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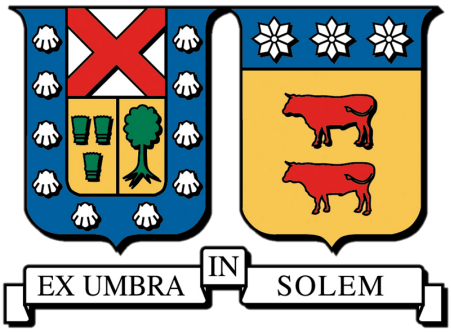

SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF RED CLOVER ISOFLAVONES USING AN AQUEOUS-ETHANOL SUSPENSION AS COSOLVENT

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THESIS FOR A MASTER'S DEGREE IN CHEMICAL ENGINEERING

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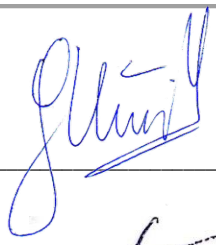

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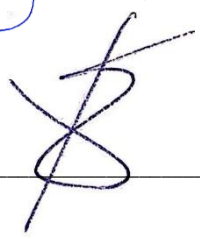

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Abstract

Increasing demand of treatments for different types of cancers and menopause symptoms in women have boosted research for natural alternatives to hormone therapy. In Chile, red clover (*Trifolium pratense* L.) is a plant known for its high content of isoflavones. These are naturally occurring nonsteroidal phenolic compounds (PCs) with high antioxidant activity (AA), which also exhibit high estrogenic or anti-estrogenic properties, due to the resemblance of its molecular structures to human steroid estrogens. Supercritical fluid extraction (SFE) with CO_2 is a non-conventional extraction method that addresses the challenges of maceration and solvent extraction for this type of compounds. Although widely applied to the extraction of phenolic compounds, such as isoflavones, the industrial-scale development of SFE is hindered by its inherently batch nature, due to the high-pressure handling of solids. This work applies a novel supercritical CO_2 extraction format that performs the extraction to finely disrupted red clover leaves suspended in a hydroethanolic mixture acting as a static cosolvent or modifier. The validated hypotheses of this work was that the overall yield and selectivity of isoflavones from red clover extracted from suspensions will exceed those of the packed-bed SFE at the same conditions. Thus, the objective of this study was to evaluate the extraction yield and selectivity of isoflavones from ground red clover leaves, using modified supercritical CO_2 under different pressure (20-35 MPa), temperature (40-50 °C) and liquid phase compositions (35-85 wt% water, CO_2-free basis), comparing packed bed and hydroethanolic suspension approaches. Total solids, phenolics, flavonoids, isoflavones and AA were quantified. Between packed-bed and suspension extraction, total phenolics peaked at 6.16 *vs.* 9.68 mg gallic acid equivalent (GAE)/g substrate, flavonoids at 7.80 *vs.* 8.15 mg quercetin equivalent (QE)/g substrate, isoflavones at 5.31 *vs.* 7.41 mg formononetin + biochanin A (F+BA)/g substrate and AA at 40.33 *vs.* 33.25 µmol Trolox equivalent (TE)/g substrate. Lastly, the extraction of suspensions was far more selective for PCs, flavonoids and isoflavones. Isoflavone selectivity for suspensions peaked at 89.76 mg (F+BA)/g dry extract, exceeding the benchmark of 40-80 mg isoflavones per capsule in commercially available red clover supplements. Results showed that the transfer mechanism of solutes was mainly dependent on the overall ethanol composition in the solvent mixture and differed fundamentally from the packed-bed extractions, as extractions at the same conditions had contrasting trends between both extraction approaches.

Acknowledgements

Funding by the Agencia Nacional de Investigación y Desarrollo (ANID) from Chile, through Fondecyt project 1221451 (JMdV) and the Tuition Scholarship for the Master of Science in Chemical Engineering Program by Universidad Técnica Federico Santa María are deeply acknowledged. Also, greatly acknowledge the Laboratorio de Extracción de Materiales Biológicos (LEMaB) for hosting and supporting the thesis work. Finally, sincere appreciation is extended to all members of the Fondecyt project 1221451, particularly to Gonzalo Núñez and José Manuel del Valle for their guidance, to Marco Mamani for conducting the spectrophotometric and chromatographic analyses and to Laura Cuellar for her valuable training on equipment operation.

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Nomenclature

Acronym	Meaning
AA	Antioxidant activity
ANID	Agencia Nacional de Investigación y Desarrollo
BA	Biochanin A
BC	Bioactive compounds
BPR	Back-Pressure Regulator
CCSFE	Countercurrent supercritical fluid extraction
CXDES	CO_2-expanded deep eutectic solvent
D	Daidzein
DES	Deep eutectic solvent
EoS	Equation of state
ESE	Ethanol-solvent extraction
F	Formononetin
Fl	Red clover flowers
G	Genistein
GAE	Gallic acid equivalent
GRAS	Generally recognised as safe
GXL	Gas-expanded liquids
HPLC	High-performance liquid chromatography
INIA	Instituto de Investigación Agropecuaria
L	Red clover leaves
LEMaB	Laboratorio de Extracción de Materiales Biológicos
PB-SFE	Packed-bed supercritical fluid extraction
PC	Phenolic compounds
PC-SAFT	Perturbed-Chain Statistical Associating Theory
PR	Peng-Robinson
PUC	Pontificia Universidad Católica de Chile

Continued on the next page

Acronym	**Meaning**
QE	Quercetin equivalent
$scCO_2$	Supercritical CO_2
SE	Solvent extraction
SFE	Supercritical fluid extraction
SL	Solid-liquid extraction
S-SFE	Supercritical fluid extraction of suspensions
St	Red clover stems
TFC	Total flavonoid content
TIC	Total isoflavone content
TPC	Total phenolic content
TSC	Total solid content
WSE	Water-solvent extraction
Variable	**Meaning, units**
K	Distribution coefficient, -
m	Mass of hydroethanolic mixture loaded in the vessel, g
m_{CO_2}	CO_2 mass recovered during depressurization, g
m^G_{EtOH}	Ethanol mass recovered in extracts, g
m^L_{EtOH}	Ethanol mass recovered from inside the vessel, g
$m^G_{H_2O}$	Water mass recovered in extracts, g
$m^L_{H_2O}$	Water mass recovered from inside the vessel, g
P	Pressure, MPa
P_c	Critical pressure, MPa
Q_{CO_2}	CO_2 flow rate, g/min
Q^S_{CS}	Coolvent flow rate, cm^3/min
T	Temperature, °C
T_c	Critical temperature, °C
t_d	Dynamic stage time, h
t_s	Static stage time, min
w'_{3G}	Water fraction in the gaseous phase on a CO_2-free basis, wt%
w'_{3L}	Water fraction in the liquid phase on a CO_2-free basis, wt%
w'_{3S}	Overall water fraction in the solvent mixture on a CO_2-free basis, wt%

Chapter 1

Introduction

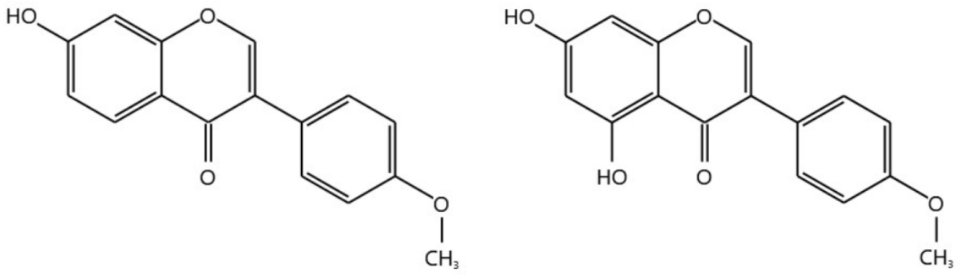
Women going through menopause experience negative symptoms associated to the low levels of estrogen and progesterone produced by their bodies [1]. The most common symptoms include hot flashes, sleep disorders, depression, headaches, sweating, low libido, osteoporosis and sexual dysfunction [2, 3]. Consequently, a variety of treatments have been developed for the management or prevention of these adverse effects, such as, isoflavone intake as an alternative to hormone therapy [2, 4].

Isoflavones are naturally occurring nonsteroidal phenolic compounds (PCs) with high *in-vitro* and *in-vivo* antioxidant capacity, which resemble vertebrate steroid estrogens, due to their molecular structure [5]. Accordingly, many studies have reported the potential health benefits of isoflavone consumption for certain types of cancers and hormonal disorders, including prostate cancer [6], breast cancer [7] and various menopause symptoms [2]. In the human diet, the main sources of isoflavones are soybean (*Glycine max*), soy-based products and legumes, which are rich in daidzein, genistein, and their glycosides [8]. Additionally, many plants, such as, red clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.), and alfalfa (*Medicago sativa*) contain formononetin, biochanin A and their glycosides [5, 9].

Red clover is a short-lived (2-3 years) perennial forage herb, native to Europe, that grows in temperate climates [10]. In Chile, the Instituto Nacional de Investigación Agropecuaria (INIA) has developed red clover strains characterized for having higher endurance and isoflavone content (Figure 1.1) that other cultivars: Quiñequeli-INIA, Redqueli-INIA and Superqueli-INIA [11].

The growing interest in red clover extract as a natural source of isoflavones for human consumption, rather than competing with the food market for soybean extract, has driven R&D efforts towards green and eco-friendly extraction techniques. Conventionally, maceration and solvent extraction (SE) with alcohols (methanol or ethanol) have been employed for the extraction of red clover antioxidants, due to their simplicity and the good solubility of isoflavones in mildly polar solvents. However, these methods present significant drawbacks, including high operating

temperatures, excessive solvent and energy consumption, generation of unwanted liquid waste and poor selectivity. Consequently, the pharmaceutical and food industries seek alternative approaches to obtain stable and pure natural products, while avoiding conventional extraction techniques.



(a) (b)

Figure 1.1: Molecular structure of formononetin (a) and biochanin A (b), the main isoflavones in red clover.

Supercritical fluid extraction (SFE) is a non-conventional extraction technique in which pressure and temperature conditions must exceed the solvent's critical point, developed to overcome the aforementioned limitations of SE and maceration. The most commonly used solvent is carbon dioxide, due to its relatively low critical temperature and pressure (T_c = 31.1 °C, P_c = 7.38 MPa), the possibility of finely tuning its physical properties with small variations in pressure and temperature, its non-toxic nature, low cost and wide availability. Moreover, at room temperature and atmospheric pressure it vaporizes, leaving a solvent-free product. Initially, CO_2-SFE was applied to processes of tea decaffeination, coffee decaffeination, lipid extraction, essential oils extraction and other light fatty acids from various seeds and spices [12], but further development shifted its focus into a wider substrate range and target extracts [13].

