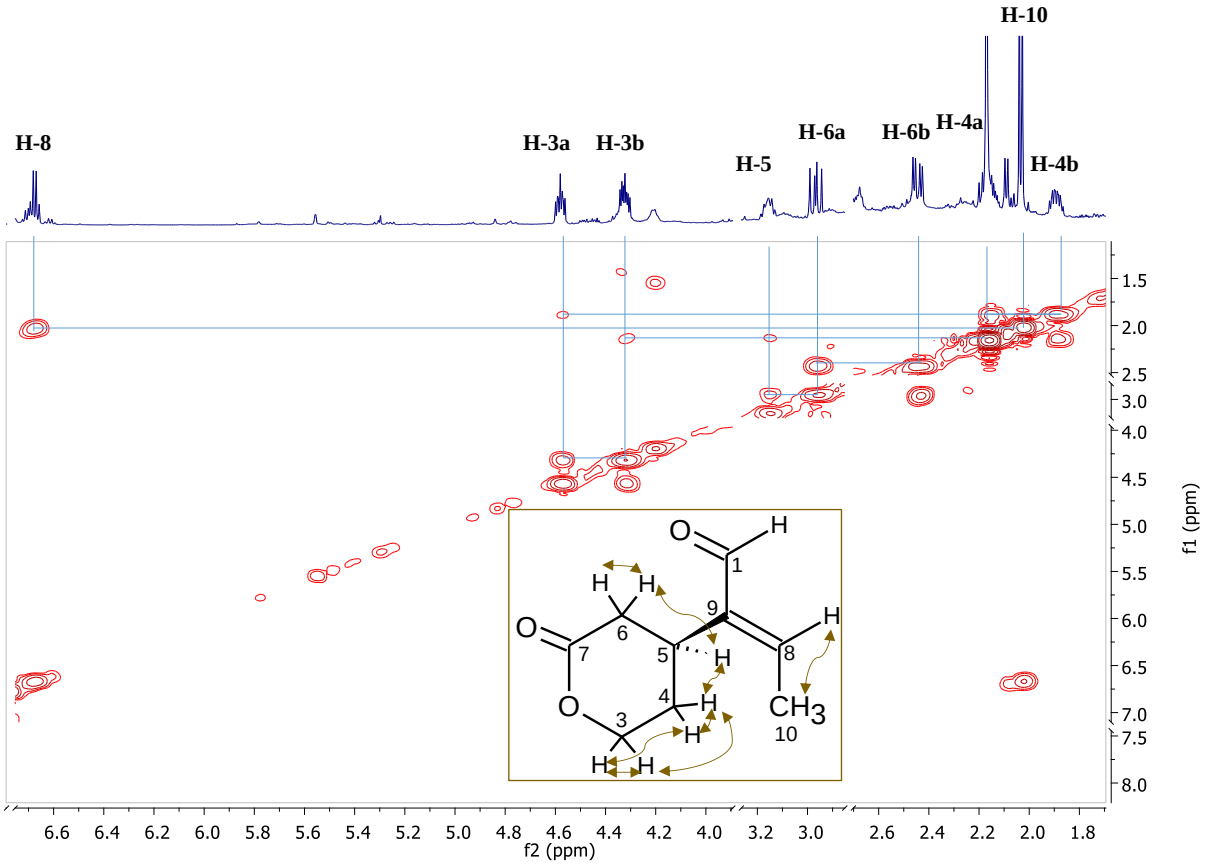


Figure 8b: COSY-NMR spectrum of EDA lactone.

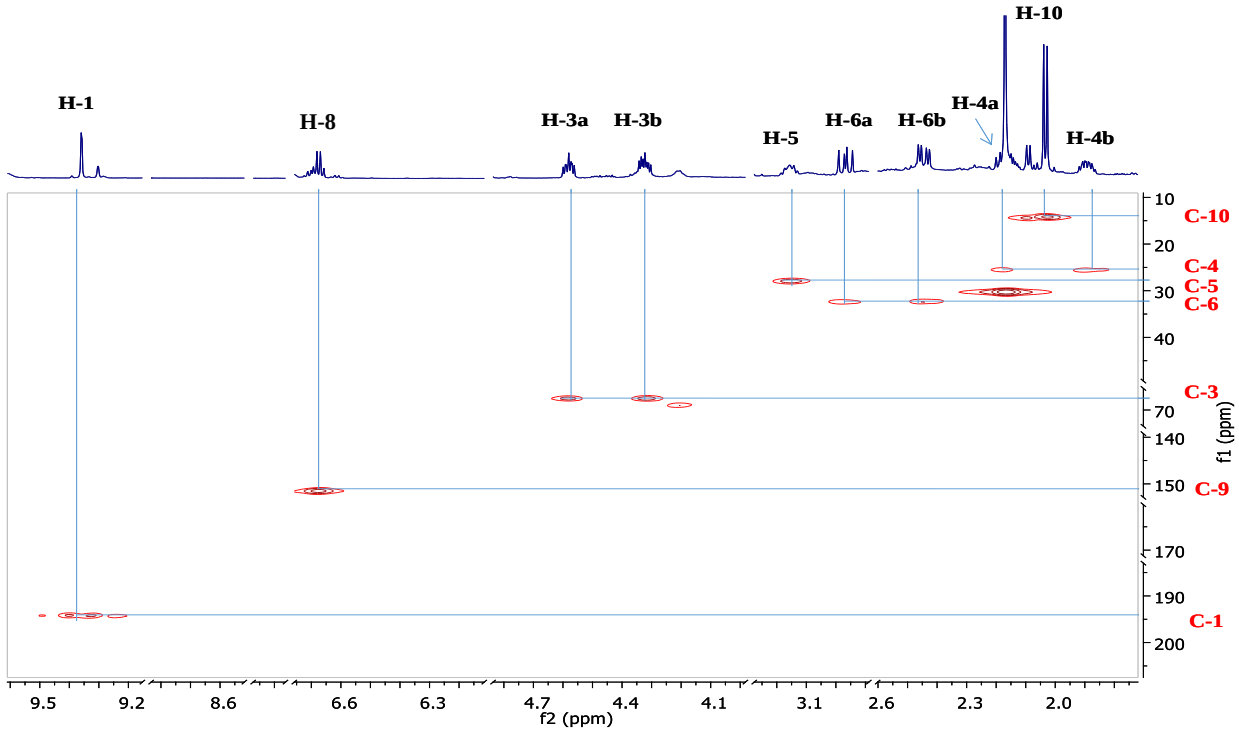


Figure 8c: HSQC-NMR spectrum of EDA lactone.

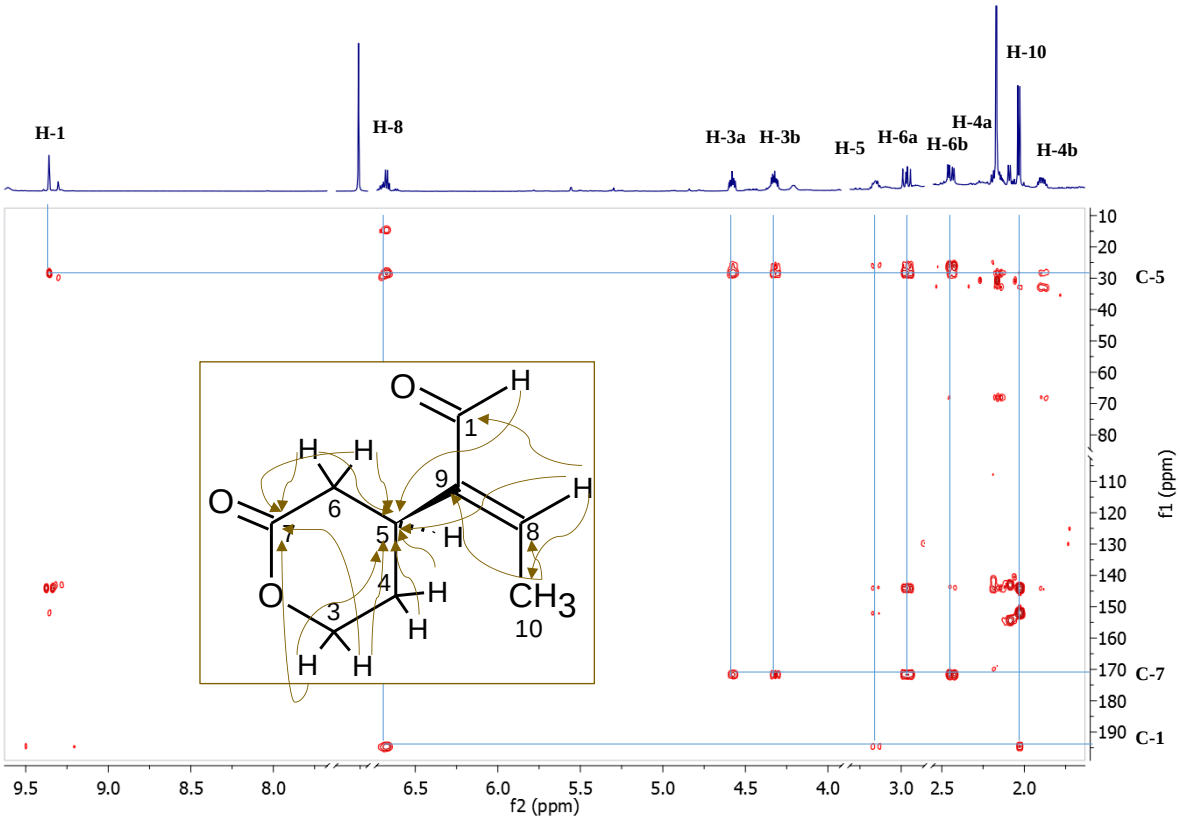


Figure 8d: HMBC-NMR spectrum of EDA lactone.

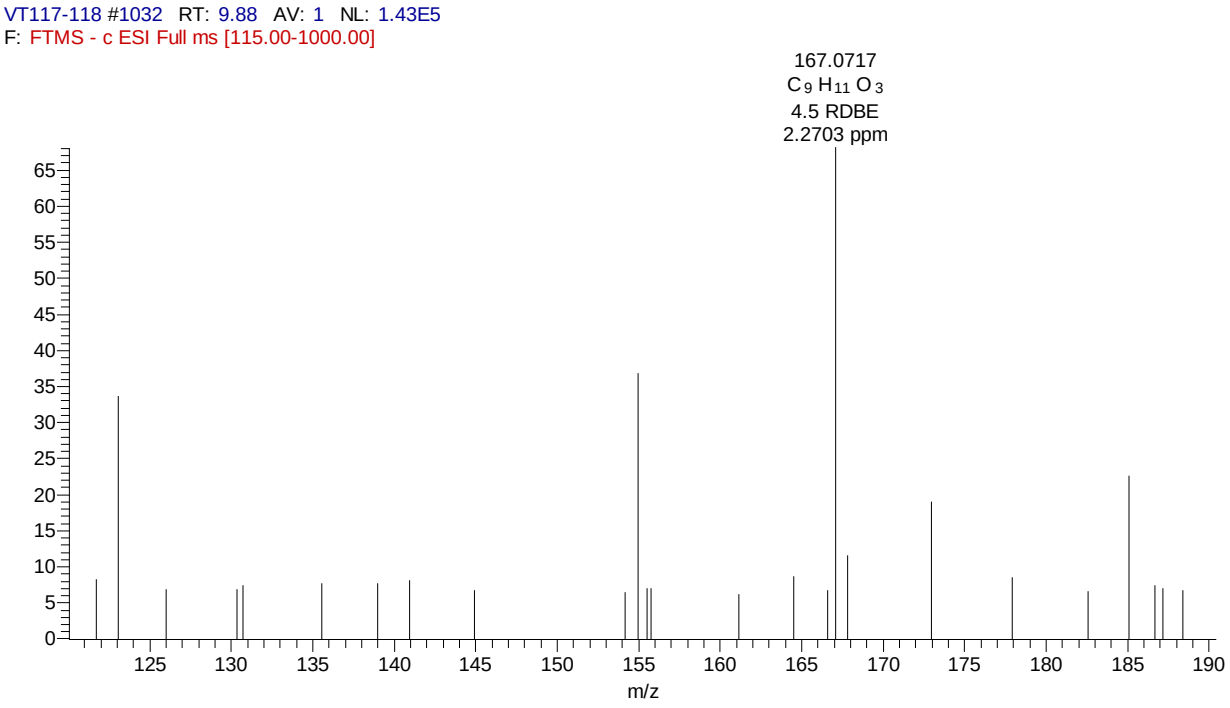


Figure 8e: HRMS spectrum of EDA lactone.

Moreover, two minor picks were collected during the preparative HPLC purification of MFLA from the corresponding enriched CPC fraction. The NMR and HRMS analysis (Figures 9a-9d) of the first peak (12.5 mg) revealed the presence of an unsaturated fatty acid which was identified as

(*9E, 11E*)-13-oxotrideca-9,11-dienoic acid (mc-2: fig. 7). The ESI (-)-HRMS spectrum determined the length of the fatty chain showing a pseudomolecular ion $[M-H]^-$ at *m/z* 223.1341 with suggested EC of $C_{13}H_{19}O_3$ and ERDeq of 4 (Figure 9d). The complete ^{1}H and ^{13}C NMR data of **mc-2** are presented in table 3. This compound was initially identified as product of hydroperoxide lyase reaction of *Chlorella pyrenoidosa* with 13- hydroperoxylinolenic acid as a substrate [45] and subsequently was isolated as antifungal substance from the leaves of *Chenopodium album* L. *var. album* (pigweed) treated with a cupric chloride solution [46]. The direct isolation from natural sources is presented here for the first time.

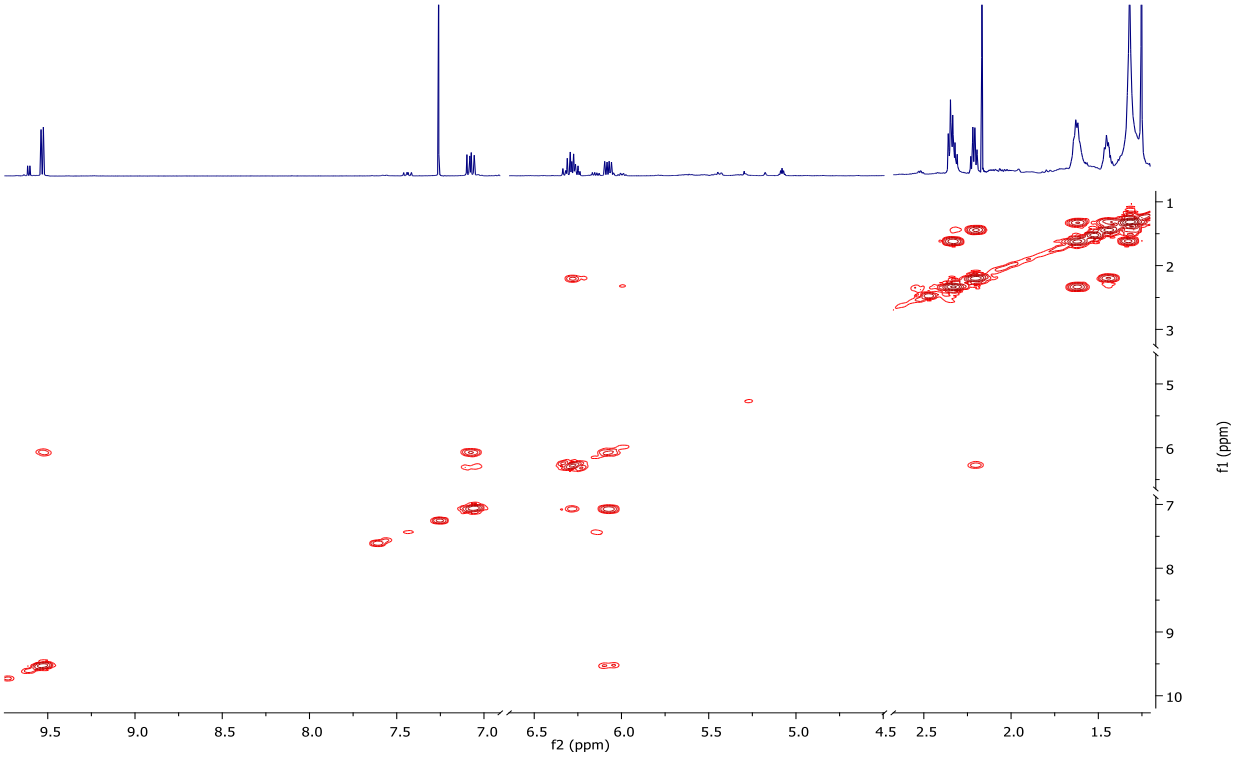


Figure 9a: COSY-NMR spectrum of (*9E, 11E*)-13-oxotrideca-9,11-dienoic acid.

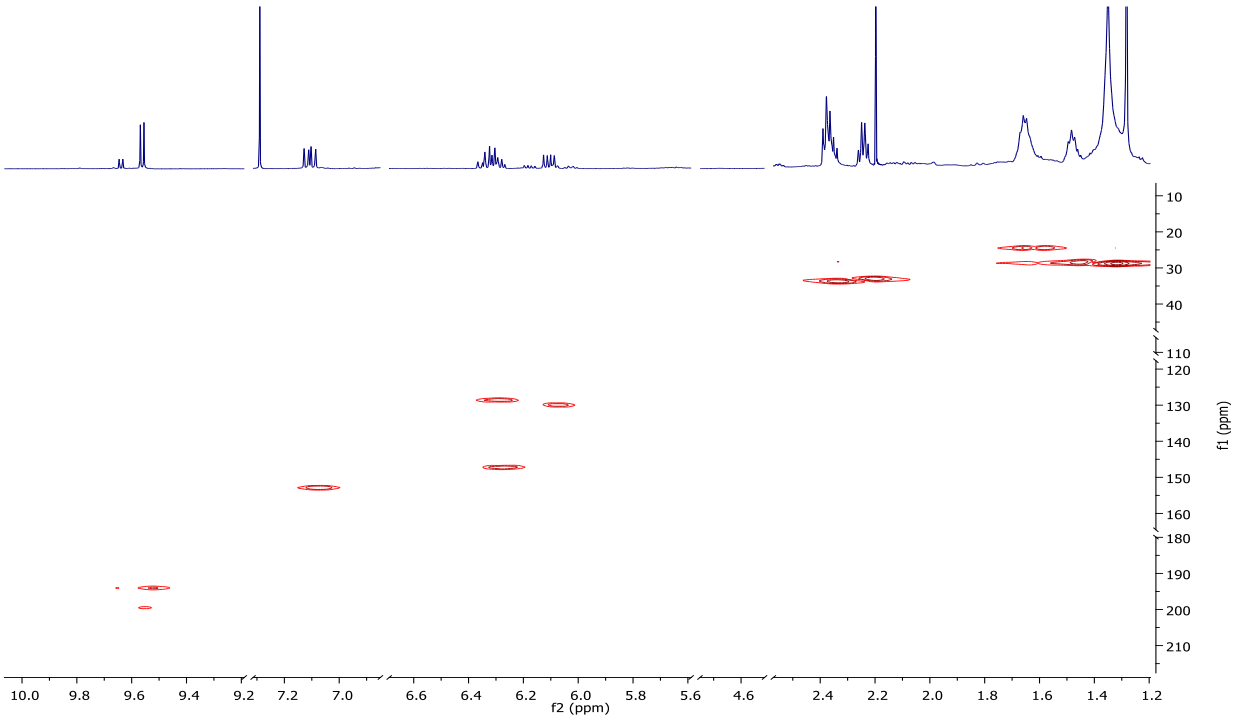


Figure 9b: HSQC-NMR spectrum of (9E, 11E)-13-oxotrideca-9,11-dienoic acid.

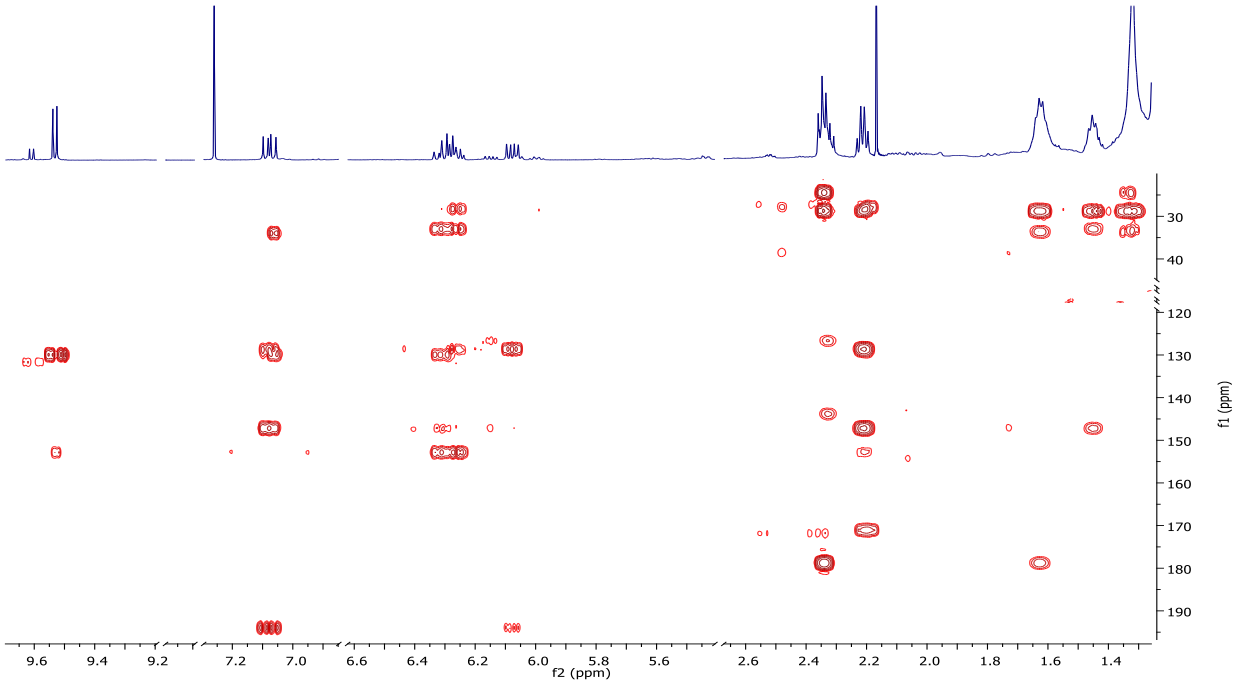


Figure 9c: HMBC-NMR spectrum of (*9E, 11E*)-13-oxotrideca-9,11-dienoic acid.

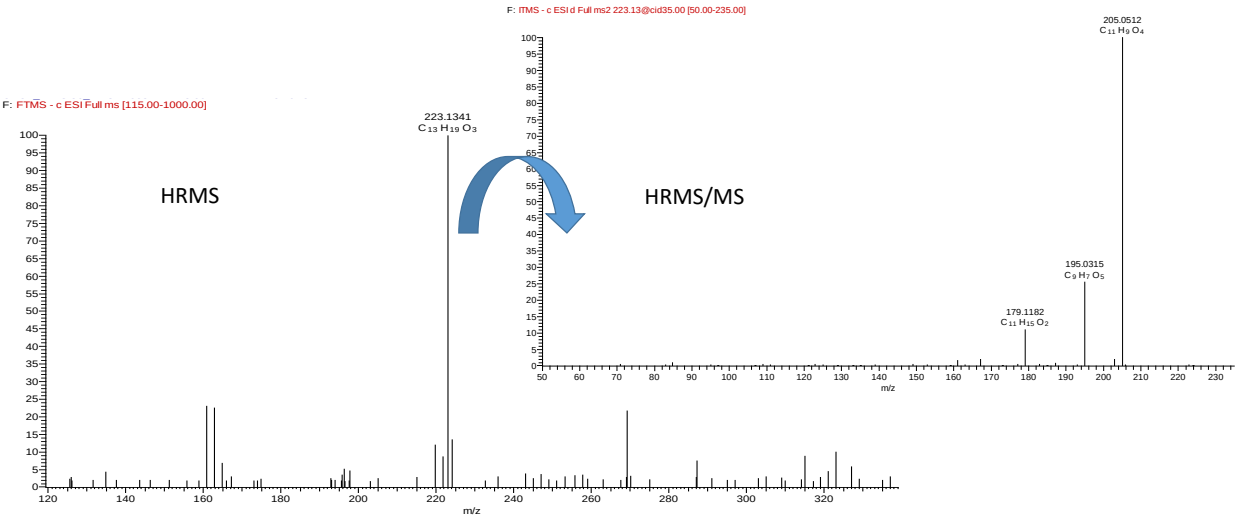


Figure 9d: HRMS and HRMS/MS spectra of (*9E, 11E*)-13-oxotrideca-9,11-dienoic acid.

The second collected minor peak of preparative HPLC analysis (9.5 mg) contained a mixture of two secoiridoid isomers which were identified as (*1R,8E*) 1-ethoxy-ligstroside aglycon (**mc-3**: fig. 7) and (*1S, 8E*) 1-ethoxy-ligstroside aglycon (**mc-4**: fig. 7). The NMR data of the above isomers (Figures 10a-10e) are very close to aglycon moiety of ligstroside. The only spectroscopic difference concerns the spin system of the ethyloxy moiety which replace the sugar moiety in C-1 of ligstroside (Table 3). The stereochemistry of those isomers was based on 2D NOESY NMR spectrum. The off-diagonial cross-peak between H-5 and H-10, for both isomers, suggests close proximity of the involved hydrogen atoms indicating thus *E* configuration of C8-C9 double bond. On the other hand, in the NOESY spectrum a cross-peak between the H-1 and H-6b was appeared for 1*R* isomer and a correlation peak between H-1 and H-8 for 1*S* isomer verifying thus the suggested structures (Figures 10c). The complete ^{1}H-NMR and ^{13}C-NMR spectroscopic data of the above compounds are presented in table 3. To the best of our knowledge, there are not references data regarding both (*1R, 8E*)- and (*1S, 8E*)- 1-ethoxy-ligstroside aglycons. Recently, Cavaca *et al.*, published the formation of both (1*S*)- and (1*R*)- 1-methoxy – oleuropein aglycon after acid-promote methanolisis of oleuropein[47]. These isomers have the same secoiridoid subunit with the isolated 1-ethoxy-ligstroside aglycons. The spectroscopic data referring to monoterpen moieties of (1*S*)- and (1*R*)-1-methoxy – oleuropein aglycons are similar to experimental data of the corresponding subunits of (1*S*)- and (1*R*)-1-ethoxy-ligstroside aglycons verifying thus the suggested structures [47].

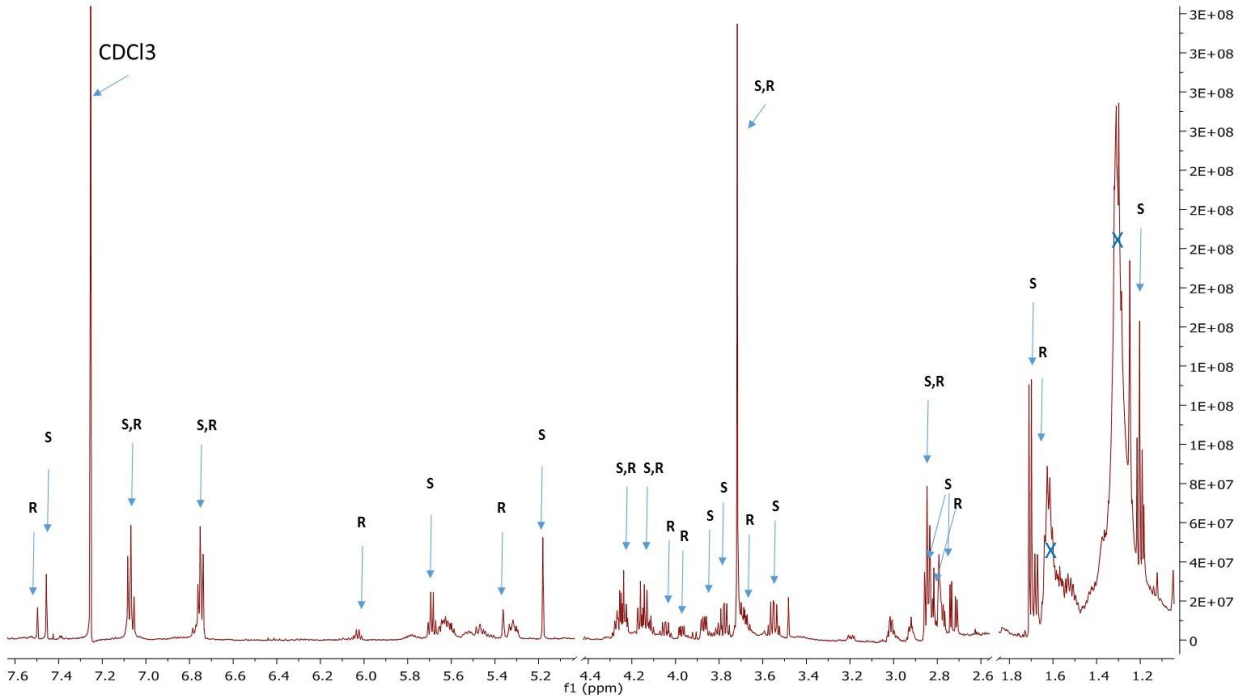


Figure 10a: ^{1}H-NMR spectrum of (1S)- and (1R)- (8E) 1-ethyloxy-ligstroside aglycon.

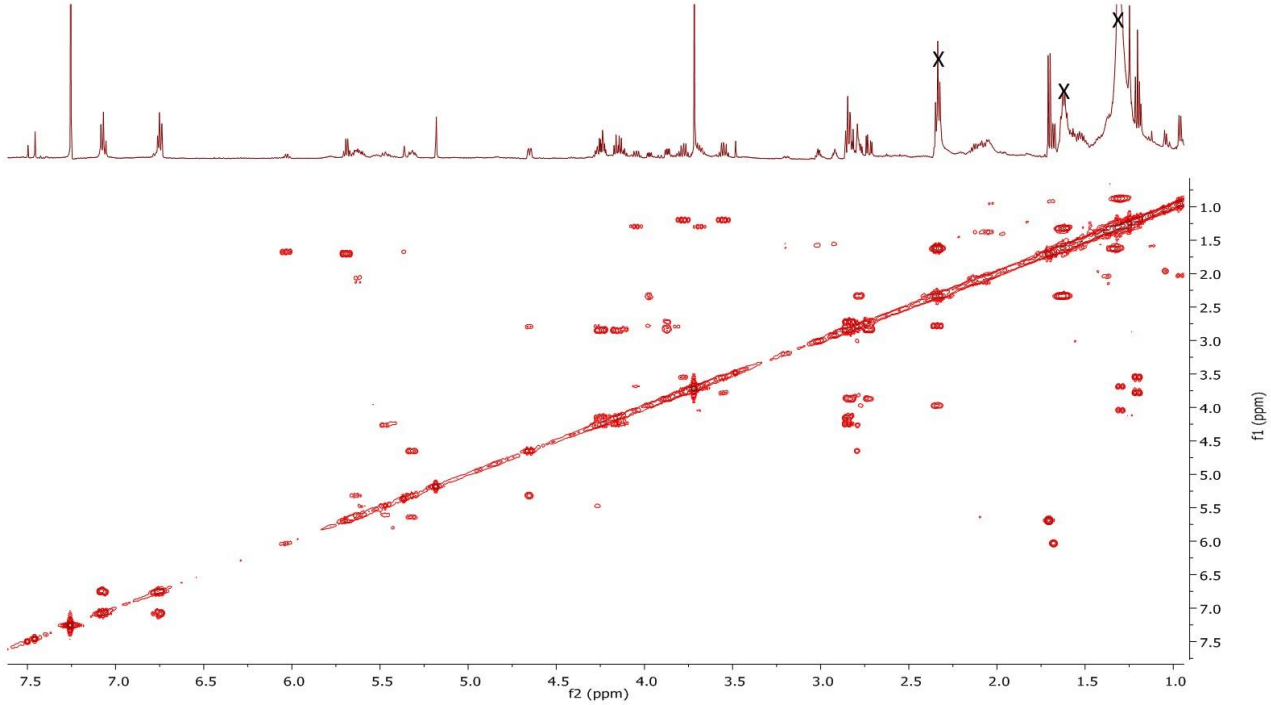


Figure 10b: COSY-NMR spectrum of (1*S*)- and (1*R*)- (8E) 1-ethyloxy-ligstroside aglycon.

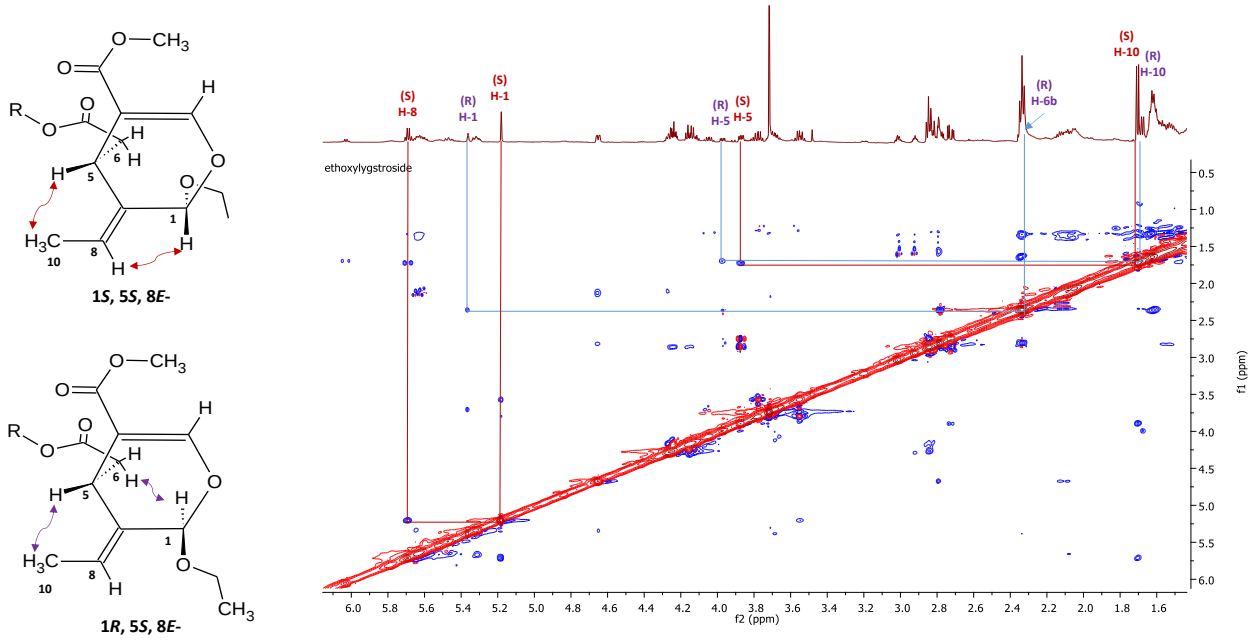


Figure 10c: NOESY-NMR spectrum of (1*S*)- and (1*R*)- (8E) 1-ethyloxy-ligstroside aglycon.

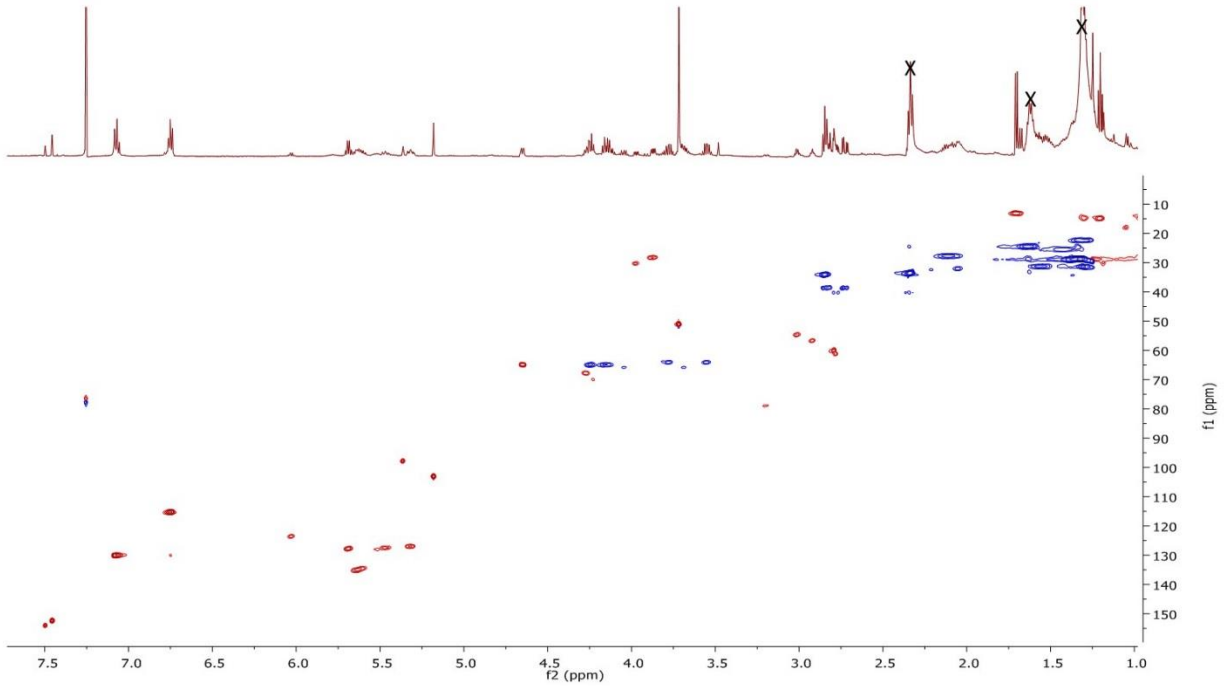


Figure 10d: HSQC-DEPT NMR spectrum (1*S*)- and (1*R*)- (8E) 1-ethyloxy-ligstroside aglycon.

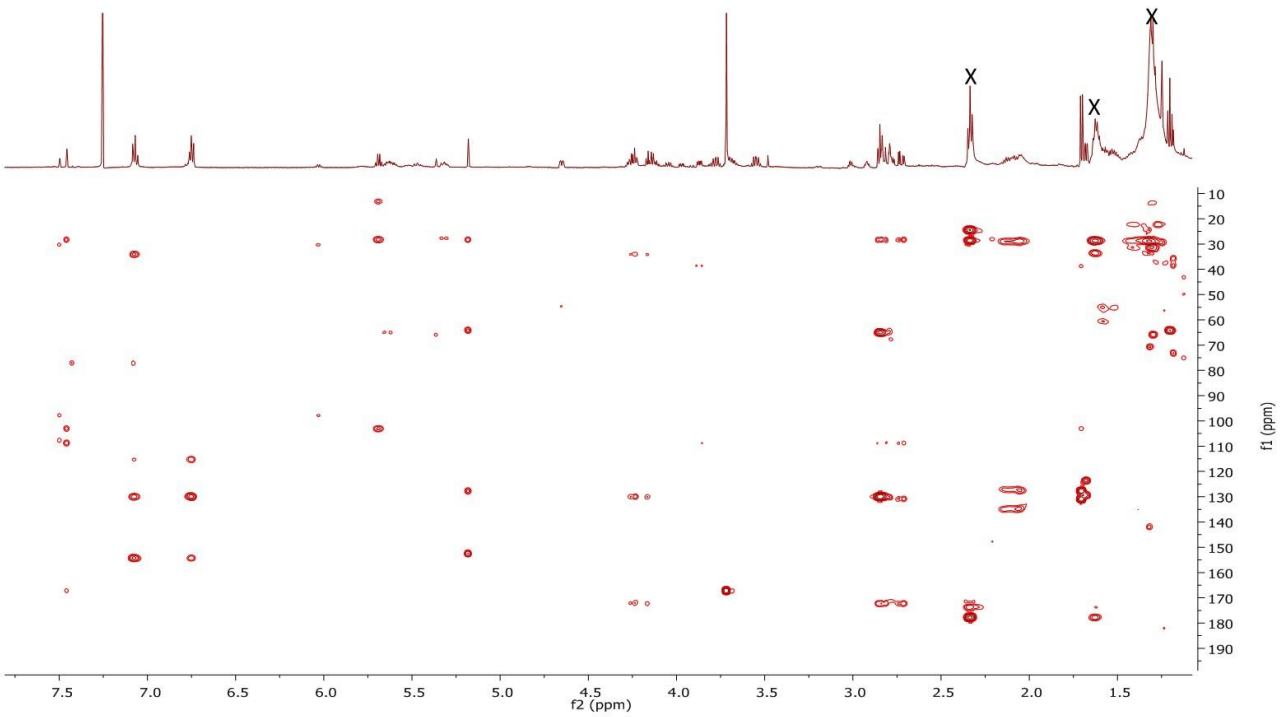


Figure 10e: HMBC NMR spectrum (1*S*)- and (1*R*)- (8E) 1-ethyloxy-ligstroside aglycon.