The SFE of PCs with pure carbon dioxide its greatly limited by the chemical nature of CO_2. Heavy and/or polar compounds have negligent solubility in supercritical CO_2 ($scCO_2$). Therefore, cosolvents or modifiers are used (mixed with the main solvent) to improve the solubility of these compounds, since, among other properties, the polarity of the medium is modified and the intermolecular interactions of the solute and the $scCO_2$ are increased [14, 15, 16]. The most studied cosolvents, for the SFE of polar bioactive compounds (BC) from plant matrices, are water and ethanol [13], due to their polar nature [16] and Generally Recognised as Safe (GRAS) classification.

SFE has been widely applied to numerous substrates and a variety of cosolvents that achieved generally positive results. However, its industrial applications remain limited, as it is an inherently batch process that requires handling solids at high pressure. Furthermore, there is scarce information on costs and on the scaling of laboratory or pilot plant equipment to industrial scale operations [17]. Transforming its batch-wise nature into a continuous process could enhance the overall economic

feasibility of SFE, fostering its establishment as a robust green industrial process for natural products.

This work focuses on the evaluation of red clover extract composition, isoflavone selectivity and antioxidant activity through a novel extraction format: $scCO_2$ extraction of solid red clover suspended in different cosolvent mixtures. If a fluid-like solid exhibits favorable extraction yields and selectivity under SFE conditions, the implementation of a countercurrent SFE (CCSFE) process for red clover antioxidants could become a promising alternative. In such a scenario, the suspended solid could be continuously pumped through a packed-bed extractor or column, enabling more sustained contact with the solvent phase and potentially enhancing extraction efficiency. Moreover, this configuration could offer economic advantages by reducing solvent consumption and operational costs.

Chapter 2

Hypothesis and objectives

The hypothesis of this work is that finely disrupting red clover and suspending it in a liquid mixture, prior to supercritical extraction, will improve both the yield and selectivity of isoflavones, compared to supercritical extraction performed in a packed bed format.

The general objective is to evaluate the extraction yield and selectivity of isoflavones from ground red clover, using modified supercritical CO_2 under different pressure, temperature and gas phase compositions and comparing packed bed and hydroethanolic suspension approaches. The specific objectives are:

1. To extract phenolic compounds, flavonoids and isoflavones from suspended red clover in a hydroethanolic mixture using modified scCO_2, comparing their yield and selectivity with packed-bed SFE of red clover reported in the literature.

2. To determine the equilibrium compositions of the liquid and vapor phases within the extraction cell at the end of the suspension-based extractions.

3. To correlate the phase-equilibrium composition with the overall composition of the extracts obtained in the extraction of suspensions, evaluating their influence on the extracts' antioxidant capacity.

Chapter 3

State of the art

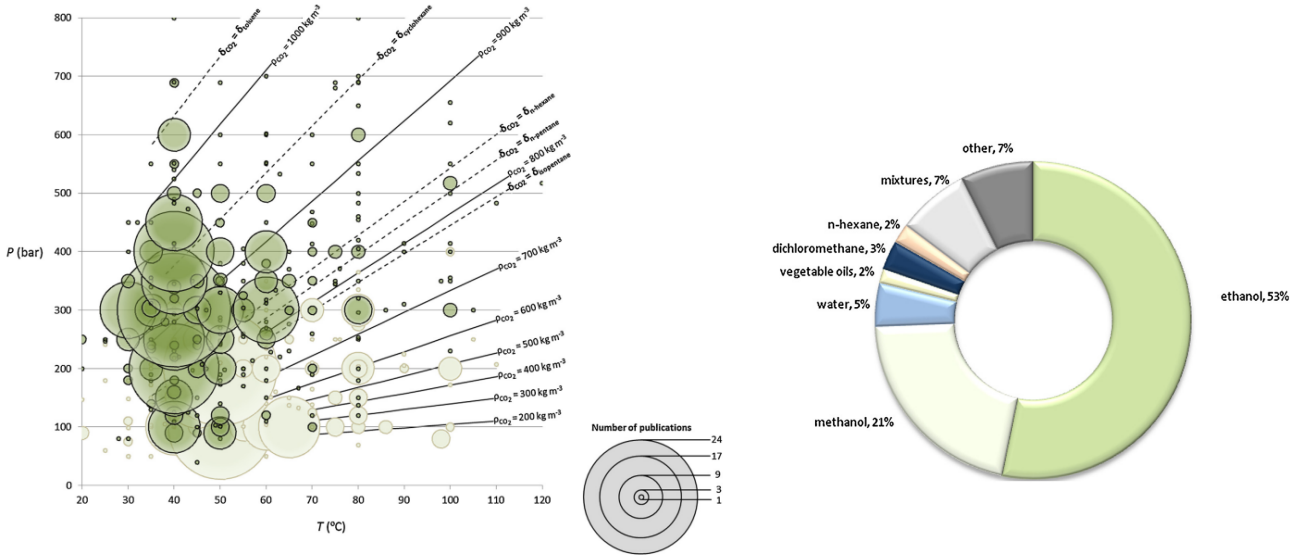
It has been reported that red clover extracts have gained popularity as alternative therapies, mainly because biochanin A and formononetin exhibit strong estrogenic activity and its standardized the extraction process. Thus, reliable compositions of the medication can be obtained [2]. Some patented processes for obtaining dry red clover extract include pressing aerial parts at 70 °C followed by two-day fermentation at 20 °C before extracting with liquid ethanol [18], solvent extraction with methanol [19] and solvent extraction with ethanol [20]. These patents also include concentration and drying steps. In the scientific literature, the extraction of red clover phenolics and isoflavones has been investigated and compared using both conventional and non-conventional techniques [9, 21, 22, 23, 24]. To date, only Mamani et al. [25] have reported SFE of red clover leaves and stems, whereas Zhou et al. [26] the SFE of its seeds. The consistency or divergence of their findings will be discussed in Section 5.2.

As it was stated before, the SFE of many substrates has been reported in literature. De Melo et al. [13] reviewed the extraction of plant BC from solid matrices between 2000-2013, where they discuss trends in common biomass used, pressure and temperature conditions for the extraction process and common cosolvents employed. Often, process conditions range from 20-40 MPa and 40-50 °C (Figure 3.1A), whereas ethanol is the most used cosolvent (Figure 3.1B), probably because it is completely miscible with CO_2 beyond 15 MPa. Moreover, SFE has been reported for extracting BC from bark, rhizomes, flowers, roots, fruits, leaves, seeds and others.

Throughout this work, "supercritical fluid extraction" will refer to extraction carried out with $scCO_2$ as the solvent, unless explicitly stated otherwise. Similarly, the terms "gaseous phase", "supercritical phase" and "vapor phase" will be regarded as synonymous for the purposes of this study.

Lycopene was extracted from tomatoes with $scCO_2$ at 45-70 °C, 33.5-45 MPa, 8-20 kg/h CO_2 and 0-10 wt% vegetable oil as cosolvent [27]. The extraction yield was greatly improved with increasing temperature, pressure, CO_2 flow rate and use of cosolvent. Maximum lycopene yield was

60%, obtained at 66 °C, 45 MPa and 20 kg/h of CO_2 in the presence of cosolvent.



(A) (B)

Figure 3.1: Typical operating conditions reported for supercritical extraction of solid plant matrices between 2000–2013. Pressure–temperature diagram (A), where green circles indicate the occurrence frequency of reported conditions. Pie chart showing the relative distribution of cosolvents employed in supercritical extraction of plant matrices (B) [13].

Fatty acids, PCs and carotenoids were extracted from *Butia capitata* fruits through a 3-step extraction with pure CO_2 (50 °C, 35 MPa), pressurized ethanol (50 °C, 35 MPa) and pressurized water (50 °C, 35 MPa). Extraction kinetics were compared with a three-step conventional extraction of Soxhlet with hexane (50 °C, 1 bar), SE with ethanol (50 °C, 1 bar) and SE with water (50 °C, 1 bar) [28]. The overall yield of the non-conventional extraction was 68.78%, 1.4 times greater than the yield of conventional methods, but extracts had lower antioxidant capacity.

Although many BCs can be extracted from fruits and vegetables, several studies indicate that agricultural waste, by-products and non-dietary plants may contain higher concentrations of PCs than their main products. Examples include apple peels, peach peels, pear peels, avocado pits, jackfruit seeds, mango pits and tamarind seeds [29, 30]. Exploring different biomass sources is worthwhile to either (1) avoid competition with the food market or (2) promote a circular economy through waste valorization.

Regarding agricultural by-products, phenolic antioxidants were extracted from grape pomace using ethanol-modified $scCO_2$ and compared with solid–liquid extraction with 96 vol% ethanol [31]. Approximately 400 ppm of PCs were obtained via SFE at 50 °C, 35 MPa and 8 mol% ethanol, nearly double the yield of solid–liquid extraction. Chromatographic analysis of both SFE and solid–liquid extracts revealed differences in the nature of the compounds obtained. SFE preferentially extracted

lighter PCs, such as catechin and epicatechin, whereas pure ethanol favored the extraction of heavier and polymerized PCs, which exhibit greater solubility in polar solvents.

Zulkafli et al. [32] investigated the effect of pressure (10–25 MPa), temperature (50–95 °C) and hydroethanolic mixtures as cosolvents (0–100 mol%) on the yield of PCs from bamboo leaves. The optimal conditions for a maximum PC yield of 7.31 mg catechin equivalents/g bamboo leaves and an antioxidant activity of 3.65 3-tert-butyl-4-hydroxyanisole equivalents/g bamboo leaves were 20 MPa, 95 °C, a 25:75 mol% ethanol:water ratio and 5 mol% cosolvent in the CO_2 stream. The results demonstrated that PC yield varied significantly, depending on the cosolvent employed, with hydroethanolic mixtures outperforming pure water and ethanol. Therefore, cosolvent composition is a key variable for tuning the extraction yield

Industrial SFE plants employ two or more extraction vessels to minimize dead time. While fresh substrate in one extractor is under operation, the vessel containing exhausted material is being reconditioned. In three-vessel extraction plants, the solvent simulates a pseudo-countercurrent contact with the substrate [33]. This configuration has been modeled to evaluate the reduction of overall operational costs in oilseed extraction as a function of particle size, solvent's superficial velocity, and the number of extractors [34]. The minimum operational cost was estimated at 4.08 USD/kg oil using 2 mm particles, CO_2 superficial velocity of 2.76 mm/s and four extraction vessels. Authors continued their work optimizing production costs as a function of number of extractors and the extractor inner diameter [35]. Production costs totaled at 7.8 USD/kg oil, but claimed great uncertainty in their estimated CAPEX (±50%). The same principle was later applied to *Cannabis sativa* L. seed oil, where plant productivity was optimized with three extractors [36].

All of the aforementioned studies have focused on the extraction of solid substrates, employed as the packed bed of the extraction device. CCSFE has only been applied to liquids, for example: in the fractionation and economic evaluation of milk fat [37], extraction of antioxidants from orange juice [38], removal of phenolic pollutants from aqueous matrices [39], ethanol purification [40] and wine aroma extraction [41, 42]. Fundamental concepts related to CCSFE, such as: graphical methods for estimating the number of theoretical stages, mass transfer correlations, flooding and pressure drop diagrams are discussed elsewhere [43, 44, 45]. Nevertheless, information regarding CCSFE, applied to the extraction of bioactive phenolics from plant matrices, remains scarce.

In the last decade, authors have contributed to the development of a CCSFE process for solid substrates by grinding and suspending the plant matrix in a mixture of the extraction cosolvent. This approach has been termed "enhanced solvent extraction", meaning, liquid solvent saturated with CO_2 at high pressure. The extraction yield of anthocyanins and total extraction yield were studied for elderberry pulp at 40 °C and 20.9 MPa, by modifying the composition of the hydroethanolic suspension liquid [46]. Under certain conditions, a liquid and gas phase split and the recovery of anthocyanins was enhanced. In these cases, the anthocyanin yield was higher

than under monophasic conditions (liquid only or vapor only) and was directly proportional to the overall water and ethanol content in the extractor.

Tong et al. [47] performed extraction of chlorophyll a from suspended *Spirulina platensis*, optimizing cosolvent composition, cosolvent volume, static extraction time, dynamic extraction time, pressure, temperature and CO_2 flow rate. Maximum yield was 6.84 mg/g at 53 °C, 48.7 MPa, 10 g/min CO_2 and 21.2 mL of 40 vol% ethanol as a static cosolvent. A similar approach was taken in SFE of astaxanthin with CO_2 from *Haematococcus pluvialis*, where SFE yield was evaluated at different solvent-to-cosolvent ratios with ethanol. As the cosolvent enhanced the overall yield, their focus shifted to evaluate extraction yield when, instead of a vapor phase, CO_2-expanded ethanol extracted astaxanthin [48]. Overall, suspended algae in expanded ethanol yielded more astaxanthin than SFE extraction, maximum extraction yield was 1.24-fold the control acetone extraction yield.

del Valle et al. [49] discussed how the vapor–liquid equilibrium of CO_2, ethanol and water takes advantage of the partial miscibility of water with the gas, allowing phase separation, while ethanol dissolves large amounts of CO_2, significantly modifying the diffusivity, surface tension, pH, and viscosity of the liquid [16]. In summary, these changes enhance transport properties. Wetting the plant matrix and allowing diffusion into the liquid alters the mass transfer mechanism. Therefore, SFE of plant suspensions becomes a two-step mass transfer process (Figure 3.2): slow diffusion of the compound from the solid matrix into bulk liquid, followed by rapid solubility-driven transport from the liquid to the gas. In their work, del Valle et al. [49] simulated *S. platensis* data [47] to validate this hypothesis. When Aravena et al. [50] extracted astaxanthin from *H. pluvialis* suspended in water, the results showed a different mechanism compared to the dry substrate. An S-shaped (lagged) extraction curve was obtained, likely due to the diffusion of astaxanthin was limited by the negligible water solubility of lipids, which acted as a physical barrier.

Kühn & Temelli [51] carried out extraction of blueberry pomace suspended in ethanol and water at 50 °C and 40 MPa, to compare the extraction yield of PCs as a function of cosolvent composition. The total phenolic content (TPC) was always higher when liquid–vapor equilibrium was present in the cell, except for one condition. The highest yields of TPC and total anthocyanins were 100.0 mg GAE/100 g dry mass and 84.6 mg cyanidin-3-galactoside equivalent/100 g dry mass, respectively, with an overall solvent composition of 31.2:4.8:64. Compositions of CO_2, ethanol and water in the liquid and supercritical phase were not measured.

Overall, integrating gas-expanded liquid (GXL) extraction with SFE in a single process has shown promising outcomes in terms of yield and selectivity of antioxidant and algae extracts. Furthermore, the fluidized suspended solids could, in principle, be pumped into a CCSFE process. However, this remains to be demonstrated.

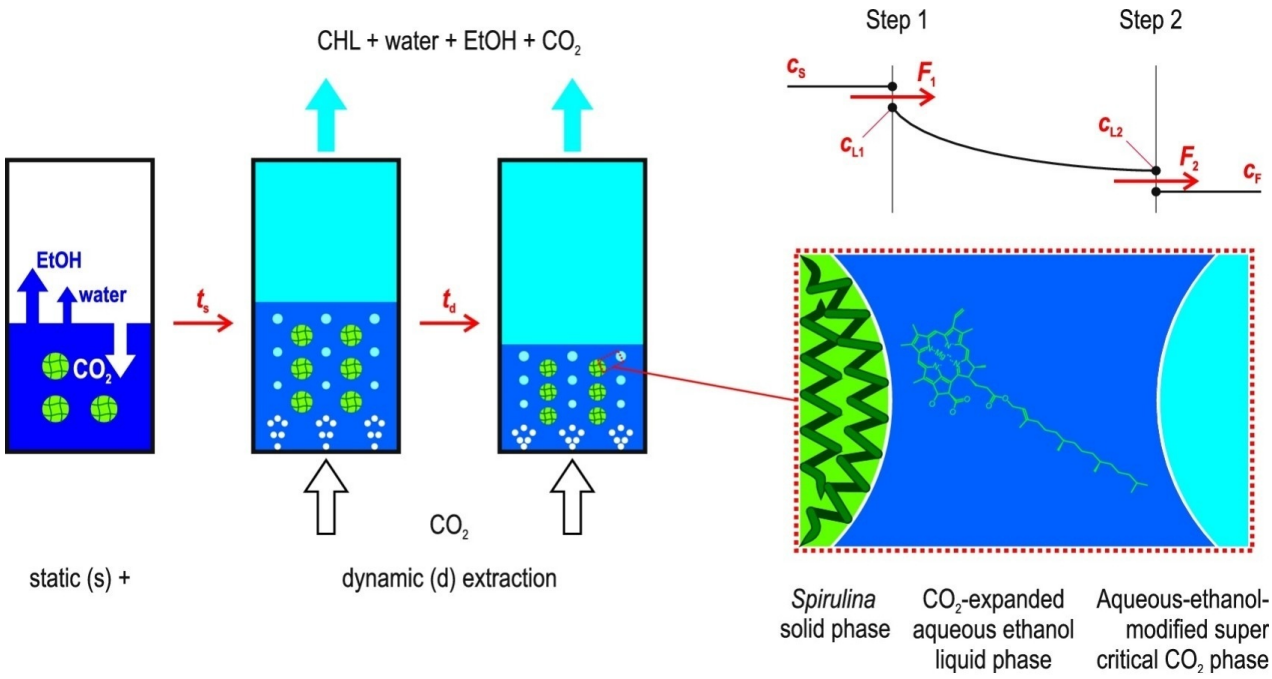


Figure 3.2: Schematic representation of supercritical CO_2 extraction of chlorophyll A from *S. platensis* suspended in a hydroetyhanolic suspension. The vessel is loaded with liquid and $scCO_2$ and left to equilibrate (t_s); The $scCO_2$ stream extracts chlorophyll A; The liquid phase decreases its volume after the gas drags cosolvent (ethanol + water) (t_d); Zoomed-in two-step mass transfer and chlorophyll A concentration profile. Image from del Valle et al. [49].

To perform the extraction of suspensions, the selected suspension liquid must: be partially miscible with $scCO_2$ at the extraction conditions and be a useful modifier to transport the targeted compounds from the solid to the $scCO_2$ stream [52]. In summary, the two aforementioned requirements ensure that a liquid phase is always in contact with the solid particles and that the target solute is effectively transferred from the substrate to the $scCO_2$ stream via a liquid suspension medium.

These requirements were implicitly adopted in the work of Dou et al. [53], who compared the extraction yield of crocin I, using deep eutectic solvents (DES) and CO_2-expanded DES (CXDES) under different operating conditions: pressure (4–12 MPa), temperature (30–50 °C), DES volume (10–40 mL) and extraction time (0–120 min). Similar to extraction of suspensions, gardenia fruit was ground to 1 mm particles and processed in a biphasic system consisting of a CXDES-rich liquid phase, mixture of choline chloride and propylene glycol (1:3 molar ratio), and a CO_2-rich supercritical phase. The presence of pressurized CO_2 dissolved in the liquid phase enhanced mass transfer and significantly improved the extraction yield of crocin I, mitigating the limitations imposed by the high viscosity of DESs. Although the CXDES extraction methodology met the restrictions mentioned before, the authors noted that the supercritical phase did not contain dissolved crocin I, due to the negligible solubility of the choline chloride and propylene

glycol mixture in $scCO_2$. To complement the miscibility requirement, it is proposed that both the suspending liquid and the supercritical solvent be partially miscible with each other.

Phase equilibrium knowledge is essential to select experimental conditions that fulfill these requirements. Apart from the study of Dou et al. [53], only water and ethanol have been employed as suspension media. However, ternary equilibrium data for $scCO_2$:ethanol:water systems under typical conditions for PCs extraction remain scarce. Durling et al. [54] reported such data at 40 °C and pressures ranging from 10-30 MPa. Sato et al. [55] provided ternary and quaternary equilibrium data for $scCO_2$:ethanol:water, $scCO_2$:ethanol:flavonoid and $scCO_2$:ethanol:water:flavonoid systems at 40–80 °C and 10–20 MPa. Distribution coefficients (K) for flavone, 6-methoxyflavone, and 7-hydroxyflavone were accurately correlated using the Peng–Robinson (PR) equation of state (EoS) with Wong-Sandler mixing rules. These coefficients increased with higher pressures or higher overall water content, ranging from 10^{-2} to 10^{-1} in magnitude.

In literature, the CCSFE process design is often solved as binary distillation with McCabe-Thiel or Ponchon-Savarit graphic methods, where the complex extract mixture is treated as a single pseudo-component extracted with pure CO_2 [43]. However, SFE of suspended vegetal matrices in diluted ethanol is composed of three major components: solvent (CO_2) and two cosolvents (ethanol + water). Mamani et al. [52] proposed a methodology to validate hydroethanolic mixtures as a modifier and a suspension medium for the extraction of finely disrupted plant tissue through ternary equilibrium diagrams. Using the Perturbed-Chain Statistical Associating Fluid Theory (PC-SAFT) EoS, they modeled the ternary CO_2:ethanol:water equilibrium at 30–35 MPa and 40–50 °C and developed a procedure to calculate the equilibrium compositions inside the vessel during extraction: the recovered liquid phase from the vessel after full depressurization has the CO_2-free composition of the liquid phase during the extraction. Then, based in the PC-SAFT model, the composition of the gaseous phase can be estimated through the tie line, as shown in Figure 3.3. Experimental conditions can be defined based on this model. Once the gaseous phase composition is known, the cosolvent composition and flow rate are adjusted to maintain a stable suspension volume and composition throughout the entire process, as highlighted by del Valle et al. [49].

Currently, South America does not have active industrial-scale SFE projects, mainly because Chile and its neighboring countries do not have industry among their main economic activities and SFE is misconceived as an expensive technology. Researching ways to minimize cost and boost economies of scale is key step toward encouraging investment and development of chilean products.