Table 3: ^{1}H- and ^{13}C NMR data of the new EVOO constituents.

EDA lactone (mc-1)			**(*9E, 11E*)-13-oxotrideca-9,11-dienoic acid (mc-2)**			**(*1S, 8E*) 1-ethoxy-ligstroside aglycon (mc-3)**			**(*1R, 8E*) 1-ethoxy-ligstroside aglycon (mc-4)**		
	^{1}H δ=ppm, (*J*=Hz)	^{13}C (ppm)		^{1}H δ=ppm, (*J*=Hz)	^{13}C (ppm)		^{1}H δ=ppm, (*J*=Hz)	^{13}C (ppm)		^{1}H δ=ppm, (*J*=Hz)	^{13}C (ppm)
1	9,36 d (1.4)	195	**1**	-	178.4	**1**	**5,19** brs	**103,5**	**1**	**5,37** brs	**98.3**
3	4,57 ddd (11.3/6.5/4.5) 4,31 ddd (11.3/7.8/4.5)	68,1	**2**	2.35 t (7.4)	33.9	**3**	7,46 brs	152,9	**3**	7,50 brs	154.5
4	2,14 m 1,88 m	26,1	**3**	1.63 m	24.5	**4**	-	108,8	**4**	-	107,8
5	3,14 m	28,6	**4-6**	1.33 m	28.8	**5**	**3,88** dd (9.4/4.6)	**28,8**	**5**	**3,98** dd (9.6/4.3)	**30.8**
6	2,96 dd (16.5/11.3) 2,43 dd (16.5/6.4)	32,9	**7**	1.45 m	28.5	**6**	**2,84** dd (14.8/9.4) **2,73** dd (14.8/4.6)	39,1	**6**	**2,78** dd (14.8/4.3) **2,34** dd (14.8/9.6)	40.7
7	-	172	**8**	2.21 m	32.9	**7**	-	172,3	**7**	-	171,4
8	6,68 q (7.2)	152,2	**9**	6.27 m	147.2	**8**	**5,70** q (7.1)	**128,4**	**8**	**6.04** q (7.1)	**124.1**
9	-	144,3	**10**	6.29 m	128.6	**9**	-	131,5	**9**	-	129,5
10	2,02 d (7.2)	14,8	**11**	7.07 dd (15.2/7.9)	152.8	**10**	1,71 d (7.0)	13,8	**10**	1,68 dd (7.1/1.5)	13,8
			12	6.08 dd (15.2/10.0)	130.0	**11**	-	167,2	**11**	-	167,2
			13	9.53 d (7.9)	194.1	**12**	3,71s	51,5	**12**	3,71s	51,5
						1'	4,25 m 4,16 m	65,5	**1'**	4,25 m 4,16 m	65,5
						2'	2,85 t (7.2)	34,6	**2'**	2,85 t (7.2)	34,6
						3'	-	130,1	**3'**	-	130,1
						4'/8	7,08 d (8.5)	130,6	**4'/8**	7,07 d (8.5)	130,6
						5'/7	6,75 d (8.5)	115,8	**5'/7**	6,76 d (8.5)	115,8
						6'	-	154.4	**6'**	-	154.4
						1''	**3.78** dq (9.4/7.0) **3.55** dq (9.4/7.0)	**64.7**	**1''**	**4.04** dq (9.4/7.0) **3.68** dq (9.4/7.0)	**66.3**
						2''	1.21 t (7.0)	15.4	**2''**	1.29 t (7.0)	15.3

Conclusion

A pioneering ACE-based extraction was developed from laboratory to pilot scale, leading for the first time to the recovery of EVOO phenolic fraction in large quantities. Prep-CPC analysis was conducted on TPF providing certain amounts of enriched fractions with EVOO biophenols. Furthermore, prep-HPLC was applied for the gram-scale isolation of OLEA, OLEO, HT, MFOA and MFLA in pure form. In parallel, the availability of considerable amounts of enriched CPC fractions provided the opportunity for in depth analysis and made posible the isolation of three novel natural products (EDA lactone, (*1R, 8E*)-1-ethoxy-ligstroside aglycon, (*1S, 8E*)-1-ethoxy-ligstroside aglycon) and a fatty acid that was isolated for the first time from EVOO.

This highly productive methodology, with the advantage of industrial scale potentials, provide in short period of time, huge amounts of EVOO TPF and high-added value compounds in pure form. It should be underlined that is the first time where OLEA, OLEO, MFOA and MFLA are available in quantities able to meet the needs of *in vivo* experiments.

References

[1] F. Luchetti, Importance and future of olive oil in the world market — an introduction to olive oil, Eur. J. Lipid Sci. Technol. 104 (2002) 569–563. https://doi.org/10.1002/1438-9312(200210)104:9/10<559::AID-EJLT559>3.0.CO;2-Q.

[2] R.R. Muñoz, M.L. Moya, J.M. Gil, Market values for olive oil attributes in Chile: A hedonic price function, Br. Food J. 117 (2015) 358–370. https://doi.org/10.1108/BFJ-01-2014-0009.

[3] D. Capogna, M.I. Gomez, Olive oil: an overview of the Japanese market, Oilseeds Fats Crop. Lipids. 23 (2016) 21–23. https://doi.org/10.1051/ocl/2016041.

[4] C.J. Su, J. Sun, W. Zhu, L. Peng, History, distribution, and potential of the olive industry in China: A review, Sustain. 10 (2018) 1–19. https://doi.org/10.3390/su10051426.

[5] F. Visioli, M. Franco, E. Toledo, J. Luchsinger, W.C. Willett, F.B. Hu, M.A. Martinez-Gonzalez, Olive oil and prevention of chronic diseases: Summary of an International conference, Nutr. Metab. Cardiovasc. Dis. 28 (2018) 649–656. https://doi.org/10.1016/j.numecd.2018.04.004.

[6] F.B. Hu, The Mediterranean Diet and Mortality — Olive Oil and Beyond, N. Engl. J. Med. 348 (2003) 2595–2596. https://doi.org/10.1056/NEJMp030069.

[7] N.B. Ray, K.D. Hilsabeck, T.C. Karagiannis, D.E. McCord, Bioactive Olive Oil Polyphenols in the Promotion of Health, in: Role Funct. Food Secur. Glob. Heal., Academic Press, 2019: pp. 623–637. https://doi.org/10.1016/B978-0-12-813148-0.00036-0.

[8] A. Angelis, M. Hamzaoui, N. Aligiannis, T. Nikou, D. Michailidis, P. Gerolimatos, A. Termentzi, J. Hubert, M. Halabalaki, J.H. Renault, A.L. Skaltsounis, An integrated process for the recovery of high added-value compounds from olive oil using solid support free liquid-liquid extraction and chromatography techniques, J. Chromatogr. A. 1491 (2017) 126–136. https://doi.org/10.1016/j.chroma.2017.02.046.

[9] N. and A. (NDA) EFSA Panel on Dietetic Products, Scientific Opinion on the substantiation of a health claim related to polyphenols in olive and maintenance of normal blood HDL cholesterol concentrations (ID 1639, further assessment) pursuant to Article 13(1) of Regulation (EC) No 1924/2006, EFSA J. 10 (2012) 2848. https://doi.org/10.2903/j.efsa.2012.2848.

[10] L. Martinez, G. Ros, G. Nieto, Hydroxytyrosol: Health Benefits and Use as Functional Ingredient in Meat., Med. (Basel, Switzerland). 5 (2018). https://doi.org/10.3390/medicines5010013.

[11] P. Kouka, A. Priftis, D. Stagos, A. Angelis, P. Stathopoulos, N. Xinos, A.-L. Skaltsounis, C. Mamoulakis, A.M. Tsatsakis, D.A. Spandidos, D. Kouretas, Assessment of the antioxidant activity of an olive oil total polyphenolic fraction and hydroxytyrosol from a Greek Olea europea variety in endothelial cells and myoblasts, Int. J. Mol. Med. 40 (2017) 703–712. https://doi.org/10.3892/ijmm.2017.3078.

[12] G.K. Beauchamp, R.S.J. Keast, D. Morel, J. Lin, J. Pika, Q. Han, C.H. Lee, A.B. Smith, P.A.S. Breslin, Ibuprofen-like activity in extra-virgin olive oil, Nature. 437 (2005) 45–46. https://doi.org/10.1038/437045a.

[13] A.H. Abuznait, H. Qosa, B.A. Busnena, K.A. El Sayed, A. Kaddoumi, Olive-Oil-Derived Oleocanthal Enhances β-Amyloid Clearance as a Potential Neuroprotective Mechanism against Alzheimer's Disease: In Vitro and in Vivo Studies, ACS Chem. Neurosci. 4 (2013) 973–982. https://doi.org/10.1021/cn400024q.

[14] B. Polini, M. Digiacomoa, S. Carpi, S. Bertini, F. Gado, G. Saccomanni, M. Macchia, P. Nieri, C. Manera, S. Fogli, Oleocanthal and oleacein contribute to the in vitro therapeutic

potential ofextra virgin oil-derived extracts in non-melanoma skin cancer, Toxicol. Vitr. 52 (2018) 243–250. https://doi.org/10.1016/j.tiv.2018.06.021.
[15] M. Czerwińska, A.K. Kiss, M. Naruszewicz, A comparison of antioxidant activities of oleuropein and its dialdehydic derivative from olive oil, oleacein, Food Chem. 131 (2012) 940–947. https://doi.org/10.1016/j.foodchem.2011.09.082.
[16] A. Filipek, M.E. Czerwińska, A.K. Kiss, M. Wrzosek, M. Naruszewicz, Oleacein enhances anti-inflammatory activity of human macrophages by increasing CD163 receptor expression, Phytomedicine. 22 (2015) 1255–1261. https://doi.org/10.1016/j.phymed.2015.10.005.
[17] M. Naruszewicz, M. Czerwinska, A. Kiss, Oleacein. Translation from Mediterranean Diet to Potential Antiatherosclerotic Drug, Curr. Pharm. Des. 21 (2015) 1205–1212. https://doi.org/10.2174/1381612820666141007141137.
[18] R. Masella, A. Cardilli, L. Gennaro, A. Bocca, D. Modesti, E. Coni, A. Cantafora, Antioxidant activity of 3,4-DHPEA-EA and protocatecuic acid: a comparative assessment with other olive oil biophenols, Redox Rep. 4 (2004) 113–121. https://doi.org/10.1179/135100099101534792.
[19] F. Paiva-Martins, M.H. Gordon, P. Gameiro, Activity and location of olive oil phenolic antioxidants in liposomes, Chem. Phys. Lipids. 124 (2003) 23–36. https://doi.org/10.1016/S0009-3084(03)00032-X.
[20] K.-C. Lai, S.-F. Peng, C.-C. Liu, J.-Y. Huang, J.-Y. Kuo, Z.-Y. Cheng, R.S.-C. Wu, C.-C. Lin, J.-K. Chen, J.-G. Chung, Maslinic Acid Enhances Immune Responses in Leukemic Mice Through Macrophage Phagocytosis and Natural Killer Cell Activities In Vivo., In Vivo (Brooklyn). 33 (2019) 65–73. https://doi.org/10.21873/invivo.11440.
[21] K. Shimazu, S. Fukumitsu, T. Ishijima, T. Toyoda, Y. Nakai, K. Abe, K. Aida, S. Okada, A. Hino, The Anti-Arthritis Effect of Olive-Derived Maslinic Acid in mice is Due to its Promotion of Tissue Formation and its Anti-Inflammatory Effects, Mol. Nutr. Food Res. 1800543 (2018) 1800543. https://doi.org/10.1002/mnfr.201800543.
[22] T. Nikou, V. Liaki, P. Stathopoulos, A. D.Sklirou, E.N. Tsakiri, T. Jakschitz, G. Bonn, I.P. Trougakos, M. Halabalaki, L.A. Skaltsounis, Comparison survey of EVOO polyphenols and exploration of healthy aging-promoting properties of oleocanthal and oleacein, Food Chem. Toxicol. 125 (2019) 403–412. https://doi.org/doi.org/10.1016/j.fct.2019.01.016.
[23] X. Jing, P. Ning, H. Cao, J. Wang, Z. Sun, A review of application of annular centrifugal contactors in aspects of masstransfer and operational security, Hydrometallurgy. 177 (2018) 41–48. https://doi.org/10.1016/j.hydromet.2018.02.005.
[24] S.S. Deshmukh, J.B. Joshi, S.B. Koganti, Flow visualization and three-dimensional CFD simulation of the annular region of an annular centrifugal extractor, Ind. Eng. Chem. Res. 47 (2008) 3677–3686. https://doi.org/10.1021/ie070959w.
[25] J. Zhou, W. Duan, X. Zhou, C. Zhang, Application of annular centrifugal contactors in the extraction flowsheet for producing high purity yttrium, Hydrometallurgy. 85 (2007) 154–162. https://doi.org/10.1016/j.hydromet.2006.08.010.
[26] L.L.M. Meikrantz D.H. WD Flim, C. J. Heald, G.Mendoza, S. B.Meikrantz, A new annular centrifugal contactor for pharmaceutical processes, Chem. Eng. Comm. 189 (2002) 1629–1639.
[27] W. Murayama, T. Kobayashi, Y. Kosuge, H. Yano, Y. Nunogaki, K. Nunogaki, A new centrifugal counter-current chromatograph and its application, J. Chromatogr. A. 239 (1982) 643–649. https://doi.org/10.1016/S0021-9673(00)82022-1.

[28] A.P. Foucault, Centrifugal partition chromatography, M. Dekker, New York, 1995.
[29] N. Fumat, A. Berthod, K. Faure, Effect of operating parameters on a centrifugal partition chromatography separation, J. Chromatogr. A. 1474 (2016) 47–58. https://doi.org/10.1016/j.chroma.2016.10.014.
[30] I.A. Sutherland, Recent progress on the industrial scale-up of counter-current chromatography, J Chromatogr A. 1151 (2007) 6–13. https://doi.org/10.1016/j.chroma.2007.01.143.
[31] H. Zhou, J. Lv, Q. Yuan, Preparative isolation and purification of punicalin from pomegranate husk by high-speed countercurrent chromatography, Sep. Purif. Technol. 72 (2010) 225–228. https://doi.org/10.1016/j.seppur.2010.01.018.
[32] M. Bojczuk, D. Żyżelewicz, P. Hodurek, Centrifugal partition chromatography – A review of recent applications and some classic references, J. Sep. Sci. Sci. 40 (2017) 1597–1609. https://doi.org/10.1002/jssc.201601221.
[33] A. Berthod, M.J. Ruiz-ángel, S. Carda-broch, Countercurrent chromatography : People and applications, J. Chromatogr. A. 1216 (2009) 4206–4217. https://doi.org/10.1016/j.chroma.2008.10.071.
[34] O. Shpigun, T. Maryutina, A. Berthod, B. Spivakov, I.A. Sutherland, Countercurrent chromatography in analytical chemistry (IUPAC Technical Report), Pure Appl. Chem. 81 (2009) 355–387. https://doi.org/10.1351/pac-rep-08-06-05.
[35] J. Kang, D. Gu, T. Wu, M. Wang, H. Zhang, H. Guo, Y. Yin, Y. Yang, J. Tian, An approach based upon the consecutive separation and the economical two-phase solvent system preparation using UNIFAC mathematical model for increasing the yield of high-speed counter-current chromatography, Sep. Purif. Technol. 162 (2016) 142–147. https://doi.org/10.1016/j.seppur.2016.02.026.
[36] A. Kotland, S. Chollet, C. Diard, J. Autret, J. Meucci, J. Renault, L. Marchal, Industrial case study on alkaloids purification by pH-zone refining centrifugal partition chromatography, J. Chromatogr. A. 1474 (2016) 59–70. https://doi.org/10.1016/j.chroma.2016.10.039.
[37] F. Chemat, M. Abert Vian, A.S. Fabiano-Tixier, M. Nutrizio, A. Režek Jambrak, P.E.S. Munekata, J.M. Lorenzo, F.J. Barba, A. Binello, G. Cravotto, A review of sustainable and intensified techniques for extraction of food and natural products, Green Chem. 22 (2020) 2325–2353. https://doi.org/10.1039/c9gc03878g.
[38] F. Chemat, M. Abert-Vian, A.S. Fabiano-Tixier, J. Strube, L. Uhlenbrock, V. Gunjevic, G. Cravotto, Green extraction of natural products. Origins, current status, and future challenges, TrAC - Trends Anal. Chem. 118 (2019) 248–263. https://doi.org/10.1016/j.trac.2019.05.037.
[39] International Olive Council, Determination of Biophenols in Olive Oils by HPLC, COI/T.20/Doc No 29/Rev.1. 29 (2017) 1–8.
[40] D. Michailidis, A. Angelis, N. Aligiannis, S. Mitakou, L. Skaltsounis, Recovery of Sesamin, Sesamolin, and Minor Lignans From Sesame Oil Using Solid Support-Free Liquid-Liquid Extraction and Chromatography Techniques and Evaluation of Their Enzymatic Inhibition Properties, 10 (2019). https://doi.org/10.3389/fphar.2019.00723.
[41] P.J. Fellows, Food processing technology-principles and practice, 4th ed., Woodhead Publishing, Elsevier Ltd., 2017.
[42] A. Termentzi, M. Halabalaki, A.L. Skaltsounis, From Drupes to Olive Oil: An Exploration of Olive Key Metabolites, AOCS Press, 2015. https://doi.org/10.1016/B978-1-63067-041-2.50012-4.

[43] K.L. Tuck, P.J. Hayball, Major phenolic compounds in olive oil: metabolism and health effects, J. Nutr. Biochem. 13 (2002) 636–644. https://doi.org/10.1016/S0955-2863(02)00229-2.
[44] A. Angelis, L. Antoniadi, P. Stathopoulos, M. Halabalaki, L.A. Skaltsounis, Oleocanthalic and Oleaceinic acids: New compounds from Extra Virgin Olive Oil (EVOO), Phytochem. Lett. 26 (2018) 190–194. https://doi.org/10.1016/j.phytol.2018.06.020.
[45] B.A. Vick, D.C. Zimmerman, Metabolism of Fatty Acid Hydroperoxides by Chlorella pyrenoidosa , Plant Physiol. 90 (1989) 125–132. https://doi.org/10.1104/pp.90.1.125.
[46] S. Tahara, S. Kasai, M. Inoue, J. Mizutani, Identification of mucondialdehyde as a novel stress metabolite, Experientia. 50 (1994) 137–141. https://doi.org/10.1007/BF01984952.
[47] L.A.S. Cavaca, C.A.B. Rodrigues, S.P. Simeonov, R.F.A. Gomes, J.A.S. Coelho, G.P. Romanelli, A.G. Sathicq, J.J. Martinez, C.A.M. Afonso, Valorization of Oleuropein via Tunable Acid-Promoted Methanolysis., ChemSusChem. 11 (2018) 2300–2305. https://doi.org/10.1002/cssc.201800980.

Part II: Limited production oils

Chapter 1: Green extraction of edible seed pastes and oils from Greek flora. Evaluation of their *in vitro* antioxidant and dermo-cosmetic activity.

Abstract

Vegetables seed oils (VSOs) are regarded as key components of human diet from ancient times. In addition, VSOs are used for the improvement of human life quality and some of them possess leading role in cosmetics industry due to their dermo-cosmetic advantages. In the current study, seven different seed species from plant of Greek flora (*Vitis vinifera, Cucurbita pepo, Punica granatum, Helianthus annuus, Cannabis sativa, Foeniculum vulgare* and *Prunus dulcis*) were selected and cold pressed in order to obtain high quality seed oils. During the oil produce process, the generated by-product pastes (VSPs) were collected and evaluated as potential sources of high added value extracts and/or compounds. The dermo-cosmetic properties of VSOs and VSPs directed the study to the development of green methodologies for their extraction. Specifically, VSOs were treated with food grade XAD-7, while for VSPs two different protocols were applied, the Ultrasound Assisted Extraction (UAE) and the Supercritical Fluid Extraction (SFE). Regarding UAE, by-product pastes were firstly defatted with *n*-Hexane and then ethanol or aqueous solution of ethanol/water 1:1 v/v were used to obtain two different extracts form each starting material. For the second extraction protocol, SFE technique was adopted. Extraction with pure CO_2 was employed to remove the fatty fraction of VSPs. Afterwards, ethanol was used as co-solvent in 10% and 20% to obtain two more extracts from each paste. All the extracts were analyzed with HPLC-DAD and LC-HRMS, while GC-MS was further employed on VSOs for determination of their fatty acid composition. After the characterization of VSOs and VSPs chemical composition the obtained extracts were evaluated for their dermo-cosmetic biological activity and their inhibition against collagenase, elastase and tyrosinase enzymes.

Keywords

Edible seed oils, seed by-products, adsorption resins, green extraction, UAE, SFE, antioxidant, tyrosinase, elastase, collagenase.

Introduction

The recent advancements of natural products chemistry and the dissemination of their beneficial properties for human health have led to the increasing consumers' demand for medical plant-based products. Among them, vegetable seed oils (VSO) have an extensive use from ancient times, for their edible attributes, as well as, for dermo-cosmetic and pharmaceutical uses [1]. Most of them are characterized by high nutritional value, which has established them as predominant

components of pharmaceutical and food industries. VSOs derive from seeds which are usually by-products of fruit processing industries, like pomegranate seeds from beverage industries and grape seeds from wine industries. Vegetable seeds and seed based products are a nutritional pool for animal and human diet. Moreover, they stand as a potent source for the improvement of human well-being [2] since their chemical composition, is rich in important molecules like lipids, vitamins, sterols, tannins and glucosides [3]. During pressing process of seeds, lipophilic compounds are transferred to the produced oil and more polar solutes remain at the by-product seed paste (VSPs).

The last decade, cosmetic industry has developed numerous applications of VSO in cosmetic formulations due to their natural and non-toxic character. Usually, they are used as moisturizers and emollients for increasing the hydration of the skin [4]. Edible oils are characterized by a complex chemical profile, consisted mainly of triglycerides, diglycerides, monoglycerides and free fatty acids. Also, VSOs contain monounsaturated fatty acids like ω-9, ω-6 and ω-3. Furthermore, they are a good source of polyunsaturated fatty acids that human organism cannot produce, like linoleic acid [5]. Moreover, 2-5% of their composition is consisted of molecules with high bioactive interest like terpenes, phenols, vitamins and phytosterols [6]. These ingredients are known for their antioxidant, antiaging, skin moisturizing activity and many other positive impacts to human well being and health [7]. Therefore, the last decade has witnessed a great industrial interest towards VSOs; numerous cosmetic formulations of VSOs have been developed, especially moisturizers and emollients for increasing the hydration of the skin [4].

During the oil production procedure, seeds are pressed providing VSOs, lipophilic compounds are transferred to the produced oil and more polar solutes remain at the generated dry pellets, which consist the by-product seed paste (VSPs). These byproducts are regarded as wastes of the procedure. Though, they could serve as potential sources of health beneficial compounds and extracts [8–10], because they are enriched with the polar constituents of vegetable seeds, named biophenols, known for their strong antioxidant properties. Scientific and industrial interest has focused on exploiting these by-products, since they are reusable and low-cost material suitable for further profitable industry applications, which potentially hold numerous pharmacological activities.

In parallel, another need in the industry has arisen in order to fit their production processes with eco-friendly criteria and green methodologies. The last years, cosmetics domain adopt the use of

non-toxic solvents and materials for the environment and human, while at the same time animal tests from research and development methodologies are switching from *in vivo* experiments to *in vitro* assays.

Nowadays, several green methodologies have been proposed for the treatment of raw materials like edible oils and their byproducts. Specifically, adsorption resins are used from industries, in order to obtain enriched extracts in bioactive compounds. Low cost, simplicity of procedure and the flexibility of application in a huge range of starting materials, are some of their basic advantages [11]. Ultrasound Assisted Extraction (UAE) is another very useful technique to obtain extracts from natural sources [12] UAE applies acoustic cavitation that produces cavitation bubbles, which increase the shear forces [13]. By this way the solvent is diffused through the walls of the cells and provokes their collapse, leading at the dispersion of their compounds in the extraction solvent [14]. This methodology provides the ability of using green solvents, like ethanol (EtOH) and water (H_2O), leading on the production of safe (nontoxic) edible extracts and products [15].

Likewise, Supercritical Fluid Extraction (SFE) is an entirely green procedure, applicable from lab to industrial scale, with noteworthy advantages. The most common solvent of SFE procedures is carbon dioxide (CO_2), a non-toxic solvent which is gas at room temperature so it does not contaminate the final extract [16]. The gaseous nature of CO_2 is altered on supercritical conditions when the pressure and temperature are adjusted over 72.9 atm and 31.3 ^{o}C, respectively [17]. Supercritical form of solvents, provide the ability to increase their diffusion properties, while at the same time their viscosity is decreased, compared to their liquid form [18]. In this way, the solvent penetrates effectively the extraction material and as a result, the mass transfer is increased and the time of extraction is reduced [19]. Another advantage of SFE is that CO_2, which is considered as a non-polar solvent, can be combined with other more polar green solvents, like EtOH, in order to enrich the extracts with higher polarity molecules [20]. The SFE finds application in a huge range of fields and it has been established as suitable technique for waste and byproduct management [21].

Among these lines, in the current study, we aimed to implement green methodologies for the exploitation of seven different VSOs and the corresponding VSPs deriving from plants which are typical cultivated in the area of Greece. In particular, seeds originated from: *Vitis vinifera* (grape seeds), *Cucurbita pepo* (pumpkin seeds), *Punica granatum* (pomegranate seeds), *Helianthus*

annuus (sunflower seeds), *Cannabis sativa* (hemp seeds), *Foeniculum vulgare* (fennel seeds) and *Prunus dulcis* (almond seeds). All of them were cold pressed, squished and finally VSO and the corresponding VSPs were produced.

Moreover, we sought to employ three different extraction methods and investigate the chemical composition of the produced extracts. On VSOs, liquid-solid XAD-7 adsorption resin was applied with ethanol (EtOH) as elution solvent, while VSPs were treated with UAE and SFE methodologies. On both experimental procedures, the first step was the defatting with *n*-Hexane (*n*-Hex). Afterwards, two different extracts were produced from each extraction process. The defatted raw materials that derived from UAE, were extracted firstly with EtOH and then with EtOH/H_2O in ratio 1:1 v/v. The defatted raw materials that resulted from SFE, were treated with CO_2 and 10% EtOH, as co-solvent. Afterwards, one more extraction was carried out with CO_2 and 20% EtOH. In total, 5 extracts were obtained from each kind of seed, reaching a total number of 35. LC-HRMS and HPLC-DAD analysis were performed on all the extracts, while GC-MS analysis was employed on VSOs after esterification procedure.

Finally, we aimed to evaluate the produced extracts for their antioxidant activity and for their ability to prevent skin related processes such as pigmentation and aging by performing several bioassays in vitro. In specific, the total phenolic content (TPC) of each extract was calculated in correlation with gallic acid equivalents and the radical scavenging activity assay using DPPH (1,1-diphenyl-2-picrylhydrazyl) was performed, in order to have a first screening of extracts antioxidant activities. Furthermore, enzymatic assays were applied on multiple concentrations of all the extracts, in order to evaluate their inhibitory activity on tyrosinase, collagenase and elastase enzymes.

Methods and Materials

Reagents

The extraction solvents were analytical grade and purchased from Fisher Scientific (Pennsylvania, USA). The solvents used in UPLC-HRMS analysis were LC-MS grade and supplied also from Fisher Scientific (Pennsylvania, USA). All the VSOs and their corresponding VSPs were kindly provided from Medi Oils (Athens, Greece). Gallic acid, sodium carbonate (99% purity), 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95% purity), Folin–Ciocalteu reagent and the reagents of the enzymatic assays were purchased from Sigma-Aldrich. In detail, mushroom tyrosinase

(lyophilized powder, ≥1000 units/mg solid, EC Number: 1.14.18.1), 3,4-dihydroxy-L-phenylalanine, sodium phosphate monobasic, sodium phosphate dibasic, kojic acid, elastase type IV from porcine pancreas (EC Number 254-453-6), N-Succinyl-Ala-Ala-Ala-p-nitroanilide (EC Number 257-823-5), Trizma base reagent grade, elastatinal, collagenase from Clostridium histolyticum (released from physiologically active rat pancreatic islets Type V, ≥1 FALGPA units/mg solid, > 125 CDU/mg solid (EC Number: 232-582-9), MMP 2 fluorogenic substrate (MCAPro-Leu-Ala-Nva-DNP-Dap-Ala-Arg-NH_2) and phosphoramidonwere purchased also from Sigma-Aldrich.

Cold press production of seed oils and seed paste by-products

Seven edible seeds provided from producers of Greek area (Table 1) were selected and cold pressed in a KK40 F Special (Oil Press GMPH, Reut, Germany) food-safe mill. The seeds are placed in the stainless steel hopper and passed through a system of hard seed screws and sieves where the material is pressed producing the seed oil and pellets of oil-free paste. The specification of mill enabled a seed capacity of up to 40 kg/h, depending on seed morphological characteristics (seed size, hardness, oil percentage, etc.). In the present work, the screws speed was properly adjusted to cover treatment capacity of 15 kg/h for the hardest seeds (*GrpS* and *PmgS*), and 25 kg/h for the softer seeds (*PmpS*, *AlmS*, *SnfS*, *HmpS* and *FnnS*). Table 1 summarizes the procedure parameters following for the cold press production of seed oils (VSO) and remaining seep paste by-products (VSP).

Table 1: Details for the cold press procedure

Plant	***Raw material***	***Treated amound***	***Treatment speed***	***Produsedoil***	***% of oil production (v/w of seed)****	***Oil Dencity***
Vitis vinifera	***Grape seed** (GrpS)*	*30 Kg*	*15 kg/h*	*2.1 L*	*7%*	*824.4 mg/mL*
Cucurbita pepo	***Pumpkin seed** (PmpS)*	*20 Kg*	*25 kg/h*	*4.2 L*	*21%*	*788.4 mg/mL*
Punica granatum	***Pomegranate seed** (PmgS)*	*25Kg*	*15 kg/h*	*1.75 L*	*7%*	*729.1 mg/mL*
Helianthus annuus	***Sunflower seed** (SnfS)*	*20 Kg*	*25 kg/h*	*4.4 L*	*22%*	*823.4 mg/mL*
Cannabis sativa	***Hemp seed** (HmpS)*	*20 Kg*	*25 kg/h*	*3.8 L*	*19%*	*836.3 mg/mL*
Foeniculum vulgare	***Fennel seed** (FnnS)*	*40 Kg*	*25 kg/h*	*0.6 L*	*1.5%*	*913.4 mg/mL*
Prunus dulcis	***Almond seed** (AlmS)*	*20 Kg*	*25 kg/h*	*5.6 L*	*28%*	*793.4 mg/mL*

** the remain percentage constituting the by-products*

Production of green extracts from VSOs and VSPs using adsorption resins, UAE and SFE

VSOs were extracted using adsorption resins XAD-7. 30 g of resin XAD-7 were embedded in each column with dimensions of 65 cm x 1.8 cm. Resin was activated with three column volumes of EtOH. 100 mL of each seed oil were loaded in different column with a flow rate of 1 drop per second. Then, the columns were rinsed with 200 mL of food grade *n*-Hex (per column), in order to remove the remained lipophilic components of the starting material. The elution was conducted with 150 mL of EtOH using again a flow rate of 1 drop/sec. The final extracts were evaporated until dryness in Rota vapor, at 40^{o}C. The generated VSPs were extracted with two different methods, UAE and SFE. Ultrasound extractions took place in an ultrasonic P300H bath of Elma Schmidbauer (Singen, Germany). 10 g of each VSP were placed in erlenmeyers and extracted successively with 30 mL of *n*-Hex, EtOH and EtOH/H_2O 1:1 (v/v). Each ultrasound extraction lasted 20 min and the corresponding eluents were evaporated to dryness and weighted. For the supercritical fluid experiments, analytical scale apparatus of SEPAREX (Nancy, France) was used. It was consisted of a force ventilation oven, which was equipped with a 100 mL stainless steel extraction vessel, a separator, a back-pressure regulation valve, a CO_2 chiller unit, a co-solvent and a CO_2 liquid pump. The sample vessel has a total volume of 100 mL and 50 g from each paste were grounded to powder and initially extracted with 100% CO_2 a flow rate of 15 g/min. After the defatting step, two more successive extractions were applied. The first one was accomplished with CO_2 at 15 g/min and 10% EtOH w/w as co-solvent and the second one with 15 g/min CO_2 and 20% EtOH w/w. In all the SFE procedures, pressure was set at 300 bar and extracts were treated until exhaustiveness.

Esterification process and GC-MS analysis of VSOs

Esterification process of fatty acids contacted as described by *K. Eder et al.* [22], with slight modifications. 30 mg of each VSO, 6 mL of toluene and 12 mL of sulfuric acid diluted in MeOH (1% v/v) were mixed in a flask under stirring, heated at 65 ^{o}C for 2 hours. Afterword, 30 mL of NaCl aqua solution (5% w/v) were added and the mixture was extracted with 30 mL of *n*-Hex three times. In the final 90 mL of *n*-Hex was added 25 mL of $NaHCO_3$ aqua solution (2% w/v). To dehydrate the organic phase was used anhydrous Na_2SO_4. Finally, the mixture was filtrated and

the organic phase was evaporated until dryness. 0,5 mg of each VSO was diluted in 1 mL of dichloromethane and was transferred in analysis vial.

GC-MS analysis were obtained in a Hewlett–Packard 6890-5973 (Thermo-Scientific, USA). The analysis was conducted at EI mode-70 eV. For compounds separation a capillary column TP-5MS (30 m × 0.25 mm × 0.25 μm) (Thermo-Scientific, USA) was used. Helium was used with as carrier gas in a flow rate of 1 mL/min. The injection was conducted with a volume of 1 μL in splitless mode. Inlet temperature was set at 220°C. The method was started from 60°C. After the fifth minute temperature started to be increased, reaching 280°C with heating step of 3 C/min. Temperature was kept stable for 10 min at 280 °C. The software Xcalidur 2.0 (Thermo-Scientific, USA) was used. The identification of compounds was based on correlation with mass spectra data with those of Wiley and NBS libraries.

LC-HRMS and HPLC-DAD analysis of the extracts

All the produced extracts were analyzed by UPLC-HRMS/MS. The experiments were accomplished to an H class Acquity UPLC system (Waters, USA) coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, USA). For the chromatographic separation, a Fortis C-18 column (1.7 μm, 150 × 2.1 mm) was used temperature was set at 40°C. The elution solvent system consisted of acidified water with 0.1% formic acid (A) and acetonitrile (B). Gradient started with 2% B for 2 min, which reached 100% at 18 min and stayed for 2 more min. At the 21th minute system returned to the initial conditions and stayed for 4 min for system equilibration. The flow rate was at 400 μL/min, the injection volume was 10 μL and samples were kept at 7°C. Ionization was carried out in negative and positive ion mode (ESI±). The mass spectrometric parameters were: capillary temperature 350°C; sheath gas 40 units; aux gas 10 units; capillary voltage 30 V; and tube lens 100 V for the positive mode. For negative ionization capillary voltage was adjusted at −20 V and tube lens of −80 V, while all the other parameters remained stable. Data were recorded in full scan from 113 to 1,000 *m/z* and HRMS/MS experiments were carried out with data dependent method with collision energy 35.0% (q = 0.25).

HPLC analysis was conducted on a Thermo Finnigan HPLC system (Ontario, Canada) equipped with a SpectraSystem P4000 pump, a SpectraSystem 1000 degasser, a SpectraSystem AS3000 automated injector, and a UV SpectraSystem UV6000LP detector. Data acquisition was controlled by the ChromQuest™ 5.0 software (ThermoScientific™). HPLC-DAD experiments were run on a Supelco Analytical (Sigma-Aldrich) HS C18 column, with dimensions 25 × 4.6 mm, 5 μm, at

room temperature. As elution solvent system, acidified H_2O (0.1% v/v of formic acid) and acetonitrile were used. Gradient was started with 98% of H_2O and reached 2% after 60 min. At 61 min elution system returned to the initial conditions and stayed for 4 min. Flow rate was set at 1 mL/min and injection volume at 10 μL. Chromatograms of 254 nm, 280 nm and 366nm, were recorded.

TPC and DPPH Assays

Total Phenolic Content (TPC) of extracts was evaluated via Folin Ciocalteu colorimetric assay. Gallic acid was used for TPC evaluation and eight different concentrations were applied for the construction of the calibration curves (2.5 μg/ml, 5 μg/ml, 10 μg/ml, 12.5 μg/ml, 20 μg/ml, 25 μg/ml, 40 μg/ml and 50 μg/ml) mixed with Folin Ciocalteu reagent (ten-fold dilution) and sodium carbonate solution (7.5 % w/v). Extracts were tested in two different concentrations (1000 and 500 μg/mL in the wells) and analyzed twice in triplicates. The absorptions were measured on an Infinite 200 PRO series reader (Tecan Group, Switzerland) at 765 nm. TPC values were expressed as mg of gallic acid equivalent/mg of extract using the resulted calibration curve (R^2=0.9978).

DPPH radical scavenging assay was performed using a previously described protocol [23] with some modifications. DPPH stock solution was prepared by diluting 12.4 mg of DPPH reagent in 100 mL absolute ethanol concluding in final concentration of 314 μM. Stock solution was vortexed and protected in dark at room temperature until its use. As positive control gallic acid was used, in a concentration of 29.4 μM. All the extracts were diluted in DMSO providing four different concentrations of 10 mg/mL, 5 mg/mL, 1 mg/mL and 0.5 mg/mL. Briefly, 190 μL of DPPH solution were mixed with 10 μL of gallic acid or the samples in a 96-well plate. Negative control was composed from 190 μL of DPPH solution and 10 μL of DMSO. For the blanks 190 μL of EtOH and 10 μL of sample were used. All the experiments were conducted twice in triplicates. After 30 minutes incubation in dark place and at room temperature, the absorbance (abs) was measured using an Infinite 200 PRO series reader (Tecan Group, Switzerland) at 517 nm.

The calculation formula of radical scavenging activity percentage is:

$$\text{radical scavenging activity (\%)} = [1 - ((X\ \text{sample} - X\ \text{blank})/X\ \text{control})] \times 100$$

X control symbolizes the absorbance of the negative control, while X sample the absorbance after the reaction of samples with DPPH. X blank symbolizes the absorbance of samples with EtOH.

Tyrosinase, Elastase and Collagenase Activity Assays

The enzymatic assays for tyrosinase, elastase and collagenase enzymes were applied as previously described by *Michailidis et al* and *Angelis et al* [24,25] with few modifications. Experiments were performed twice in triplicates. The calculation formula of inhibition percentage is:

Inhibition (%) = [((X control − X control's blank) − (X sample − X sample's blank))/(X control − X control's blank)] × 100

X control symbolizes the absorbance or fluorescence value of the mixture consisting of buffer, enzyme, sample solvent, and substrate, while X sample stands forthe absorbance or fluorescence value of the mixture of buffer, enzyme, sample or positive control solution and substrate. Blanks contained all the above-mentioned components except the enzyme.

As standard of comparison for tyrosinase, elastase, and collagenase enzymatic assays the half maximal inhibitory concentration (IC50) of each positive control was used.

Tyrosinase enzymatic assay: This assay evaluates the action of the tested samples at the catalytic oxidation of L-DOPA to dopachrome by tyrosinase. Kojic acid (IC_{50} = 50 µM) was used as positive control. In a 96-well microplate, 80 µl of phosphate-buffered saline (PBS) (0.067 M, pH = 6.8), 40 µl of the tested sample, and 40 µl of mushroom tyrosinase (EC Number: 1.14.18.1), 100 U/mL dissolved in PBS buffer were mixed and incubated in the dark for 10 min at room temperature. Afterwards, 40 µl of 2.5 mM L-DOPA (substrate) dissolved in PBS buffer were added and the mixture was incubated for 10 min so that the dopachrome is formed. The tyrosinase activity was determined at 475 nm using the reader Infinite 200 PRO series (Tecan). The final concentrations of the extracts on the plate were 500µg/mL, 150 µg/mL and 20µg/mL diluted in PBS with 1% DMSO.

Elastase enzymatic assay: The evaluation of elastase activity is based on the release of p-nitroaniline from N-succinyl-Ala-Ala-Ala-p-nitroanilide that is stimulated by elastase. Elastatinal (IC_{50} = 0.5 µg/ml) was used as positive control. In a 96-well microplate, 70 µl of Tris-Hlbuffer (50 mM, pH = 7.5), 10 µl of tested sample, and 5 µl of elastase (0.45 U/ml) (dissolved in Tris-HCl buffer) were mixed and incubated in the dark for 15 min at room temperature. Then, 15 µl of 2 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide (substrate) dissolved in Tris-HCl buffer was added, and the mixture was incubated for 30 min at 37°C. The production of p-nitroaniline was determined at 405 nm using the reader Infinite 200 PRO series (Tecan). The final concentrations of the extracts were 300µg/mL, 150µg/mL and 30µg/mL dissolved in Tris-HCl buffer with

0.5%DMSO. Collagenase enzymatic assay: Collagenase activity was determined with a spectrofluorimetric method using a fluorogenic metalloproteinase-2 (MMP2) substrate (MCAPro-Leu-Ala-Nva-DNP-Dap-Ala-Arg-NH2) which is enzymatically degraded by collagenase to produce fluorescence. Phosphoramidon (IC_{50} = 16 μM) was used as positive control. In a 96-well black microplate, 120 μl of Tris-HCl buffer (50 mM, pH = 7.3), 40 μl of tested sample, and 40 μl of collagenase (50 μg/ml) from C. histolyticum (dissolved in Tris-HCl buffer) were incubated for 10 min at 37°C avoiding light exposure. Then, 40 μl of 50.0 μM fluorogenic substrate dissolved in Tris-HCl buffer were added, and the mixture was incubated in dark for 30 min at 37°C. The fluorescence intensity of was measured at an excitation maximum of 320 nm and an emission maximum of 405 nm.

All the measurements took part in the Infinite 200 PRO series reader (Tecan Group, Switzerland).

Results and Discussion

Production of seed oils and GC-MS analysis

All selected seeds were cold pressed on temperatures between 25-30 ºC, in order to produce oil fractions and their corresponding by-products. Generally, oil production varies for each kind of seed, depending on the concentration of their saponifidied content. The highest oil yield was obtained from Alm seeds (30% v/w of raw material), following from Snf seeds (22% v/w of raw material), Hmp seeds (20% v/w of raw material) and Pmp seeds (20% v/w of raw material). Pmp seeds and Grp seeds provided 7% v/w of oil, while Fnn seeds had the lowest oil production with only, 1.5% v/w (Table 1). The duration of oil extraction process varied for each kind of seed, due to the differences on raw material hardness. Pressing speed of the seeds was strong correlated with the final quality of oils. Because of the friction between the seeds and the smashing spiral-screws of the mill the temperature was increased. Although, the specification of mill enabled a treatment seed capacity up to 40 kg/h, the operation parameters were set in lower speed in order to avoid thermal degradation of the produced oils and by-products. The cold pressing mill treated 15 kg/h regarding the hardest seeds (Grp and Pmg), while for the softer seeds (Pmp, Alm, Snf, Hmp and Fnn seed) the crashing rate was 25 kg/h for each one (Table 1). The cold VSOs are considered as functional products, because their constituents do not cover only nutritional needs, but also enhance additional health benefits for human [26]. Avoiding the thermal and refining procedure, cold press VSOs retain their natural characteristic flavors and the valuable bioactive substances that are decomposed with classic intensive production methodologies [27]. The disadvantage of

lower production volume of cold pressed oils, in contrast to the refined, is equilibrated with the high added value of the final quality [28].

GC technique is widely used for the analysis of oils. Since many years it is considered as one of the most efficient methods for the detection of volatile and hydrophobic compounds [29]. Previous studies with GC-MS analysis have been published discussing the VSOs of the current work [30–33]. Their results have significant similarities with the results of this study with minor differences, probably due to the different cultivation areas and conditions.

Data from GC-MS analysis of VSOs revealed their profiles concerning fatty acids and in some cases of volatile compounds as well. After the required esterification process (described in section 2.4), triglycerides, diglycerides and monoglycerides were decomposed to the corresponding fatty acids. Via GC-MS experiments, the percentages of poly-unsaturated (PUFA), mono-unsaturated (MUFA) and saturated fatty acids (SFA) can be calculated for each VSO (Table 2). In our studied seeds oils, AlmSO had the highest percentage of MUFA (64.3%), with 64.0% being oleic acid and traces of palmitoleic acid (0.3%). FnnSO provided the second highest percentage of MUFA (57.1%) with oleic acid being the only representative. PmpSO arrived third on the rank of MUFA content (56.5%) with only representative, vaccenic acid, an isomer of oleic acid (Table 2). According to EFSA, the content of MUFA in oils is of high significance because their consumption is correlated to the reduction of LDL-cholesterol concentration [34]. Moreover, it has been proved that oleic acid has a strong positive impact to the protection of cardiovascular diseases [35]. SnfSO was also rich in MUFA (44.2%-oleic acid), followed by GrpSO (32.0%), HmpSO (22.0%) and PmgSO (9.6%). In addition, PmgSO provided an extremely high amount of PUFA (84.1%) with punicic acid being the main representative (76.3%). Punicic acid is recognized as a compound with health beneficial properties like anticarcinogenic, antioxidant, anti-inflammatory and antidiabetic effects [36]. Also, GrpSO and SnfSO had significant amount of PUFA, 49.7% and 42.5%, respectively. Lastly, linoleic acid is the most abundant representative of PUFA among the studied samples, with only exception the PmgSO.

Table 2: GC-MS analysis data with the percentages of the compounds identified in grape seed oil (GrpSO), pumpkin seed oil (PmpSO), pomegranate seed oil (PmgSO), sunflower seed oil (SnfSO), cannabis seed oil (HmpSO) fennel seed oil (FnnSO) and almond seed oil (AlmSO).

Fatty acids	GrpSO	PmpSO	PmgSO	SnfSO	HmpSO	FnnSO	AlmSO
Palmitic acid C16:0	9.3%	26.7%	2.8%	7.7%	43.5%	4.2%	8.3%

Palmitoleic acid C16:1 (n9Z)	-	-	-	-	-	-	0.3%
Stearic acid C18:0	9.0%	11.6%	3.5%	5.6%	13.8%	-	2.5%
Oleic acid C18:1 (n9Z)	32.0%	-	9.6%	44.2%	21.6%	57.1%	64.0%
Vaccenic acid C18:1 (n11E)	-	56.5%	-	-	-	-	-
Linoleic acid C18:2 (n9Z, n12Z)	49.7%	4.1%	-	42.5%	3.6%	-	24.1%
Linolelaidic acid C18:2 (n9E, n12E)	-	-	4.9%	-	-	5.6%	-
Punicic acid C18:3 (n9Z, n11E, n13E)	-	-	76.3%	-	-	-	-
Linolenic acid C18:3 (n9Z, n12Z, n15Z)	-	-	2.9%	-	-	-	-
Arachidic acid C20:0	-	1.1%	-	-	3.7%	-	-
Eicosenoic acid C20:1 (n11Z)	-	-	-	-	0.4%	-	-
Behenic acid C22:0	-	-	-	-	1.5%	-	-
Mono-unsaturated fatty acids	32.0%	56.5%	9.6%	44.2%	22.0%	57.1%	64.3%
Poly-unsaturated fatty acids	49.7%	4.1%	84.1%	42.5%	3.6%	5.6%	24.1%
Saturated fatty acids	18.3%	39.4%	6.3%	13.3%	62.5%	4.2%	10.8%
Other compounds	-	-	-	-	11.9%	33.1%	0.8%

In more details, the two most abundant fatty acids in the studied VSOs were oleic and linoleic acid ranging from 9.6-64% and 3.6-49.7%, respectively. Only PmgSO and PmpSO had different fatty acids as major components. As it is already mentioned, PmpSO had high concentration of vaccenic acid (56.5%) and PmgSO was rich in punicic acid (76.3%). Moreover, palmitic and stearic acid were found in the studied VSOs, but in lower quantities. As minor fatty acids palmitoleic, linolenic, linolelaidic and arachidic were noticed, in some of VSOs profiles. Except of fatty acids, VSOs contains some other lipophilic compounds with significant biological properties which have been previously reported were found. It should be highlighted that AlmSO had amounts of vitamin E

(0.8%), which is considered as one of the most effective antioxidant compounds in nature [37]. FnnSO was rich in anethole (17.1%) and estragole (16.0%), two compounds with antioxidant activity and broad applications in food industry [38,39]. It should be noticed that profile GC-MS profile of HmpSO revealed abundance of azelaaldehydric acid (11.9%). The table 2 summarizes the above mentioned information of GC-MS analysis.

Recovery of phenolic fraction from seed oils and by-product pastes

Extraction of seed oils using resin technology

Resins is a spread technique for the recovery of bioactive compounds from natural sources, not only in laboratory field but also in industry [40,41]. Usually, adsorption resins are used to bind compounds from the liquid by-products of the oil processes [42–44] and there are scientific data regarding the recovery of lignans with direct elution of sesame seed oil through adsorption resin [45]. However, to the best of our knowledge, this is the first time that adsorption resins were used for the recovery of bioactive molecules from GrpSO, PmpSO, PmgSO, SnfSO, HmpSO, FnnSO and AlmSO.

Solid-liquid extraction was conducted on all the VSOs, using absorption resin XAD-7. We chose to use a food grade resin, with ethanol as elution solvent, in order to have a totally green procedure and to transfer the edible properties from the raw materials to the extracts. The final seed oil extracts cover a wide range of yields. The highest yield was provided by PmgSO (2173.9 mg/100 mL), following with descending order the yields of FnnSO (2001.0 mg/100 mL of oil), HmpSO (1854.6 mg/100 mL), AlmSO (1268.1 mg/100 mL), PmpSO (416.8mg/100 mL of oil), SnfSO (388.3 mg/100 mL of oil) and GrpSO (213.1 mg/100 mL) (Table 2).

Recovery of biophenols from seed paste by-products using UAE and SFE

During the cold pressed VSOs production, certain amounts of paste byproducts (VSPs) were produced. The following extraction processes were focused on green criteria in order to produce high quality edible extracts. Two green techniques, ultrasound assisted extraction and supercritical fluid extraction, have been applied. Firstly, 10 g of each different VSPs were defatted with food grade *n*-Hex, providing a lipophilic fraction of 37.8 mg/g paste for GrpSP, 162.1 mg/g paste for PmpSP, 70.6 mg/g paste for PmgSP, 95.0 mg/g paste for SnfSP, 67.5 mg/g paste for HmpSP, 112.6 mg/g paste for FnnSP and 88.6 mg/g paste for AlmSP. Furthermore, ethanolic extracts of VSPs were prepared, providing 55.1 mg/g from GrpSP, 27.2 mg/g from PmpSP, 22.7 mg/g from PmgSP,

16.6 mg/g from SnfSP, 20.3 mg/g from HmpSP, 34.7 mg/g from FnnSP and 35.4 mg/g from AlmSP. The last UAE extracts of VSPs were produced using as extraction solvent H_2O/EtOH in ratio 1:1 v/v. Their corresponding yields were 456 mg, 252.6 mg, 239.8 mg, 1053.7 mg, 230.6 mg, 762.5 mg and 658.0 mg for GrpSP, PmpSP, PmgSP, SnfSP, HmpSP, FnnSP and AlmSP, respectively (Table 3).

SFE, which is a wide spread technique in food industry [46], was also applied on seed pastes, in order to obtain two kind of extracts. Firstly, a defatting step was necessary to remove the oil from the raw by-product. As it is observed from table 3, all the pastes have a certain amount of fatty fraction. PmpSP provided the highest (190.2 mg/g paste) and GrpSP gave the lowest weight (44.1 mg/g paste). All the others were among 89.5 and 77.8 mg/g paste, with only exception the FnnSP yield that reached 123.8 mg/g paste. The defatted seed pastes were extracted with CO_2 and 10% w/w of EtOH, as co-solvent. The highest yield was provided by PmpSP (74.2 mg/g paste) and followed with descending order by FnnSP (31.1 mg/g paste), GrpSP (19.5 mg/g paste), PmgSP (19.0 mg/g paste), AlmSP (16.1 mg/g paste), SnfSP (14.8 mg/g paste) and HmpSP (2.6 mg/g paste).

The last extraction was conducted with CO_2 and 20% w/w of EtOH. The richest extract was produced from FnnSP (49.1 mg/g paste), followed by PmgSP (14.2 mg/g paste), AlmSP (10.9 mg/g paste), GrpSP (10.0 mg/g paste), SnfSP (7.5 mg/g paste), PmgSP (5.2 mg/g paste) and HmpSP (3.9 mg/g paste). In table 3 the extract yields of seed by-products that were obtained with UAE and SFE techniques are presented.

Table 3: Yields of seed paste (VSP) extracts (mg/g of raw material) from grape (GrpSP), pumpkin (PmpSP), pomegranate (PmgSP), sunflower (SnfSP), cannabis (HmpSP), fennel (FnnSP) and almond (AlmSP) after UAE with n-Hex, EtOH and EtOH/H_2O 1:1 v/v and SFE with CO_2, CO_2 with 10%EtOH w/w and CO_2 with 20%EtOH w/w. Yields of seed oil extracts from 100mL of grape (GrpSO), pumpkin (PmpSO), pomegranate (PmgSO), sunflower (SnfSO), cannabis (HmpSO), fennel (FnnSO) and almond (AlmSO) after solid-liquid extraction with resin XAD-7.

	VSPs						*VSOs*
	UAE			*SFE*			*XAD-7*
Extraction solvent	*n-Hex defatting (mg/g raw material) (w/w percentage of raw material)*	*EtOH (mg/g raw material)*	*EtOH/H_2O 1:1 v/v (mg/g raw material)*	*CO_2 defatting (mg/g raw material) (w/w percentage of raw material)*	*CO_2 + 10%EtOH w/w (mg/g raw material)*	*CO_2 + 20%EtOH w/w (mg/g raw material)*	*EtOH (mg/100 mL raw oil)*
GrpSP	37.8 (3.8%)	55.1	45.6	44.1 (4.4%)	19.5	10.0	213.1 (2.1%)
PmpSP	162.1 (16.2%)	27.2	25.3	190.2 (19.0%)	74.2	5.2	416.8 (4.2%)
PmgSP	70.6 (7.1%)	22.7	24.0	89.5 (9.0%)	19.0	14.2	2173.9 (21.7%)
SnfSP	95.0	16.6	105.4	80.9	14.8	7.5	388.3

	(9.5%)			(8.1%)			(3.9%)
HmpSP	67.5 (6.8%)	20.3	23.1	77.8 (7.8%)	2.6	3.9	1854.6 (18.5%)
FnnSP	112.6 (11.3%)	34.7	76.3	123.8 (12.4%)	31.1	49.1	2001. (20.0%)
AlmSP	88.6 (8.9%)	35.4	65.8	89.0 (8.9%)	16.1	10.9	1268.1 (12.7%)

At this point it should be noticed that after the defatting procedure similar percentage of fatty fractions were obtained both with SFE and UAE techniques for all the VSPs (Figure 1). Taking the above-mentioned results together, it seems that the defatting process was highly effective for both techniques and for all the raw materials. HPLC data were investigated and they are in accordance with the yields of each extraction (Appendix figures 1-21).

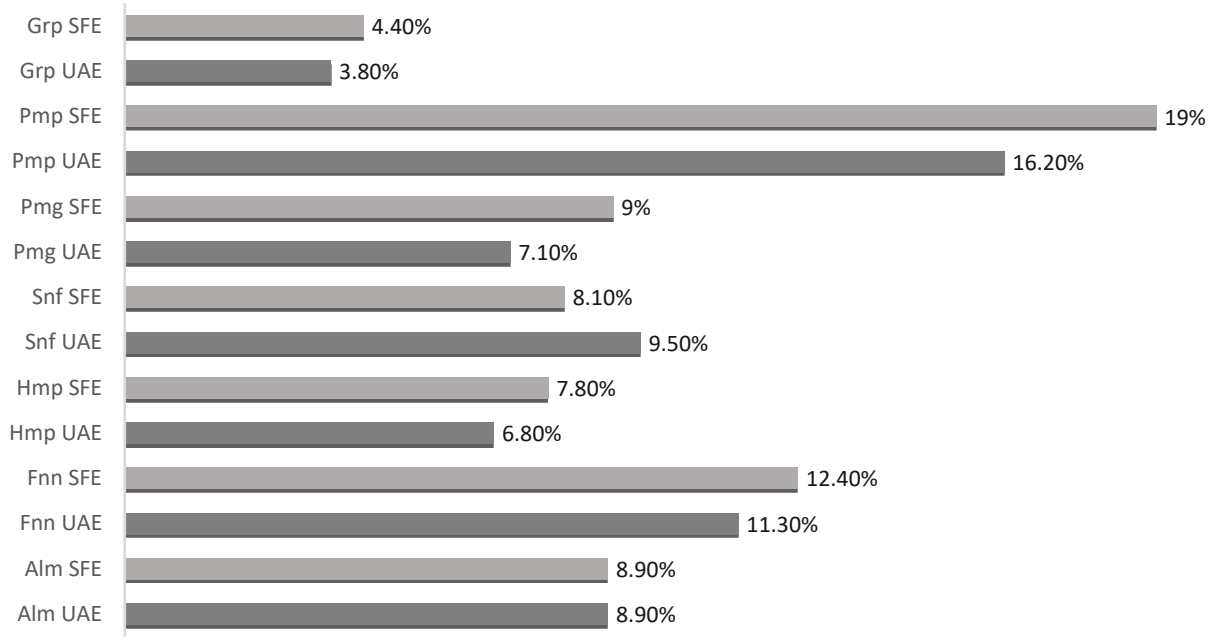


Figure 1: Percentages of fatty fraction from seed paste extracts using SFE (light grey) and UAE (dark grey) techniques.

Characteristic example is the chromatograms of FnnS extracts, where the CO_2 20% EtOH is richer in metabolites (more eluted peaks, with high absorbance) in comparison to CO_2 10% EtOH extract (Appendix figures 10-12). Furthermore, HPLC peak shaping and baseline, of GrpS extracts showed the existence of polymeric compounds (Appendix figures 7-9), probably proanthocyanins that have been already identified in the composition of GrpS [47].

Biological evaluation of VSO and VSP extracts

TPC and DPPH of extracts

Both DPPH and TPC are two established methodologies for the evaluation of the antioxidant capacity of extracts and/or pure compounds, widely used in the field of natural products [48,49].

TPC is a methodology for determination of polyphenols. This technique measures the total reducing capacity of a sample and results are expressed as mg of gallic acid equivalents (GAE) per g of extracts [48]. The extracts were tested in two concentrations, 500 and 1000 μg/mL and our results demonstrated the proper correlation between the estimated values in all cases. The estimated mean values showed a considerable variation -1.07-324.84 mg GAE/g. In more detail, the highest values were recorded for GrpS. More specifically, UAE EtOH/H_2O presented the highest value (324.84 mg GAE/g) followed by UAE EtOH (253.19 mg GAE/g) and SFE 20% (147.26 mg GAE/g). SFE 10% and XAD-7 revealed lower values (14.2 mg GAE/g and 7.81 mg GAE/g, however being from the highest of the dataset for these extractions, indicating the high phenolic content of GrpS in comparison to the other tested raw materials. It has to be noted that from the seven tested seed extracts, the four of them revealed the highest TPC values for UAE extracts, while the other three for the SFE extracts, underlining the efficiency of both extraction methodologies for the obtainment of phenolic compounds. Regarding XAD-7 extracts that were obtained from the VSO of seeds, FnnSO extract revealed the highest TPC value (19.13 mg GAE/g) (Figure 2).

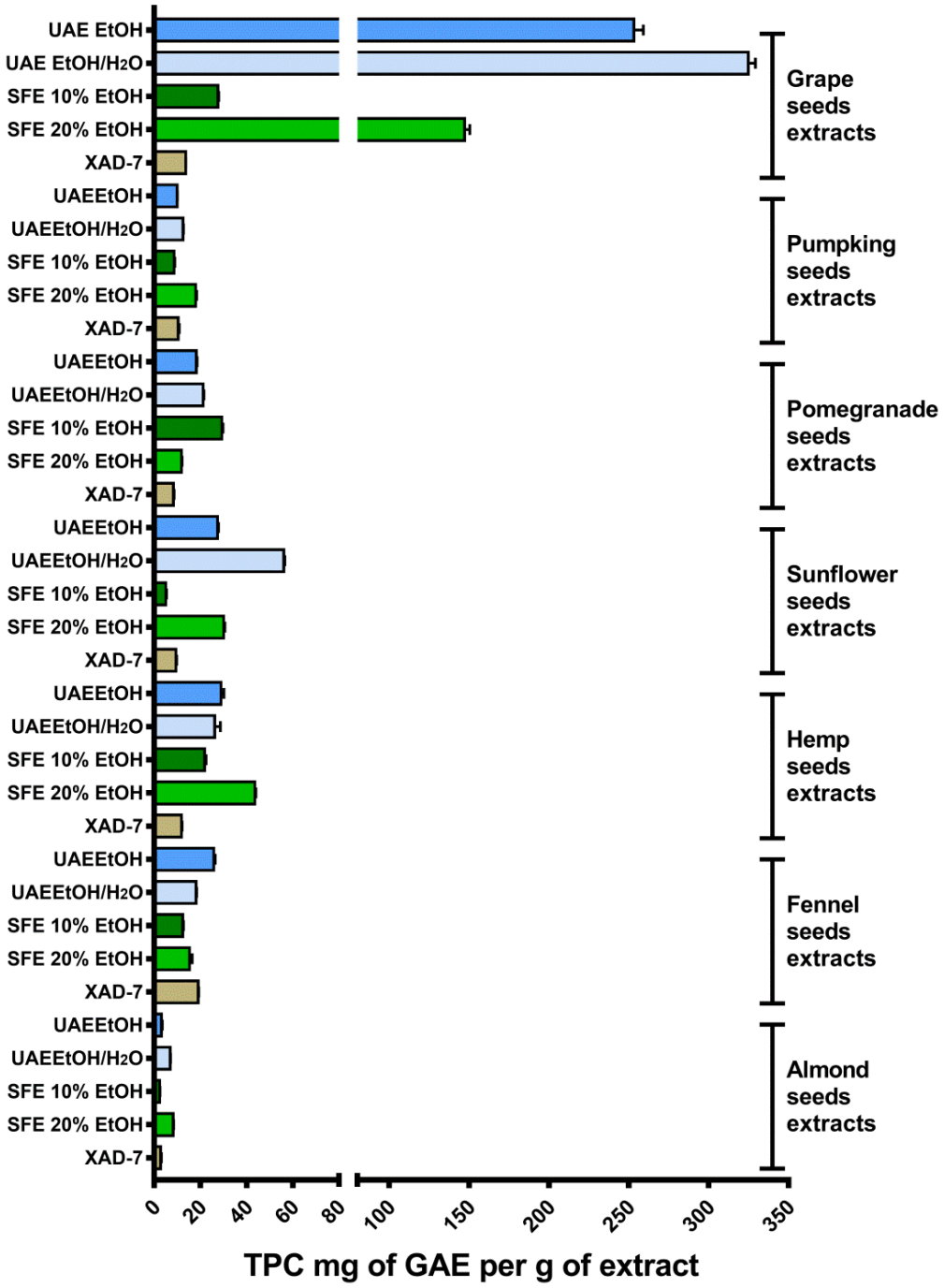


Figure 2: Results of TPC assay. The results are presented as mg of gallic acid equivalent (GAE) per g for the produced extracts from grape seeds, pumpkin seeds, pomegranate seeds, sunflower seeds, hemp seed, fennel seeds and almond seeds at the concentration of 10mg/mL. Each extraction method is depicted on the Y axis.

DPPH is considered as an accurate methodology for the evaluation of the radical scavenging activity of antioxidants and it is expressed as percentage (%) of oxidative inhibition [49]. In our results, four different concentrations (500, 250, 50 and 25 μg/mL) of each extract were tested reaching an inhibition range of 3.06% to 63.5%. As it was expected from TPC assays, the most effective radical scavenging activity was found in GrpSP extracts (Figure 3). In specific, 500 μg of UAE EtOH/H_2O 1:1 v/v extract showed 63.5±5.2% inhibition, followed by 55.9±5.6%, 47.9±1.2% and 31.2±1.5% for the 250, 50 and 25 μg/mL, respectively. Furthermore, the GrpSP extracts of UAE EtOH and SFE 20% EtOH exhibited significant inhibition. The highest concentration of HmpSP SFE 20% EtOH extract and SnfSP UAE EtOH, UAE EtOH/H_2O and SFE 20% EtOH gave positive results as well (Figure 3).

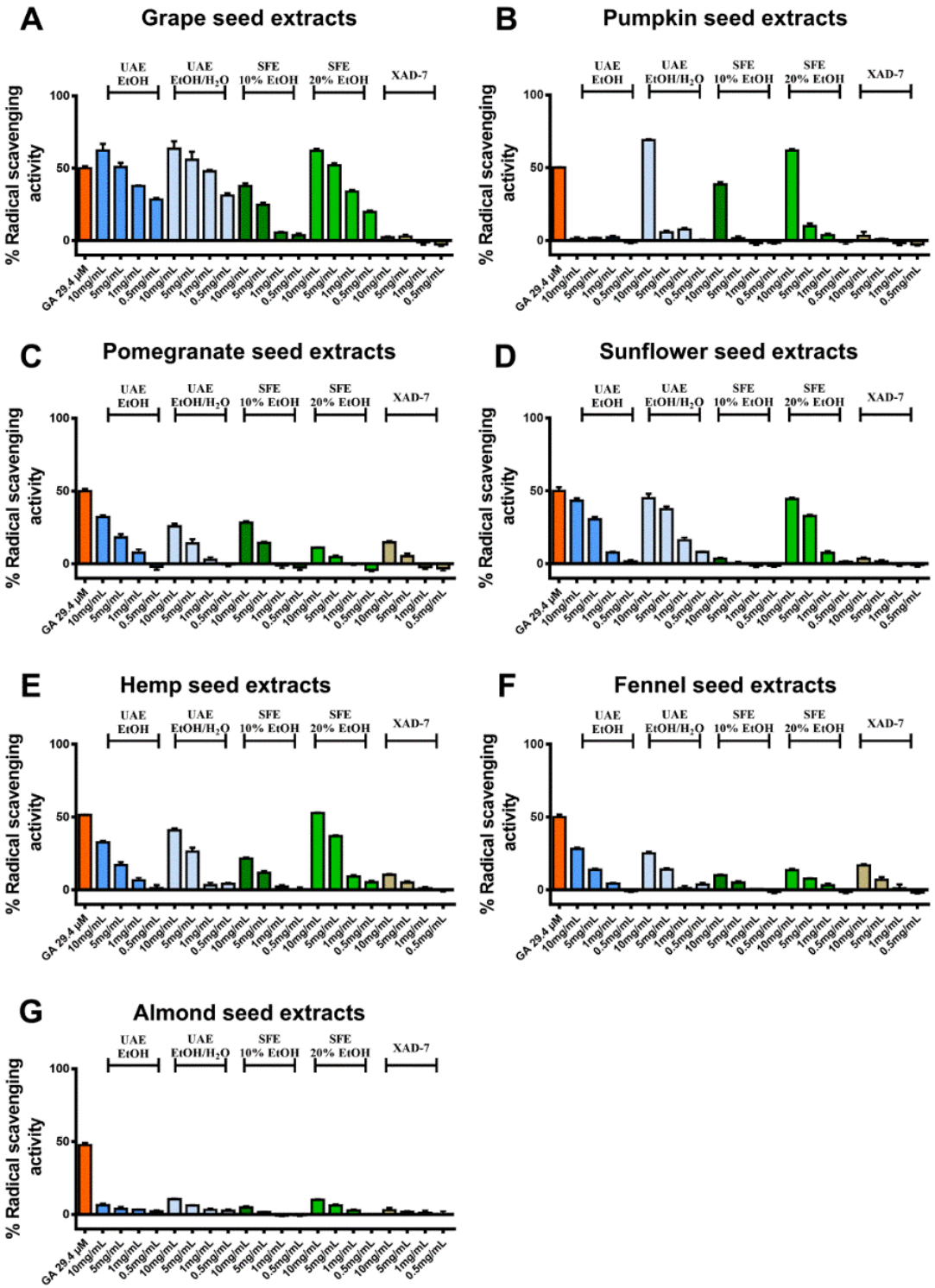


Figure 3: Results of the DPPH radical scavenging activity assay. The percentage of DPPH radical scavenging activity (%) is presented for the produced extracts from (A) grape seeds, (B) pumpkin seeds, (C) pomegranate seeds, (D) sunflower seeds, (E) hemp.

Enzymatic inhibition activity of extracts

Tyrosinase, collagenase and elastase enzymatic assays are used to test the efficiency of extracts and pure compounds for dermo-cosmetic applications. All the above-mentioned extracts from VSOs and byproducts were evaluated for their inhibition properties on these three enzymes.

Pomegranate fruit concentrated solutions and peels are well-studied products of *Punica granatum* and many studies have evaluated their potent inhibition skill against collagenase, elastase and tyrosinase [50–52]. However, there are no reports available for the inhibitory activity, against these enzymes, of PmgSO and PmgSP extracts. Additionally, AlmSO is known for its anti-aging effects at skin [53]. Based on *Yuan et al* almond hulls MeOH extract provides a potent anti-tyrosinase activity [52]. Concerning fennel fruits, they have been found as inactive inhibitors for elastase and tyrosinase enzymes in previous studies [54,55]. Moreover, cannabis leaves provide low tyrosinase inhibition activity while seeds have no significant activity against this enzyme [56,57]. Several studies have proven that grape seed and pomace have anti-collagenase, anti-elastase and anti-tyrosinase properties due to their high proanthocyanidins content [58–61]. Sunflower stem extract seems to have a potent anti-tyrosinase activity, based *on Kim et al.* [62]. Regarding, *Curcubita pepo* no studied were found for the investigation of its potent anti-tyrosinase, anti-elastase and anti-collagenase activity. Based on the above literature data, information regarding enzymatic inhibition studies of the seven VSOs and VSPs investigated at this work are scarce. Towards this purpose, all the investigated materials (seed oils and by-products extracts) were evaluated concerning their inhibitory properties against tyrosinase, collagenase and elastase enzymes.

Starting with tyrosinase activity assay, only the two UAE extracts (EtOH and EtOH/H_2O 1:1 v/v) of GrpSP revealed significant inhibition on tyrosinase enzyme (Figure 4). Specifically, the concentration of 500 μg/mL provided 75.0±0.7% and 72.4±0.3% inhibitory activity, while 150 μg/mL of the UAE EtOH and UAE EtOH/H_2O extracts showed 48.0±1.0% and 60.4±2.7% inhibition, respectively, suggesting the anti-pigmentation properties of GrpS (Figure 4A).

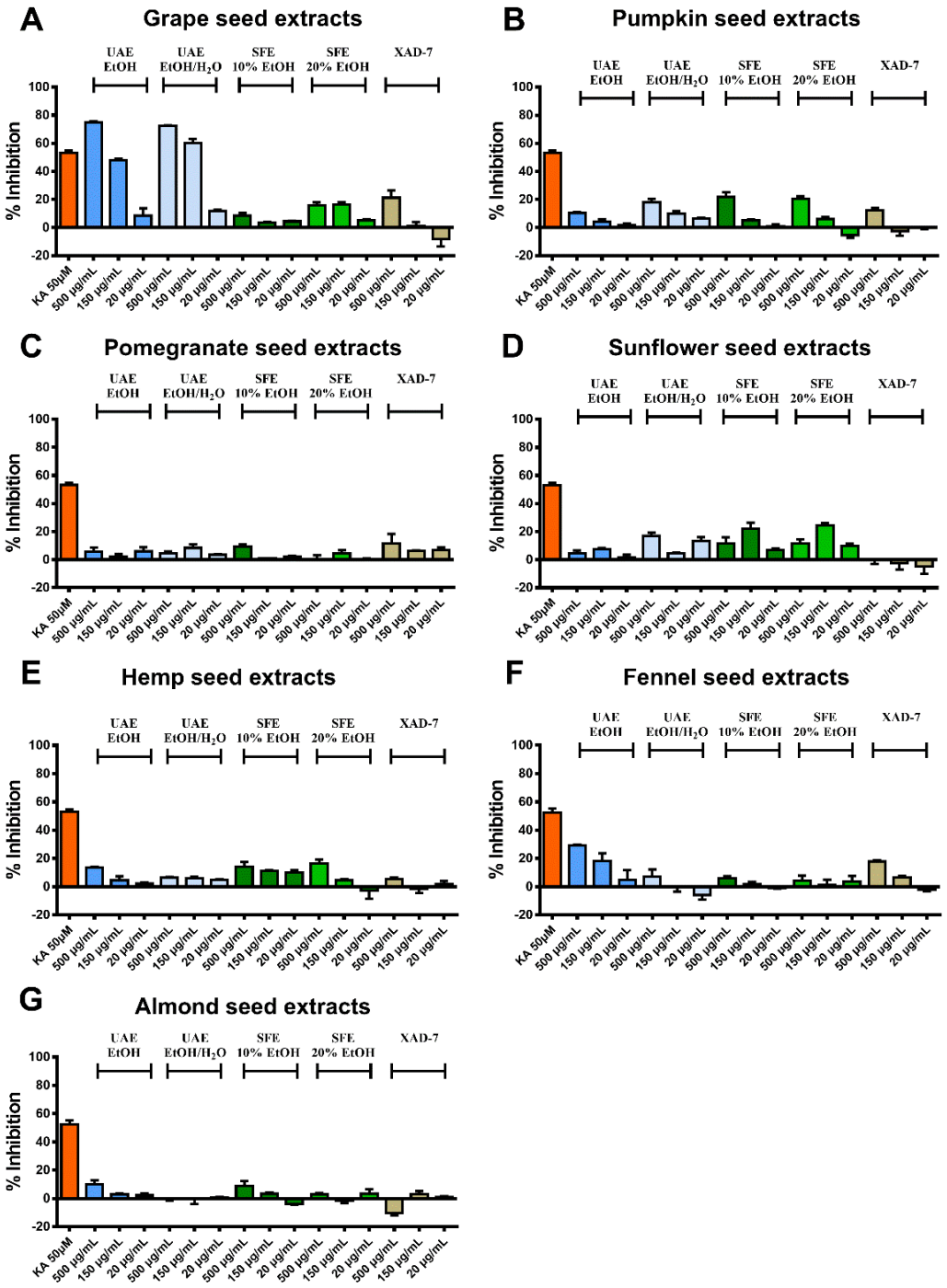


Figure 4: Results of the tyrosinase activity assay. The percentage of tyrosinase enzyme inhibition (%) is presented for the produced extracts from (A) grape seeds, (B) pumpkin seeds, (C) pomegranate seeds, (D) sunflower seeds, (E) hemp seed, (F) fennel seeds and (G) almond seeds. Kojic acid (KA) was used as positive control.

All the other tested pastes showed inhibition activities below the positive control (kojic acid), even at higher concentrations (Figures 4B-4G).

Regarding the elastase activity assay, the two GrpSP UAE extracts (EtOH and EtOH/H_2O 1:1 v/v) exhibited remarkable high inhibitory activity on the elastase enzyme at all tested concentrations (30, 150 and 300 μg/mL) (Figure 5). Specifically, GrpSP UAE EtOH at 30, 150 and 300 μg/mL showed inhibition of 67.8±0.5%, 74.0±2.1% and 91.3±1.4%, respectively and GrpSP UAE EtOH/H_2O 51.3±6.4%, 62.8±8.2% and 83.2±1.0%, at the respective concentrations. It should be noted that at GrpSO XAD-7 reached the inhibition value of 49.8±7.4% the higher concentration (300μg/mL) against elastase enzyme (Figure 5A). Moreover, PmpSP SFE 20% EtOH revealed significant 60.3% inhibition at the highest concentration (300 μg/mL) while the other extracts (Figures 5B-5G). Taken our results together, GrpS extracts and PmpSP SFE 20% EtOH strongly inhibit elastase enzyme and could be used for their anti-aging properties.

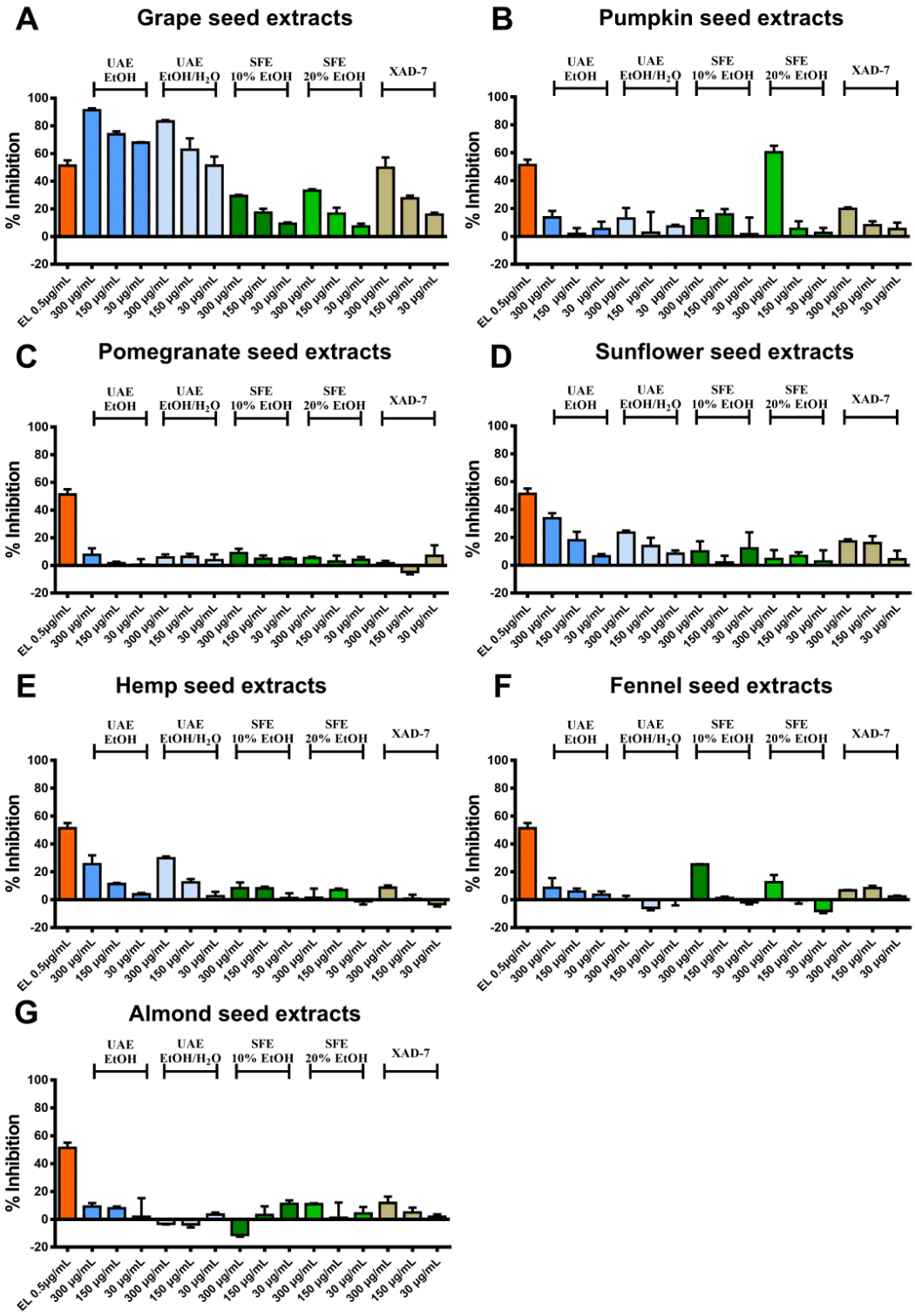


Figure 5: Results of the elastase activity assay. The percentage of elastase enzyme inhibition (%) is presented for the produced extracts from (A) grape seeds, (B) pumpkin seeds, (C) pomegranate seeds, (D) sunflower seeds, (E) hemp seed, (F) fennel seed.