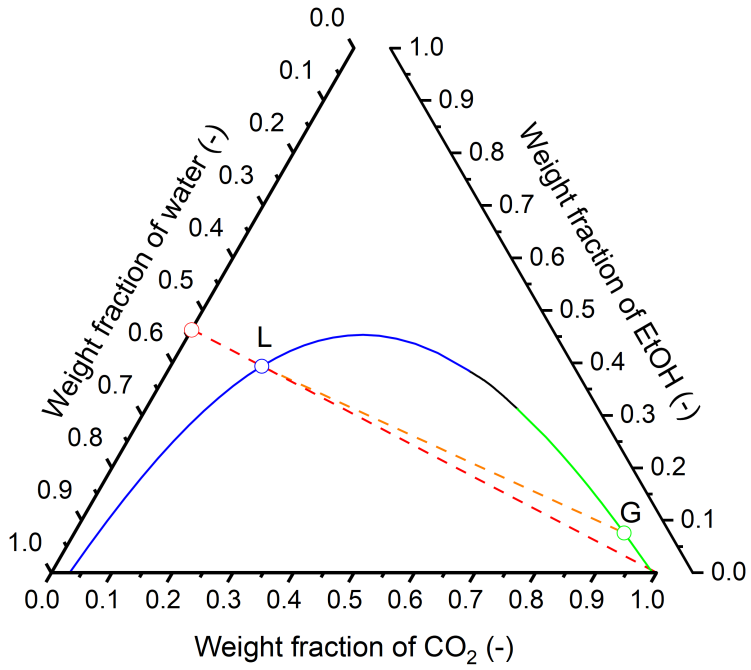


Figure 3.3: Ternary phase equilibrium diagram for the CO_2:ethanol:water system at 40 °C and 30 MPa. The solid line represents the binodal curve that defines the two-phase region, with blue indicating the liquid phase (−) and green the gaseous phase (−). A straight line is extended from the recovered liquid phase after full depressurization (○) toward the CO_2 vertex (−−). Its intersection with the binodal curve (○) corresponds to the liquid-phase composition during extraction. Finally, the gaseous-phase composition (○) is estimated using the PC-SAFT-simulated tie line (−−).

Chapter 4

Materials and methods

4.1 Substrate and sample preparation

The red clover variety Superqueli-INIA was supplied by INIA in February 2023. The material was dried at 50 °C for 48 h, ground and the stems were removed. The resulting powder was sieved to obtain particles within the 425–500 µm range, vacuum-packed and stored at -20 °C until extraction.

A rotor–stator dispersing device was employed to finely disrupt the red clover leaves and prepare the suspensions. From previous experience, the optimal conditions were identified to prevent sedimentation of the plant material at the bottom of the vessel. One gram of ground substrate was mixed with a hydroethanolic mixture of the desired composition, prepared with technical-grade ethanol (96 vol%, Winkler, Santiago, Chile) and distilled water (Q341-25, Quimis, São Paulo, Brazil), and processed using an Ultra-Turrax T-25 (Ika Works Inc., Wilmington, NC) operating at 24,000 rpm for 20 min. Each suspension was immediately used for extraction experiments.

4.2 Analysis reagents and standards

HPLC-grade water and methanol, along with potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4), were obtained from J.T. Baker (Xalostoc, Mexico). HPLC-grade acetic acid and Trolox were supplied by Sigma-Aldrich (Buchs, Switzerland), while the Folin–Ciocalteu reagent, by Merck KGaA (Darmstadt, Germany). Sodium carbonate (Na_2CO_3), aluminum chloride hexahydrate ($AlCl_3{\cdot}6H_2O$) and sodium nitrite ($NaNO_2$) were provided by CTR Scientific (Monterrey, Mexico). Sodium hydroxide (NaOH), fluorescein, AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride), hydrochloric acid (HCl, 0.1 N) and sodium chloride (NaCl) were supplied by Desarrollo de Especialidades Químicas (Monterrey, Mexico). Carbon dioxide (99.8%, GasLab, Santiago, Chile), technical-grade ethanol (96 vol%, Winkler, Santiago, Chile) and distilled water (Q341-25, Quimis, São Paulo, Brazil) were used as

solvent and cosolvents during the extraction experiments, respectively. Finally, gallic acid, quercetin, formononetin and biochanin A (Sigma-Aldrich, Buchs, Switzerland) were used as standards for spectrophotometric and high-performance liquid chromatography (HPLC) analyses.

4.3 Supercritical CO_2 extractions of suspensions

Extraction of suspensions was performed at LEMaB of Pontificia Universidad Católica de Chile (PUC) in the experimental device shown in Figure 4.1. Prepared suspensions (Section 4.1) were loaded into a 100 cm^3 extraction vessel. The CO_2 was pumped using a P-50A piston pump (Thar Technologies, Inc.) and the cosolvent using a 260 D Syringe Pump (Teledyne Isco, Lincon, NE).

Following the loading of the extraction vessel, it was tightly sealed, pressurized with carbon dioxide and cosolvent to the required extraction pressure and the convection oven set to the required extraction temperature.

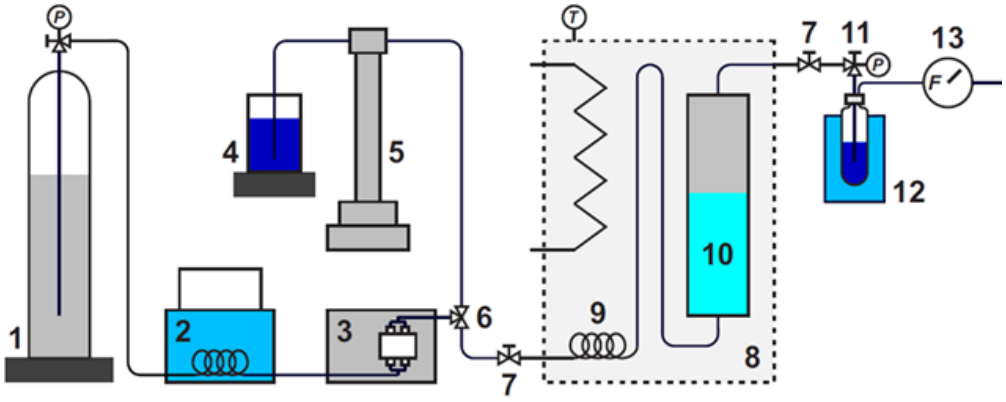


Figure 4.1: Process flow diagram of LEMaB extraction system for suspensions: (1) CO_2 cylinder; (2) refrigerated circulating bath; (3) high-pressure piston pump; (4) cosolvent reservoir; (5) high-pressure syringe pump; (6) T-union; (7) on-off valve; (8) convection oven; (9) preheater coil and syphon tubing; (10) extraction vessel; (11) BPR; (12) cold trap; (13) wet gas meter. Image from Mamani et al. [52].

After a 30-min static extraction, a 5-h dynamic extraction period was initiated. Samples were taken every 30 min in a cold trap, located immediately after the Back-Pressure Regulator (BPR), to help condensate the extract as it was being collected.

After completing each extraction, the depressurization protocol described by Mamani et al. [52] was applied. Briefly, the vessel was depressurized through the BPR, cold trap and gas meter, similarly to the dynamic stage, but with a continuous pressure decrease until reaching atmospheric conditions. This step aimed to recover as much ethanol and water, dissolved in the gaseous phase, as possible and nearly all the CO_2 from the solvent mixture.

4.4 Characterization of extracts

4.4.1 Total solids content (TSC)

After each extraction, the extracts were dried in a convection oven at 50 °C until dried solids were visible. Then, they were placed on a hot plate at 40 °C and further dried under a gentle flow of warm nitrogen. Finally, the samples were stored in the dark inside a desiccator for 24 h.

Dry pre-weighed vials were used to determine extract recovery in each experiment. After solvent evaporation, the vials were reweighed and the total extracted solids (TSC) were calculated from the mass difference. The extraction yield was expressed as the percentage of recovery relative to the initial dry weight of the sample.

4.4.2 Total Phenolic Content (TPC)

TPC was determined using the Folin-Ciocalteu assay, described by Escobedo-Avellaneda et al. [56] with modifications. Red clover dry extract aliquots were resuspended in 10 cm^3 of a hydroethanolic solution containing 35 wt% water, using an ultrasonic device (VWR International LLC, Radnor, PA, USA) for 10 min. The total phenolic content (TPC) was then analyzed by mixing 50 µL of the resuspended extract with 600 µL of distilled water and 50 µL of Folin–Ciocalteu reagent in a 48-well plate. After 5 min, 250 µL of an aqueous 5.3% w/v Na_2CO_3 solution were added, and the samples were kept in the dark for 120 min at 37 °C. Finally, absorbance was measured at 765 nm using a Synergy HT spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Gallic acid was used as standard in concentrations from 0.1 to 0.5 mg/cm^3 ($y = 3.810x - 0.072$, $R^2 = 0,9996$). Total phenolics were reported as milligrams of Gallic Acid Equivalent (GAE) per gram of dry sample (mg GAE/g red clover).

4.4.3 Total Flavonoid Content (TFC)

TFC was determined using the Aluminum Chloride assay, described by Esnaeili et al. [57] with modifications. Briefly, 60 µL of water, 120 µL of resuspended extract, 60 µL of 0.5 M $NaNO_2$ and 60 µL of 0.3 M $AlCl_3 \cdot 6H_2O$ were placed on the 48-well plate. After a 5-min incubation, 200 µL of 2 M NaOH was added and the absorbance, read at 320 nm in the Synergy HT spectrophotometer. A standard curve of quercetin (0.1-0.5 mg/cm^3) ($y = 8.409x + 0.084$, $R^2 = 0.9998$) was used to quantify TFC. Total flavonoids were reported as milligrams of Quercetin Equivalent (QE) per gram of dry sample (mg QE/g red clover).

4.4.4 Isoflavone content (TIC)

The content of isoflavones in red clover extracts (formononetin and biochanin A) was determined by HPLC analysis using an Agilent 1260 chromatograph (Santa Clara, CA) and the assay described by Aboushanab et al. [58]. For this, 2 cm^3 of resuspended extract was filtered using a 13 mm diameter, 0.45 µm openings PTFE syringe filter. Then, a 5 µL aliquot of filtered extract was analyzed by HPLC-DAD using a Zorbax SB-C18 column (100 × 3 mm, 3.5 µm particles) at 30 °C. Detection was performed at 249 and 261 nm. HPLC-grade acidified water and methanol, using 0.1 vol% acetic acid, were used as mobile phases A and B, respectively. The flow rate was 0.6 cm^3/min using the gradient: 0 min - 51% B; 7 min - 58% B; 14 min 65% B; 21 min - 72% B. The identification was based on standards (formononetin and biochanin A) by comparing the retention times the absorption spectrum. Calibration curves of each standard (formononetin: $y = 160.68x + 71.53$, $R^2 = 0.9997$; biochanin A: $y = 175.88x - 4.490$, $R^2 = 0.9996$) at concentrations ranging from 5 to 50 µg/cm^3 of hydroethanolic solution (65 wt% water) were used to quantify the formononetin and biochanin A concentration. The results were expressed as mg of isoflavones per gram of dry sample (mg (formononetin + biochanin A)/g red clover).