In respect of collagenase activity assay, numerous of the tested extracts showed inhibitory activity. As it was expected for GrpS all the tested extracts (including GrpSO XAD-7) revealed inhibitory activity, with the UAE extracts (EtOH and EtOH/H_2O 1:1 v/v) reaching approximately 100% inhibition (Figure 6A). Also, all HmpS extracts revealed inhibitory activity over 80% (600 μg/mL of UAE EtOH, UAE EtOH/H_2O, SFE 10%, SFE 20% and XAD-7 for 95.2±0.3%, 98.0%±1.3, 94.2±0.5%, 98.5±0.2% and 79.5±0.3%, respectively), as well as FnnSO XAD-7 (93.6±0.7% at 600 μg/mL) and FnnSP UAE EtOH (98.3±1.0% at 600 μg/mL). Significant inhibition was also evident for FnnSP UAE EtOH/H_2O (62.4±0.8% at 600 μg/mL), FnnSP SFE10% (57.3±4.3% at 600 μg/mL), SnfSP UAE EtOH/H_2O (73.1±3.4% at 600 μg/mL), SnfSP SFE 20% (56.2±0.6% at 600 μg/mL), PmgSP UAE EtOH (63.0±1.9% at 600 μg/mL) and PmgSP SFE10% (70.3±2.3% at 600 μg/mL) showed inhibition activity over IC_{50} of the positive control (Figures 6B-6G).

It has to be highlighted that GrpS extracts, both GrpSO and all GrpSP extracts revealed significant inhibition against all enzymes.

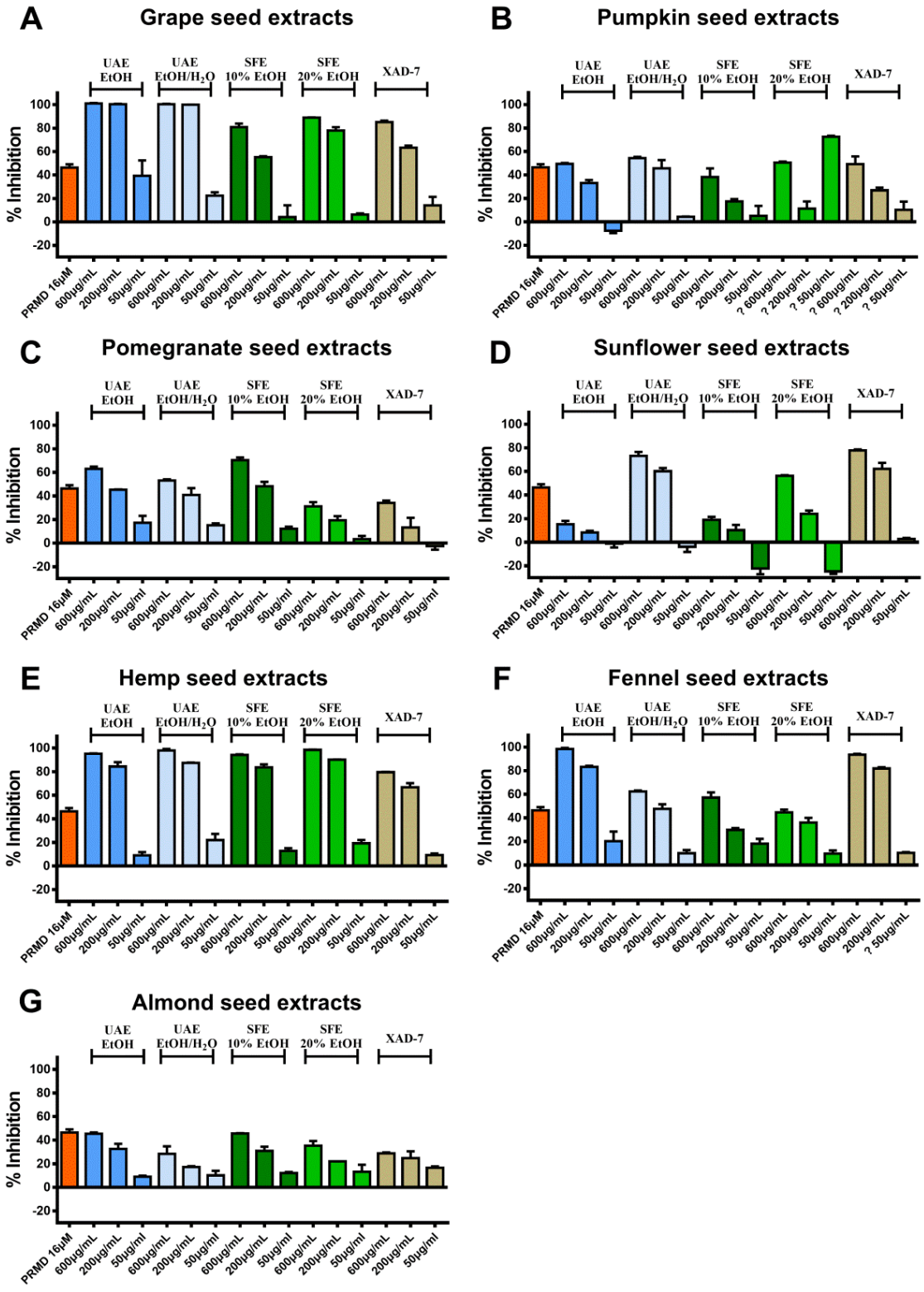


Figure 6: Results of the collagenase activity assay. The percentage of collagenase enzyme inhibition (%) is presented for the produced extracts from (A) grape seeds, (B) pumpkin seeds, (C) pomegranate seeds, (D) sunflower seeds, (E) hemp seed, (F) fennel seeds and (G) almond seeds. Phosphoramidon (PRMD) was used as positive control.

LC-HRMS analysis

For LC-HRMS analysis, 200 µg/mL of all the extracts were prepared and analyzed with the method described in section method and material. The generated chromatograms were studied in parallel with the corresponding spectra and $HRMS^2$ results. For the identification of compounds extraction ion method (XIC) and peak-to-peak selection were followed. The tools of elemental composition (EC), ring and double bonds equivalence (RDBeq) and the isotopic patterns were incorporated. For the analyzed extracts, all the detected *m/z* were registered in combination with the above-mentioned information of the used tools. In Table 4 the detected and identified metabolites of the 35 extracts are illustrated.

Table 4: Major compounds that were found via LC-HRMS analysis in the seed extracts.

	Grape seed		Pumpkin seed		Pomegranate seed		Sunflower seed		Cannabis seed		Fennel seed		Almond seed	
Compounds	**GrSP** (UAE EtOH)	**GrSO**	**PmpSP** (UAE EtOH/H_2O)	**PmpSO**	**PomSP** (SFE 10%)	**PomSO**	**SnfSP** (UAE EtOH/H_2O)	**SnfSO**	**HmpSP** (UAE EtOH)	**HmpSO**	**FnnSP** (UAE EtOH)	**FnnSO**	**AlmSP** (UAE EtOH)	**AlmSO**
Malic acid													✓	
Glucopyranose													✓	
Isorhamnetin-3-O-rutinoside													✓	
Isorhamnetin-3-O-glucoside													✓	
Naringenin													✓	
Trihydroxyoctadecenoic acid	✓	✓			✓				✓	✓		✓	✓	
Punicic acid						✓								
Petroselic acid				✓								✓		
Octadecanedioic acid		✓	✓	✓	✓			✓	✓	✓			✓	
Dihydroxypaimitic acid												✓		
Dihydroxystearic acid		✓	✓	✓	✓			✓	✓			✓	✓	
Hydroxylinoleic acid		✓	✓			✓			✓			✓	✓	✓
Dihydroxylinoleic acid		✓						✓		✓		✓		✓
Hydroxyoleic acid				✓									✓	
Linoleic acid					✓				✓				✓	
Oleic acid					✓					✓		✓	✓	
Oleanolic acid										✓				
Cannabinolic acid										✓				
Cannabichromevarinic acid										✓				
Caffeoyl tyramine									✓					

Cannabisin A									✓					
Cannabisin B									✓					
Cannabisin C									✓					
CBD A									✓	✓				
(±)-6,7-cis/trans-epoxycannabigerolic acid										✓				
α/β-cannabielsoic acid										✓				
2-(Hydroxymethyl)-1,2,3,4-butanetetrol or deoxytetritol											✓			
Casearicoside A											✓			
Corchorifatty acid											✓			
Dihydroxy-octadecenoic acid	✓	✓									✓			
myristicin											✓			
Fenchanediol											✓			
2,4-Thujanediol											✓			
2,4-Thujanediol 4-Ob-D-glucopyranoside											✓			
Gulonic acid					✓									
Glucopyranose			✓		✓									
gallic acid					✓									
Quinic acid					✓									
Protocatechuic acid			✓		✓									
4-Hydroxybenzoic acid			✓		✓									
Butanoic acid					✓									
Vanillic acid					✓									
Coumaric acid					✓									

Kaempherol					✓									
Trihydroxyoctadecadienoic acid					✓									
Trihydroxyoctadecenoic acid			✓		✓			✓						
Asiatic acid					✓									
Citric acid			✓											
Benzyl 2-O-beta-D-glucopyranosyl beta-D-glucopyranoside			✓											
2-(D-glucosyloxy)benzoic acid			✓											
Chlorogenic acid							✓							
Chlorogenic acid quinone							✓							
Coumaroylquinic acid							✓							
3-O-Feruloylquinic acid							✓							
Caffeic acid							✓	✓						
Hydroxycaffeic acid								✓						
Di-O-caffeoylquinic acid							✓							
Isoferulic acid							✓							
Dimer epicatechin-epicatechin/epicatechin-catechin/catechin-catechin/catechin-epicatechin	✓													
Epicatechin	✓													
Dimer proanthocyanidin galloylated	✓													
Trimer proanthocyanidin	✓													
Nonanedioic acid	✓													

Taking into account the metabolites that are present in each extract and their biological activity several conclusions may be drawn. As it is already mentioned, Grp seeds were the only raw material, extracts of which showed inhibition activity against all enzymes. Studying the table 4 and in parallel with bibliographic investigation, it can be assumed that GrpSP extracts are rich in proanthocyanidins like dimer of epicatechin/catechin, dimer proanthocyanidin galloylated and trimer proanthocyanidin. Based on literature data these metabolites are characterized by anti-elastase, anti-collagenase and anti- tyrosinase activity [63–65]. Hmp seed extracts (HmpSP and HmpSO) showed inhibition activity against collagenase enzyme. Based on table 4, the extract was rich in CBD A, cannabisin A, B and C, compounds that seems to enchance the anti-collagenase activity. It has to be noted that these compounds despite being followed by a long bibliographic research for their biological activities and their promising results for human health [66,67] they have never been investigated before for their tyrosinase, elastase and collagenase activity. Moreover, Fnn seeds (FnnSP and FnnSO) showed anti-collagenase activity. In these extracts, casearicoside A, corchorifatty acid, dihydroxy-octadecenoic acid and myristicin were identified and the potent activity of the extracts could be attributed to some of these molecules and/or synergistic effect of these compounds. PmgSP SFE 10% was mostly enriched in phenolic acids (gallic acid, quinic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic and coumaric acid) and kaempherol, agents that could account for its inhibitory effect on collagenase enzyme. Lastly, SnfSP UAE EtOH/H_2O and PmgSP SFE 10% were characterized by phenolic acids (chlorogenic acid, coumaroylquinic acid, 3-O-feruloylquinic acid, caffeic acid, hydroxycaffeic acid, Di-O-caffeoylquinic acid, isoferulic acid).

Conclusions

A broad range screening of seven VSOs, with dermo-cosmetic interest, and their corresponding byproducts was conducted. Fatty acid profile and non-polar compounds of VSPs were investigated with GC-MS. Moreover, all the extracts were analyzed with HPLC-DAD and LC-HRMS. Combination of these techniques provided information about the total chemical composition of the starting raw materials. Starting materials were all edible, even the VSPs, consisted of compounds with high nutraceutical value. It should be mentioned that all the extraction methodologies, XAD-7, UAE and SFE, were based on low environmental fingerprint techniques in combination with nontoxic solvents, providing totally green extracts. All the above-mentioned extracts were applied on enzymatic assays in order to evaluate their dermo-cosmetic properties. Most of the extracts

were found to be strong anti-collagenase inhibitors, while UAE grape seeds show significant results and as anti-elastase and anti-tyrosinase factors.

References

[1] R.D. Reichert, Oilseed medicinals: In natural drugs, dietary supplements and in new functional foods, Trends Food Sci. Technol. 13 (2002) 353–360. https://doi.org/10.1016/S0924-2244(02)00183-8.

[2] M.E. Snodgrass, World food: an encyclopedia of history, culture, and social influence from hunter-gatherers to the age of globalization, 2013. https://doi.org/10.5860/choice.50-3042.

[3] M.B. Copeland, L. O., & McDonald, Principles of Seed Science and Technology, 4th ed., SPRINGER SCIENCE+BUSINESS MEDIA, LLC, 2001.

[4] G. Badea, I. Lăcătuşu, N. Badea, C. Ott, A. Meghea, Use of various vegetable oils in designing photoprotective nanostructured formulations for UV protection and antioxidant activity, Ind. Crops Prod. 67 (2015) 18–24. https://doi.org/10.1016/j.indcrop.2014.12.049.

[5] M. Michalak, A. Kiełtyka-Dadasiewicz, Oils from fruit seeds and their dietetic and cosmetic significance, Herba Pol. 64 (2018) 63–70. https://doi.org/10.2478/hepo-2018-0026.

[6] K. Wołosik, M. Knas, A. Zalewska, M. Niczyporuk, A.W. Przystupa, The importance and perspective of plant-based squalene in cosmetology, J. Cosmet. Sci. 64 (2013) 59–65.

[7] R. Sarkar, I. Podder, N. Gokhale, S. Jagadeesan, V.K. Garg, Use of vegetable oils in dermatology: an overview, Int. J. Dermatol. 56 (2017) 1080–1086. https://doi.org/10.1111/ijd.13623.

[8] B. Adaramola, A. Onigbinde, S. Oluwatosin, Physiochemical properties and antioxidant potential of Persea Americana seed oil, Chem. Internatioanl. (2016) 168–175.

[9] J. Berbel, A. Posadillo, Review and analysis of alternatives for the valorisation of agro-industrial olive oil by-products, Sustain. 10 (2018) 1–9. https://doi.org/10.3390/su10010237.

[10] U. Thiyam, A. Kuhlmann, H. Stöckmann, K. Schwarz, Prospects of rapeseed oil by-products with respect to antioxidative potential, Comptes Rendus Chim. 7 (2004) 611–616. https://doi.org/10.1016/j.crci.2004.02.011.

[11] M.K. Khan, M. Abert-Vian, A.S. Fabiano-Tixier, O. Dangles, F. Chemat, Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (Citrus sinensis L.) peel, Food Chem. 119 (2010) 851–858. https://doi.org/10.1016/j.foodchem.2009.08.046.

[12] F. Chemat, Zill-E-Huma, M.K. Khan, Applications of ultrasound in food technology: Processing, preservation and extraction, Ultrason. Sonochem. 18 (2011) 813–835. https://doi.org/10.1016/j.ultsonch.2010.11.023.

[13] S. Dey, V.K. Rathod, Ultrasound assisted extraction of β-carotene from Spirulina platensis, Ultrason. Sonochem. 20 (2013) 271–276. https://doi.org/10.1016/j.ultsonch.2012.05.010.

[14] M. Vinatoru, An overview of the ultrasonically assisted extraction of bioactive principles from herbs, Ultrason. Sonochem. 8 (2001) 303–313. https://doi.org/10.1016/S1350-4177(01)00071-2.

[15] B.K. Tiwari, Ultrasound: A clean, green extraction technology, TrAC - Trends Anal. Chem. 71 (2015) 100–109. https://doi.org/10.1016/j.trac.2015.04.013.

[16] X. Ye, S.J. Xue, J. Shi, Advances in Food Processing Technology, Springer Singapore, 2019. https://doi.org/10.1007/978-981-13-6451-8.

[17] M. Herrero, A. Cifuentes, E. Iba??ez, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review, Food Chem. 98 (2006) 136–148.

[18] S.K. Singh, M.S. Pavan, S.P. N, R. Kant, Applications of Super Critical Fluid Extraction in Milk and Dairy Industry: A Review, J. Food Process. Technol. 9 (2018).

https://doi.org/10.4172/2157-7110.1000769.
[19] Q. Lang, C.M. Wai, Supercritical fluid extraction in herbal and natural product studies — a practical review, Talanta. 53 (2001) 771–782. https://doi.org/http://dx.doi.org/10.1016/S0039-9140(00)00557-9.
[20] R. Gallego, M. Bueno, M. Herrero, Sub- and supercritical fluid extraction of bioactive compounds from plants, food-by-products, seaweeds and microalgae – An update, TrAC - Trends Anal. Chem. 116 (2019) 198–213. https://doi.org/10.1016/j.trac.2019.04.030.
[21] K. Li, Z. Xu, A review of current progress of supercritical fluid technologies for e-waste treatment, J. Clean. Prod. 227 (2019) 794–809. https://doi.org/10.1016/j.jclepro.2019.04.104.
[22] K. Eder, Gas chromatographic analysis of fatty acid methyl esters, J. Chromatogr. B Biomed. Sci. Appl. 671 (1995) 113–131. https://doi.org/10.1016/0378-4347(95)00142-6.
[23] A. Angelis, J. Hubert, N. Aligiannis, R. Michalea, A. Abedini, J.M. Nuzillard, S.C. Gangloff, A.L. Skaltsounis, J.H. Renault, Bio-guided isolation of methanol-soluble metabolites of common spruce (Picea abies) bark by-products and investigation of their dermo-cosmetic properties, Molecules. 21 (2016) 1–15. https://doi.org/10.3390/molecules21111586.
[24] D. Michailidis, A. Angelis, N. Aligiannis, S. Mitakou, L. Skaltsounis, Recovery of Sesamin, Sesamolin, and Minor Lignans From Sesame Oil Using Solid Support-Free Liquid-Liquid Extraction and Chromatography Techniques and Evaluation of Their Enzymatic Inhibition Properties, 10 (2019). https://doi.org/10.3389/fphar.2019.00723.
[25] A. Angelis, P. Mavros, P.E. Nikolaou, S. Mitakou, M. Halabalaki, L. Skaltsounis, Phytochemical analysis of olive flowers' hydroalcoholic extract and in vitro evaluation of tyrosinase, elastase and collagenase inhibition activity, Fitoterapia. 143 (2020). https://doi.org/10.1016/j.fitote.2020.104602.
[26] D. Boskou, Edible Cold Pressed Oils and Their Biologically Active Components, J. Exp. Food Chem. 3 (2017) 1–2. https://doi.org/10.4172/2472-0542.1000e108.
[27] A. Prescha, M. Grajzer, The Antioxidant Activity and Oxidative Stability of Cold-Pressed Oils, (2014) 1291–1301. https://doi.org/10.1007/s11746-014-2479-1.
[28] S.C. Manchanda, J.S. Passi, Selecting healthy edible oil in the Indian context, Indian Heart J. 68 (2016) 447–449. https://doi.org/10.1016/j.ihj.2016.05.004.
[29] J. Blomberg, P.J. Schoenmakers, U.A.T. Brinkman, Gas chromatographic methods for oil analysis, J. Chromatogr. A. 972 (2002) 137–173. https://doi.org/10.1016/S0021-9673(02)00995-0.
[30] B. Uba, Determination of fatty acid composition and physicochemicacal properties of cucurbita Maxima (pumpkin) seed oil cultivated in Northeast Nigeria Determination of Fatty Acid Composition and Physicochemical Properties of Cucurbita Maxima (Pumpkin) Seed O, (2019).
[31] D.F. Alfekaik, S.A. Al-Hilfi, Fatty Acids Composition by (GC-MS) and Most Important Physical Chemicals Parameters of Seed Oil Pomegranate and Grape Seeds, 6 (2016). www.iiste.org.
[32] J. Garavaglia, M.M. Markoski, A. Oliveira, A. Marcadenti, Grape seed oil compounds: Biological and chemical actions for health, Nutr. Metab. Insights. 9 (2016) 59–64. https://doi.org/10.4137/NMI.S32910.
[33] P. Viorica-mirela, R.D. Nicoleta, M. Camelia, The possibilities of obtaining , characterizing and valorification of almond oil (Prunus Amygdalus), (2013) 3–6.

[34] Scientific Opinion on the substantiation of a health claim related to “low fat and low trans spreadable fat rich in unsaturated and omega-3 fatty acids” and reduction of LDL-cholesterol concentrations pursuant to Article 14 of Regulation (EC) No 1924/200, EFSA J. 9 (2011) 2168. https://doi.org/10.2903/j.efsa.2011.2168.
[35] L. Perdomo, N. Beneit, Y.F. Otero, Ó. Escribano, S. Díaz-Castroverde, A. Gómez-Hernández, M. Benito, Protective role of oleic acid against cardiovascular insulin resistance and in the early and late cellular atherosclerotic process, Cardiovasc. Diabetol. 14 (2015) 1–12. https://doi.org/10.1186/s12933-015-0237-9.
[36] H.A. Bedel, N.T. Turgut, A.U. Kurtoğlu, C. Usta, Effects of the nutraceutical, punicic acid, Indian J. Pharm. Sci. 79 (2017) 328–334. https://doi.org/10.4172/pharmaceutical-sciences.1000234.
[37] V. Böhm, Vitamin E, Antioxidants. 7 (2018) 11–12. https://doi.org/10.3390/ANTIOX7030044.
[38] P. Vera, E. Canellas, C. Nerín, Compounds responsible for off-odors in several samples composed by polypropylene, polyethylene, paper and cardboard used as food packaging materials, Food Chem. 309 (2019) 125792. https://doi.org/10.1016/j.foodchem.2019.125792.
[39] R.S. Freire, S.M. Morais, F.E.A. Catunda, D.C.S.N. Pinheiro, Synthesis and antioxidant, anti-inflammatory and gastroprotector activities of anethole and related compounds, Bioorganic Med. Chem. 13 (2005) 4353–4358. https://doi.org/10.1016/j.bmc.2005.03.058.
[40] L. Papaoikonomou, K. Labanaris, K. Kaderides, A.M. Goula, Adsorption–desorption of phenolic compounds from olive mill wastewater using a novel low-cost biosorbent, Environ. Sci. Pollut. Res. (2019). https://doi.org/10.1007/s11356-019-07277-2.
[41] Z. Xu, Q. Zhang, H.H.P. Fang, Applications of Porous Resin Sorbents in Industrial Wastewater Treatment and Resource Recovery, Crit. Rev. Environ. Sci. Technol. 33 (2003) 363–389. https://doi.org/10.1080/10643380390249512.
[42] A. Bianco, F. Bonadies, G. Romeo, M.L. Scarpati, A. Soriero, N. Uccella, The recovery of biophenols from wastewaters of olive oil production, Nat. Prod. Res. 20 (2006) 259–264. https://doi.org/10.1080/14786410500062163.
[43] K. Rusanov, E. Garo, M. Rusanova, O. Fertig, M. Hamburger, I. Atanassov, V. Butterweck, Recovery of polyphenols from rose oil distillation wastewater using adsorption resins - A pilot study, Planta Med. 80 (2014) 1657–1664. https://doi.org/10.1055/s-0034-1383145.
[44] A.P. Ericson, R.F. Matthews, A.A. Teixeira, H.A. Moye, Recovery of Grapefruit Oil Constituents from Processing Waste Water using Styrene-divinylbenzene Resins, J. Food Sci. 57 (1992) 186–189. https://doi.org/10.1111/j.1365-2621.1992.tb05452.x.
[45] J.C. Zhou, L.M. Zhao, M. Zhang, Y.F. Peng, Designed modification on macroporous resin for extracting sesamin from sesame oil, Desalin. Water Treat. 51 (2013) 2068–2075. https://doi.org/10.1080/19443994.2013.734532.
[46] N.L. Rozzi, R.K. Singh, Supercritical fluids and the food industry, Compr. Rev. Food Sci. Food Saf. 1 (2002) 33–44. https://doi.org/10.1111/j.1541-4337.2002.tb00005.x.
[47] C. Saucier, M. Mirabel, F. Daviaud, A. Longieras, Y. Glories, Rapid fractionation of grape seed proanthocyanidins, J. Agric. Food Chem. 49 (2001) 5732–5735. https://doi.org/10.1021/jf010784f.
[48] J.C. Sánchez-Rangel, J. Benavides, J.B. Heredia, L. Cisneros-Zevallos, D.A. Jacobo-Velázquez, The Folin-Ciocalteu assay revisited: Improvement of its specificity for total phenolic content determination, Anal. Methods. 5 (2013) 5990–5999.

https://doi.org/10.1039/c3ay41125g.
[49] S.B. Kedare, R.P. Singh, Genesis and development of DPPH method of antioxidant assay, J. Food Sci. Technol. 48 (2011) 412–422. https://doi.org/10.1007/s13197-011-0251-1.
[50] S.J. Kang, B.R. Choi, S.H. Kim, H.Y. Yi, H.R. Park, S.J. Park, C.H. Song, J.H. Park, Y.J. Lee, S.K. Ku, Inhibitory effects of pomegranate concentrated solution on the activities of hyaluronidase, tyrosinase, and metalloproteinase, J. Cosmet. Sci. 66 (2015) 145–159. https://doi.org/10.1016/j.clinthera.2015.05.337.
[51] T.S.A. Thring, P. Hili, D.P. Naughton, Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants, BMC Complement. Altern. Med. 9 (2009) 1–11. https://doi.org/10.1186/1472-6882-9-27.
[52] Y. Yuan, W. Jin, Y. Nazir, C. Fercher, M.A.T. Blaskovich, M.A. Cooper, R.T. Barnard, Z.M. Ziora, Tyrosinase inhibitors as potential antibacterial agents, Eur. J. Med. Chem. 187 (2020) 111892. https://doi.org/10.1016/j.ejmech.2019.111892.
[53] P.K. Mukherjee, N. Maity, N.K. Nema, B.K. Sarkar, Bioactive compounds from natural resources against skin aging, Phytomedicine. 19 (2011) 64–73. https://doi.org/10.1016/j.phymed.2011.10.003.
[54] K.K. Lee, J.H. Kim, J.J. Cho, J.D. Choi, Inhibitory effects of 150 plant extracts on elastase activity, and their anti-inflammatory effects, Int. J. Cosmet. Sci. 21 (1999) 71–82. https://doi.org/10.1046/j.1467-2494.1999.181638.x.
[55] J.H. Nam, D.U. Lee, Foeniculum vulgare extract and its constituent, trans-anethole, inhibit UV-induced melanogenesis via ORAI1 channel inhibition, J. Dermatol. Sci. 84 (2016) 305–313. https://doi.org/10.1016/j.jdermsci.2016.09.017.
[56] K.T. Lee, B.J. Kim, J.H. Kim, M.Y. Heo, H.P. Kim, Biological screening of 100 plant extracts for cosmetic use (I): Inhibitory activities of tyrosinase and DOPA auto-oxidation, Int. J. Cosmet. Sci. 19 (1997) 291–298. https://doi.org/10.1046/j.1467-2494.1997.171725.x.
[57] A. Manosroi, C. Chankhampan, B.O. Kietthanakorn, W. Ruksiriwanich, P. Chaikul, K. Boonpisuttinant, M. Sainakham, W. Manosroi, T. Tangjai, J. Manosroi, Pharmaceutical and cosmeceutical biological activities of hemp (cannabis sativa L var. sativa) leaf and seed extracts, Chiang Mai J. Sci. 46 (2019) 180–195.
[58] C.M. Galanakis, ed., Grape Processing By-Products as Active Ingredients for Cosmetic Proposes, in: Handb. Grape Process. By-Products Sustain. Solut., Academic Press, an imprint of Elsevier, 2017: pp. 267–292. https://doi.org/10.1016/B978-0-12-809870-7.00011-9.
[59] C. Beres, G.N.S. Costa, I. Cabezudo, N.K. da Silva-James, A.S.C. Teles, A.P.G. Cruz, C. Mellinger-Silva, R. V. Tonon, L.M.C. Cabral, S.P. Freitas, Towards integral utilization of grape pomace from winemaking process: A review, Waste Manag. 68 (2017) 581–594. https://doi.org/10.1016/j.wasman.2017.07.017.
[60] J. Wittenauer, S. MäcKle, D. Sußmann, U. Schweiggert-Weisz, R. Carle, Inhibitory effects of polyphenols from grape pomace extract on collagenase and elastase activity, Fitoterapia. 101 (2015) 179–187. https://doi.org/10.1016/j.fitote.2015.01.005.
[61] P.K. Mukherjee, R. Biswas, A. Sharma, S. Banerjee, S. Biswas, C.K. Katiyar, Validation of medicinal herbs for anti-tyrosinase potential, J. Herb. Med. 14 (2018) 1–16. https://doi.org/10.1016/j.hermed.2018.09.002.
[62] Y. Kim, S. Lee, J.H. Ryu, K.D. Yoon, S.S. Shin, Effect of Aurea Helianthus stem extract on anti-melanogenesis, Biosci. Biotechnol. Biochem. 82 (2018) 1871–1879.

https://doi.org/10.1080/09168451.2018.1506311.
[63] S.E. Song, B.K. Choi, S.N. Kim, Y.J. Yoo, M.M. Kim, S.K. Park, S.S. Roh, C.K. Kim, Inhibitory effect of procyanidin oligomer from elm cortex on the matrix metalloproteinases and proteases of periodontopathogens, J. Periodontal Res. 38 (2003) 282–289. https://doi.org/10.1034/j.1600-0765.2003.02604.x.
[64] W.M. Chai, C. Ou-Yang, Q. Huang, M.Z. Lin, Y.X. Wang, K.L. Xu, W.Y. Huang, D.D. Pang, Antityrosinase and antioxidant properties of mung bean seed proanthocyanidins: Novel insights into the inhibitory mechanism, Food Chem. 260 (2018) 27–36. https://doi.org/10.1016/j.foodchem.2018.04.001.
[65] N.F. Brás, R. Gonçalves, N. Mateus, P.A. Fernandes, M.J. Ramos, V. De Freitas, Inhibition of pancreatic elastase by polyphenolic compounds, J. Agric. Food Chem. 58 (2010) 10668–10676. https://doi.org/10.1021/jf1017934.
[66] S. Wang, Q. Luo, P. Fan, Cannabisin F from hemp (Cannabis sativa) seed suppresses lipopolysaccharide-induced inflammatory responses in BV2 microglia as SIRT1 modulator, Int. J. Mol. Sci. 20 (2019). https://doi.org/10.3390/ijms20030507.
[67] S. Frassinetti, E. Moccia, L. Caltavuturo, M. Gabriele, V. Longo, L. Bellani, G. Giorgi, L. Giorgetti, Nutraceutical potential of hemp (Cannabis sativa L.) seeds and sprouts, Food Chem. 262 (2018) 56–66. https://doi.org/10.1016/j.foodchem.2018.04.078.

Appendinx

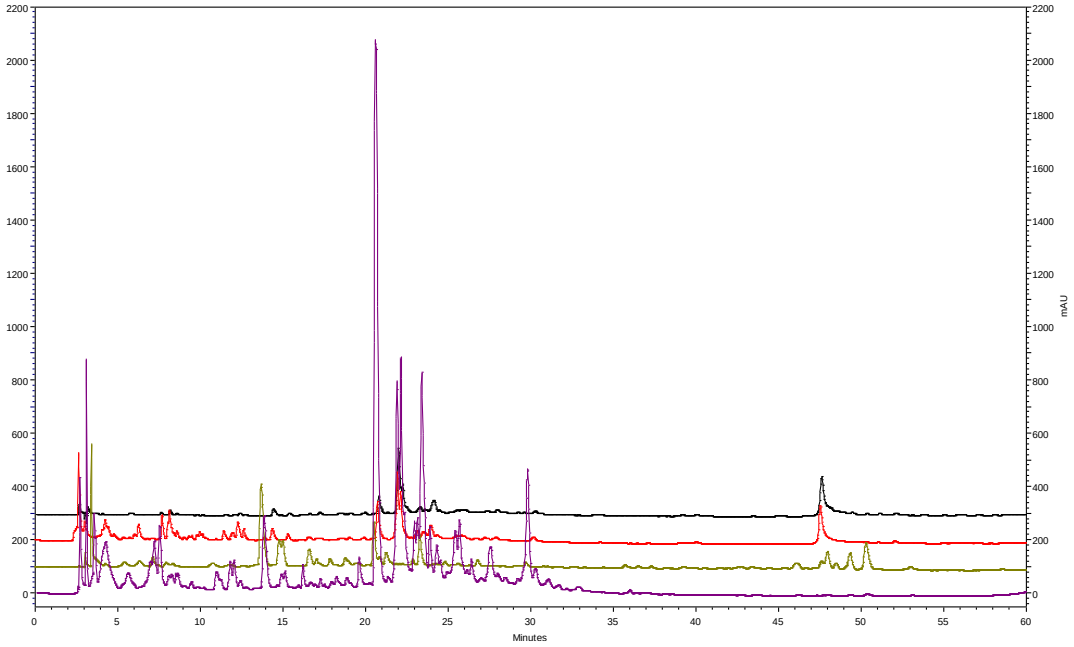


Figure 1: Chromatograph of hemp seed paste extracts at 254 nm (black: UAE EtOH, red: UAE EtOH/H_2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

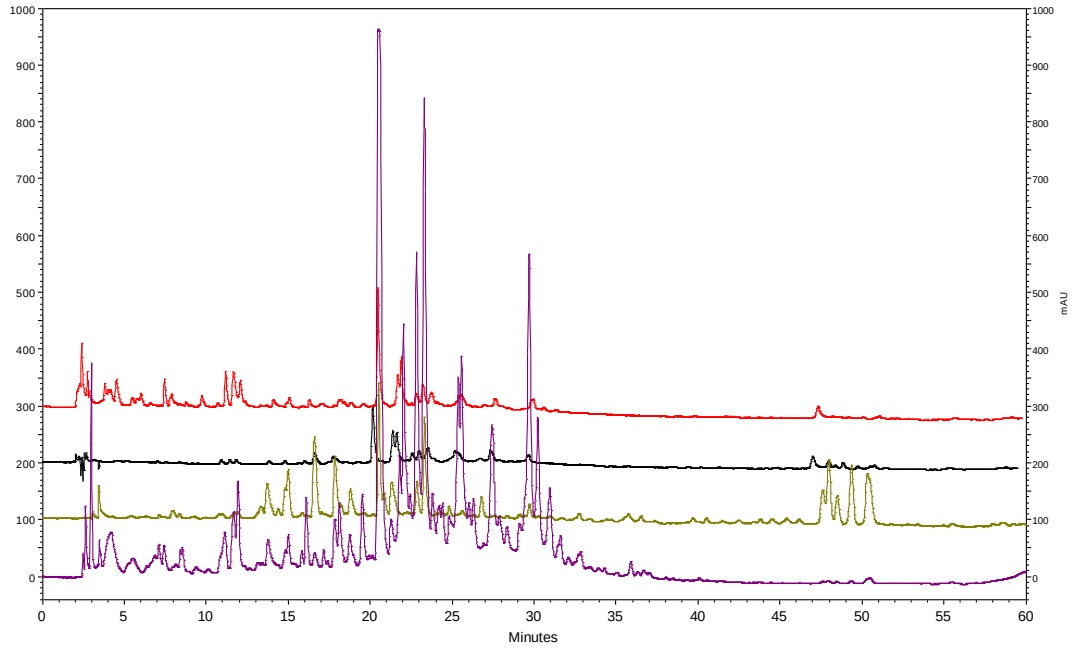


Figure 2: Chromatograph of hemp seed paste extracts at 280 nm (black: UAE EtOH, red: UAE EtOH/H_2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

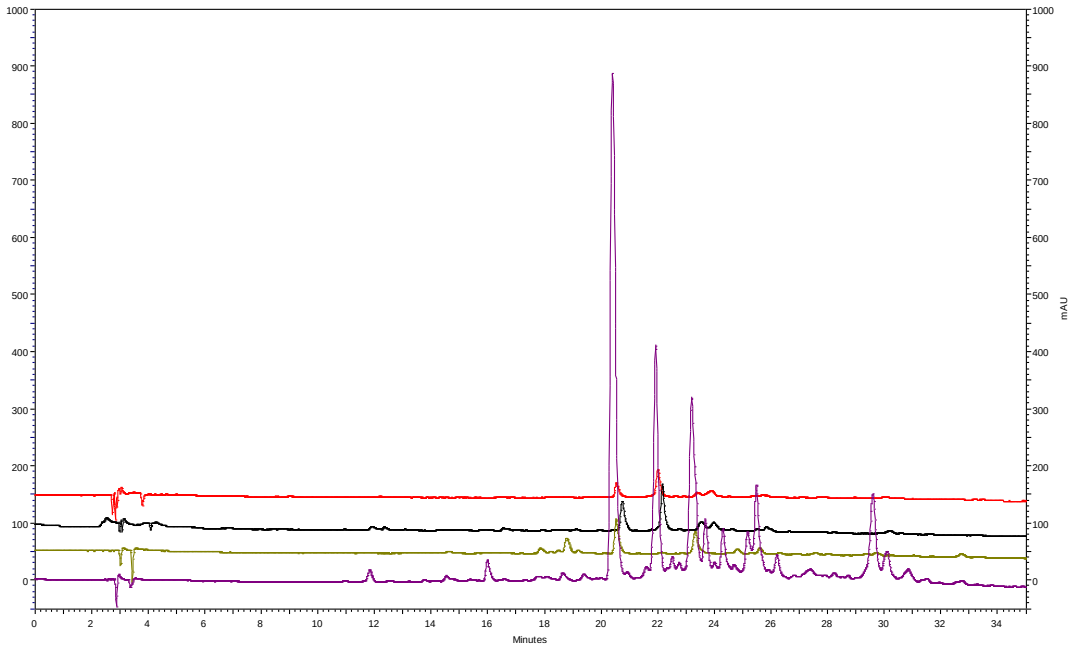


Figure 3: Chromatograph of hemp seed paste extracts at 350 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

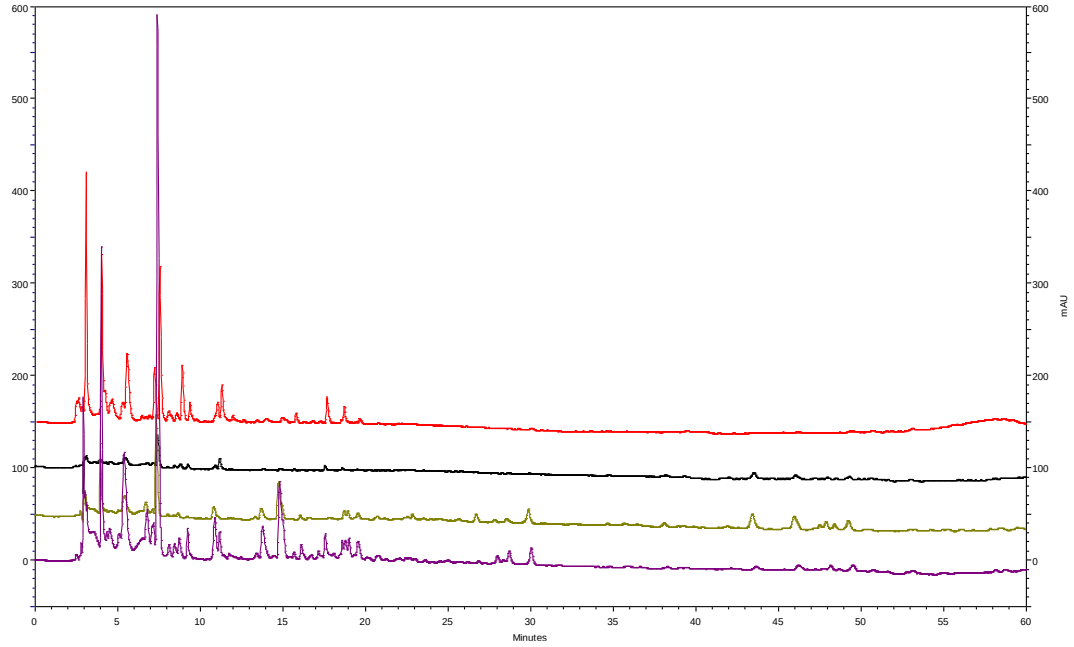


Figure 4: Chromatograph of almond seed paste extracts at 254 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

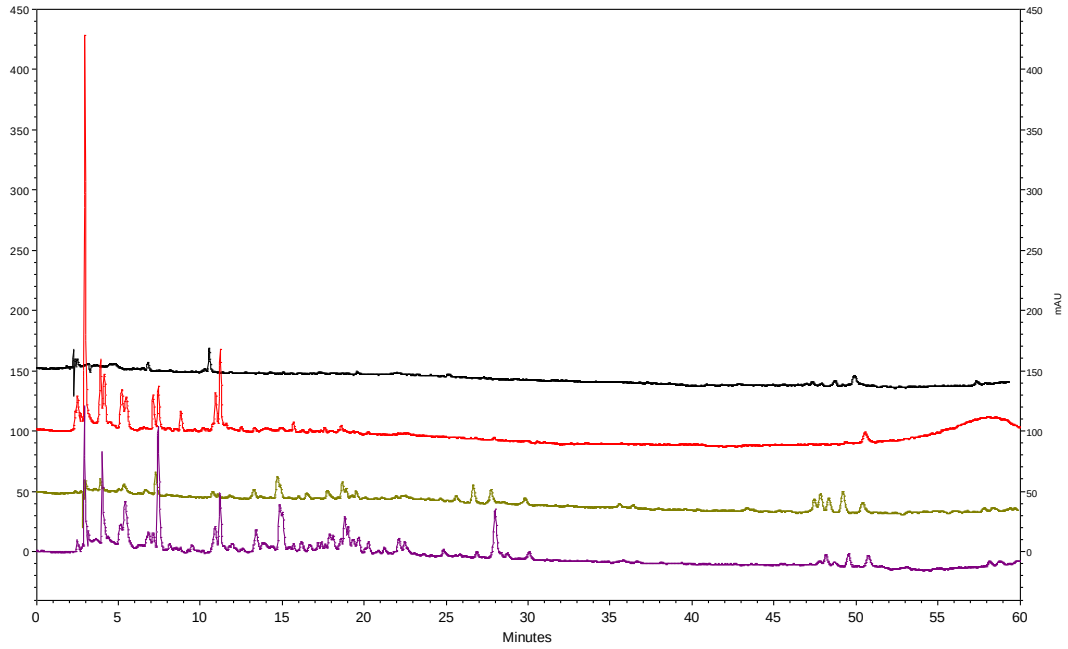


Figure 5: Chromatograph of almond seed paste extracts at 280 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

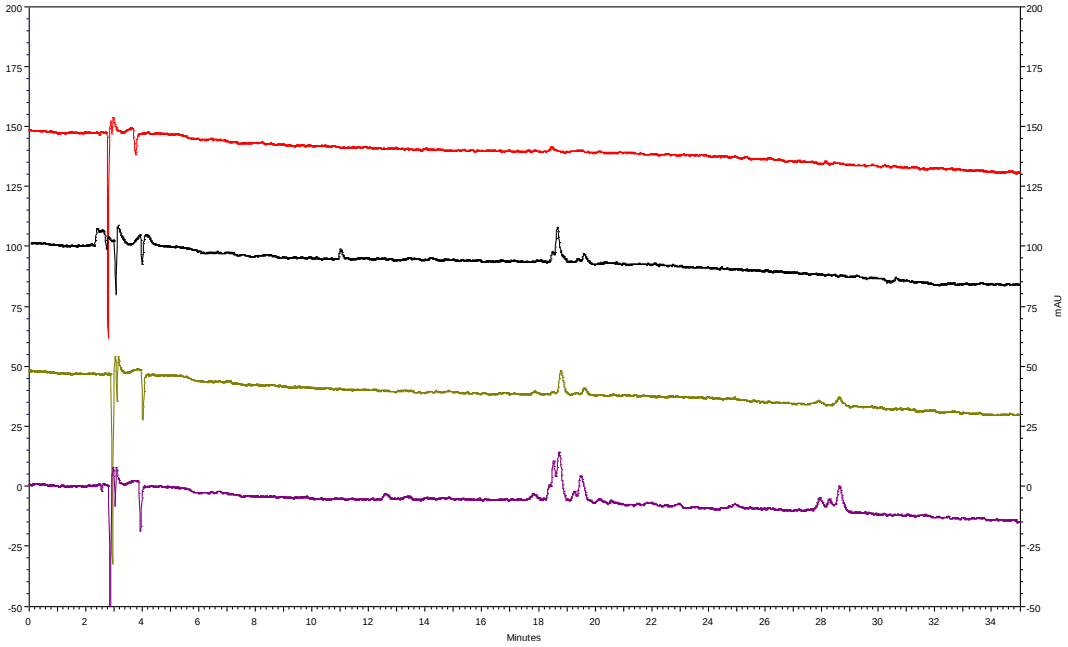


Figure 6: Chromatograph of almond seed paste extracts at 350 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

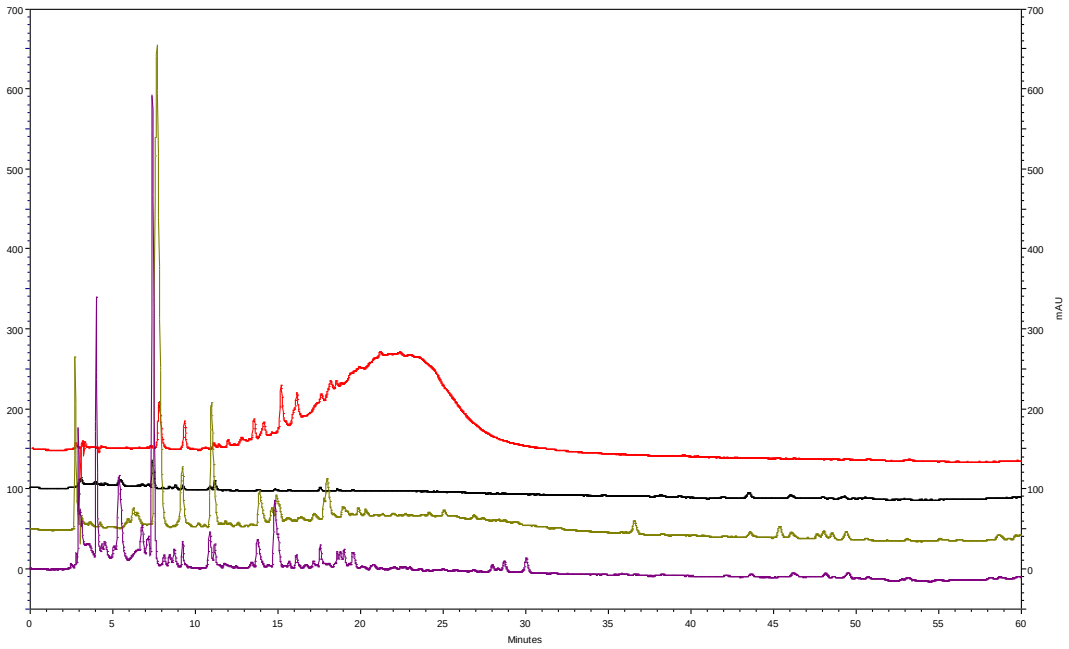


Figure 7: Chromatograph of grape seed paste extracts at 254 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

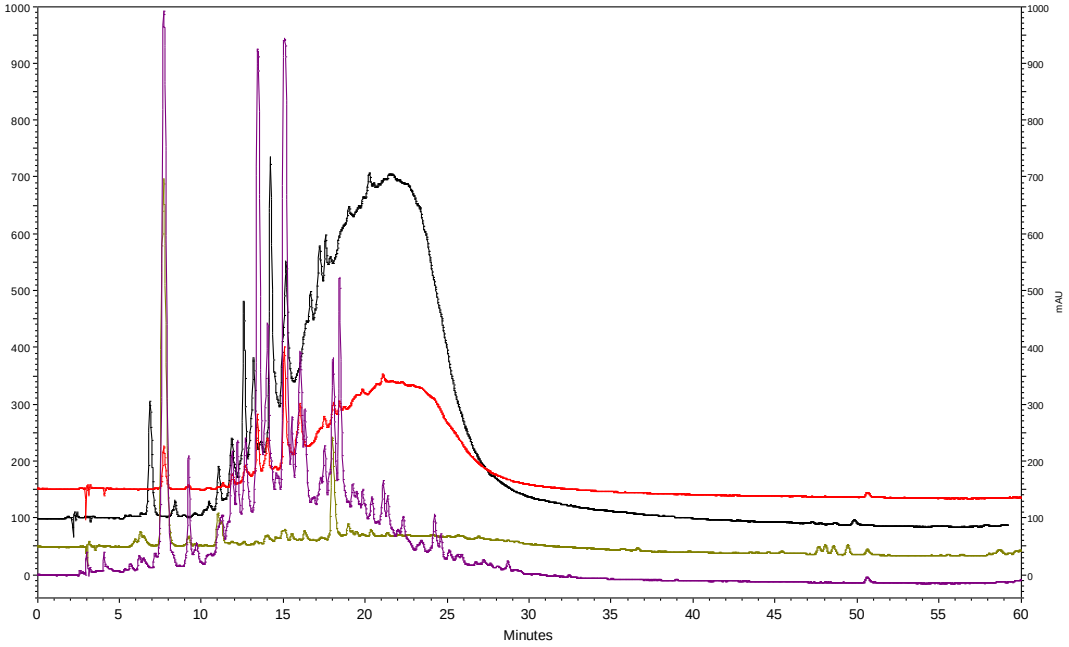


Figure 8: Chromatograph of grape seed paste extracts at 280 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

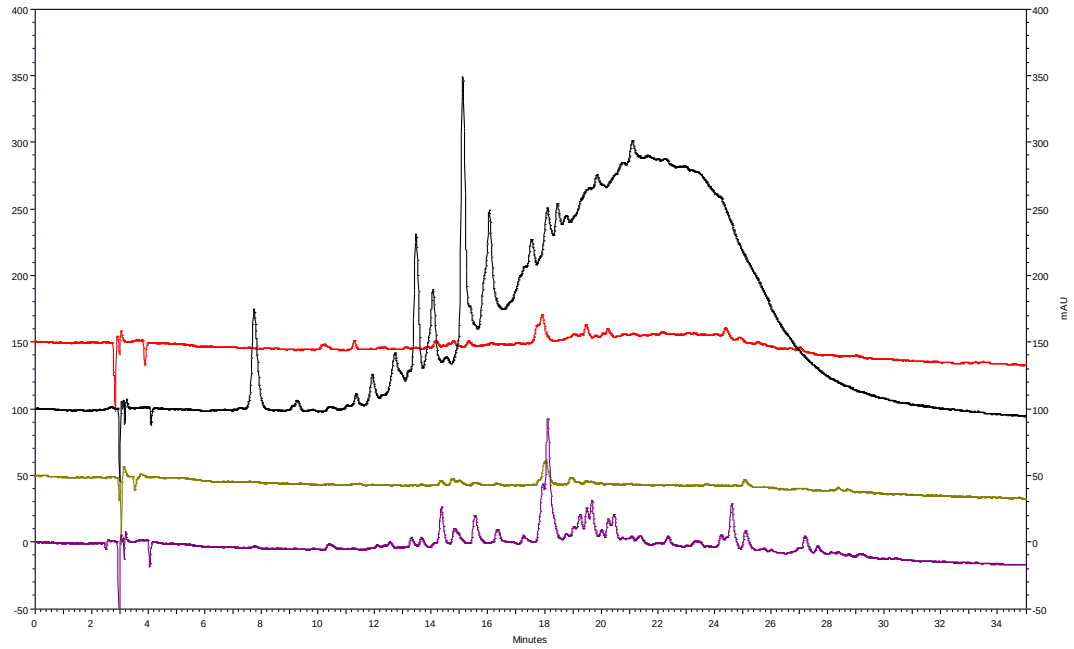


Figure 9: Chromatograph of grape seed paste extracts at 350 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

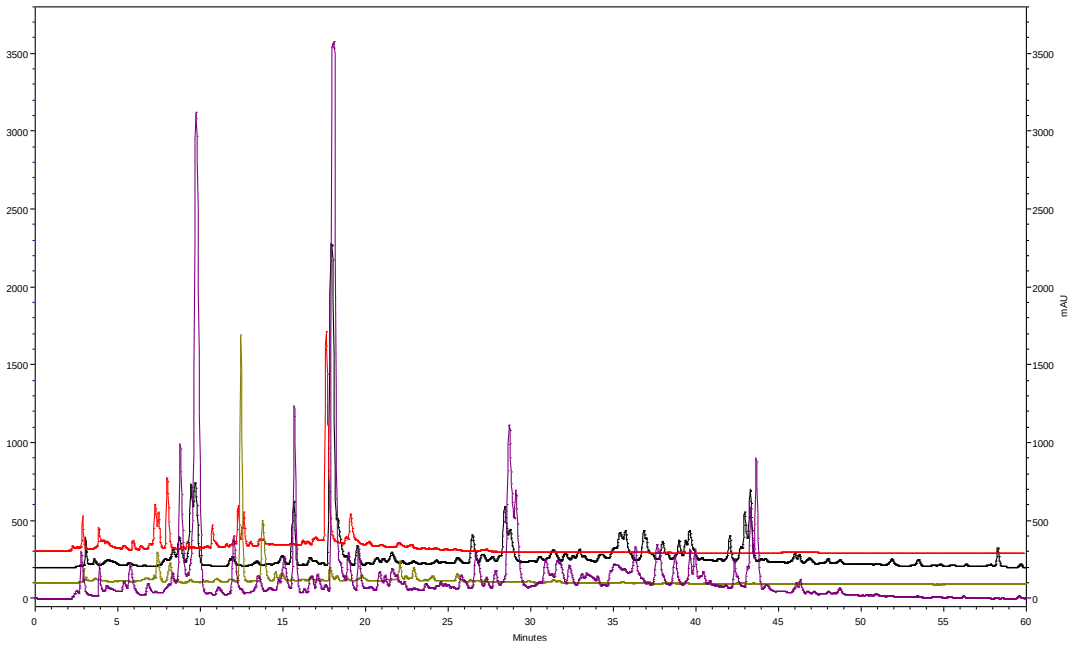


Figure 10: Chromatograph of fennel seed paste extracts at 254 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

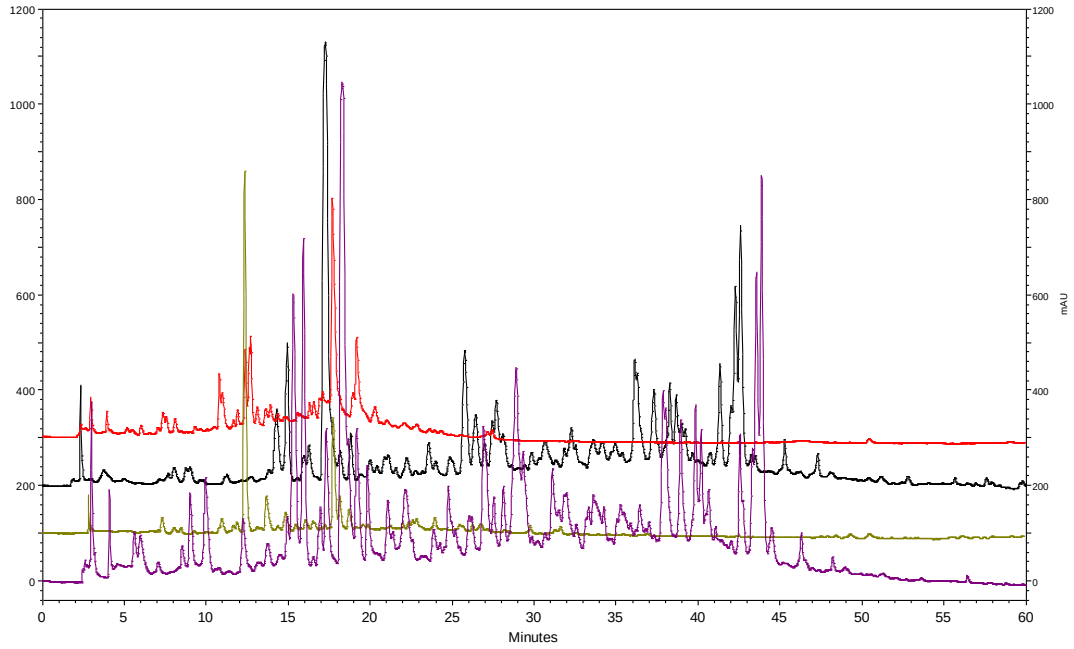


Figure 11: Chromatograph of fennel seed paste extracts at 280 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

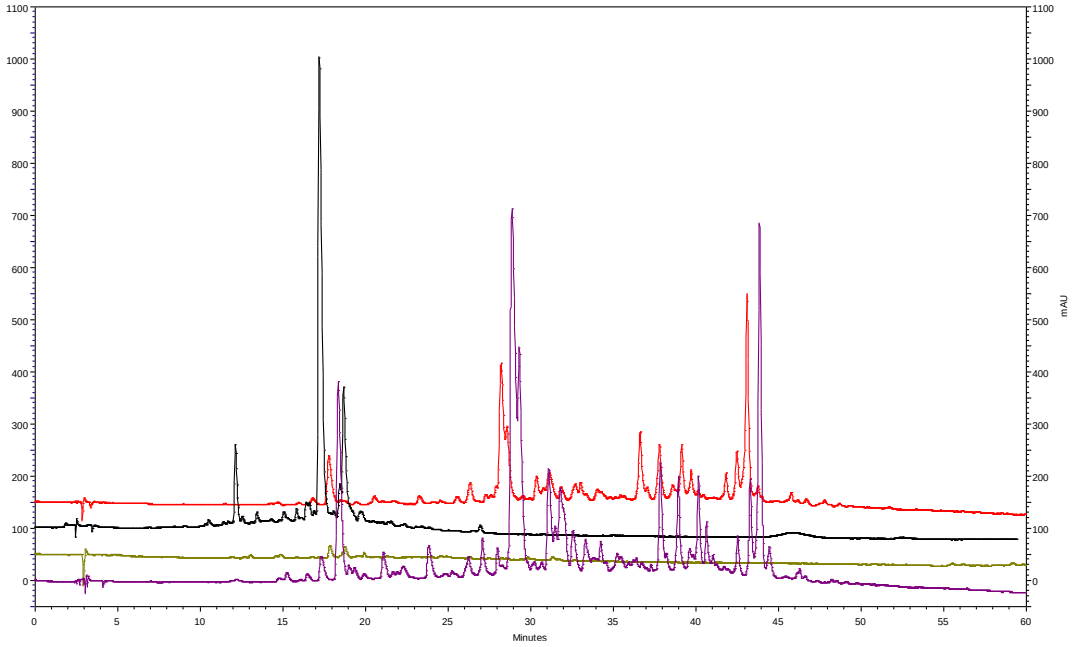


Figure 12: Chromatograph of fennel seed paste extracts at 350 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

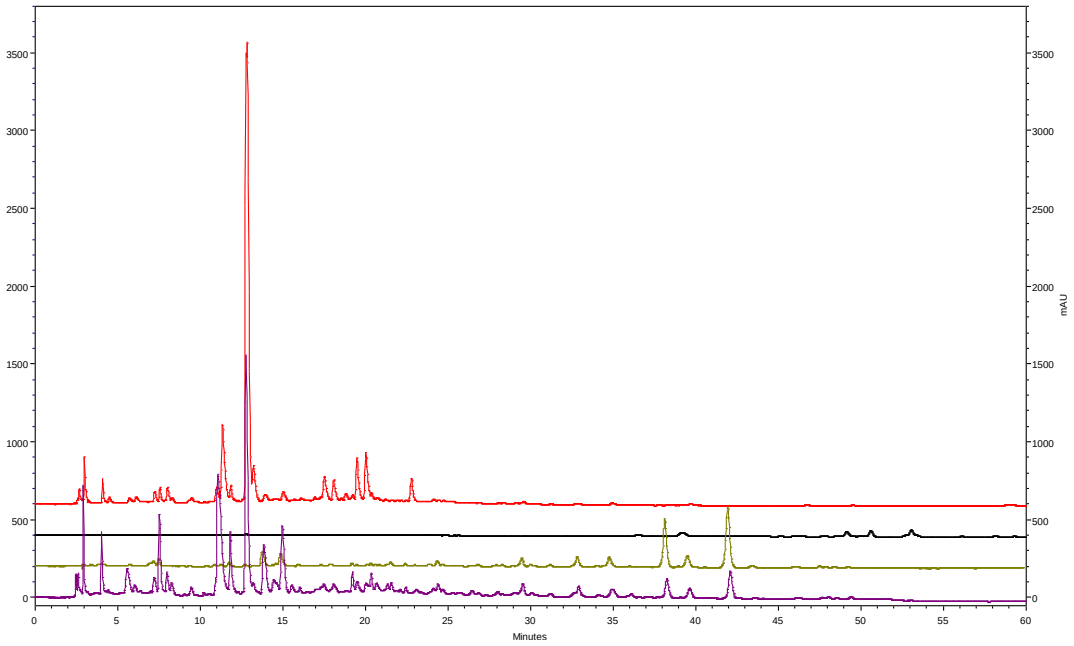


Figure 13: Chromatograph of sunflower seed paste extracts at 254 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

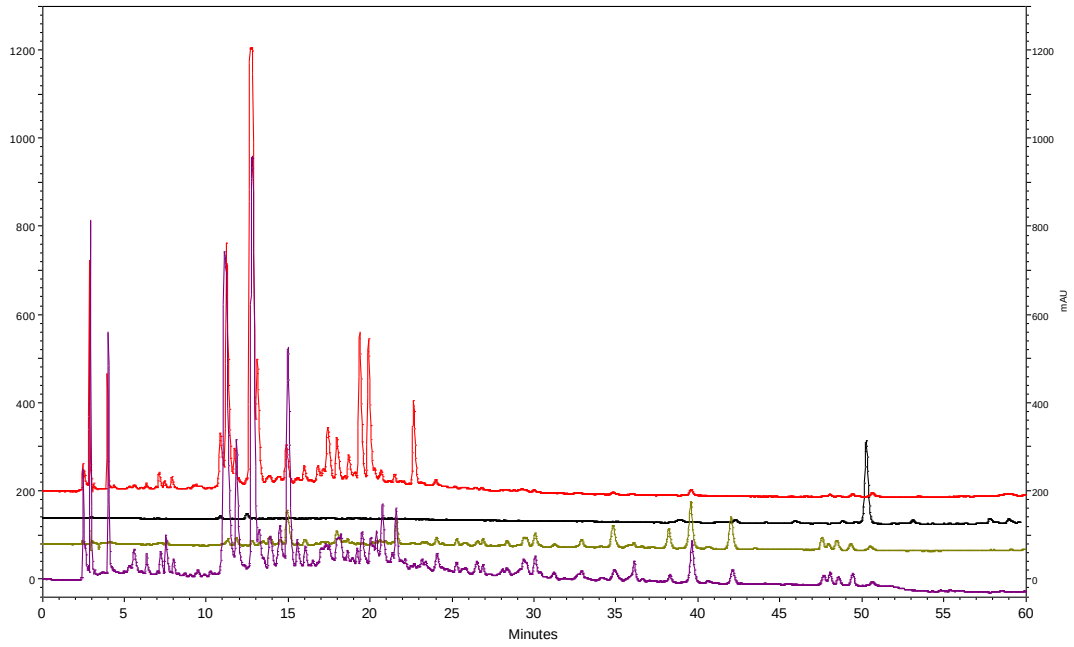


Figure 14: Chromatograph of sunflower seed paste extracts at 280 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

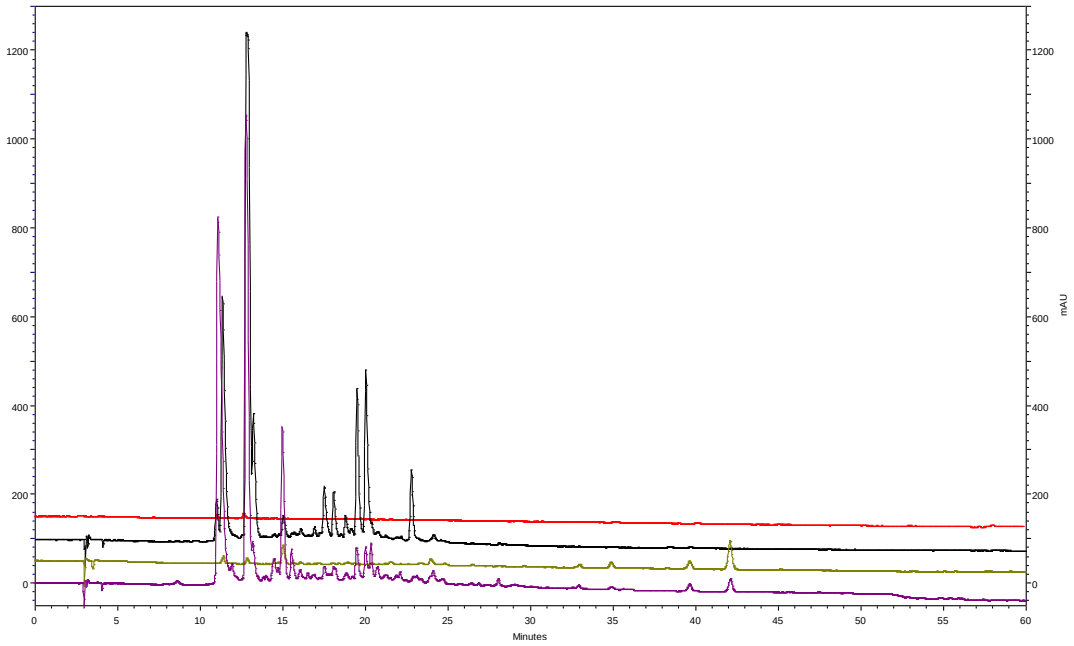


Figure 15: Chromatograph of sunflower seed paste extracts at 350 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

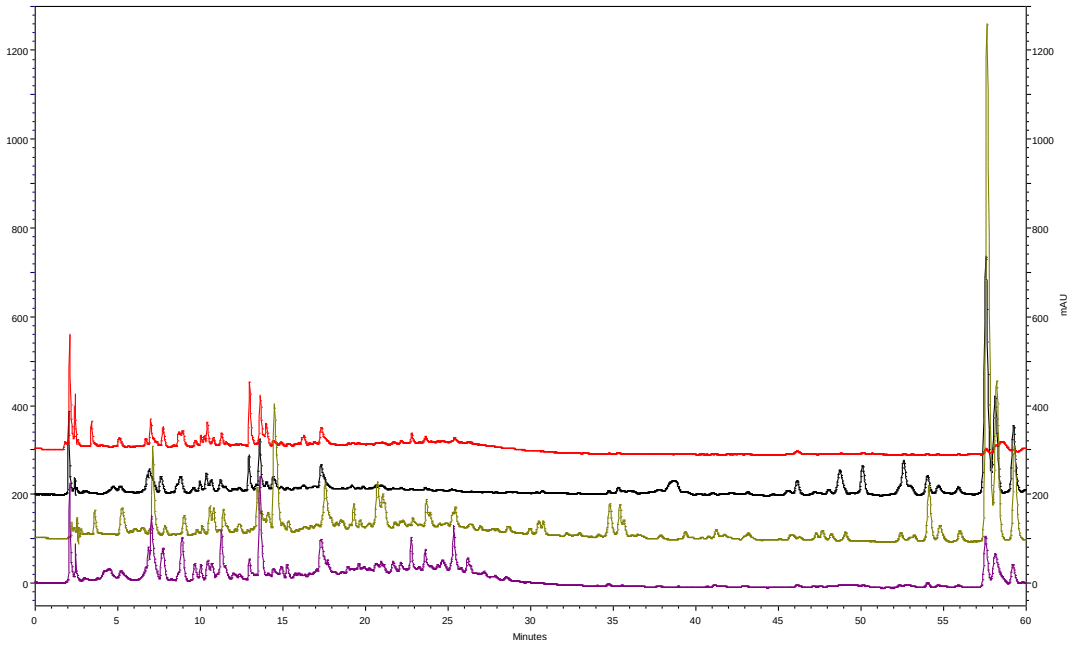


Figure 16: Chromatograph of pomegranate seed paste extracts at 254 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

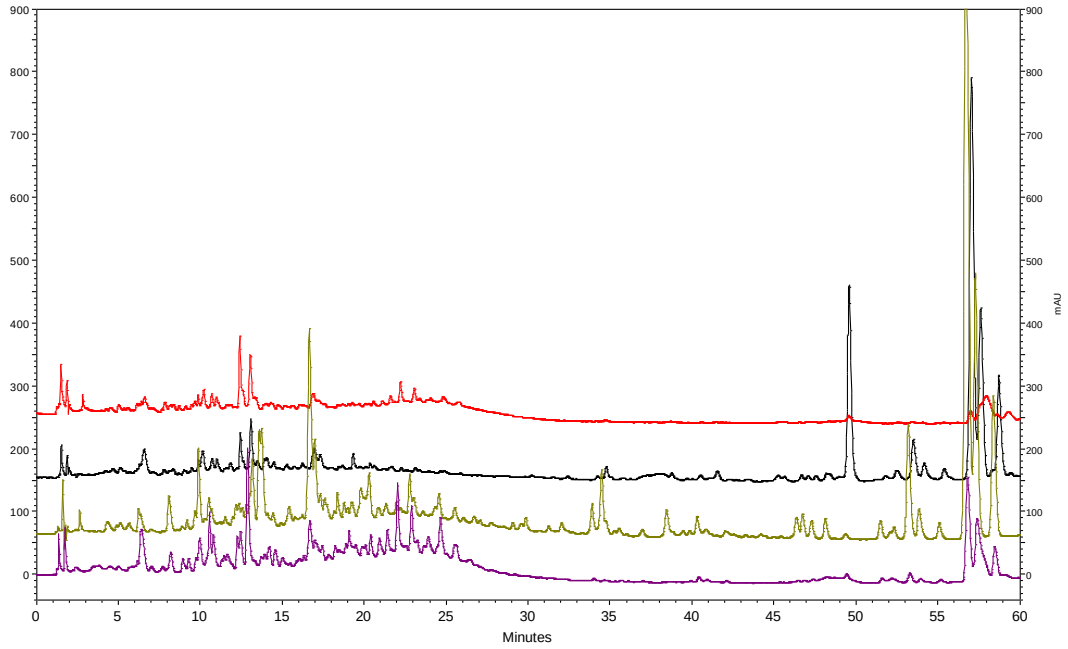


Figure 17: Chromatograph of pomegranate seed paste extracts at 280 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

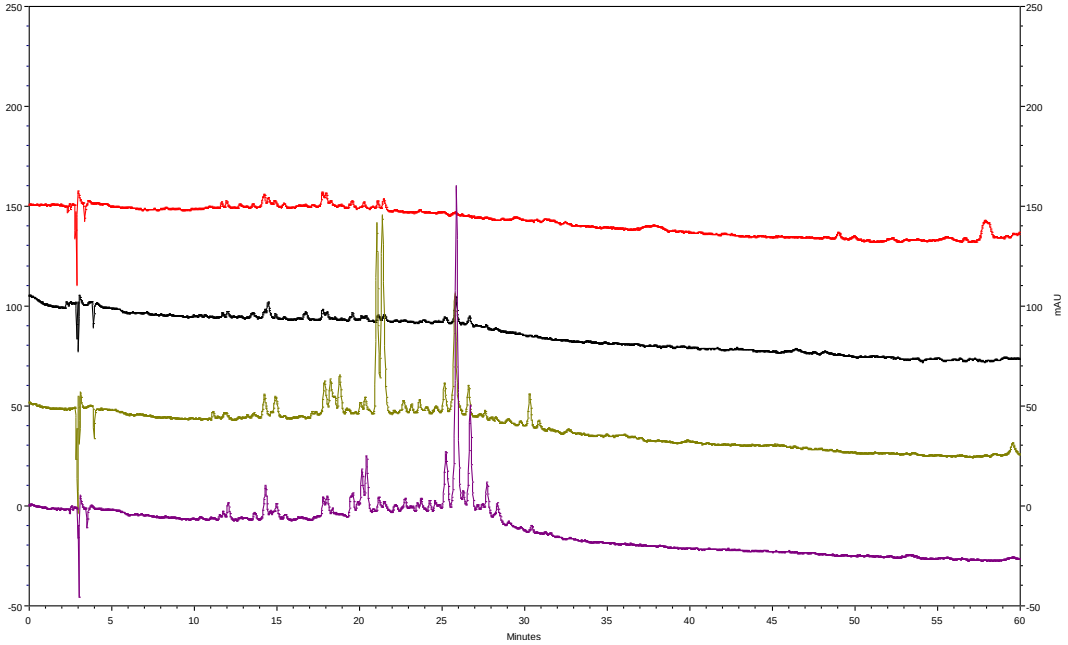


Figure 7: Chromatograph of pomegranate seed paste extracts at 350 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

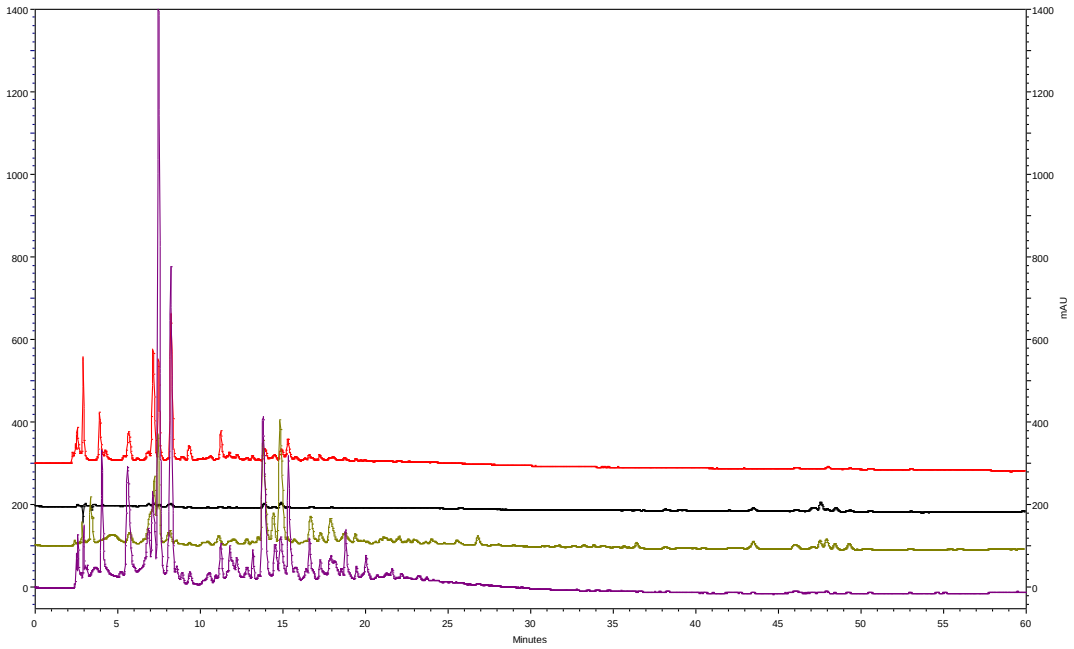


Figure 19: Chromatograph of pumpkin seed paste extracts at 254 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

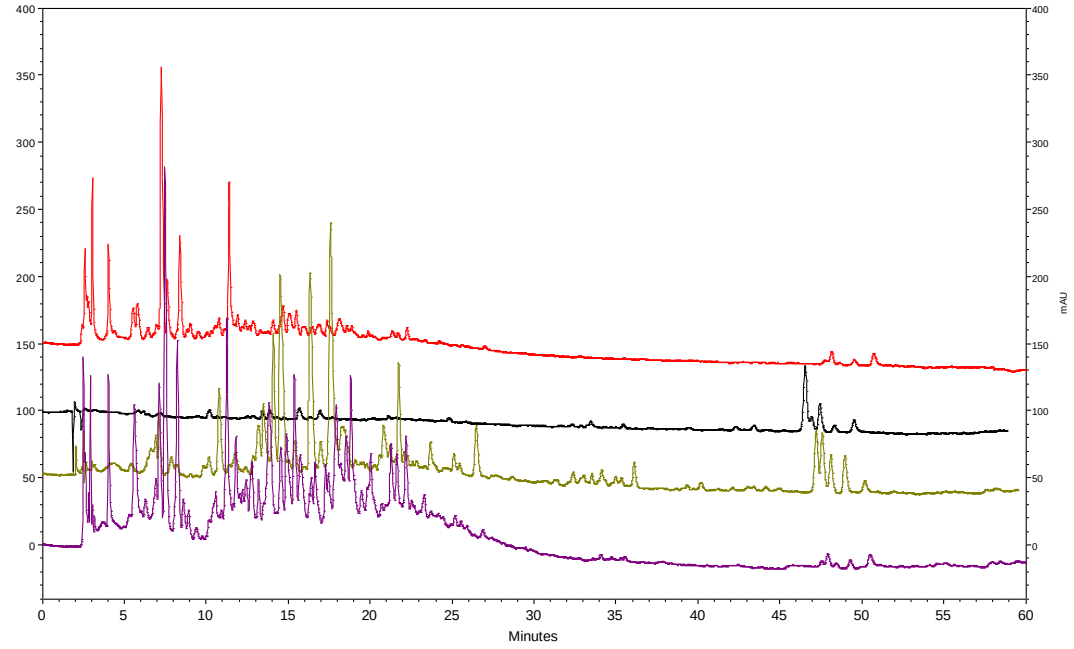


Figure 20: Chromatograph of pumpkin seed paste extracts at 280 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

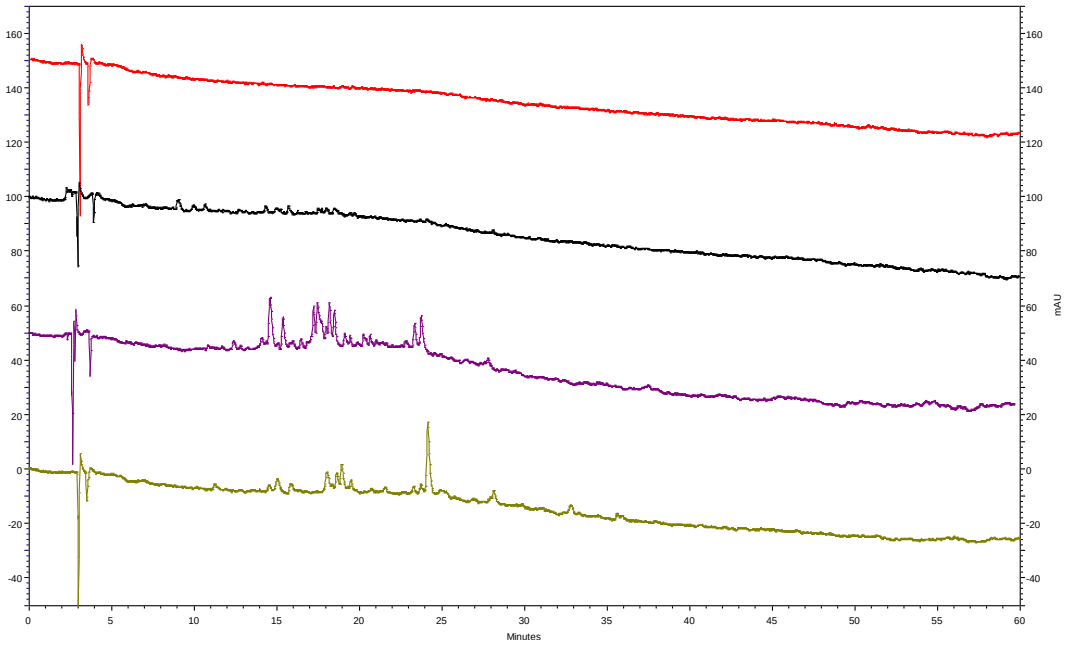


Figure 21: Chromatograph of pumpkin seed paste extracts at 254 nm (black: UAE EtOH, red: UAE EtOH/H2O 1:1 v/v, brown: SFE 10% EtOH, purple: SFE 20% EtOH).