4.4.5 Antioxidant activity (AA)

Antioxidant activity (AA) was determined using the oxygen radical absorbance capacity (ORAC) method, following the procedure described by Escobedo-Avellaneda et al. [56]. Briefly, 100 µL of the resuspended extract were diluted in 900 µL of 75 mM sodium phosphate buffer (pH 7.4). Then, 25 µL of this solution were transferred to a Costar polystyrene black 96-well round-bottom plate. A microplate reader (Synergy HT, BioTek Instruments, Inc., Bad Friedrichshall, Germany) was used to automatically dispense 150 µL of 1 µM fluorescein solution, followed by 25 µL of 153 mM AAPH after a 30 min incubation at 37 °C. Fluorescence (485/20 nm excitation and 528/20 nm emission) was monitored at 37 °C for 1 h at 2 min intervals using the same instrument. The area under the curve obtained for 25–100 µM Trolox standards was used to construct a five-point calibration curve ($y = 268244x + 5 \times 10^{-6}$, $R^2 = 0.9990$), and the results were expressed as µmol Trolox equivalents (TE) per gram of dry sample (µmol TE/g red clover).

4.5 Statistical analysis

One extraction was performed by duplicate (40 °C, 30 MPa and 85 wt% water content on a CO_2-free basis). All compositional analyses were made by triplicate. These results were expressed as the average ± standard deviation. The statistical differences between means were determined using Tukey tests ($\alpha = 0.05$, Minitab 21, Coventry, United Kindom).

4.6 Experimental design

The diagram in Figure 4.2 exemplifies conditions for one experiment in the experimental design for the extraction of suspensions (30 MPa, 40 °C and liquid phase with $100 \cdot w'_{3L} = 35$ wt% water on a CO_2-free basis). The orange symbol in Figure 4.2 represents the global composition of the solvent mixture during the static extraction period (containing $100 \cdot w'_{3S}$ wt% water on a CO_2-free basis). During the 30-min static/equilibration period, the solvent mixture splits into a water-rich liquid phase containing $100 \cdot w'_{3L}$ wt% water on a CO_2-free basis (blue symbol on Figure 4.2) and a CO_2-rich gaseous phase containing $100 \cdot w'_{3G}$ wt% water on a CO_2-free basis (green symbol in Figure 4.2).

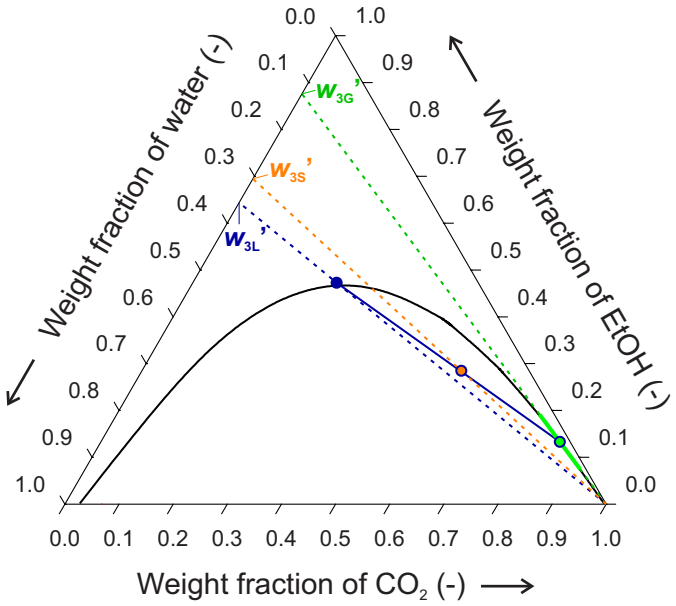


Figure 4.2: Experimental conditions for the extraction of suspensions shown in a ternary equilibrium diagram (CO_2 + ethanol + water), where w'_{3L} (blue line and dot), w'_{3G} (green lines and dot) and w'_{3S} (orange line and dot) correspond the fractions of water, on a CO_2-free basis, in the liquid phase, gaseous phase and overall solvent mixture in the extraction vessel. Due to limitations of the cosolvent feeding device, the actual composition of the solvent mixture during dynamic extraction was in the solid green line, instead of the target green dot.

The experimental conditions were selected following the methodology proposed by Mamani et al. [52], which involves modeling the ternary (CO_2 + ethanol + water) system using the PC-SAFT EoS [59, 60]. Table 4.1 summarizes the experiments conducted in this study at pressures between 20-35 MPa and temperatures of 40-50 °C, using different loadings and overall compositions of the suspensions to achieve the desired liquid and gaseous phase conditions. A CO_2 flow rate of 10 or 20 g/min was applied, with the cosolvent flow rate adjusted as indicated in Table 4.1, depending on the liquid-phase composition and CO_2 mass flow rate. The same experimental conditions were

reported by Mamani et al. [25] for packed-bed SFE of red clover. Due to experimental challenges in achieving the target gaseous-phase composition by adjusting the CO_2-to-cosolvent mass-to-volume ratio, the actual solvent mixture composition remained within a narrow range, as represented by the solid green line in Figure 4.2. The experimental conditions of the packed-bed extractions are shown in Appendix A, Table A.1.

Table 4.1: Experimental conditions used in this work for the extraction of suspensions. T and P are the extraction temperature and pressure, w'_{3L}, w'_{3G} and w'_{3S} are the mass fractions, on a CO_2-free basis, of water in the liquid and gaseous phase during dynamic extraction and in the suspension loaded in the extraction vessel prior to the static extraction stage, respectively; m is the mass of hydroethanolic mixture loaded in the extraction vessel with the ground red clover in the extraction of suspensions. Q_{CO_2} and Q^S_{CS} are the mass flow rate of CO_2 and volume flow rate of cosolvent mixture during dynamic extraction, respectively.

P	T	$100 \cdot w'_{3L}$	Suspension		Q_{CO_2}	Dynamic extraction	
(MPa)	(°C)	(wt%)	m (g)	$100 \cdot w'_{3S}$ (wt%)	(g/min)	Q^S_{CS} (cm^3/min)	$100w'_{3G}$ (wt%)
20	40	55	38.6	50.5	10	0.70	16.0
30	40	85	39.8	83.3	20	0.25	36.7
30	40	55	37.6	50.5	10	0.76	17.2
30	40	35	36.3	29.8	10	2.03	15.2
35	50	55	37.1	50.0	10	0.87	18.7

Chapter 5

Results and discussion

5.1 Results

Mamani et al. [25] reported the extraction yields for packed-bed SFE and ethanol solid-liquid extraction of Superqueli-INIA red clover strain. For solid-liquid extraction with ethanol, the TSC, TPC, TFC, TIC and AA were: 43.19 ± 0.31 wt%, 27.10 ± 0.94 mg GAE/g substrate, 8.11 ± 0.56 mg QE/g substrate, 8.11 ± 0.15 mg (formononetin + biochanin A)/g substrate and 45.33 ± 0.26 µmol TE/g substrate, respectively.

This work applies the methodology developed to validate hydroethanolic mixtures as suspensions for SFE [52]. It is necessary to evaluate the performance of the computational procedure to quantify equilibrium compositions and the developed PC-SAFT model, because accurate interpretation of each result relies on evaluating both extraction yields and the solvent composition after dynamic extraction.

On this section it is described the composition of the gaseous and liquid phase of the ternary system, composed of CO_2:ethanol:water, after dynamic extractions. Then, the extraction kinetics and yield for each analyzed components is presented: total solids, total phenolics, total flavonoids, total isoflavones, antioxidant activity and selectivity.

5.1.1 Phase composition in the extraction of suspensions

To calculate the composition of CO_2:ethanol:water in the liquid and gaseous phase after dynamic extraction, the metodology of Mamani et al. [52] was used. It required experimental measurements of the ethanol (m^G_{EtOH}) and water ($m^G_{H_2O}$) weight recovered during full depressurization, the weight of evacuated CO_2 during full depressurization (m_{CO_2}) and the weights of ethanol (m^L_{EtOH}) and water ($m^L_{H_2O}$) in the remaining liquid inside the vessel. It is important to note that, in every experiment, a liquid phase remained in the vessel, indicating that the methodology successfully

achieved phase separation even in the presence of solid substrate.

Figure 5.1 compares the experimental design with the achieved compositions and the model predictions of the equilibrium compositions for the ternary CO_2:ethanol:water system after the dynamic extraction. The equilibrium slightly deviated from the experimental design, enriching the liquid phase with water when performing the extractions at 20 MPa and 40 °C (Figure 5.1A) and 35 MPa and 50 °C (Figure 5.1C), but at 30 MPa and 40 °C (Figure 5.1B) an exception was observed only with 35 wt% water in the liquid phase on a CO_2-free basis (Figure 5.1B). This may be related to the challenges of accurately measuring the compositions of the gaseous phase in the available SFE equipment. Overall, similar trends were seen by Mamani et al. [52], who studied the same ternary system at similar pressure and temperature without solid substrate. Thus, it would be correct to assume that the low concentration of dissolved solutes has negligible effect on phase equilibrium.