Chapter 2: Green approaches for the extraction of bioactive compounds from grape seed by-products for cosmetic purposes.

Abstract

The current study focuses on the exploitation of grapevine industry by-products, which mainly represented by grape seeds, with final goal the discovery of extracts with beneficial dermo-cosmetic properties. Grape seeds are considered as a rich pool of bioactive compounds with beneficial effects for human health. Moreover, it is the raw material for the production of grape seed oil which finds a broad range of applications in cosmetic and pharmaceutical area. The first step of this study, was the cold press extraction of oil from grape seeds. The produced oil as well as the produced paste, were subsequently extracted with green methodologies. Adsorption resins were applied on oil with elusion solvent ethanol. For the seed paste, two different extraction techniques were tested. The first one, was ultrasound assisted extraction with ethanol and ethanol/water in ratio 1:1 v/v as diffusion solvents. Moreover, supercritical fluid extraction, with CO_2 and ethanol in percentages of 10% and 20% were employed on paste. All the extracts were analyzed with LC-HRMS to record their chemical profiles. Resin extract was fractionated with centrifugal partition chromatography following the systems *n*-Hex/EtOAc/EtOH/H_2O in ratio 8:2:8:2 and 8:2:9:1 v/v/v/v. Furthermore, ultrasound ethanolic paste extract was also treated with centrifugal partition chromatography using the system *n*-Hex/EtOAc/MeOH/H_2O in ratio 1:10:3:7 v/v/v/v and its final fractions were analyzed with HPTLC and were finally evaluated concerning their inhibition activity against tyrosinase, collagenase and elastase enzymes.

Introduction

Vitis vinifera, commonly known as grapevine, belongs botanically to Vitaceae family and is considered as one of the most wide spread cultivated plants in human history [1]. Based on scientific evidence, grapevine plant appeared in Eurasia 65 million years ago, approximately [2]. The first reports of its cultivation were traced back to the seventh millennium BC, in the area of Iran [3]. The main reason of grapevine exploitation is its fruits, grapes, that are used for direct consumption as well as the production of wines, spirits and other alcoholic beverages [4]. An important product that derives from grapevine are the grape seeds (GS), which compose the main solid by-product (38-54% w/w) of wineries [5]. The high amounts of GrpS generated by wineries have established them as the most efficient starting material for the production of grape seed oil (GrpSO), an edible oil with profuse applications in food and cosmetic industries [6,7]. The most common method for the production of GrpSO is the extraction of GRPS and subsequent refining with organic solvents [8]. Generally, GrpSO composes the 8 to 20% of dry GrpS [1]. The rest residue is the grape seed paste (GrpSP), which is considered as by-product of the GrpSO production procedure [5]. It has to be noted that both materials are regarded as agricultural by-products of grapevine.

Grapevine plant is characterized as a rich source of bioactive compounds and consequently many cosmetic products have been developed and realized to the market with grape-based chemical composition. Similarly, the corresponding products and by-products are rich in these effectual compounds. Namely, unsaturated fatty acids are the most abundant chemical category (90%) of GrpSO with major representatives, linoleic acid and then oleic acid [9,10]. Furthermore, compounds with significant antioxidant activity like tocopherols and phytosterols are encompassed in GrpSO chemical composition [11]. GrpSP is characterized additionally by hydrophilic compounds like catechin, epicatechin and their corresponding dimers, trimers and tetramers [12]. The most common polymers of GrpSP are procyanidins, a category of proanthocyanidins (condensed tannins) [13]. Based on previous studies, proanthocyanidins of grape seed have numerous beneficial bioactivities. Series of experiments have proved the antioxidant, anti-inflammatory, anti-allergic, anti-cancer, immune-stimulating, anti-viral, cardio-protective, and antithrombotic properties of proanthocyanidins [14–17]. Cosmetic industry is

highly attracted from proanthocyanidins due to their antiaging properties [18]. Enzymatic assays have shown a potential inhibition activity of proanthocyanidins from *Punica granatum*, *Ginko biloba* and other woody plants against collagenase, tyrosine and elastase. However, further investigation is required for the establishment of grape seed proanthocyanidins as inhibitors of the above mentioned enzymes [19,20].

The treatment of these kind of starting materials like grapevine by-products and generally natural products is a challenging procedure for the fields of research and industry as well. It is widely known that the steps of extraction and fractionation play crucial role to the quality of the final extract/product. Many procedures and techniques have been bibliographically proposed for this purpose and few of them have been industrially applied. Adsorption resins are an established eco-friendly method for the removal of pollutants mainly from waterwastes [21]. Moreover, it is a useful tool for the recovery of targeted metabolites from liquid solutions [22]. It is a widespread methodology in the field of natural products and due to the easiness of operation conditions and the simplicity of application, it corresponds perfect from laboratory to pilot scale uses [23]. Likewise, Ultrasound Assisted Extraction (UAE) is a familiar technique for natural products and edible raw materials handling [24]. Plenty of advantages characterize this technique starting from the low time consumption in correlation with other green, modern or conventional methodologies like Microwave Assisted Extraction and Soxhlet [25,26]. The low pressure and temperature of the operation conditions of UAE are critical for user's safety and the protection of thermo-sensitive compounds [27]. As it is already mentioned, UAE is considered as an eco-friendly approach due to the perfect functionality of the technique with non-toxic solvents and the corresponding low volume requirement [28]. The principal of ultrasound extraction is based on the dispersed compressions and expansions that were produced in the solvent [29]. The captivation occurring during the expansion periods of solvent increases their polarity and the extraction efficiency [25]. Another suitable technique for the investigation of natural products is Supercritical Fluid Extraction (SFE) [30]. The low viscosity and the high diffusion nature of supercritical fluids contribute to the obtainment of high extract yields, while the low organic solvent consumption and the recirculation of CO_2 render SFE at the top of the rank of green extraction methodologies [31]. Also, SFE is an efficient method to obtain thermo-sensitive compounds, due to the mild thermal conditions of operation process [32]. The fractionation of natural based extracts could be a demanding operation as well. For this reason, state of the art techniques, like Centrifugal Partition

Chromatography (CPC), have been employed [33]. The solid support free liquid-liquid nature of this chromatographic technique enables the management of high amounts of extracts, providing the maximum recovery [34]. Another advantage of CPC is that broad solvent categories can be adjusted as mobile or stationary phase, giving the opportunity to run experiments in a wide range of polarities [35]. It is notable, that the high resolution of CPC can even separates stereoisomers or mixtures of oligomers and polymers [36–38].

Motivated by the above observations and the experience of our research team in natural products field, the chemical profile of GrpSO and GrpSP were investigated. Promising green extraction methodologies were tested and the ultrasound GrpSP ethanolic extract, which provided the most efficient results was developed in pilot scale and extract was further fractionated. Final goal was the exploration of the inhibitory effect of the obtained fractions against enzymes with applications in cosmetic industry and the proposal of green methodologies for the treatment and exploitation of agricultural by-products.

More specifically, in our research approach grape seeds were provided from producers of the area of Greece, were cold pressed, as described in previous work (*Part II Chapter 1*), for the generation of GrpSO and the corresponding GrpSP as starting materials. In order to investigate the chemical profile of GrpSO, food grade adsorption resin XAD-7 was selected for the recovery of its phenolic fraction, followed from CPC analysis. In parallel, taking in consideration that by-products of food industry originated from processes with no chemical treatment could be potential pools of bioactive natural compounds, we focused on the development of green methodologies for the utilatation of GRSP. Two different extraction methods were applied on GRSP, the first one was based on Ultrasound Assisted Extraction (UAE), while the second one on Supercritical Fluid Extraction (SFE). In both cases, after the defatting process, the extraction with different solvents were performed. EtOH and EtOH/H_2O 1:1 v/v were the selected solvents of UAE, while for the SFE were chosen CO_2 with 10% of EtOH on the first extraction step and then the concentration of EtOH was increased at 20%. Finally, four different extracts (UAE: EtOH-***UE1*** and EtOH/H_2O 1:1 v/v-***UE2***, SFE: CO_2 + EtOH10%-***SE1*** and CO_2 + EtOH20%-***SE2***) were obtained. UE1 was the most efficient extract based on yield and chromatographic analysis (LC-HRMS) and was produced on preparative scale and fractionated with CPC. Moreover, for the CPC fractions High Performance Thin Layer Chromatography (HPTLC) and LC-HRMS analysis were employed.

Ultimately, CPC fractions were evaluated concerning their inhibition activity on tyrosinase, collagenase and elastase.

Methods and Materials

Reagents

All the solvents were purchased from Fisher Scientific (Pennsylvania, USA). Analytical grade solvents were used for extraction, separation and HPTLC experiments. UPLC-HRMS analysis was conducted with LC-MS grade solvents. Adsorption resin XAD-7 Ambrerlite was purchased from Sigma Aldrich (Missouri, USA). HPTLC silica gel 60 F_{254} 20 × 20 cm plates were provided from Merck Millipore (Massachusetts, USA). Reagents for the enzymatic assays were supplied from Sigma Aldrich (Missouri, USA). Specifically, for the enzymatic assays mushroom tyrosinase (lyophilized powder, ≥1000 units/mg solid, EC Number: 1.14.18.1), 3,4-dihydroxy-L-phenylalanine, sodium phosphate monobasic, sodium phosphate dibasic, kojic acid, elastase type IV from porcine pancreas (EC Number 254-453-6), N-Succinyl-Ala-Ala-Ala-p-nitroanilide (EC Number 257-823-5), Trizma base reagent grade, elastatinal, collagenase from Clostridium histolyticum (released from physiologically active rat pancreatic islets Type V, ≥1 FALGPA units/mg solid, > 125 CDU/mg solid, EC Number: 232-582-9), MMP 2 substrate fluorogenic, chlorexidine, bovine serum albumin (BSA), acetic acid glacial, p-(dimethylamino) benzaldehyde, sodium tetraborate, hyaluronidase (released from bovine testes Type I-S, lyophilized powder, 400–1,000 units/mg solid, EC Number: 3.2.1.35), hyaluronic acid, and tannic acid were used.

Instrumentation

Ultrasonic P300H bath of Elma Schmidbauer (Singen, Germany) was used for the analytical scale UAE. Preparative UAE was carried out using a PEXE3N-Type ultrasound extractor of R.E.U.S (Darp, France) with capacity volume up to 3 L and embedded coolant system. For supercritical fluid experiments an analytical scale apparatus of SEPAREX (Nancy, France) was used, consisted of an embedded 100 mL stainless steel extraction vessel in a ventilated oven. Also, the apparatus carried CO_2 chiller unit, control valve of back-pressure regulators separator, co-solvent and a CO_2 liquid pump. For the fractionation, an A-CPC apparatus of Rousselet-Robatel-Kromaton (Anonay, France) was icorporated, equipped with a chromatography semi-preparative column of 200 mL (FCPC200®) for GRSO and a preparative column of 1000 mL (FCPC1000®) for GRSP analysis. Solvents were pump through the column with preparative Lab Alliance Series III P300 pump (Pennsylvania, USA). HPTLC analysis run on Camag instrumentation (Muttenz, Switzerland).

VisionCats (28.3000) was used as software to connect and coordinate the Automatic TLC Sampler 4 (ATS 4) equipped with 100 μl syringe, twin-trough development chamber (10x10 cm^2), TLC Scanner III, TLC Plate Heater 3 and TLC Visualizer 2. LC-HRMS experiments were accomplished to an H class Acquity UPLC system (Waters, USA) coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, USA). All the enzymatic assays were measured on Infinite 200 PRO series reader (Tecan Group, Switzerland).

Recovery of GrpSO phenolic fraction using XAD-7

30 g of XAD-7 were embedded in flash column of 65 cm x 1.8 cm and activated with three column volumes of EtOH. Crude GRSO (100 mL) was eluted through the column with flow rate of 1 drop/sec. Afterwards, 200 mL of *n*-Hex rinsed the column to sweep along the lipophilic remnants. Recovery of targeted compounds took place with the elution of resin column with 150 mL of EtOH. The extract was evaporated until dryness under vacuum condition at 40 ^{O}C.

Extraction of GrpSP using UAE and SFE

The extraction process of GRSP with UAE was performed in three stages. Initially, the GRSP was pulverized to powder and 10 g of them were defatted with 30 mL of *n*-Hex for 20 min. Afterwards, the raw material was extracted with 30 mL of EtOH for 20 more min. For the last extraction an aqueous solution of EtOH/H_2O 1:1 v/v was prepared and used for extraction again for 20 min. The extracts were evaporated until dryness with RotaVap at 40^{o}C. For the preparative scale UAE, 1.4 kg were added in the ultrasound extraction basket and again 1.5 liters of *n*-Hex were used for the defatting process, while the targeted extract was obtained with 1.5 L of EtOH.

For the SFE experiments 50 g of grounded GrpSP powder was loaded in the extraction basket. The defatting procedure was conducted with pure CO_2. The flow rate of CO_2 pump was set at 15 g/min, oven temperature at 40^{o}C, and system pressure at 300 bar. After 4 hours the fatty fraction was collected. The first SFE extract was obtained using CO_2 and 10% of EtOH as co-solvent. For the second one co-solvent (EtOH) was increased at 20%.

CPC fractionation of GrpSO and GrpSP

Fractionation of GrpSO XAD-7 extract was performed with CPC analysis. An elution-extrusion methodology, in descending mode of two step gradient solvent system of *n*-Hex/EtOAc/EtOH/H_2O in ratio 8:2:8:2 and 8:2:9:1 v/v/v/v was followed. At the apparatus, a 200 mL semi-preparative column had been installed and loaded with stationary phase at 200 rpm and

10 mL/min. Before mobile phase was started to pump through the column, rotation speed was increased at 950 rpm and mobile phase flow rate was adjusted at 5 mL/min during the elution, while for the extrusion flow rate was increased at 10 mL/min. Sample injection was 200 mg diluted in 5 mL of the biphasic system. In total 3 column volume run and 60 fractions of 10 mL were collected, 20 from the first solvent system, 20 from the second and 20 from the extrusion process. Concerning GRSP, UE1 extract was selected for fractionation with CPC technique (discussed in detail in section 3.1). Ascending mode was applied and as solvent system was chosen *n*-Hex/EtOAc/MeOH/H_2O in ratio 1:10:3:7 v/v/v/v. For the chromatography, a preparative CPC column of 1000 mL was used. During the loading of stationary phase (MeOH/H_2O) rotation was set at 200 rpm and flow rate at 15 mL/min. Afterwards, rotation was increased at 800 rpm and mobile phase (*n*-Hex /EtOAc) was pumped through the column. After the chemical equilibration of the biphasic system, 6 g of UE1 were injected. For the elution, flow rate remained at 15 mL/min, while during the extrusion the flow was maximized at 20 mL/min. Finally, 160 fractions of 20 mL were collected.

HPLTLC and LC-HRMS analysis

All the fractions of CPC were spotted and developed with HPTLC technique, in order to obtain a clear view of GrpSP constituents. The fractions were diluted in methanol (4 mg/mL) and 25 μL of each one spotted with a length of 8 mm. As development solvent system acetone/chlorophorm/water/methanol 1:7:0.5:3 v/v/v/v was chosen. All the developed TLC plates were pictured at 254 nm, 366 nm and visible after sprayed with sulfuric vanillin (vanillin 2.5% w/v, H_2SO_4 2.5% v/v diluted in MeOH) and heated at 130^{o}C for 1 min. In parallel, for each fraction a concentration of 200 μg/mL was prepared and analyzed with LC-HRMS. Samples were analyzed with a LC gradient consisted of H_2O with 0.1% formic acid (FA) (solvent A) and ACN (solvent B). The elution method started with 2% of B which stayed for 2 min. In the next 16 min B reached 100% and stayed for 2 min. Finally, at 21st min, system returned to the initial conditions and stayed for 4 min for system equilibration. An Ascentis Express C18 (150 mm x 2.1 mm, 2.7 μm) column was used for the separation, with stable temperature of 40°C. Measurements were performed with a total acquisition time of 25 min and a flow rate of 400 μL/min. The injection volume was 10 μL and the autosampler temperature was at 7°C.

Mass spectra were obtained in negative and positive ion mode using an electrospray ionization source (ESI). For the negative ionization, capillary temperature was set at 350°C, capillary voltage

at -30 V and tube lens at -100 V. Sheath and auxiliary gas were adjusted at 40 and 10 arb, respectively. For the positive ionization, capillary temperature was set at 350°C, capillary voltage at +40 V, tube lens at +120 V, sheath gas 40 arb and auxiliary gas 10 arb. All mass spectra were recorded in full scan mode in the range of 115-1000 *m/z*, with resolving power 30,000 at 500 *m/z* and scan rate 1 microscan per second. HRMS/MS experiments were obtained in data-depending method with collision energy 35.0% (q = 0.25).

Enzymatic assays

CPC analysis of UE1 provided 19 fractions that were evaluated concerning their inhibition activity against tyrosinase, elastase and collagenase enzymes based on the protocols that were described by *Michailidis et al.* [35] (Appendix), with few modifications at samples concentration. UE1 CPC fractions were diluted at three different concentrations of 300 μg/mL, 150 μg/mL and 75 μg/mL in order to evaluate their tyrosinase and collagenase inhibition activity. For elastase inhibition assay, concentrations of 300 μg/mL, 100 μg/mL and 50 μg/mL of each fraction were tested. In all experiments, DMSO concentration in each well did not exceed 5% of the final volume. All the enzymatic assays were designed to provide the competitive inhibition activity of the compounds. For all the enzymatic assays, the half maximal inhibitory concentration (IC50) of each positive control was used as standard of comparison. All the experiments were conducted in triplicates and twice in total.

Calculation of inhibition percentages was based on the formula:

Inhibition (%) = [((X control − X control's blank) − (X sample − X sample's blank))/(X control − X control's blank)] × 100

X control symbolizes the absorbance or fluoresces of the mixture consisting of buffer, enzyme, sample solvent, and substrate. X sample symbolizes the absorbance or fluoresces of the mixture of buffer, enzyme, sample, or positive control solution and substrate. Blanks constituted by all the above-mentioned components except the enzyme.

Results and Discussion

Extraction of GrpSO and GrpSP

Based on previous studies (*Part II Chapter 1*), GrpSO treatment with XAD-7 provided yield of 213.1 mg/100 mL. Concerning GrpSP extracts, different yields were obtained depending on the extraction process and the used solvent. It should be noticed that between the two techniques UAE and SFE, the first one is less time consuming and produces higher amount of yields per gram of

raw material. More specifically, after 20 min of extraction for each extract, UE1 and UE2, were weighted 551 mg (55.1 mg/g of raw material) and 456 mg (45.6 mg/g of raw material), respectively. The production of SFE extracts required more than 4 hours. Their yields (mg/g of raw material) were lower in correlation with UAE extracts. In detail, SE3 and SE4 were weighted 975 mg (19.5 mg/g of raw material) and 500 mg (10.0 mg/g of raw material), respectively. All the extract yields are presented in detail in table 1.

Table 1: Yields of GrpSO and GrpSP extracts from UAE, SFE and XAD-7. Results for UAE and SFE are presented as mg/g of raw material and for XAD-7 as mg/100 mL of oil.

Extraction method	Extraction solvent	*Yield*
UAE	*n-Hex defatting*	37.8 mg/g
	EtOH	55.1 mg/g
	EtOH/H_2O 1:1 v/v)	45.6 mg/g
SFE	*CO_2 defatting*	44.1 mg/g
	CO_2 + 10%EtOH w/w	19.5 mg/g
	CO_2 + 20%EtOH w/w	10.0 mg/g
XAD-7	*EtOH*	213.1 mg/100 mL

Selection of GRSP extract for scale up

In total, five extracts were obtained; one from GrpSO and four for GrpSP (UE1, UE2, SE3 and SE4). GrpSO and one extract of GrpSP were chosen for further investigation and fractionation. The selection of the appropriate GRSP extract was based on method efficiency and the chemical profile of the extracts. The aim of the preparative scale extraction was the obtainment of high amount of extracts, rich in bioactive compounds. Thus, we focused on methodologies that provide extracts with high yields and their chemical profiles are rich in secondary metabolites. Also, crucial role for the selection of the most suitable technique was the adaptation of the chosen methodology on pilot and industrial scale.

In more detail, the extraction of GrpSP was accomplished in laboratory scale with two green methodologies, SFE and UAE. For both techniques, the first step was the defatting of the raw material. For the defatting process with SFE, 4 hours were required, while for UAE only 20 min. Similarly, UE1 and UE2 needed only 20 minutes to be obtained. As indicated, extracts derived from SFE needed more time to be obtained, specifically, for SE3 2 hours and for SE4 3 hours were needed. SFE is a suitable method to obtain low polarity molecules and with the proper co-solvent can recover middle polar solutes. On the other hand, UAE can be used and for the extraction of more polar compounds, depending on the polarity of the applied solvents. In order to extract the

maximum chemical categories of compounds, SFE experiments were focused on low and middle polarities, while UAE extractions were targeted to polar and middle polarity molecules. Both of the SFE extracts had lower yield than those of UAE.

SE1 yielded 19.5 mg/g of raw material and SE2 10.0 mg/g of raw material. In addition, 55.1 mg/g of raw material and 45.6 mg/g of raw material were provided from UE1 and UE2, respectively. These differences on yields are a first sign that the chemical profile of GrpSP does not include high amounts of low and middle polarities molecules, probably because during the oil pressing procedure the majority of hydrophobic compounds found a more chemical related environment on GRSO. The four extracts (SE1, SE2, UE1 and UE2) were analyzed with LC-HRMS (Appendix Figures 1-4). Their profiles showed that the extracts SE2 and UE2 were poor in metabolites, while UE1 and SE1 gave similar spectra. The difference between UE1 and SE1 allocated at the concentration of their solutes. UE1 is richer in polar compounds, but with presence of middle and low polarity molecules. At SE1 the reverse analogies of compounds are observed; polar are the minority while non polar represent the majority of constituents. Based on previous study of our research group, extracts that were originated from UAE extraction had higher dermocosmetic benefits in contrast with them of SFE. The high yield rate, the chemical profile and the evaluated inhibition activities of UE1, revealed that it is the most efficient extract for further investigation. Thus, UE1 was produced in preparative scale. 1.4 kg of GrpSP powder were defatted with *n*-Hex and afterwards extracted with 1.5 L of EtOH. The obtained extract, after the evaporation reached the weight of 61.75 g.

Fractionation of GrpSO with semi prep-CPC and UE1 with prep-CPC

GrpSO extract of XAD-7 was analyzed with CPC. A step gradient solvent system, on descending mode was chosen due to the nonpolar nature of the extract. Specifically, 400 mL of Hex/EtOAc/EtOH/H_2O 8:2:8:2 v/v/v/v were prepared in a separation funnel. The upper phase was loaded to the column and after the run of a heavy phase column volume, the mobile phase was changed and 200 mL from the heavy phase of system Hex/EtOAc/EtOH/H_2O 8:2:9:1 v/v/v/v were pumped through the column. Afterwards, extrusion process was started with the upper phase of the second biphasic system. Experimental Sf was estimated at 67.5%. In total, 60 fractions of 10 mL were collected and were combined in 14 final fraction. Among the CPC fractions oleanolic acid, monoglycarides and unsaturated fatty acids were detected via NMR.

Extract UE1 was the most promising among pastes extracts. For this reason, UE1 was prepared on preparative scale in order to obtain high amounts of extract. Afterwards, 6 g of UE1 were treated with prep-CPC using the solvent system *n*-Hex/EtOAc/MeOH/H_2O, in ratio 1:10:3:7 v/v/v/v and ascending mode. Elution step was completed after passing of 3.2 column volumes of mobile phase, followed by extrusion step in order to obtain faster the more polar compounds of the UE1. Sf of the experiment was calculated at 70%. The 160 obtained fractions were grouped to 19 final fractions and analyzed on HPTLC (Figure 1). It should be noticed that observing HPTLC analysis, the separation was efficient, with clear grouping of the solutes. Also, as it will be discussed in section 3.4, LC-HRMS analysis revealed the successful fractionation of the extract.

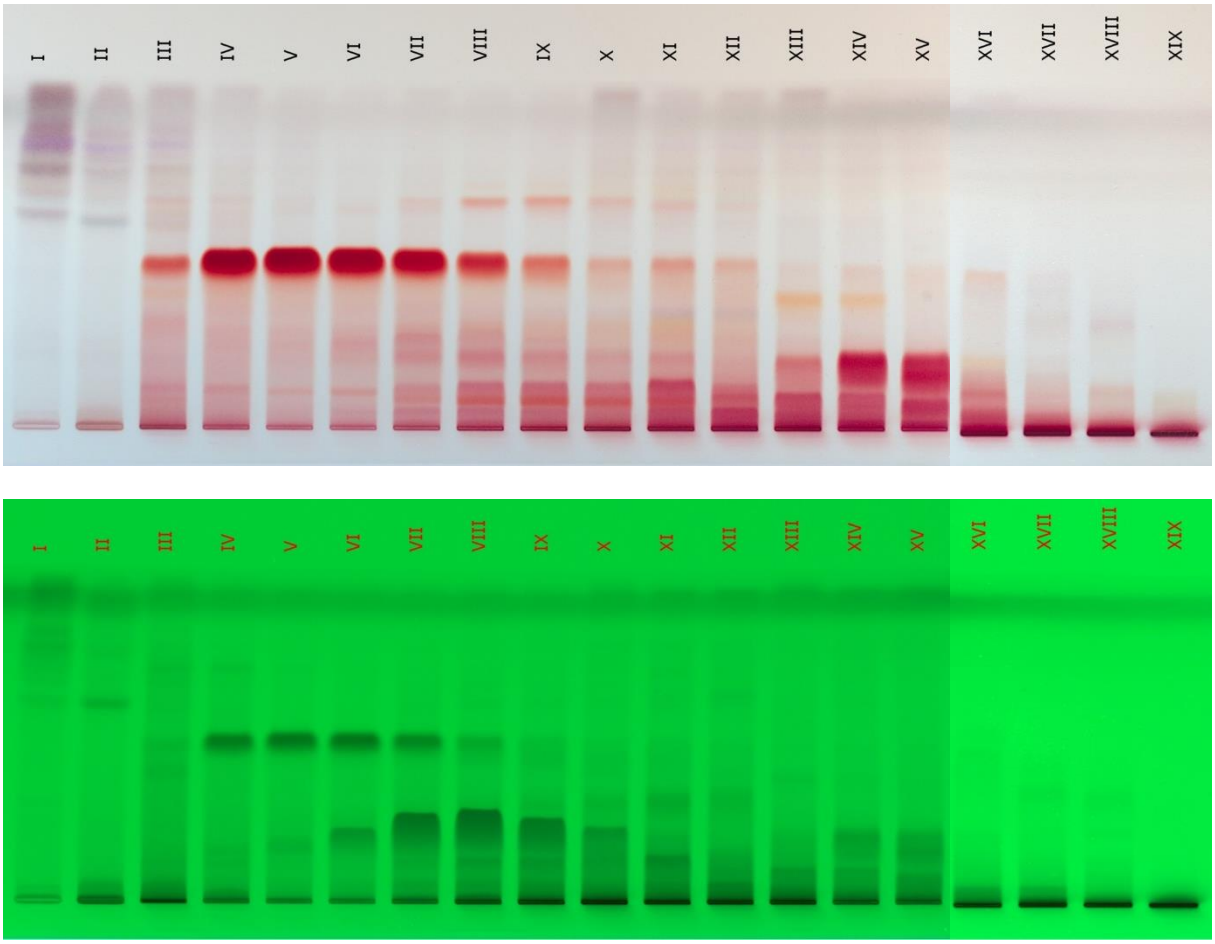


Figure 8:HPTLC of GprSP UAE EtOH CPC fractions at visible sprayed with sulfuric vanillin (up) and at 254 nm (down).

LC-HRMS analysis of UE 1 CPC fractions

The major peaks of grape seed paste UE 1 extract were dereplicated and correlated with literature data [13,39–42] (Tables 2-3). Chemical structure of catechin/epicatechin was identified on

negative ($C_{15}H_{13}0_6$, *m/z*=289.0720) and positive ($C_{15}H_{15}0_6$, *m/z*=291.0863) ion mode. Moreover, dimer of epicatechin was detected on negative mode ($C_{30}H_{27}O_{12}$, *m/z*=579.1519). Epicatechin-3-gallate/catechin-3-gallate was also recorded on negative mode ($C_{22}H_{17}O_{10}$, *m/z*=441.0828). Furthermore, negative mode revealed the appearance of monogallate epicatechin dimer ($C_{37}H_{29}O_{16}$, *m/z*=729.1459) and trimer of epicatechin ($C_{45}H_{37}O_{18}$, *m/z*=865.1988). Trimer of epicatechin was recorded also in positive mode ($C_{45}H_{39}O_{18}$, *m/z*=867.213). Two more compounds were detected in positive ionization, trimer of epicatechin gallate ($C_{52}H_{43}O_{22}$, *m/z*=1019.225) and tetramer of epicatechin ($C_{60}H_{51}O_{24}$, *m/z*=1155.279). As it is noticed on table 2-3, most of the compounds that were identified from HRLC-MS were proanthocyanins and smaller fragment of them, like catechin and epicatechin, which have been previously reported in GrpPS chemical composition. Epicatechin was the most abundant molecule in the grape seed polymers. To the best of our knowledge, oligomers and polymers of proanthocyanins were identified, while molecules with unknown structure were also recorded.

Table 2: LC-HRMS negative ionization analysis of grape seed UAE extract.

$[M-H]^-$ *(m/z)*	Chemical formula	Identified compounds	$[M-H]^{2-}$ *(m/z)*	RDB	Delta
169.0145	$C_7H_5O_5$	gallate	not recorded	5.5	1.677
289.072	$C_{15}H_{13}0_6$	catechin/epicatechin	245.08, 205.05, 179.04	9.5	0.895
313.2388	$C_{18}H_{33}O_4$	Fatty acid derivative	295.23, 277.22, 183.14, 167.87	2.5	1.172
327.2179	$C_{18}H_{31}O_5$	Fatty acid derivative	291.20, 229.14, 171.10	3.5	0.619
329.2334	$C_{18}H_{33}O_5$	Fatty acid derivative	311.22, 229.14, 211.13	2.5	0.433
331.0672	$C_{13}H_{15}0_{10}$	Unknown 1	169.01	6.5	0.393
335.0772	$C_{16}H_{15}0_8$	Unknown 2	289.07	9.5	-0.091
441.0828	$C_{22}H_{17}O_{10}$	epicatechin-3-gallate/catechin-3-gallate	331.04, 289.07, 169.01	14.5	0.159
476.2789	$C_{27}H_{40}O_7$	Unknown 3	279.23	8	1.928
569.2245	$C_{27}H_{37}O_{13}$	Unknown 4	523.22,478.22, 286.28, 243.15, 196.55	9.5	1.029
577.1357	$C_{30}H_{25}O_{12}$	Unknown 5	478..27, 425.09, 309.02, 287.00, 196.55	18.5	0.919

579.1519	$C_{30}H_{27}O_{12}$	dimer epicatechin	not recorded	17.5	1.935
585.2189	$C_{27}H_{37}O_{14}$	Unknown 6	478.25, 390.39, 286.98, 196.55	9.5	0.087
729.1459	$C_{37}H_{29}O_{16}$	dimer epicatechin-2-gallate	670.73, 577.13, 559.12, 407.08, 286.25, 196.55	23.5	-0.23
865.1988	$C_{45}H_{37}O_{18}$	trimer epicatechin	739.16, 695.14, 637.31, 577.13, 478.2502, 407.08, 286.25	27.5	0.292
1153.2579	$C_{60}H_{49}O_{24}$	tetramer epicatechin	not recorded	36.5	-3.464

Table 3: LC-HRMS positive ionization analysis of grape seed UAE extract.

$[M-H]^+$ *(m/z)*	**Chemical formula**	**Identified compounds**	**$[M-H]^{2+}$ *(m/z)***	**RDB**	**Delta**
291.0863	$C_{15}H_{15}O_6$	catechin/epicatechin	273.08, 165.05, 139.04	8.5	-0.153
317.102	$C_{17}H_{17}O_6$	Unknown 1	286.97, 196.49, 165.05	9.5	0.08
559.1305	$C_{23}H_{27}O_{16}$	Unknown 2	538.44, 449.83, 286.8, 196.48	10.5	2.019
593.2751	$C_{34}H_{41}O_9$	Unknown 3	553.25	14.5	0.979
731.1609	$C_{37}H_{31}O_{16}$	Unknown 4	579.11, 443.0969, 409.09, 351.37, 289.07, 196.49	22.5	0.313
867.213	$C_{45}H_{39}O_{18}$	trimer epicatechin	715.16, 577.13, 409.09	26.5	0.216
1019.225	$C_{52}H_{43}O_{22}$	trimer epicatechin gallate	891.28, 822.54, 731.16, 688.70, 566.79, 456.58, 356.73, 287.05	31.5	0.609
1155.279	$C_{60}H_{51}O_{24}$	tetramer epicatechin	not recorded	4.5	0.076

Furthermore, the 19 UE1 CPC fractions were dereplicated separately (Table 4). At the first two fraction were eluted less polar compounds. Fraction III was rich on catechin/epicatechin (*m/z*=289.0720) and epicatechin gallate (*m/z*=441.0828). Fractions IV, V and VI have as major compound catechin/epicatechin (*m/z*=289.0720) and the dimmers of them (*m/z*=579.1519). In fraction VII catechin/epicatechin (*m/z*=289.0720), gallate (*m/z*=169.0145) and non galloylated proanthocyanidins dimers (*m/z*=577.1357) were detected. Fraction VIII provided the same profile with VII plus the abundance of mono-galloylated dimer (*m/z*=729.1459). High concentration of gallate (*m/z*=169.0145) was allocated in fraction IX. Fraction X was rich in mono-galloylated

dimer (m/z=729.1459) non galloylated proanthocyanidins dimers (*m/z*=577.1357) and gallate (*m/z*=169.0145). At fraction XI major signal of mono-galloylated dimer (*m/z*=729.1459) and minor of gallate (*m/z*=169.0145), were detected. Mono-galloylated dimer (*m/z*=729.1459) was detected also in fraction XII. XIII and XIV were rich in proanthocyanidins dimers (*m/z*=577.1357) and mono-galloylated dimer (*m/z*=729.1459). In fraction XV proanthocyanidins dimers (*m/z*=577.1357), mono-galloylated dimer (*m/z*=729.1459) and trimer epicatechin (*m/z*=865.1988) was found. At fraction XVI proanthocyanidins dimers (*m/z*=577.1357 and trimer epicatechin (*m/z*=865.1988) were eluted. In fraction XVII proanthocyanidins dimers (*m/z*=577.1357), trimer epicatechin (*m/z*=865.1988) and proanthocyanidin tetramers (*m/z*=1153.2579) were obtained. Fractions XVIII and XIX were rich on sugars (*m/z*=133.0147).

Table 4: Major compounds of UE1 CPC fractions that identified with LC-HRMS/MS.

Number of fraction	Compounds	Number of fraction	Compounds	Number of fraction	Compounds
I	non polar compounds	**VII**	catechin/epicatechin gallate non galloylated proanthocyanidins dimers	**XIII**	proanthocyanidins dimers mono-galloylated dimer
II	non polar compounds	**VIII**	catechin/epicatechin gallate non galloylated proanthocyanidins dimers mono-galloylated dimer	**XIV**	proanthocyanidins dimers mono-galloylated dimer
III	catechin/epicatechin epicatechin gallate	**IX**	gallate	**XV**	proanthocyanidins dimers mono-galloylated dimer trimer epicatechin
IV	catechin/epicatechin catechin/epicatechin dimer	**X**	mono-galloylated dimer gallate non galloylated proanthocyanidins dimers	**XVI**	proanthocyanidins dimers trimer epicatechin
V	catechin/epicatechin catechin/epicatechin dimer	**XI**	mono-galloylated dimer gallate	**XVII**	proanthocyanidins dimers trimer epicatechin proanthocyanidin tetramers
VI	catechin/epicatechin catechin/epicatechin dimer	**XII**	mono-galloylated dimer	**XVIII & XIX**	sugars (HPTLC shown also proanthocyanidin polymers, probably with higher mass than 2000 m/z)

Enzymatic evaluation of CPC fractions

All the final fractions of UE1 CPC analysis were evaluated concerning their inhibition activity against tyrosinase, elastase and collagenase enzymes (Figure 2). Focusing on tyrosinase inhibition assay, at the employed protocol the enzyme reacts with L-Dopa (substrate) and produces dopaquinone, a molecule that its concentration is measured at 475 nm and it is correlated with tyrosinase activity. Experimental results revealed that Fractions VIII, XIII and XIV at concentration of 300 μg/mL, provided inhibition values of 43.30±3.25%, 46.03±1.76% and 44.95±1.88%, respectively). The other fractions had lower inhibition activity against tyrosinase enzyme. It should be underlined that fractions IV, V and VI seemed to trigger tyrosinase activity. Based on LC-HRMS analysis, fractions IV, V and VI were rich in catechin/epicatechin and their corresponding dimers, compounds with high anti-tyrosinase properties [43]. Experimental results of our study were in confrontation with the bibliography data, concerning the tyrosinase inhibition activity of catechin/epicatechin. In order to investigate these contradictory observations, the above mentioned CPC fractions (IV, V and VI) were tested again on tyrosinase inhibition assay, with a crucial modification on protocol; at the wells of the tested fractions no substrate was added. The three tested fractions provided again tyrosinase activation. Based on these evidences, it can be suggested that fractions IV, V and VI contained molecules with structures close to dopaquinone, which is the substrate of tyrosinase inhibition assay, and in this way provided fault tyrosinase activity results.

Fractions of UE1 revealed significant anti-collagenase activity. All the fractions have higher inhibition properties than positive control at 300 μg/mL. More specifically, fractions I, II, III, VII, VIII, X, XII, XIII, XIV, XV, XVI, XVII, XVIII and XIX inhibited collagenase activity more than 80% (82.23%, 87.35%, 89.58%, 80.24%, 81.74%, 92.56%, 94.96%, 91.73%, 81.23%, 87.79%, 85.43%, 86.38% and 95.63%, respectively). Based on literature data, previous studies of grape pomace have also shown the anti-collagenase effect of the polyphenolic extract [44,45].

Concerning elastase inhibition activity many studies have highlighted the anti-elastase properties of grape pomace [44,45]. In our study, CPC fractions XIV, XV, XVI, XVII, XVIII and XIX of grape seed paste provided high inhibition activity at 300 μg/mL, with inhibition values of 66.62±2.44%, 53.07±2.17%, 88.17±4.903%, 60.37±2.59%, 49.68±2.81% and 58.50±2.96%. Based on LC-HRMS analysis (section 3.4) the major compounds of these fractions are

proanthocyanidins dimers, trimer of epicatechin, mono-galloylated dimers and at the last fractions proanthocyanidins polymers (constituted by more than four monomers) were identified.

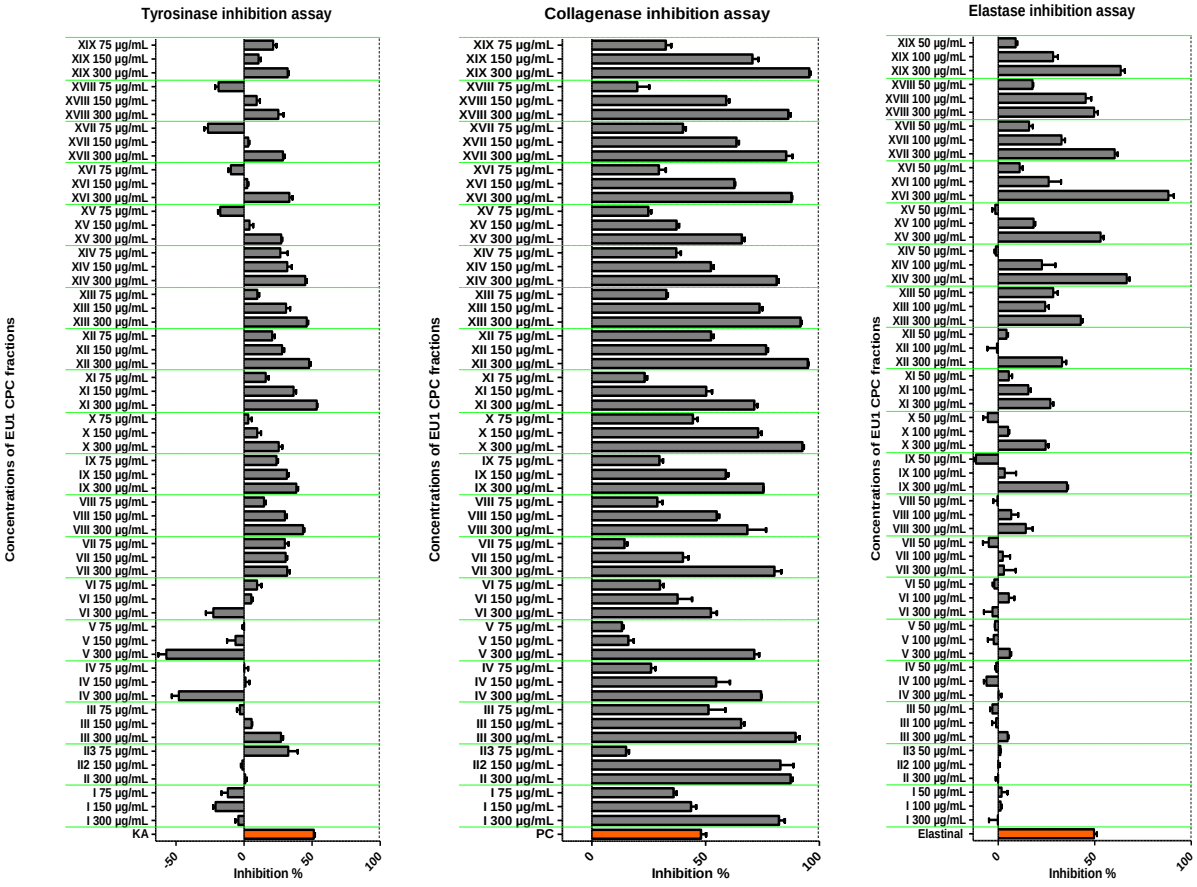


Figure 9: Inhibition assays of UE1 CPC fractions for the evaluation of their activity.

Conclusions

The current study proposes an effective green exploitation plan of grape seeds that are generally considered as agricultural by-products. After cold press extraction of seeds, grape seed oil and a seed paste were produced. Both of them were extracted with eco-friendly techniques, suitable for industrial applications. Food grade resins were employed for the extraction of GrpSO, while for GrpSP two different extraction approaches were used, UAE and SFE. GrpSO XAD-7 extract and the most efficient GrpSP extract, in terms of chemical profile and the required production time, were fractionated via CPC for further investigation of their chemical profile. The 19 CPC fractions of GrpSP were subsequently analyzed via LC-HRMS for compounds identification. The identified compounds were correlated with evaluation tests against the enzymes tyrosinase, collagenase and elastase in order to locate compounds with dermo-protective properties. Most of CPC fractions of

UAE extract revealed strong anti-collagenase effect, while specific fractions were characterized by anti-tyrosinase and anti-elastase activity. To conclude, in the current research a holistic green strategy is proposed for the production of a GrpPS-based extract with industrial potentials and promising dermo-protective properties.

References

[1] N. Rombaut, R. Savoire, B. Thomasset, T. Bélliard, J. Castello, É. Van Hecke, J.L. Lanoisellé, Grape seed oil extraction: Interest of supercritical fluid extraction and gas-assisted mechanical extraction for enhancing polyphenol co-extraction in oil, Comptes Rendus Chim. 17 (2014) 284–292. https://doi.org/10.1016/j.crci.2013.11.014.

[2] P. This, T. Lacombe, M.R. Thomas, Historical origins and genetic diversity of wine grapes, Trends Genet. 22 (2006) 511–519. https://doi.org/10.1016/j.tig.2006.07.008.

[3] J.F. Terral, E. Tabard, L. Bouby, S. Ivorra, T. Pastor, I. Figueiral, S. Picq, J.B. Chevance, C. Jung, L. Fabre, C. Tardy, M. Compan, R. Bacilieri, T. Lacombe, P. This, Evolution and history of grapevine (Vitis vinifera) under domestication: new morphometric perspectives to understand seed domestication syndrome and reveal origins of ancient European cultivars., Ann. Bot. 105 (2010) 443–455. https://doi.org/10.1093/aob/mcp298.

[4] M.T. Prats-Llinàs, H. Nieto, T.M. DeJong, J. Girona, J. Marsal, Using forced regrowth to manipulate Chardonnay grapevine (Vitis vinifera L.) development to evaluate phenological stage responses to temperature, Sci. Hortic. (Amsterdam). 262 (2020) 109065. https://doi.org/10.1016/j.scienta.2019.109065.

[5] T. Maier, A. Schieber, D.R. Kammerer, R. Carle, Residues of grape (Vitis vinifera L.) seed oil production as a valuable source of phenolic antioxidants, Food Chem. 112 (2009) 551–559.

[6] P. Glampedaki, V. Dutschk, Stability studies of cosmetic emulsions prepared from natural products such as wine, grape seed oil and mastic resin, Colloids Surfaces A Physicochem. Eng. Asp. 460 (2014) 306–311. https://doi.org/10.1016/j.colsurfa.2014.02.048.

[7] Y. Nakamura, S. Tsuji, Y. Tonogai, Analysis of proanthocyanidins in grape seed extracts, health foods and grape seed oils, J. Heal. Sci. 49 (2003) 45–54. https://doi.org/10.1248/jhs.49.45.

[8] N. Rombaut, R. Savoire, B. Thomasset, J. Castello, E. Van Hecke, J.L. Lanoisellé, Optimization of oil yield and oil total phenolic content during grape seed cold screw pressing, Ind. Crops Prod. 63 (2015) 26–33. https://doi.org/10.1016/j.indcrop.2014.10.001.

[9] C.P. Passos, R.M. Silva, F.A. Da Silva, M.A. Coimbra, C.M. Silva, Supercritical fluid extraction of grape seed (Vitis vinifera L.) oil. Effect of the operating conditions upon oil composition and antioxidant capacity, Chem. Eng. J. 160 (2010) 634–640. https://doi.org/10.1016/j.cej.2010.03.087.

[10] B. Matthäus, Virgin grape seed oil: Is it really a nutritional highlight?, Eur. J. Lipid Sci. Technol. 110 (2008) 645–650. https://doi.org/10.1002/ejlt.200700276.

[11] G. Davidov-Pardo, D.J. McClements, Nutraceutical delivery systems: Resveratrol encapsulation in grape seed oil nanoemulsions formed by spontaneous emulsification, Food Chem. 167 (2015) 205–212. https://doi.org/10.1016/j.foodchem.2014.06.082.

[12] A.I. Mandic, S.M. Dilas, G.S. Ćetković, J.M. Čanadanović-Brunet, V.T. Tumbas, Polyphenolic composition and antioxidant activities of grape seed extract, Int. J. Food Prop. 11 (2008) 713–726. https://doi.org/10.1080/10942910701584260.

[13] B. Gabetta, N. Fuzzati, A. Griffini, E. Lolla, R. Pace, T. Ruffilli, F. Peterlongo, Characterization of proanthocyanidins from grape seeds, Fitoterapia. 71 (2000) 162–175. https://doi.org/10.1016/S0367-326X(99)00161-6.

[14] N. Martinez-Micaelo, N. González-Abuín, A. Ardèvol, M. Pinent, M.T. Blay, Procyanidins and inflammation: Molecular targets and health implications, BioFactors. 38 (2012) 257–265. https://doi.org/10.1002/biof.1019.

[15] M.J. Salvadó, E. Casanova, A. Fernández-Iglesias, L. Arola, C. Bladé, Roles of proanthocyanidin rich extracts in obesity, Food Funct. 6 (2015) 1053–1071. https://doi.org/10.1039/c4fo01035c.
[16] M. Pinent, A. Castell-Auví, M.I. Genovese, J. Serrano, A. Casanova, M. Blay, A. Ardévol, Antioxidant effects of proanthocyanidin-rich natural extracts from grape seed and cupuassu on gastrointestinal mucosa, J. Sci. Food Agric. 96 (2016) 178–182. https://doi.org/10.1002/jsfa.7079.
[17] N. Unusan, Proanthocyanidins in grape seeds: An updated review of their health benefits and potential uses in the food industry, J. Funct. Foods. 67 (2020) 103861. https://doi.org/10.1016/j.jff.2020.103861.
[18] Z.F. Ma, H. Zhang, Phytochemical constituents, health benefits, and industrial applications of grape seeds: Amini-review, Antioxidants. 6 (2017) 1–11. https://doi.org/10.3390/antiox6030071.
[19] A.K. Ghimeray, U.S. Jung, H.Y. Lee, Y.H. Kim, E.K. Ryu, M.S. Chang, In vitro antioxidant, collagenase inhibition, and in vivo anti-wrinkle effects of combined formulation containing Punica granatum, Ginkgo biloba, Ficus carica, and Morus alba fruits extract, Clin. Cosmet. Investig. Dermatol. 8 (2015) 389–396. https://doi.org/10.2147/CCID.S80906.
[20] K. Takagi, T. Mitsunaga, Tyrosinase inhibitory activity of proanthocyanidins from woody plants, J. Wood Sci. 49 (2003) 461–465. https://doi.org/10.1007/s10086-002-0496-4.
[21] C. Xu, W.Z. Sun, X.L. Qin, Y.X. Jia, S.T. Yu, M. Xian, Effective adsorption of phenolic compounds by functional group modified resins: behavior and mechanism, Sep. Sci. Technol. 54 (2019) 467–477. https://doi.org/10.1080/01496395.2018.1518331.
[22] G. Bringmann, Large-Scale Biotechnological Production of the Antileukemic Marine Natural Product Sorbicillactone A, Mar. Drugs. 5 (2007) 23–30. https://doi.org/10.3390/md20070003.
[23] B. Borina, A. Pavko, Adsorption of vancomycin on Amberlite XAD-16 in a packed bed column, Chem. Biochem. Eng. Q. 23 (2009) 479–483.
[24] F. Chemat, N. Rombaut, A.G. Sicaire, A. Meullemiestre, A.S. Fabiano-Tixier, M. Abert-Vian, Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review, Ultrason. Sonochem. 34 (2017) 540–560. https://doi.org/10.1016/j.ultsonch.2016.06.035.
[25] S.C. Mandal, V. Mandal, A.K. Das, Classification of Extraction Methods, in: Essentials Bot. Extr., Elsevier, 2015: pp. 83–136. https://doi.org/10.1016/b978-0-12-802325-9.00006-9.
[26] Chapter 3 Analytical uses of ultrasounds, in: Tech. Instrum. Anal. Chem., Elsevier, 2002: pp. 43–82. https://doi.org/10.1016/S0167-9244(02)80005-1.
[27] C. Bendicho, I. Lavilla, Ultrasound Extractions, in: Ref. Modul. Chem. Mol. Sci. Chem. Eng., Elsevier, 2018. https://doi.org/10.1016/b978-0-12-409547-2.04571-6.
[28] C. McDonnell, B.K. Tiwari, Ultrasound: A Clean, Green Extraction Technology for Bioactives and Contaminants, Compr. Anal. Chem. 76 (2017) 111–129. https://doi.org/10.1016/bs.coac.2017.03.005.
[29] R. Vardanega, D.T. Santos, M.A. De Almeida, Intensification of bioactive compounds extraction from medicinal plants using ultrasonic irradiation, Pharmacogn. Rev. 8 (2014) 88–95. https://doi.org/10.4103/0973-7847.134231.
[30] S. Kumar, Supercritical Fluid Extraction, in: Anal. Tech. Nat. Prod. Res., 2016: p. 21.

https://doi.org/10.1124/dmd.117.079962.
[31] J.M. Prado, R. Vardanega, I.C.N. Debien, M.A. de A. Meireles, L.N. Gerschenson, H.B. Sowbhagya, S. Chemat, Conventional extraction, in: Food Waste Recover. Process. Technol. Ind. Tech., Elsevier Inc., 2015: pp. 127–148. https://doi.org/10.1016/B978-0-12-800351-0.00006-7.
[32] A. Capuzzo, M.E. Maffei, A. Occhipinti, Supercritical fluid extraction of plant flavors and fragrances, Molecules. 18 (2013) 7194–7238. https://doi.org/10.3390/molecules18067194.
[33] A. Marston, C. Borel, K. Hostettmann, Separation of natural products by centrifugal partition chromatography, J. Chromatogr. A. 450 (1988) 91–99. https://doi.org/10.1016/S0021-9673(00)90718-0.
[34] A. Angelis, M. Hamzaoui, N. Aligiannis, T. Nikou, D. Michailidis, P. Gerolimatos, A. Termentzi, J. Hubert, M. Halabalaki, J.H. Renault, A.L. Skaltsounis, An integrated process for the recovery of high added-value compounds from olive oil using solid support free liquid-liquid extraction and chromatography techniques, J. Chromatogr. A. 1491 (2017) 126–136. https://doi.org/10.1016/j.chroma.2017.02.046.
[35] D. Michailidis, A. Angelis, N. Aligiannis, S. Mitakou, L. Skaltsounis, Recovery of Sesamin, Sesamolin, and Minor Lignans From Sesame Oil Using Solid Support-Free Liquid-Liquid Extraction and Chromatography Techniques and Evaluation of Their Enzymatic Inhibition Properties, 10 (2019). https://doi.org/10.3389/fphar.2019.00723.
[36] A. Foucault, K. Nakanishi, Comparison of several aqueous two phase solvent systems (ATPS) for the fractionation of biopolymers by centrifugal partition chromatography (CPC), J. Liq. Chromatogr. 13 (1990) 2421–2440. https://doi.org/10.1080/01483919008049043.
[37] C.S. Lau, K.A. Bunnell, E.C. Clausen, G.J. Thoma, J.O. Lay, J. Gidden, D.J. Carrier, Separation and purification of xylose oligomers using centrifugal partition chromatography, J. Ind. Microbiol. Biotechnol. 38 (2011) 363–370. https://doi.org/10.1007/s10295-010-0799-1.
[38] A.. Foucault, Enantioseparations in counter-current chromatography and centrifugal partition chromatography, J. Chromatogr. A. 906 (2001) 365–378.
[39] Y. Hayasaka, E.J. Waters, V. Cheynier, M.J. Herderich, S. Vidal, Characterization of proanthocyanidins in grape seeds using electrospray mass spectrometry, Rapid Commun. Mass Spectrom. 17 (2003) 9–16. https://doi.org/10.1002/rcm.869.
[40] L. Lin, J. Sun, P. Chen, M.J. Monagas, J.M. Harnly, Uhplc-pda-esi/hrms, (2014).
[41] J. Mata, J.A.F. Machado, Counterfactual decomposition of changes in wage distributions using quantile regression, J. Appl. Econom. 20 (2005) 445–465. https://doi.org/10.1016/S0031-9422(98)00107-1.
[42] C. Saucier, M. Mirabel, F. Daviaud, A. Longieras, Y. Glories, Rapid fractionation of grape seed proanthocyanidins, J. Agric. Food Chem. 49 (2001) 5732–5735. https://doi.org/10.1021/jf010784f.
[43] X. Song, X. Hu, Y. Zhang, J. Pan, D. Gong, G. Zhang, Inhibitory mechanism of epicatechin gallate on tyrosinase: inhibitory interaction, conformational change and computational simulation, Food Funct. 11 (2020) 4892–4902. https://doi.org/10.1039/d0fo00003e.
[44] J. Wittenauer, S. MäcKle, D. Sußmann, U. Schweiggert-Weisz, R. Carle, Inhibitory effects of polyphenols from grape pomace extract on collagenase and elastase activity, Fitoterapia. 101 (2015) 179–187. https://doi.org/10.1016/j.fitote.2015.01.005.
[45] N. Mohd Maidin, N. Michael, M.J. Oruna-Concha, P. Jauregi, Polyphenols extracted from

red grape pomace by a surfactant based method show enhanced collagenase and elastase inhibitory activity, J. Chem. Technol. Biotechnol. 93 (2018) 1916–1924. https://doi.org/10.1002/jctb.5459.

Appendix

Tyrosinase enzymatic assay: This assay measures the inhibition of the tested samples at the catalytic oxidation of L-DOPA to dopachrome by tyrosinase. Kojic acid (IC50 = 50 μM) was used as positive control. In a 96-well microplate, 80 μl of phosphate-buffered saline (PBS) (1/15 M, pH = 6.8), 40 μl of the tested sample (100 U/ml) (dissolved in PBS buffer) were mixed and incubated in the dark for 10 min at room temperature. Afterward, 40 μl of 2.5 mM L-DOPA (substrate) dissolved in PBS buffer was added, and the mixture was incubated for 15 min. The 96-well microplate was measured at 475 nm.

Elastase enzymatic assay: Elastase protocol monitors the release of *p*-nitroaniline from N-succinyl-Ala-Ala-Ala-p-nitroanilide that is stimulated by elastase. Elastatinal (IC50 = 0.5 μg/ml) was used as a positive control. In a 96-well microplate, 70 μl of Trizma buffer (50 mM, pH = 7.5), 10 μl of tested sample (dissolved in Trizma buffer), and 5 μl of elastase (0.45 U/ml) (dissolved in Trizma buffer) were mixed and incubated in the dark for 15 min at room temperature. Afterward, 15 μl of 2 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide (substrate) dissolved in Trizma buffer was added, and the mixture was incubated for 30 min at 37°C. The 96-well microplate was measured at 405 nm.

Collagenase enzymatic assay: Collagenase fragmentates the fluorescence molecule MMP2. The inhibition of the enzyme was measured concerning the reduction of the fluorescent intensity that was produced. Chlorhexidine (IC50 = 50 μM) was used as a positive control. In a 96-well dark microplate, 120 μl of Tris-HCl buffer (50 mM, pH = 7.3), 40 μl of tested sample, and 40 μl of collagenase (50 μg/ml) from *C. histolyticum* (dissolved in Tris-HCl buffer) were incubated for 10 min at 37°C avoiding light exposure. Afterward, 40 μl of 50.0 μM MMP2 (substrate) (MCA-Pro-Leu-Ala-Nva-DNP-Dap-Ala-Arg-NH_2) dissolved in Tris-Cl buffer was added, and the mixture was incubated in dark for 30 min at 37°C. The fluorescent intensity of 96-well microplate was measured at an excitation maximum of 320 nm and an emission maximum of 405 nm.

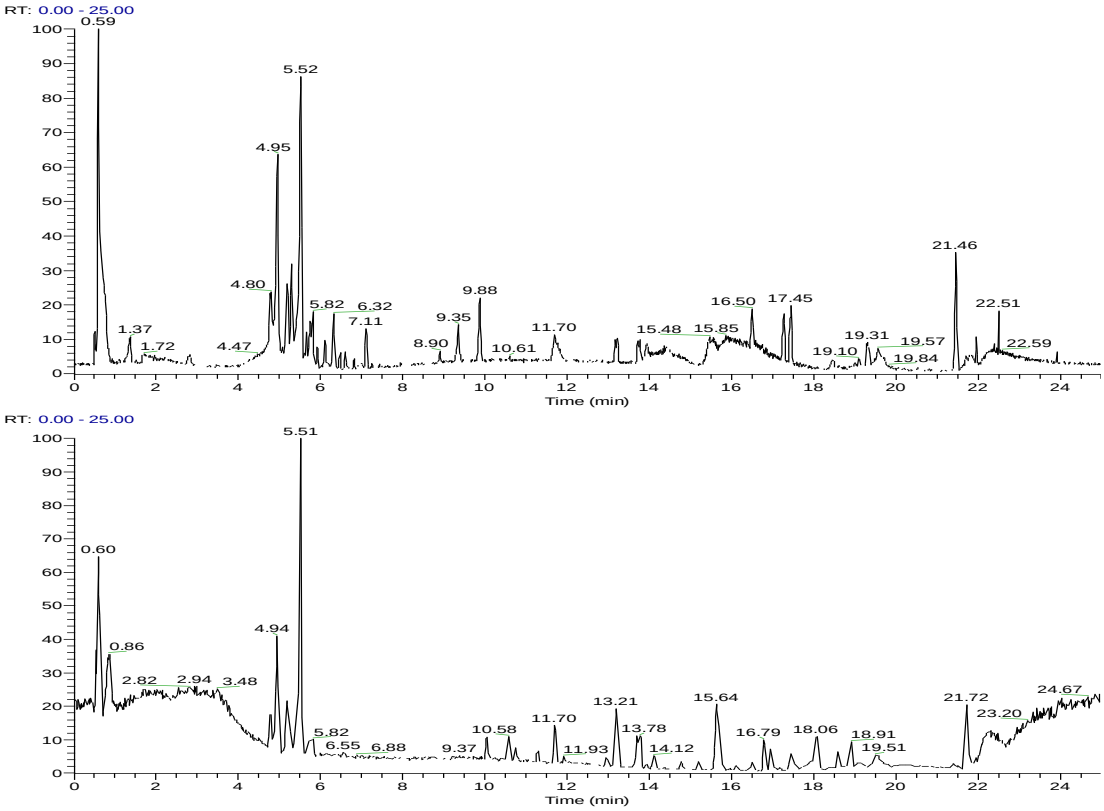


Figure 1: UPLC-HRMS chromatograms of UE1 in negative mode (up) and positive mode (down).

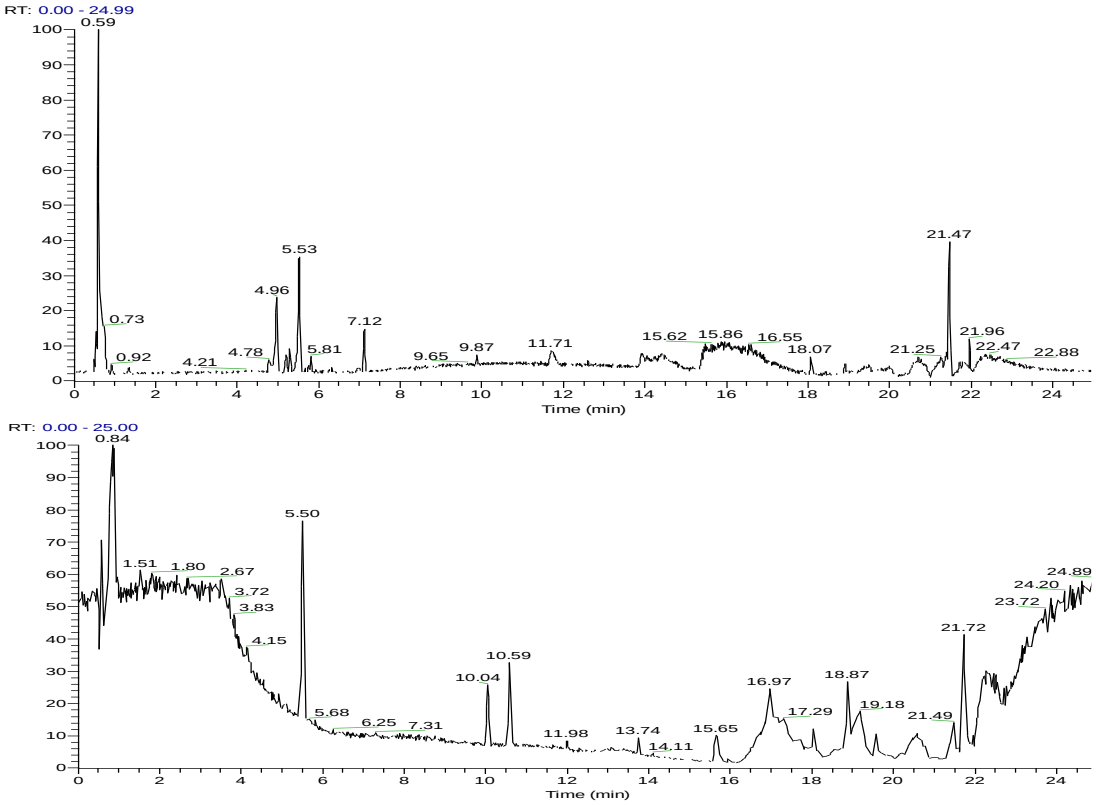


Figure 2: UPLC-HRMS chromatograms of UE2 in negative mode (up) and positive mode (down).

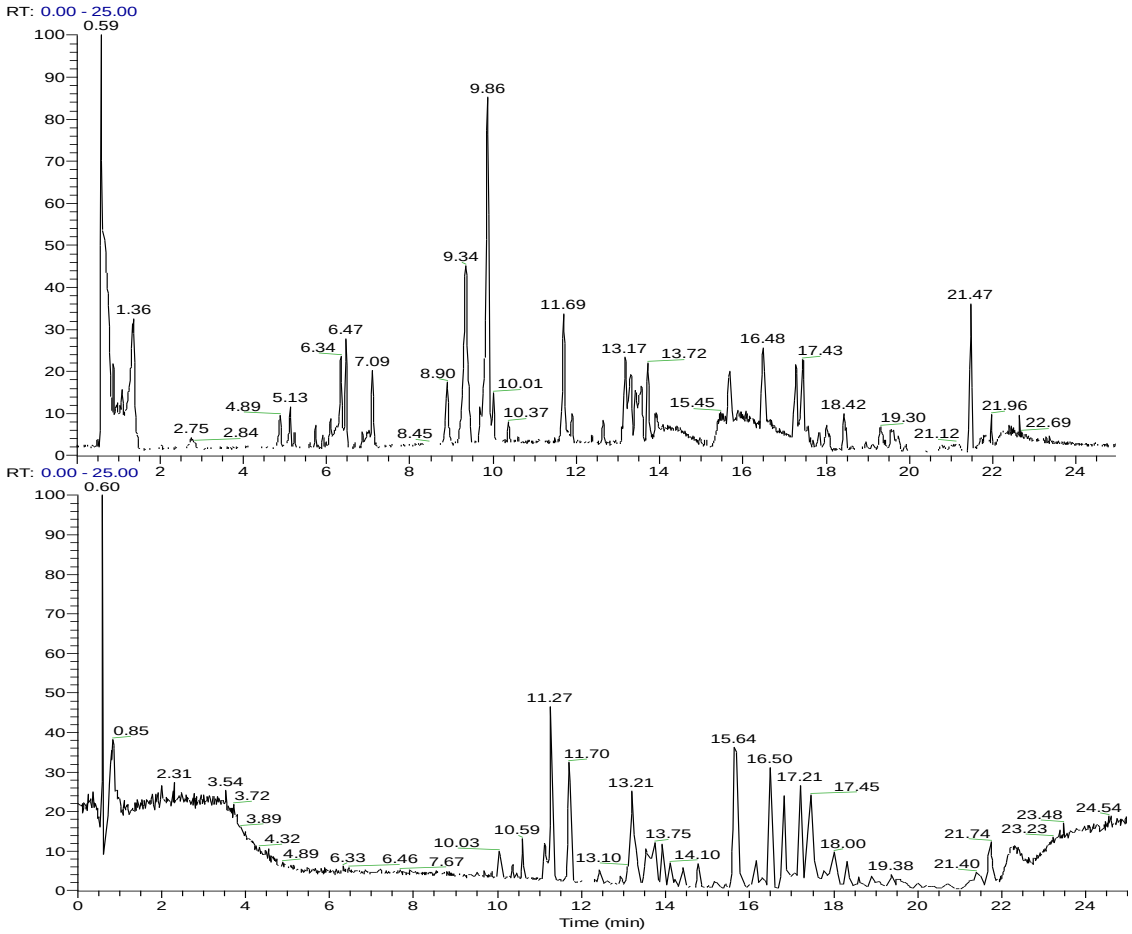


Figure 10:UPLC-HRMS chromatograms of SE1 in negative mode (up) and positive mode (down).

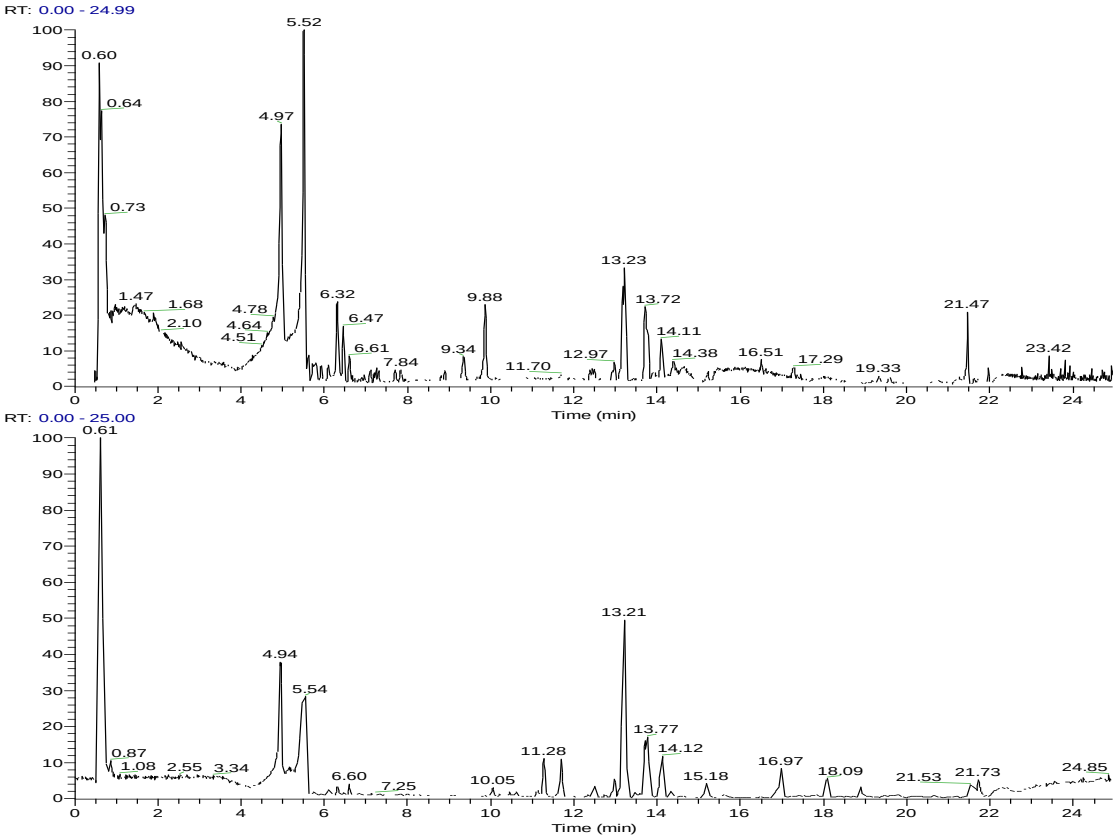


Figure 11:UPLC-HRMS chromatograms of SE2 in negative mode (up) and positive mode (down).

Chapter 3: Integration of a holistic green strategy for the isolation of significant compounds from fennel seed oil and its by-products, with pilot scale potentials.

Abstract

The current study focused on the recovery of bioactive compounds from *Foeniculum vulgare* (fennel) cold pressed seed oil (FnnSO) and its corresponding by-product seed paste (FnnSP), using green methodologies compatible with pilot scale processes. Pure FnnSO was eluted on food grade adsorption resins XAD-7, while FnnSP was treated with two different eco-friendly extraction methods; ultrasound assisted extraction (UAE) and supercritical fluid extraction (SFE). For UAE procedure, a defatting step was applied at the beginning of the process with *n*-Hexane, which was followed by the extraction of FnnSP with ethanol and afterwards with ethanol/water in ratio 1:1 v/v. SFE experiments started with the application of pure CO_2, in order to remove the fatty fraction from the FnnSP, and continued with two more extractions; one with ethanol as co-solvent in 10% and the other with 20%. All the extracts were analyzed via LC-HRMS and the most efficient extract was selected for further purification. XAD-7 extract and SFE with ethanol 10% extract were analyzed with Centrifugal Partition Chromatography (CPC). Pure anethole was isolated in considerable amounts from the chromatography of XAD-7 extract, while a fraction of CPC was rich in isomeric forms of 1-(4-methoxyphenyl) propane-1,2-diol. From the CPC experiment of SFE extract, fraction with high concentration of syringin was obtained. 1-(4-methoxyphenyl) propane-1,2-diol isomers were separated with supercritical fluid chromatography (SFC), while syringin was purified with prep-HPLC. Minor compounds were identified after prep-TLC analysis of CPC fractions.

Introduction

Foeniculum vulgare, commonly known as fennel, is an aromatic plant of Umbelliferae (Apiaceae) family, natively grown in the Mediterranean countries [1,2]. Nowadays it is worldwide cultivated due to its extensive use and its globally high economic importance [3]. Fennel seeds are widely used in food industry as ingredient of edible products, mainly to enhance their flavor [4]. In traditional medicine, fennel was used as anti-hypertension, carminative and diuretic agent as well as to increase mother breast production milk [5]. Plenty of studies provide evidence about the antioxidant, antimicrobial, antibacterial and antifungal activity of fennel extracts and compounds [6]. It should be noted that especially for fennel seeds several published researches focusing on their biological importance shown anti-oxidant, anti-microbial and acaricidal activities [1,7–9]. All the above facts underline that fennel and specially fennel seeds are a potential pool of natural products with high biological interest.

In order to be able to investigate deeper the field of natural products, a huge range of methodologies has been developed, during the years. Moreover, the current demand of industry to adopt green strategies and eco-friendly solutions in the production processes [10,11], has directed the design of different techniques and methodologies for the treatment of natural products. Characteristic example is the food grade resin XAD-7 that is commonly used in industry due to its low cost, simplicity of the extraction procedure, high efficiency and low pollution fingerprint [12]. In parallel, other advanced extraction procedures have been industrialized based on supercritical fluids technology [13]. Characteristic example is Supercritical Fluid Extraction (SFE) which provides the ability to obtain extracts without chemical or thermal degradation [14]. On SFE several types of gas and liquid solvents can be employed, capable to cover wide range of polarities [15]. Usually, CO_2 is used as major solvent and ethanol as co-solvent [16]. The above mentioned flexibility offers the advantage of a low cost process totally non-toxic [17]. Another useful tool of green processes arsenal is Ultrasound Assisted Extraction (UAE). Ultrasounds' use has been established from laboratory to industrial scale applications, due to their extensive extraction capabilities and their combination with green solvents [18].

It is really important to adapt an eco-friendly approach not only on extraction step of a process but also during the isolation and purification steps. Advanced techniques like Centrifugal Partition Chromatography (CPC) can be employed characterized by minimal environmental fingerprint [19]. CPC is included at the family of liquid-liquid techniques with the pioneering advantage of

solid support free stationary phase [20]. This technique requires the development of an efficient solvent system with two or more non mixable phases, in which solutes all partitioned based according on their chemical properties [21]. Stationary phase is immobilized in the column with centrifugal forces, while mobile phase is pumped through immobilized phase [22]. Due to the liquid nature of the two phases, a theoretical total recovery of the sample can be provided, while the mild operation conditions make the technique efficient for the chemically sensitive compounds [23]. The low solvent and time consumption and the high sample injection volume demonstrate CPC suitable for a huge range of applications [24].

Concerning the purification step, preparative Reverse Phase High Performance Liquid Chromatography (prep-RP-HPLC) is regarded as the dominant technique [25]. The low cost of operation and the limited solvent consumption, in comparison to the classic isolation techniques, are the basic advantages that prep-RP-HPLC offers [26]. As it is already mentioned, supercritical fluids are applicable not only for extraction but also for purification procedures with noteworthy results [27]. Supercritical Fluid Chromatography (SFC) is a ground breaking technique, interrelated to HPLC philosophy, that levels up its advantages with the introduction of supercritical conditions [28]. This pioneering technique, mostly used for the isolation of non and/or middle polar compounds, mainly consist of the use of CO_2 in supercritical conditions as solvent, accompanied with a liquid co-solvent [29]. The advantages of SFC are several. Most important are the minor environmental fingerprint, the low viscosity and the high density of supercritical solvents, factors that minimize the analysis time and make this technique attractive to the industry [30,31]. It is also considered as a exploitable tool for the efficient separation of polymers and isomeric structures [32,33].

Herein, a holistic green strategy was designed, from the extraction step to final compounds purification, using techniques totally suited to the industrial sector [34–37]. The treatment began with cold pressing method of fennel seeds, in order to produce fennel seed oil (FnnSO) and a solid paste (FnnSP) as by-product. FnnSO was extracted using adsorption resin XAD-7. FnnSO was loaded in a column embedded with resin XAD-7, in order to obtain the extract of interest. Moreover, UAE and SFE were used for the extraction of FnnSP. Specifically, on UAE ethanol and ethanol/water in ratio 1:1 v/v were used as solvents. Concerning SFE, pure CO_2 and two different concentrations of ethanol as co-solvent (10%, 20%) were employed on laboratory apparatus, while

pure CO_2 and ethanol 10% as co-solvent were scaled up on pilot instrumentation to obtain extracts from seed oil by-products (FnnSP). All the extracts were analyzed with LC-HRMS.

The next step was the isolation of pure solutes from the FnnSO and FnnSP extracts. In both extracts, the primary fractionation/isolation were accomplished with CPC. Systems of *n*-Hex/EtOAc/EtOH/H_2O in reverse phase and different ratios were employed on both CPC analyses. Ultimately, a profound natural product of fennel, anethole was isolated in pure form, while other compounds were also identified in CPC fractions such as syringin and 1-(4-methoxy-phenyl)-propane-1,2-diol isomers mixture that were isolated for the first time, from *Foeniculum vulgare.* Further purification was performed with prep-RP-HPLC and SFC experiments, ended up to the isolation of pure syringin and the separation of an isomer from the above-mentioned mixture, respectively. Additionally, minor compounds of CPC fractions were identified after prep-TLC analysis. For the identification of all the compounds NMR spectroscopy was used.