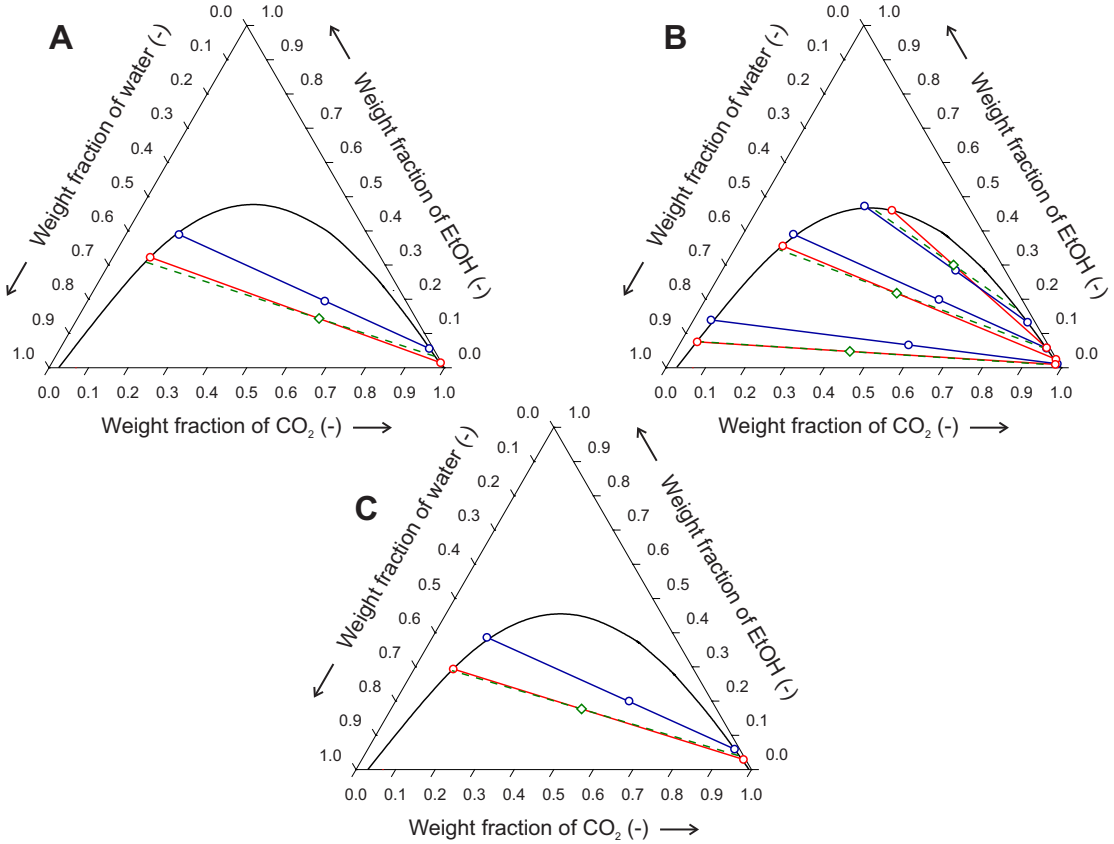


Figure 5.1: Comparison between the experimental design (○ and solid blue line) and estimates of equilibrium compositions (○ and solid red line) for the water-rich liquid phase (to the left and up) and the CO_2-rich gaseous phase (lower right, near the CO_2 vertex) after 5 h of dynamic extraction followed by depressurization. Code letters identify experiments performed at (**A**) 20 MPa and 40 °C; (**B**) 30 MPa and 40 °C; and (**C**) 35 MPa and 50 °C. The figures show the predicted overall solvent composition (◊) and tie lines (−−) by the PC-SAFT model developed by Mamani et al. [52].

Figure 5.2 shows the evolution of the water content in the liquid extract throughout the extraction process. For reference, the specific solvent consumption was always greater than 0.5 kg solvent/g substrate after 30–60 min of dynamic extraction. As expected, equilibrium was reached within 1 h into the dynamic period, which was consistent with the literature [52]. For experiments that use a liquid phase containing 55 wt% water (on a CO_2-free basis), the liquid extract composition stabilized around 13 wt%, with overlapping values at 35 MPa and 50 °C (□) and at 30 MPa and 40 °C (◊).

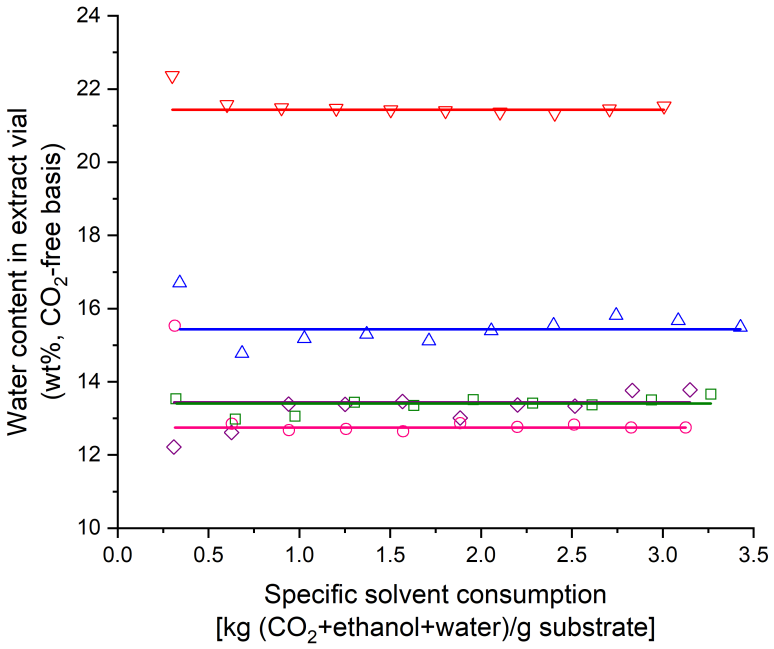


Figure 5.2: Value of water composition, on a CO_2-free basis, of extract collected in the extraction of suspensions during the dynamic period for all experiments. Symbols represent experimental measurements, corrected to account for the mass balance during full experiments, when using 55 wt% water in the liquid phase, on a CO_2-free basis, at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; (□) 35 MPa and 50 °C; and when performing extractions at 30 MPa and 40 °C, using a liquid phase containing (▽) 85, (◊) 55 and (△) 35 wt% water on a CO_2-free basis. Straight lines represent the mean composition of water in the extracts after 1 h of dynamic extraction.

Table 5.1 summarizes the phase compositions and densities at the end of the extractions. At 30 MPa and 40 °C, higher ethanol contents in the liquid phase enabled greater dissolution of CO_2. Density variations in the liquid phase (0.940–0.994 g/cm^3) were smaller than those in the gaseous phase (0.867–0.952 g/cm^3), since liquids are largely incompressible and water, the main component in the liquid phase, does not substantially alter its physical properties upon CO_2 saturation [16]. The goal was to reproduce the gaseous composition obtained in packed-bed extractions. However, slight differences were seen, related to model and equipment limitations.

Table 5.1: Composition and density of the liquid and gaseous phase after equilibrium is reached during dynamic extraction, where w_1, w_3 and ρ are the mass composition of CO_2, the mass composition of water in a CO_2-free basis and the mass density. Symbols represent the experimental design conditions: when using a 55 wt% water content in the liquid phase, on a CO_2-free basis, at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; and (□) 35 MPa and 35 °C. When performing extraction at 30 MPa and 40 °C, symbols represent (▽) 85 wt%, (◊) 55 wt% and (△) 35 wt% water on a CO_2-free basis in the liquid phase.

		○	▽	◊	△	□
		Liquid phase, extraction of suspension				
$100 \cdot w_1$	(wt%)	9.5	4.1	11.7	34.1	9.9
$100 \cdot w_3$	(wt%, CO_2-free)	64.3	92.3	59.9	30.5	67.4
ρ	(g/cm^3)	0.957	0.994	0.957	0.940	0.962
		Gaseous phase, extraction of suspensions				
$100 \cdot w_1$	(wt%)	94.3	97.7	91.6	83.4	92.3
$100 \cdot w_3$	(wt%, CO_2-free)	12.8	21.4	13.3	15.3	13.4
ρ	(g/cm^3)	0.867	0.913	0.932	0.952	0.918
		Gaseous phase, packed-bed extraction				
$100 \cdot w_1$	(wt%)	93.2	97.2	93.0	85.7	93.0
$100 \cdot w_3$	(wt%, CO_2-free)	16.0	36.7	17.2	15.2	18.7
ρ	(g/cm^3)	0.851	0.912	0.915	0.922	0.904

5.1.2 Total solid yield

Figure 5.3 presents the cumulative extraction curves of total solids for packed-bed (Figure 5.3A) and suspension extraction (Figure 5.3B) with modified scCO$_2$. Table 5.2 compares both methodologies with the reference solid–liquid extraction of red clover. Suspension extraction yields were substantially lower than the other two methods, corresponding to only 0.17–0.64 times the packed-bed yield and 0.17–0.47 times the solid–liquid extraction yield.

The solid extraction curve for packed-bed extractions show a distinct "S" pattern (Figure 5.3A), except at 30 MPa, 40 °C and 35 wt% water on a CO_2-free basis (△). This can be related to slow a slow initial diffusion from the solid to the CO_2 stream up until the cosolvent filled the extractor. The most noticiable contidion was at 30 MPa, 40 °C and 85 wt% water in the liquid on a CO_2-free basis (▽), which had the lowest cosolvent flow rate (0.68 cm^3/min, Appendix A). As cosolvent filled up the extractor, wetting and swelling the solid substrate, the extraction rate steeply increased until the substrate was exhausted. This was not the case of suspensions (Figure 5.3B), although the same trend was reported by Aravena et al. [50] for astaxanthin on an aqueous suspension. In this work, due to the 30-min static period, compositions and properties of the CO_2-expanded suspension were reached before the dynamic period. Thus, a more efficient and mass transfer process occurred.

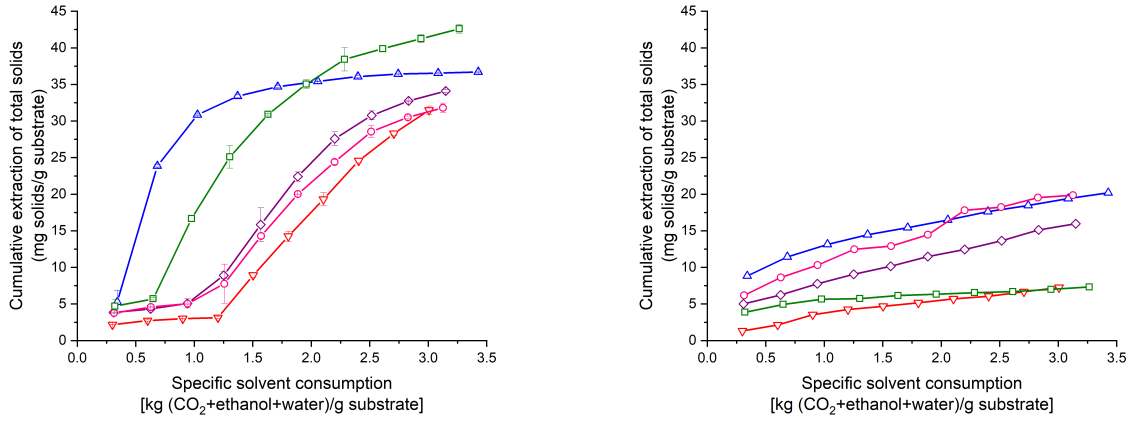


Figure 5.3: Cumulative extraction curves for total solids in (A) packed-bed and (B) suspension, expressed as percent solids extracted of the total dry weight of red clover loaded into the extractor. Symbols represent experimental measurements corrected to account for mass balance during full experiments when using 55 wt% water (CO_2-free basis) in the water-rich liquid phase at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; or (□) 35 MPa and 50 °C. When performing extractions at 30 MPa and 40 °C using a liquid phase containing (▽) 85 wt%; (◊) 55 wt%; and (△) 35 wt% water (CO_2-free basis).

In the extraction of suspensions, for the same water content in the liquid phase (55 wt% on a CO_2-free basis), lowering the pressure and temperature had a pronounced positive effect on the overall extraction yield. The TSC at 35 MPa and 50 °C was 7.33 wt%, which doubled at 30 MPa and 40 °C. Further lowering the pressure to 20 MPa increased the yield to 19.89 wt%. This trend is counterintuitive, as higher pressures are generally associated with increased CO_2 solvent power [61]. However, the gaseous phase at 35 MPa and 50 °C (□) was not the densest condition (Table 5.1). In fact, the highest solid yield was achieved by the least dense gas phase condition (0.867 g/cm^3) that occurred at 20 MPa and 40 °C (○). This suggests that the mass transfer mechanism in suspensions differs fundamentally from that in packed-bed extractions. At the same pressure and temperature, 30 MPa and 40 °C, the water content in the liquid phase also showed a pronounced effect. It was inversely proportional to the overall solid yield, probably due higher solubility of the extracted compounds in mildly polar solvents. Table 5.1 shows that the gaseous phase contained 97.7 wt%, 91.6 wt% and 83.4 wt% CO_2 when using 85 wt% (▽), 55 wt% (◊) and 35 wt% (△) water in the liquid phase, on a CO_2-free basis, respectively. The amount of dissolved cosolvent in the gaseous phase determined the overall solid yield. Moreover, the yield followed the same trend as the cosolvent flow rate. High cosolvent flow rates, due to the mass balance, produced larger amounts of extract and, generally, enhanced the yield. However, the composition of water and ethanol in the liquid and gaseous phases ultimately determined the composition and concentration of the extracts.

Table 5.2: Overall yield of total solids (TSC), total phenolic content (TPC) and total flavonoid content (TFC) for the packed-bed, suspensions and solid-liquid (SL) extractions of red clover. Symbols represent experimental conditions for experiments when using 55 wt% water (CO_2-free basis) in the water-rich liquid phase at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; or (□) 35 MPa and 50 °C. When performing extractions at 30 MPa and 40 °C using a liquid phase containing (▽) 85 wt%; (◊) 55 wt%; and (△) 35 wt% water (CO_2-free basis).

	TSC (wt%)		TPC (mg GAE/g substrate)		TFC (mg QE/g substrate)	
Exp.	Packed bed	Suspension	Packed bed	Suspension	Packed bed	Suspension
○	31.81 ± 0.50^{E}	19.86	5.64 ± 0.40^{C}	5.02	6.52 ± 0.74^{B}	5.37
▽	31.49 ± 1.00^{F}	7.24 ± 0.37^{G}	4.45 ± 0.09^{E}	2.84 ± 0.03^{F}	3.39 ± 0.10^{E}	3.43 ± 0.08^{E}
◊	34.10 ± 1.46^{D}	15.96	6.13 ± 0.16^{B}	4.58	5.72 ± 0.91^{D}	5.27
△	36.71 ± 1.49^{C}	20.13	5.33 ± 0.06^{D}	9.68	5.97 ± 0.64^{C}	7.80
□	42.61 ± 0.61^{B}	7.33	6.16 ± 0.14^{B}	4.54	8.15 ± 0.19^{A}	3.79
SL	43.19 ± 0.31^{A}		27.10 ± 0.94^{A}		8.11 ± 0.56^{A}	

5.1.3 Phenolic yield

Figure 5.4 shows the cumulative extraction curves of TPC. The overall yields of packed-bed extractions (Figure 5.4A) and the extraction of suspensions (Figure 5.4B) are shown in Table 5.2. Except for the extraction performed at 30 MPa, 40 °C and 35 wt% water in the liquid phase on a CO_2-free basis (△), phenolic extraction in packed-bed outperformed suspensions.

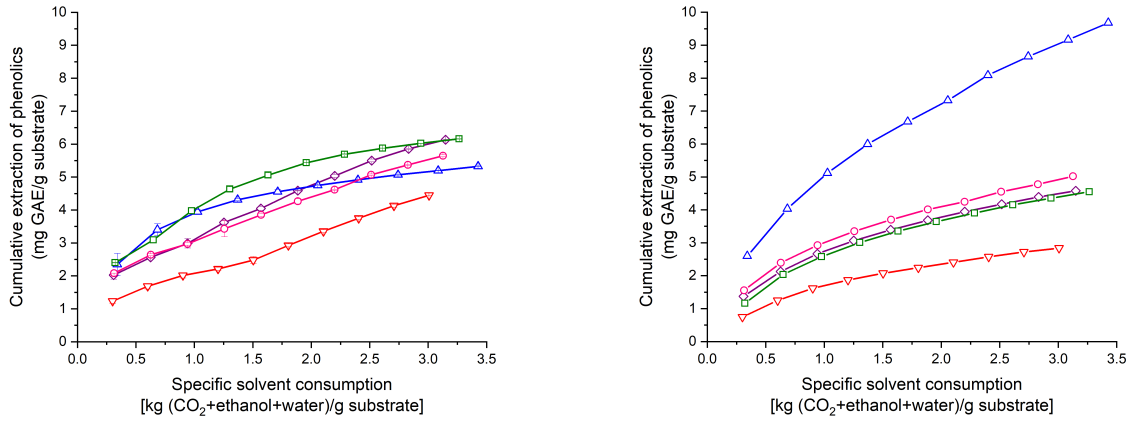


Figure 5.4: Cumulative extraction curves for total phenolic compounds in (A) packed bed and (B) suspension during the dynamic extraction stage. Symbols represent experimental measurements corrected to account for mass balance during full experiments when using 55 wt% water (CO_2-free basis) in the water-rich liquid phase at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; or (□) 35 MPa and 50 °C. When performing extractions at 30 MPa and 40 °C using a liquid phase containing (▽) 85 wt%; (◊) 55 wt%; and (△) 35 wt% water (CO_2-free basis).

For packed-bed extraction, pressure and temperature did not significantly affect the overall yield, whereas, at 30 MPa and 40 °C, the water content determined the total phenolic yield (4.45–6.13 mg GAE/g substrate) and the optimal composition was 55 wt% water on a CO_2-free basis (◊).

The extraction of suspensions clearly showed that, at 30 MPa and 40 °C, lower overall water content greatly enhanced the phenolic yield by nearly 3.5-fold from 85 wt% (▽) to 35 wt% (△) water in the liquid phase on a CO_2-free basis. Such a big difference may be explained with the affinity of phenolic compounds with mildly polar solvents and that the latter condition was the densest at constant pressure and temperature (0.952 g/cm^3). Similarly to packed-bed extractions, changes in pressure and temperature barely affected the overall yield and extraction kinetics. Overlapping curves show that, at 55 wt% in the liquid phase (CO_2-free), decreasing the pressure from 35 to 20 and the temperature from 50 to 40 °C only increased the overall phenolic yield 1.1 times.

5.1.4 Flavonoid yield

Cumulative extraction curves for total flavonoids are shown in Figure 5.5, while Table 5.2 presents the overall yields for packed-bed extractions (Figure 5.5A) and suspensions (Figure 5.5B). As with phenolic yields, suspensions were outperformed by packed-bed extractions, except in two conditions: 30 MPa, 40 °C, 35 wt% (△) and 85 wt% (▽) water in the liquid phase on a CO_2-free basis. In several conditions, for both extraction methods, total flavonoid yield surpassed the overall phenolic yield. This is unreasonable, because the Folin–Ciocalteu assay accounts flavonoids as phenolics. The quantification capacity of the assays depends on the type of flavonoid (figures B.1 and B.2, Appendix B). Also, this behavior can be influenced by the characteristics of the quantification methods. The Aluminum Chloride method employed in this work is flavonoid-specific, but Folin-Ciocalteu assay is not specific for phenolic compounds, because other reducing compounds can alter the sample readings, such as, reducing sugars and reducing amino acids [62]. These results (sections 5.1.3 and 5.1.4) are not comparable, because they may measure molar responses rather than actual concentrations.

In packed-bed extractions, the highest water content in the gaseous phase hindered the extraction of flavonoids (30 MPa, 40 °C, 85 wt% water on a CO_2-free basis), but the other conditions at the same pressure and temperature had little differences. The highest flavonoid yield was achieved at 35 MPa, 50 °C and 55 wt% water content (□), related to the relatively high cosolvent composition of the gaseous phase (7 wt% ethanol + water) and density (0.904 g/cm^3). Thus, the condition at 30 MPa, 40 °C and 85 wt% water content (▽) had the worst performance, due the low cosolvent composition of the gas phase (2.8 wt% ethanol + water).

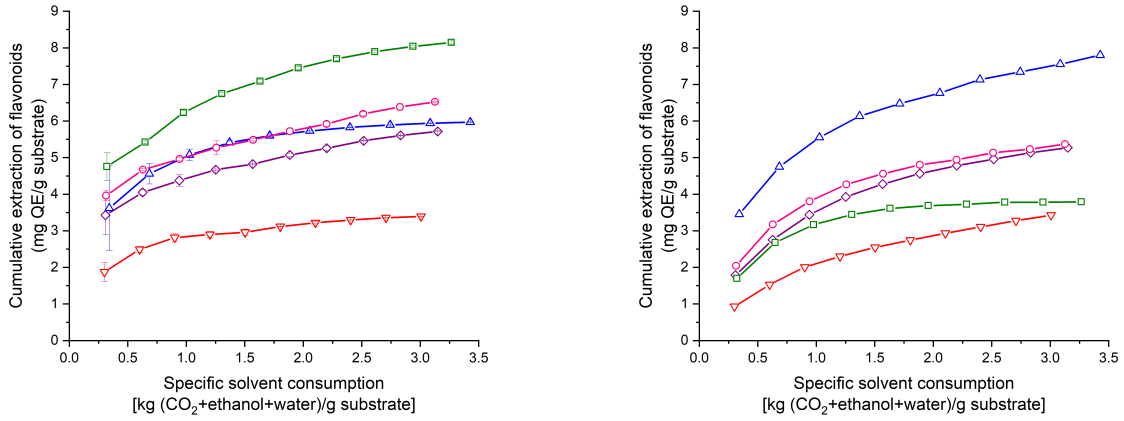


Figure 5.5: Cumulative extraction curves for total flavonoid compounds in (A) packed bed and (B) suspension during the dynamic extraction stage. Symbols represent experimental measurements corrected to account for mass balance during full experiments when using 55 wt% water (CO_2-free basis) in the water-rich liquid phase at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; or (□) 35 MPa and 50 °C. When performing extractions at 30 MPa and 40 °C using a liquid phase containing (▽) 85 wt%; (◊) 55 wt%; and (△) 35 wt% water (CO_2-free basis).

Once again, the water content in the liquid phase was the key variable in the flavonoid yield for the extraction of suspensions, showing the same trends than phenolic extraction curves. Lowering the water content from 85 wt% (▽) to 35 wt% (△) increased the overall yield from 3.43 to 7.80 mg QE/g substrate. The latter condition had, by far, the most hydroethanolic content in the gaseous phase (16.6 wt% cosolvent, Table 5.1). Therefore, it is reasonable to expect that the CO_2 stream dissolves higher amounts of flavonoids. Pressure and temperature had little effect, showing overlapping curves at 40 °C and 20 (○) to 30 MPa (◊). Nonetheless, further increasing the pressure to 35 MPa and the temperature to 50 °C lowered the overall flavonoid yield.