Methods and Materials

Reagents

The used reagents and adsorption resin XAD-7 Amberlite were purchased from Sigma-Aldrich (Missouri, USA). Analytical grade solvents were supplied from Fisher Scientific (Pennsylvania, USA). Food grade CO_2 was purchased from Revival (Athens, Greece), TLC plates of Silica gel 60 F_{254} 20cm x 20cm were purchased from Merck Millipore (Massachusetts, USA).

Instrumentation

UAE experiments were employed on an Ultrasonic P300H bath of Elma Schmidbauer (Singen, Germany). Concerning SFE, the lab scale extraction of FnnSP was performed on a Lab SFE 100 mL Apparatus of Separex (Champigneulles, France). Concerning CO_2 and co-solvent, a high-pressure CO_2 Separex LGP 50 pump (Champigneulles, France) and a SSI Series III of Scientific Systems Inc. (Woburn, United States) were used. The extraction vessel had a total volume of 100 mL and it was housed in a thermostated oven. Pilot scale SFE of FnnSP was conducted in a SFE 1-2 No. 4218 of Separex (Champigneulles, France). The apparatus was connected to a liquid preparative CO_2 pump of Separex (Champigneulles, France), with maximum capability of 10 kg/h, 2 extraction vessels of 1 L and 2 L, 3 separators of 200 mL, a co-solvent pump of Scientific Systems Inc. (Woburn, United States), with flow rate up to 40 mL/min. During the whole procedure, a continuous recycling of CO_2 was followed.

CPC instrumentation was performed for the chromatography of FnnSO and FnnSP. The instrumentation was consisting of an A-CPC apparatus of Kromaton (Anonay, France), a preparative column of 1000 mL capacity volume (FCPC1000®), a preparative Ecom ECP2000 pump (Prague, Czech Republic) and a C6-60 Buchi collector (Flawil, Switzerland).

For the SFC analysis, instrumentation of Waters (Massachusetts, USA) was selected. For the delivery of CO_2 and co-solvent, a Thar SFC pump Fluid Delivery Module was used, connected to Waters Sample Manager 2767 and Waters Column Oven. Two Waters' pumps 515 HPLC were used for the delivery of the mobile phase to detector PDA Waters 2998 and to mass SQ Detector 2 (Z-spray) Waters. A Thar SFC Heat Exchanger and a Waters Back Pressure regulator were used. Chiller Accel 500LC was equipped from Thermo Scientific. As software MassLynx v.4.1 and Aquity were used.

Prep-HPLC system was controlled with software of DataApex, ClarityTM (Prague, Czech Republic). HPLC purification was performed with two Lab Alliance preparative 36 Pumps (Dual Piston) (State College, USA), supported by UV Flash 06S DAD 800 Detector of ECOM (Prague, Czech Republic). For the purification, a preparative HPLC column Supelco supelcosil TM LC-18, 25 cm x 21.2 mm, 5μm (Pennsylvania, USA) was used. LC-HRMS experiments were conducted on an H class Acquity UPLC system (Waters, USA) coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, USA). Nuclear magnetic resonance spectra were carried out on 600MHz of Bruker AvanceAVIII-600 spectrometer (Karlsruhe, Germany) supported by TopSpin software (Bruker).

Samples production

For the production of our starting materials, fennel seeds were cold pressed. Two different materials were generated; fennel seed oil (FnnSO) and fennel seed paste (FnnSP). FnnSP is regarded as a by-product of the corresponding oil production and appears as dry solid pellets.

Solid-liquid extraction of fennel seed oil

For the extraction of FnnSO, a column with adsorption resin XAD-7 was used. Column dimensions were 65 cm x 1.8 cm. The activation of resin was employed with three bed volumes of EtOH. After the elution of the resin with distilled H_2O, 100 mL of FnnSO was passed through the column, with flow rate 1 drop/sec. Afterwards, column was rinsed with 200 mL of *n*-Hex, in order to remove the fatty components. Then, 150 mL of EtOH were used for the recovery of the extract. Elute was evaporated until dryness in RotaVap at 40^{o}C.

SFE extraction of FnnSP

50 g of grounded FnnSP were loaded at the lab scale extraction basket of analytical SFE apparatus. The oven was set at 40^{o}C. At the beginning, CO_2 was only used as extraction solvent, at the flow of 15 g/min. With this step, the starting material was defatted. Afterwards, 10% of EtOH was added as co-solvent and at the point that extract recovery was decreased, the collection erlenmeyer was changed and the percentage of co-solvent was increased at 20%. Finally, three different fractions were collected, the defatting fraction of CO_2, the CO_2 - 10% EtOH and CO_2 - 20% EtOH. For the Pilot scale extraction process, the 1L and the 2L baskets were filled with 263g and 468 g of grounded FnnSP, respectively. Flow rate of CO_2 was set at 4 kg/h. Pressure and temperature in the separators was kept constantly at 5 MPa and 35^{o}C, respectively. The extraction temperature was 40 ^{o}C, while pressure was set at 300 bar. After the defatting process with pure CO_2, co-solvent (EtOH) reached at 10%.

Centrifugal Partition Chromatography of FnnSO and FnnSP

Bioactive natural products are usually founded in small quantities on starting materials, fact that demand the treatment of high mass samples in order to isolate solutes. The gram-scale injection capability of CPC in combination with the lack of solid stationary phase make efficient the recovery of minor compounds and avoid their irreversible absorption on solid phases [38]. XAD-7 extract of FnnSO and SFE with co- solvent 10% EtOH of FnnSP were chromatographed with CPC. Reverse mode was selected for the treatment of extracts. Four different step gradient systems were used in each case, based on *n*-Hex/EtOAc/EtOH/H_2O. The injections were 3 g and 1.2 g for FnnSO and FnnSP, respectively. For FnnSO, solvent systems of *n*-Hex/EtOAc/EtOH/H_2O in ratio 8:2:4:6, 8:2:5:5, 8:2:6:4 and 8:2:7:3 v/v/v/v were used, while for FnnSP systems ratios 7:3:5:5, 7:3:6:4, 7:3:7:3 and 7:3:8:2 v/v/v/v were chosen. For both extracts, the organic mixture of *n*-Hex/EtOAc was used as stationary phase. Rotation speed was set at 650 rpm, while mobile phase run through the column with 15 mL/min. One column volume for each mobile system was passed with descending polarity. Fractions of 20 mL were collected. All the fractions were grouped at 25 final fractions for CPC of FnnSO and 30 for CPC of FnnSP.

SFC analysis

CPC fraction II of FnnSO was treated with SFC technique. For the analysis a Viridis (Waters, Massachusetts, USA) semi-prep column with silica 2-ethylpyridine 5 μm and dimensions 10 x 150 mm, was used. During the analysis, the pressure was isocratic at 150 bar. As co-solvent MeOH

was used, starting with 5% and reaching 10% after 3 min. Then percentage stayed stable for 3 more min and increased at 50% for 3 min. Finally, system returned to initial conditions for 2 min. The injection volume was 1 mL and the sample concentration reached 50 mg/mL.

Prep-HPLC and prep-TLC analysis

CPC fraction I of extract FnnSP (SFE 10% EtOH) was treated with prep-HPLC, for further purification of certain compounds. Specifically, a Supelcosil (Sigma Aldrich, Missouri, USA) column LC-18 of 25 cm x 21.2 mm and particles of 5 μm, was used. As elution solvent system a gradient mixture of H_2O (A) and AcN (B) were used. Analysis started with 2% of B and reached 40% after 30 min. After 1 min the percentage of solvents returned to initial conditions for the next 5 min. Flow rate was set at 15mL/min and PDA detector recorded at 254, 280 and 360 nm.

Selected fractions of CPC experiments were treated with prep-TLC analysis. Development solvent system was composed of DCM/MeOH in ratio 98:2 v/v for FnnSO CPC fractions. All the plates were developed twice in the same solvent system.

LC-HRMS analysis

For the LC-HRMS experiments, 200 μg/mL of each extract were prepared in MeOH/H_2O 1:1 v/v. All the samples were analysed with a LC gradient consisted of H_2O with 0.1% formic acid (FA) (solvent A) and ACN (solvent B). The elution method started with 2% of B which stayed for 2 min. In the next 16 min B reached 100% and stayed for 2 min. Finally, at 21st min, system returned to the initial conditions and stayed for 4 min for system equilibration. An Ascentis Express C18 (150 mm x 2.1 mm, 2.7 μm) column was used for the separation, with stable temperature of 40°C. Measurements were performed with a total acquisition time of 25 min and a flow rate of 400 μL/min. The injection volume was 10 μL and the autosampler temperature was at 7°C.

Mass spectra were obtained in negative and positive ion mode using an electrospray ionization source (ESI). For the negative ionization, capillary temperature was set at 350°C, capillary voltage at -30 V and tube lens at -100 V. Sheath and auxiliary gas were adjusted at 40 and 10 arb, respectively. For the positive ionization, capillary temperature was set at 350°C, capillary voltage at +40 V, tube lens at +120 V, sheath gas 40 arb and auxiliary gas 10 arb. All mass spectra were recorded in full scan mode in the range of 115-1000 *m/z*, with resolving power 30,000 at 500 *m/z* and scan rate 1 microscan per second. HRMS/MS experiments were obtained in data-depending method with collision energy 35.0% (q = 0.25).

Results and Discussion

Extraction of FnnSO with adsorption resin XAD-7

For the extraction of FnnSO, the treatment with resin XAD-7 was selected as the most appropriate methodology. Resins were commonly used for the extraction of materials with liquid nature, due to their easy applicability, the employment of simple extraction protocols and the rapidity of process. In our case, the extraction of 100 mL FnnSO, provided a yield of 2.00 g dry extract. It has to be highlighted that the intense flavour of FnnSO extract was close to the characteristic flavor of fennel seeds. This fact with combination of the totally green extraction methodology, seems to make the starting material feasible for use as ingredient in food and cosmetic products.

Extraction of FnnSP with UAE and SFE

FnnSP was extracted with two different eco-friendly methodologies, SFE and UAE. SFE is widely used on edible raw materials due to the non-toxic nature of the process.

Firstly, basket was filled with 50 g of FnnSP and pure CO_2 was applied in flow of 15 g/min for almost 5 hours, in order to remove the fatty fraction from the starting material, yielding 6.19 g. Afterwards, EtOH was added to a content of 10% and the extraction run for 4 hours at these conditions, providing 1.56 g of extract. In order to obtain the highest amount of components from the raw material, the co-solvent was increased at 20% and extraction was continued for 3 more hours. The last extract had a weight of 2.46 g. On pilot scale SFE, removal of fatty fraction lasted 3.5 h providing a yield of 87.8 g. Flow of CO_2 was set at 4 kg/h. Afterwards, 10% of EtOH was added as co-solvent for 2 h, leading at the obtainment of 34,7 g of extract (Table 1).

Untreated sample of FnnSP was also extracted with UAE following the same methodology. At the beginning, a defatting step took place in order to remove the majority of fatty fraction, yielding 1.126 g. Afterwards, FnnSP was extracted with EtOH providing 347.0 mg of extract. For the last extraction EtOH/H_2O in ratio 1:1 v/v was used yielding 760.3 mg. All the extract yields are summarized in detail on table 1.

Table 2: Yields of FnnSO and FnnSP extracts from UAE, SFE and XAD-7. Results for UAE and SFE are presented as mg/g of raw material and for XAD-7 as mg/100 mL of oil.

Sample type	Extraction method	Extraction solvent	*Yield*	*Coding*
FnnSO	**XAD-7**	EtOH	2.0 g/100 mL	E1
FnnSP	**UAE**	*n*-Hex defatting	112.6 mg/g	-
		EtOH	34.7 mg/g	E2

		EtOH/H_2O 1:1 v/v)	76.3 mg/g	E3
	SFE	CO_2 defatting	123.8 mg/g *120.1 mg/g**	-
		CO_2 + 10%EtOH w/w	31.1 mg/g *47.5 mg/g**	E4
		CO_2 + 20%EtOH w/w	49.1 mg/g	E5

**yields of Pilot scale process.*

All the extracts were compared based on their LC-HRMS profile, as it will be discussed in the next section. The intention was to scale up the most productive methodology on pilot level.

Selection of extracts for isolation process

In total, 5 different extracts were generated from fennel seeds. One from the cold pressed FnnSO and four from FnnSP. More specifically, XAD-7 (E1) from the FnnSO and UAE 100% EtOH (E2), UAE EtOH/H_2O 1:1 v/v (E3), SFE 10% EtOH (E4), SFE 20% EtOH (E5) from the FnnSP were produced.

Extract of FnnSO was the first one selected for analysis and further fractionation, in order to study the non-polar part of fennel seeds. The selection of the by-product extract forwarded for fractionation was based on their LC-HRMS profile chromatograms. E3 was the first one rejected due to its poor spectrum characterized mainly by peaks attributed to simple sugars. Contrary, extracts E2, E4 and E5 revealed richer profiles and had high chemical similarities. In order to exclude extracts from further analysis we based on the characteristics of the employed extraction techniques. Extract E2 was obtained using UAE methodology, a totally green technique, but with higher energy needs and generally UAE is considered as a rougher procedure than SFE. This fact made prominent the two SFE extracts (E4 and E5). Given the fact that both extracts had very close LC-HRMS profile and that E4 needed the half amount of EtOH for its production, E4 was finally selected to be prepared on pilot scale, for further fractionation and purification (Figure 1).

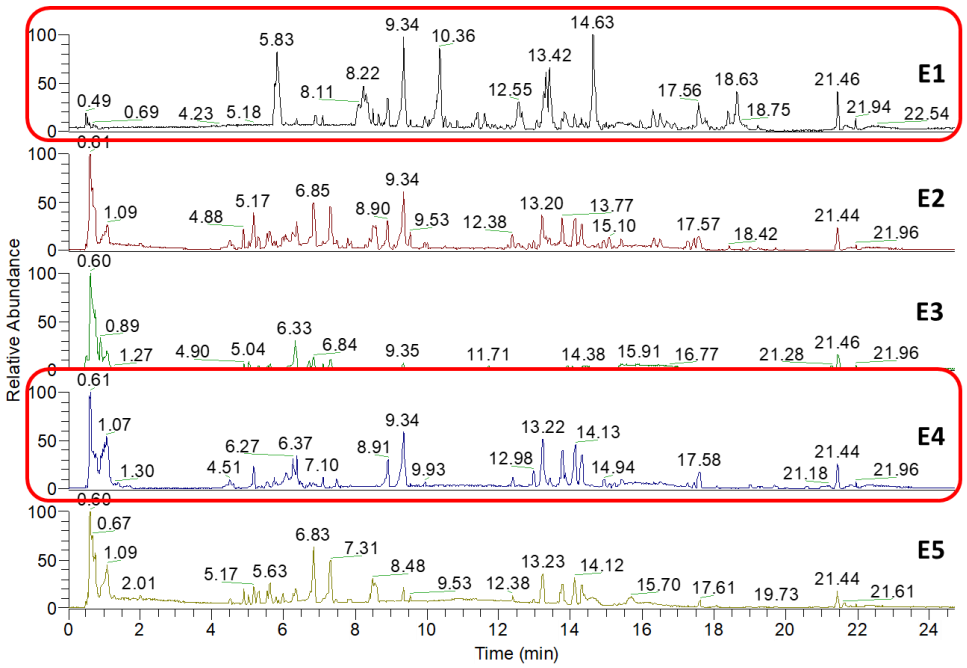


Figure 12: LC-HRMS Spectra of E1, E2, E3, E4 and E5. With red circle are marked the selected for further analysis.

CPC based fractionation and isolation of pure compounds

E1 was treated with four step gradient solvent systems in prep-CPC providing significant results. Descending mode was chosen due to the non-polar nature of the extract and experimental Sf (stationary phase retention percentage) was calculated at 71%. Following descending mode, the most hydrophobic compounds were partitioned in the stationary phase avoiding the tailing over the other solutes of the extract. During the extrusion step, non-polar compounds were eluted in pure form. Based on NMR spectral data (discussed below), fraction 21 contained high amount of pure anethole (Figure 2). Specifically, 866.1 mg of anethole were isolated. In parallel, fraction 2 weighted 154.6 mg and was enriched on isomers of 1-(4-methoxyphenyl) propane-1,2-diol, a solute that is isolated for the first time from *Foeniculum vulgare* (Figure 2).

Fractionation of E4, was also conducted with step gradient prep-CPC in descending mode, providing different fractions among which fraction 1 (150.6 mg) being noted as the most interesting due to its high concentration in syringin. Sf of this experiment after measurements, was calculated at 75%. The three above mentioned fractions, fractions 2 and 21 from CPC analysis of E1 and fraction 1 from CPC analysis of E4, were chosen for further investigation.

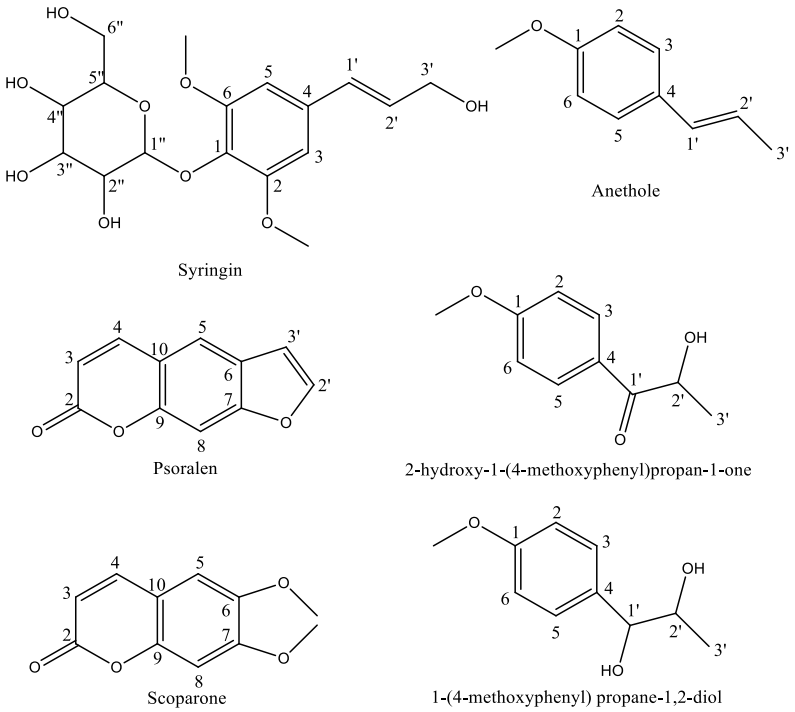


Figure 2: Chemical type of syringin, anethole, psoralen, 2-hydroxy-1-(4-methoxyphenyl)-propan-1-one, scoparone and 1-(4-methoxyphenyl) propane-1,2-diol.

SFC and prep-HPLC isolation

After the CPC analysis of E1, fraction 2 was obtained which was rich in 1-(4-methoxyphenyl) propane-1,2-diol isomers. SFC technique was decided to be performed due to its green nature, the sort analysis time and the ability to separate isomers. During the isolation process and after three injections of 50 mg, 83.4 mg of 1-(4-methoxyphenyl) propane-1,2-diol isomers and 22.9 mg of a pure isomeric form were achieved to be separated (Figure 2). For the identification process NMR spectroscopy was used. To the best of our knowledge this is the first time that this molecule was isolated from *Foeniculum vulgare,* though it has been previously chemically synthesized [40]. Moreover, it should be noticed that in previous studies, 1-(4-methoxyphenyl) propane-1,2-diol glucopyranoside was isolated from a methanolic extract of fennel fruits [40].

The non-polar compounds of fennel seed were partitioned in FnnSO, while the more polar compounds remain at the FnnSP. As a result, CPC fractions of FnnSP had higher polarity that CPC fractions of FnnSO. For this reason, HPLC was considered more suitable for compounds separation and purification in comparison to SFC, concerning CPC fractions of FnnSP. A total of three injections were run, with 50 mg/mL each one. Finally, 49.2 mg of syringin were isolated in high purity (Figure 2).

Prep-TLC analysis

In order to isolate minor compounds from specific CPC fractions of FnnSO, prep-TLC was performed. Specifically, CPC fractions 3, 4 and 5 of FnnSO were submitted to prep-TLC. After isolation and NMR experiments, the solutes scoparone, psoralen and 2-hydroxy-1-(4-methoxyphenyl)-propan-1-one were identified on fraction 3, 4 and 5, respectively (Figure 2).

Structure elucidation via 600 MHz NMR

CPC fractionation of FnnSO extract led to the isolation of pure anethole and a fraction of 1-(4-methoxyphenyl) propane-1,2-diol isomers. Further analysis of CPC fractions with prep-HPLC, SFC and prep-TLC assisted to the purification and identification of four more solutes. Precisely, pure syringin was isolated from FnnSP after treatment with prep-HPLC, while scoparone, psoralen and 2-hydroxy-1-(4-methoxyphenyl)-propan-1-one were identified from FnnSO CPC fractions, after prep-TLC analysis. All the NMR data are described in detail on table 2.

Table 3: ^{1}H NMR proton corelations of anethole, syringin, psoralen, 1-(4-methoxyphenyl) propane-1,2-diol and 2-hydroxy-1-(4-methoxyphenyl)-propan-1-one.
All the molecules were diluted in CDCl3, except of syringin which was diluted in MeOD

# of ^{1}H	Anethole	Syringin	Psoralen	Scoparone	1-(4-methoxyphenyl) propane-1,2-diol	2-hydroxy-1-(4-methoxyphenyl)-propan-1-one
2	**7.26**, d J=8.59 Hz	-	-	-	**7.26,** d J=8.57 Hz	**7.92,** d J=8.96 Hz
3	**6.84**, d J=8.81 Hz	**6.76,** s	**6.38**, d J=9.7 Hz	**6.29,** d J=9.38 Hz	**6.89,** d J=8.57 Hz	**6.98,** d J=8.96 Hz
4	-	-	**7.8**, d J=9.7 Hz	**7.61,** d J=9.38 Hz	-	-
5	**6.84**, d J=8.81 Hz	**6.76,** s	**7.68**, s	**6.95,** s	**6.89,** d J=8.57 Hz	**6.98,** d J=8.96 Hz
6	**7.26**, d J=8.59 Hz	-	-	-	**7.26,** d J=8.57 Hz	**7.92,** d J=8.96 Hz
8	-	-	**7.49**, s	**6.94,** s	-	-
1’	**6.35**, d J=15.89	**6.55**, bd J=15.84 Hz	-	-	**4.32,** d J=7.42 Hz	-
2’	**6.10**, m	**6.33**, bt J=15.84/5.69 Hz	**7.70**, d J=2.12 Hz	-	**3.84,** dq	**5.1,** m
3’	**1.86**, dd J=6.63 /1.77 Hz	**4.22**, dd J=5.69/1.61 Hz	**6.83**, dd J=2.07/0.89 Hz	-	**1.04,** d J=6.42 Hz	**1.45**, br d J=6.97 Hz
1-O-CH_3	**3.80**, s	-	-	-	-	**3.89,** s
2-O-CH_3	-	**3.86**, s	-	-	-	-
6-O-CH_3	-	**3.86**, s		**3.92**, s	-	-

7-O-CH_3	-	-		**3.95**, s	-	-
-OH	-	-	-	-	-	**3.82,** br s
1''		**4.88,** d J = 7.8 Hz	-	-	-	-
2''	-	**3.79–3.40**, m	-	-	-	-
3''	-	**3.79–3.40**, m	-	-	-	-
4''	-	**3.79–3.40**, m	-	-	-	-
5''	-	**3.79–3.40**, m	-	-	-	-
6'' a	-	**3.66**, dd J=12.10/5.32 Hz	-	-	-	-
6'' b	-	**3.78**, dd J=12.10/2.46 Hz	-	-	-	-

Conclusions

In the current study, fennel seed oil and the paste generated during the oil production were investigated for the design of a holistic green strategy starting from their extraction to the final isolation of chemical constituents. In brief, five different extracts were produced and then analyzed via LC-HRMS. Specifically, extracts were produced with XAD-7 (E1), UAE (E2, E3) and SFE (E4, E5) methodologies. Based on their LC-MS profiles, E1 and E4 were chosen for further fractionation. CPC was used to isolate the major compound of FnnSO extract, anethole in pure form, while in another fraction isomeric forms of 1-(4-methoxyphenyl) propane-1,2-diol were identified; a compound isolated for the first time from *Foeniculum vulgare*. Further purification of the isomers was completed with the support of SFC experiments. Moreover, extract E4 was chromatographed with CPC, providing fraction rich in syringin. The enriched syringin fraction was injected to prep-HPLC in order to be purified. In parallel, other minor compounds were identified from the fractions of CPC using prep-TLC and NMR. Ultimately, two different starting materials were used for the development of a holistic green strategy towards their chemical investigation. Three different extraction methodologies (XAD-7, UAE, SFE) were employed characterized by minimum ecological fingerprint and several advantages with pilot and industrial applications. Further fractionation and separation of pure compounds were conducted using state-of-the-art separation techniques i.e CPC and SFE. Moreover, the extraction methodology of the most interesting extract was scaled up to pilot extraction. The design and application of the above

research workflow characterized by green methodologies led to the final isolation of major compounds and isomeric molecules in high purity and the identification of minor solutes.

References

[1] M. Oktay, I. Gülçin, Ö.I. Küfrevioğlu, Determination of in vitro antioxidant activity of fennel (Foeniculum vulgare) seed extracts, LWT - Food Sci. Technol. 36 (2003) 263–271. https://doi.org/10.1016/S0023-6438(02)00226-8.

[2] B. Muckensturm, D. Foechterlen, J.P. Reduron, P. Danton, M. Hildenbrand, Phytochemical and chemotaxonomic studies of Foeniculum vulgare, Biochem. Syst. Ecol. 25 (1997) 353–358. https://doi.org/10.1016/S0305-1978(96)00106-8.

[3] A.E. Al-Snafi, The chemical constituents and pharmacological effects of Foeniculum vulgare- a review, Int. J. Pharmacol. Toxicol. 5 (2015) 76–81.

[4] M.C. Díaz-Maroto, M.S. Pérez-Coello, J. Esteban, J. Sanz, Comparison of the volatile composition of wild fennel samples (Foeniculum vulgare Mill.) from Central Spain, J. Agric. Food Chem. 54 (2006) 6814–6818. https://doi.org/10.1021/jf0609532.

[5] M.A. Rather, B.A. Dar, S.N. Sofi, B.A. Bhat, M.A. Qurishi, Foeniculum vulgare: A comprehensive review of its traditional use, phytochemistry, pharmacology, and safety, Arab. J. Chem. 9 (2016) S1574–S1583. https://doi.org/10.1016/j.arabjc.2012.04.011.

[6] W. He, B. Huang, A review of chemistry and bioactivities of a medicinal spice: Foeniculum vulgare, J. Med. Plants Res. 5 (2011) 3595–3600. http://www.academicjournals.org/JMPR.

[7] C.H. Lee, B.K. Sung, H.S. Lee, Acaricidal activity of fennel seed oils and their main components against Tyrophagus putrescentiae, a stored-food mite, J. Stored Prod. Res. 42 (2006) 8–14. https://doi.org/10.1016/j.jspr.2004.10.004.

[8] C. Sreemoyee, G. Nandini, B. Pradeep, Estimation of Phenolic Components and in vitro Antioxidant Activity of Fennel (Foeniculum vulgare) and Ajwain (Trachyspermum ammi) seeds, Adv. Biores. 3 (2012) 109–118.

[9] F. Anwar, M. Ali, A.I. Hussain, M. Shahid, Antioxidant and antimicrobial activities of essential oil and extracts of fennel (Foeniculum vulgare Mill.) seeds from Pakistan, Flavour Fragr. J. 24 (2009) 170–176. https://doi.org/10.1002/ffj.1929.

[10] P.J. Dunn, A.S. Wells, M.T. Williams, Asymmetric Catalysis on Industrial Scale Green Chemistry and Catalysis Handbook of Green Chemistry – Green Catalysis Microwaves in Organic Synthesis Handbook of Green Chemistry – Green Solvents Supercritical Carbon Dioxide Solvent-free Organic Synthesis, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 2010.

[11] J.H. Clark, Green chemistry: Today (and tomorrow), Green Chem. 8 (2006) 17–21. https://doi.org/10.1039/b516637n.

[12] C. Li, Y. Zheng, X. Wang, S. Feng, D. Di, Simultaneous separation and purification of flavonoids and oleuropein from Olea europaea L. (olive) leaves using macroporous resin, J. Sci. Food Agric. 91 (2011) 2826–2834. https://doi.org/10.1002/jsfa.4528.

[13] S.K. Singh, M.S. Pavan, S.P. N, R. Kant, Applications of Super Critical Fluid Extraction in Milk and Dairy Industry: A Review, J. Food Process. Technol. 9 (2018). https://doi.org/10.4172/2157-7110.1000769.

[14] 2013.Supercritical Fluid Extraction of Plant Flavors and Fragrances.pdf, (n.d.).

[15] M.D. Luque de Castro, M.M. Jiménez-Carmona, Where is supercritical fluid extraction going?, TrAC - Trends Anal. Chem. 19 (2000) 223–228. https://doi.org/10.1016/S0165-9936(99)00228-9.

[16] Y.H. Chai, S. Yusup, M.S.H. Ruslan, B.L.F. Chin, Supercritical fluid extraction and solubilization of Carica papaya linn. leaves in ternary system with CO2 + ethanol solvents, Chem. Eng. Res. Des. 156 (2020) 31–42. https://doi.org/10.1016/j.cherd.2020.01.025.

[17] N. Xynos, A. Termentzi, N. Fokialakis, L.A. Skaltsounis, N. Aligiannis, Supercritical CO2 extraction of mastic gum and chemical characterization of bioactive fractions using LC-HRMS/MS and GC–MS, J. Supercrit. Fluids. 133 (2018) 349–356. https://doi.org/10.1016/j.supflu.2017.10.011.
[18] K. Vilkhu, R. Mawson, L. Simons, D. Bates, Applications and opportunities for ultrasound assisted extraction in the food industry - A review, Innov. Food Sci. Emerg. Technol. 9 (2008) 161–169. https://doi.org/10.1016/j.ifset.2007.04.014.
[19] M. Bojczuk, D. Żyżelewicz, P. Hodurek, Centrifugal partition chromatography – A review of recentapplications and some classic references, J. Sep. Sci. 40 (2017) 1597–1609. https://doi.org/10.1002/jssc.201601221.
[20] D.I. Correa, E. Pastene-Navarrete, L. Bustamante, M. Baeza, J. Alarcón-Enos, Isolation of three lycorine type alkaloids from rhodolirium speciosum (Herb.) ravenna using ph-zone-refinement centrifugal partition chromatography and their acetylcholinesterase inhibitory activities, Metabolites. 10 (2020) 1–14. https://doi.org/10.3390/metabo10080309.
[21] G. Jerz, P. Winterhalter, The 10th international conference on countercurrent chromatography held at Technische Universität Braunschweig, Braunschweig, Germany, August 1–3, 2018, J. Chromatogr. A. 1617 (2020). https://doi.org/10.1016/j.chroma.2019.460698.
[22] N. Fumat, A. Berthod, K. Faure, Effect of operating parameters on a centrifugal partition chromatography separation, J. Chromatogr. A. 1474 (2016) 47–58. https://doi.org/10.1016/j.chroma.2016.10.014.
[23] O. Shpigun, T. Maryutina, A. Berthod, B. Spivakov, I.A. Sutherland, Countercurrent chromatography in analytical chemistry (IUPAC Technical Report), Pure Appl. Chem. 81 (2009) 355–387. https://doi.org/10.1351/pac-rep-08-06-05.
[24] D. Michailidis, A. Angelis, N. Aligiannis, S. Mitakou, L. Skaltsounis, Recovery of Sesamin, Sesamolin, and Minor Lignans From Sesame Oil Using Solid Support-Free Liquid-Liquid Extraction and Chromatography Techniques and Evaluation of Their Enzymatic Inhibition Properties, 10 (2019). https://doi.org/10.3389/fphar.2019.00723.
[25] C.F. Poole, 1.2 Family tree of chromatographic methods, in: Essence Chromatogr., Elsevier B.V., 2003: pp. 2–6.
[26] S.M. Dahimiwal, D.B. Thorat, N.P. Jain, V.B. Jadhav, P.B. Patil, A review on high performance liquid chromatography, Int. J. Pharm. Res. 5 (2013) 1–6.
[27] J.R. Williams, A.A. Clifford, A.A. Clifford, J.R. Williams, Introduction to Supercritical Fluids and Their Applications, Supercrit. Fluid Methods Protoc. 13 (2003) 1–16. https://doi.org/10.1385/1-59259-030-6:1.
[28] L.T. Taylor, Supercritical fluid chromatography for the 21st century, J. Supercrit. Fluids. 47 (2009) 566–573. https://doi.org/10.1016/j.supflu.2008.09.012.
[29] L. Liu, Y. Zhang, Y. Zhou, G. Li, G. Yang, X. Feng, The Application of Supercritical FluidChromatography in Food Quality and Food Safety: An Overview, Crit. Rev. Anal. Chem. 50 (2020) 136–160. https://doi.org/10.1080/10408347.2019.1586520.
[30] G.L. Losacco, J.L. Veuthey, D. Guillarme, Supercritical fluid chromatography – Mass spectrometry: Recent evolution and current trends, TrAC - Trends Anal. Chem. 118 (2019) 731–738. https://doi.org/10.1016/j.trac.2019.07.005.
[31] L. Khvalbota, D. Roy, M.F. Wahab, S.K. Firooz, A. Machyňáková, I. Špánik, D.W. Armstrong, Enhancing supercritical fluid chromatographic efficiency: Predicting effects of small aqueous additives, Anal. Chim. Acta. 1120 (2020) 75–84.

https://doi.org/10.1016/j.aca.2020.04.065.
[32] D.E. Knowles, L. Nixon, E.R. Campbell, D.W. Later, B.E. Richter, Industrial applications of supercritical fluid chromatography: polymer analysis, Fresenius' Zeitschrift Für Anal. Chemie. 330 (1988) 225–228. https://doi.org/10.1007/BF00515611.
[33] R.S. Hegade, F. Lynen, Chiral stationary phase optimized selectivity supercritical fluid chromatography: A strategy for the separation of mixtures of chiral isomers, J. Chromatogr. A. 1586 (2019) 116–127. https://doi.org/10.1016/j.chroma.2018.12.008.
[34] R. Margraff, O. Intes, J.H. Renault, P. Garret, Partitron 25, a multi-purpose industrial centrifugal partition chromatograph: Rotor design and preliminary results on efficiency and stationary phase retention, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 1893–1902. https://doi.org/10.1081/JLC-200063539.
[35] M. Perrut, Supercritical fluid applications: Industrial developments and economic issues, Ind. Eng. Chem. Res. 39 (2000) 4531–4535. https://doi.org/10.1021/ie000211c.
[36] D. Pradal, P. Vauchel, S. Decossin, P. Dhulster, K. Dimitrov, Integrated extraction-adsorption process for selective recovery of antioxidant phenolics from food industry by-product, Chem. Eng. Process. - Process Intensif. 127 (2018) 83–92. https://doi.org/10.1016/j.cep.2018.03.016.
[37] X. Zhang, X. Ding, J. Wang, B. Dean, Supercritical fluid chromatography-tandem mass spectrometry for high throughput bioanalysis of small molecules in drug discovery, J. Pharm. Biomed. Anal. 164 (2019) 62–69. https://doi.org/10.1016/j.jpba.2018.10.021.
[38] K.D. Yoon, Y.W. Chin, J. Kim, Centrifugal partition chromatography: Application to natural products in 1994-2009, J. Liq. Chromatogr. Relat. Technol. 33 (2010) 1208–1254. https://doi.org/10.1080/10826076.2010.484374.
[39] M.D. Esclapez, J. V. García-Pérez, A. Mulet, J.A. Cárcel, Ultrasound-Assisted Extraction of Natural Products, Food Eng. Rev. 3 (2011) 108–120. https://doi.org/10.1007/s12393-011-9036-6.
[40] T. Ishikawa, E. Fujimatu, J. Kitajima, Water-soluble constituents of anise: New glucosides of anethole glycol and its related compounds, Chem. Pharm. Bull. 50 (2002) 1460–1466. https://doi.org/10.1248/cpb.50.1460.

Thesis conclusion

VOs constitute an integral sector of human nutrition and are used from ancient time as a main source of fat as well as for food flavouring due their exceptional sensory and organoleptic properties. Additionally, their distinctive chemical characteristics have established them as agents with prominent dermo-cosmetic properties. As a result, food and pharmaceutical industry have directed their interest to the development of food and dermo-cosmetic products with VO-based composition. The employed methodology for the production of the respective products is of high significance due the current demand of the market for products coming from eco-friendly procedures with minimum environmental fingerprint.

In this study, nine different VOs were studied and investigated in terms of their chemical profile and dermo-cosmetic properties. In particular, sesame and olive oil, two worldwide commercial oils, and fennel seed, grape seed, pumpkin seed, sunflower seed, almond seed, pomegranate seed and hemp seed oils that are listed as limited production cold pressed oils, were selected. All of them were characterized by high nutritional value and dermo-cosmetic interest. Furthermore, the corresponding by-products of the limited production oils were investigated. Due to the high available portions of sesame and olive oil, both of them were treated on pilot level, while because of the low availability of the rest oils were studied on laboratory scale. The designed research pipeline for all the VO was based on green methodologies and the use of non-toxic solvents, producing extracts, fractions and pure compounds with edible properties, suitable for the food industry.

Concerning sesame oil, two different extraction methodologies were tested in laboratory scale, in combination with CPC, leading at the isolation of pure sesamin, sesamolin and other minor compounds. The isolated natural products were evaluated with enzymatic inhibition assays of collagenase, elastase, tyrosinase and hyaluronidase. Moreover, a pioneering pilot methodology for the isolation of phenolic fraction from edible oils were developed and applied for the first time. Liquid-liquid centrifugal extraction and adsorption resins were employed on sesame oil, with DMSO as extraction solvent, providing high amounts of crude extract, suitable for *in vivo* experiments.

Olive oil was also extracted in pilot scale with liquid-liquid centrifugal extraction for the first time. As a result, huge quantities of bio-phenols were obtained. After CPC and prep-HPLC analysis oleacein, oleocanthal, monoaldehydic form of oleuropein aglycone, monoaldehydic form of

ligstroside aglycone and hydroxytyrosol, compounds characterized by high pharmacological interest were isolated in gram scale. In parallel three new natural products, EDA lactone, (*1R, 8E*)-1-ethoxy-ligstroside aglycon, (*1S, 8E*)-1-ethoxy-ligstroside aglycone, were isolated for the first time, while (9*E*, 11*E*)-13-oxotrideca-9,11-diencoic acid was isolated from olive oil for the first time.

For the seven limited production oil a holistic screening green experimental workflow was designed. Fatty acid profile and non-polar compounds of VSPs were investigated with GC-MS. VOs and their by-products were extracted with totally green methodologies, food grade adsorption resins and SFE-UAE, respectively. Their chemical profiles were obtained with HPLC-DAD and LC-HRMS, while enzymatic assays were applied in order to evaluate their dermo-cosmetic properties. Their major bioactivity was the anti-collagenase effects, although many extracts revealed anti-elastase and anti-tyrosinase activity. The two most interesting raw material (grape and fennel seed oils and by-products) based on enzymatic assays results and on chemical profile, grape and fennel seeds, were investigated in deep, with green methodologies like CPC and SFC, contributing at the isolation of major compounds and identification of other minor compounds.

Concluding VOs represent an important component of human diet and are considered as a valuable source of active molecules. A plethora of experimental treatments have been suggested for their exploitation for the improvement of human life quality. However, more research is needed to establish methodologies for their extraction, isolation of pure compounds as well as elucidation of their chemical composition and evaluation of their biological properties in favour of human health.