5.1.5 Isoflavone yield

Figure 5.6 shows cumulative extraction curves of red clover isoflavones (formononetin + biochanin A). Table 5.3 shows the overall isoflavone yield for packed-bed extractions (Figure 5.6A) and the extraction of suspensions (Figure 5.6B). Appendix C provides separate extraction curves for formononetin (Figure C.1-A.B) and biochanin A (Figure C.1-C.D). The extraction of isoflavones achieved greater yields when using suspensions, reaching 81% formononetin and 89% biochanin A recovery, compared with solid-liquid extraction. Packed-bed extractions only recovered 54% and 72% formononetin and biochanin A, respectively.

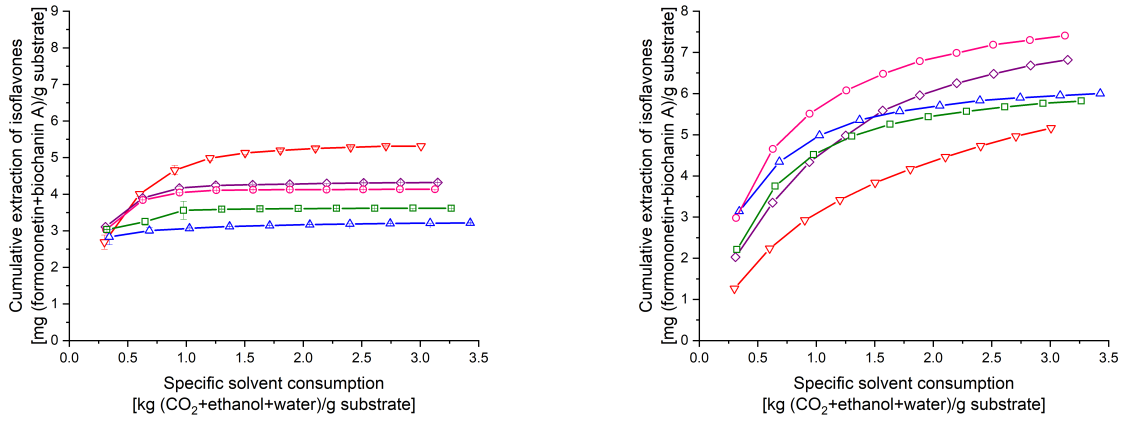


Figure 5.6: Cumulative extraction curves for total isoflavones (formononetin + biochanin A) in (**A**) packed bed and (**B**) suspension during the dynamic extraction stage. Symbols represent experimental measurements corrected to account for mass balance during full experiments when using 55 wt% water (CO_2-free basis) in the water-rich liquid phase at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; or (□) 35 MPa and 50 °C. When performing extractions at 30 MPa and 40 °C using a liquid phase containing (▽) 85 wt%; (◊) 55 wt%; and (△) 35 wt% water (CO_2-free basis).

Table 5.3: Comparison of the ratio between formononetin (F) and biochanin A (BA), total isoflavone (F+BA) yield (TIC) and antioxidant activity (AA) of extracts from packed-bed extractions, the extraction of suspensions and solid-liquid extractions (SL). Symbols represent experimental conditions for experiments when using 55 wt% water (CO_2-free basis) in the water-rich liquid phase at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; or (□) 35 MPa and 50 °C. When performing extractions at 30 MPa and 40 °C using a liquid phase containing (▽) 85 wt%; (◊) 55 wt%; and (△) 35 wt% water (CO_2-free basis).

	Formononetin / Biochanin A ratio (-)		TIC (mg (F+BA)/g substrate)		AA (µmol TE/g substrate)	
Exp.	Packed bed	Suspension	Packed bed	Suspension	Packed bed	Suspension
○	1.15 ± 0.01^{E}	1.75	4.14 ± 0.01^{E}	7.41	36.74 ± 0.15^{C}	31.59
▽	1.44 ± 0.04^{C}	1.51 ± 0.05^{B}	5.31 ± 0.08^{B}	5.16 ± 0.12^{C}	34.52 ± 0.29^{E}	32.54 ± 0.43^{F}
◊	1.21 ± 0.06^{D}	1.57	4.32 ± 0.12^{D}	6.82	36.48 ± 0.07^{D}	33.25
△	0.98 ± 0.03^{F}	1.47	3.22 ± 0.13^{G}	6.00	28.73 ± 0.14^{G}	32.09
□	0.97 ± 0.03^{G}	1.42	3.62 ± 0.17^{F}	5.82	40.33 ± 1.35^{B}	31.72
SL	1.92 ± 0.05^{A}		8.85 ± 0.15^{A}		45.33 ± 0.26^{A}	

When extracting from suspensions, at 30 MPa and 40 °C, the isoflavone yield followed a similar trend as total solids, total phenolics and total flavonoids: solvent mixtures with lower water content had better performance (35 wt% and 55 wt% water content, CO_2-free), peaking at 6.82 mg (F+BA)/g substrate. When using 55 wt% water content in the liquid (CO_2-free), decreasing the

pressure and temperature from 35 to 20 MPa and 50 to 40 °C increased isoflavone yield from 5.82 to 7.41 mg (F+BA)/g substrate.

The F/BA ratio, which peaked at 1.75 (20 MPa, 40 °C), was inversely proportional to pressure and temperature when extracting from suspensions, using 55 wt% water in the liquid phase. Biochanin A differs from formononetin by a 5- hydroxyl group, which makes it more water-soluble than formononetin [63]. Table 5.1 shows that at 55 wt% water in the liquid, the highest composition of water in the CO_2 stream was at 35 MPa and 50 °C (13.4 wt%, CO_2-free) and also the lowest F/BA ratio (1.42 F/BA), meaning that biochanin A extraction was more favorable. At 30 MPa and 40 °C, the ratio behaved similarly, but, surprisingly, the lowest ratio was achieved at 35 wt% water in the liquid phase. This can only be explained by the early exhaustion of formononetin at these conditions (Figure C.1B, Appendix C) and high fresh cosolvent flow rate, which could allow for the under-saturation of biochanin A in a ethanol-rich medium. Packed-bed extractions did not exhibited the same trends as suspensions for the isoflavone ratio.

5.1.6 Antioxidant activity

Table 5.3 shows the AA for the final cumulative extract for packed-bed extractions and suspensions. For packed-bed extractions, AA ranged from 28.73 to 40.33 µmol TE/g substrate, while for suspensions it ranged from 31.59 to 33.25 µmol TE/g substrate. The control substrate (SL) exhibited the highest AA as it simultaneously contained the highest amounts of phenolics, flavonoids and isoflavones. The AA of extracts is dependent on chemical nature and the structure of each molecule. For phenolics and flavonoids in general, the amount and position of hydroxyl functional groups strongly determine their scavenging capacity [63, 64] and, as mentioned before, hydroxyl groups also make flavonoids more water-soluble.

In suspensions, AA peaked at 30 MPa, 40 °C and 55 wt% water content (◊) at 33.25 µmol TE/g substrate, despite not corresponding to the highest phenolic, flavonoid or isoflavone levels. This condition likely provided an intermediate solvent polarity between 35 wt% and 85 wt% water, which enabled the dissolution of hydroxyl-functionalized molecules. A further increase in water content (▽) should have had even greater affinity for stronger antioxidants, but resulted in the lowest overall phenolic, flavonoid, and isoflavone yields. Probably due the water content of the liquid phase reached 92.3 wt% (CO_2-free) and it may have competed for the water-soluble compounds, explaining why this condition yielded only intermediate AA. The condition at 30 MPa, 40 °C, and 35 wt% water in the liquid phase (△) outperformed all others in phenolic and flavonoid content. Nonetheless, it showed the lowest AA performance under the same temperature and pressure (32.09 µmol TE/g substrate). This was probably because its lower overall polarity favored the extraction of ethanol-soluble O-methylated flavonoids. These are hydrophobic molecules that exhibit substantially less AA than hydroxyl-functionalized molecules [64].

For packed-bed extraction, maximum AA was reached at 35 MPa, 50 °C and 55 wt% water in the liquid phase on a CO_2-free basis (□), most likely due to its elevated phenolic and flavonoid content. However, the antioxidant capacity results from a combination of many compounds, which were not characterized in this work. Through electron transfer, metal chelation or hydrogen atom transfer, these compounds could act synergistically, although the main radical scavenging mechanism of flavonoids is the latter [63]. Overall, packed-bed extracts had higher AA than from suspensions, because phenolic and flavonoid content in extracts were greater than the extracts from suspensions. In Appendix D a comparison of phytochemical profiled throughout the different extraction techniques can explain this phenomena.

5.1.7 Selectivity

Table 5.4 compares the selectivity of phenolics, flavonoids and isoflavones per gram of dry extracted solids between packed-bed and suspension extractions. Appendix E provides the evolution of selectivity throughout the extractions for each compound (Figure E.1). As mentioned before, suspensions extracted significantly fewer total solids than packed-bed extractions, making the extracts remarkably more selective for phenolics, flavonoids and isoflavones. The control substrate was highly selective towards PCs and showed intermediate selectivity for flavonoids and isoflavones, which can be attributed to the high solubility of solutes in ethanol, combined with the highest solid yield (Table 5.2).

Table 5.4: Comparison of the selectivity of phenolics, flavonoids and isoflavones of extracts from packed-bed extractions, the extraction of suspensions and solid liquid extraction (SL). Symbols represent experimental conditions for experiments when using 55 wt% water (CO_2-free basis) in the water-rich liquid phase at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; or (□) 35 MPa and 50 °C. When performing extractions at 30 MPa and 40 °C using a liquid phase containing (▽) 85 wt%; (◊) 55 wt%; and (△) 35 wt% water (CO_2-free basis).

	Phenolics (mg GAE/g dry extract)		Flavonoids (mg QE/g dry extract)		Isoflavone (mg F+BA/g dry extract)	
Exp.	Packed bed	Suspension	Packed bed	Suspension	Packed bed	Suspension
○	17.73	27.74	20.47	29.65	13.01	40.94
▽	14.13	47.55	10.78	57.42	16.90	86.34
◊	18.00	29.81	16.68	34.32	12.68	44.42
△	14.54	48.10	16.21	38.74	8.76	29.80
□	14.47	69.96	19.13	58.47	8.48	89.76
SL	69.80		20.89		22.79	

At 30 MPa and 40 °C in suspensions, isoflavone selectivity markedly increased from 29.80 to 86.34 mg (F+BA)/g dry extract as water content rose. At 85 wt% water content, the cosolvent flow

rate was the smallest. Therefore, little amounts of concentrated extracts were recovered. Flavonoid selectivity showed a similar upward trend, whereas phenolic selectivity decreased between 35 wt% and 55 wt% water (CO_2-free) before recovering at 85 wt% water in the liquid phase (CO_2-free). At 55 wt% water in the liquid phase on a CO_2-free basis, the selectivity of phenolics, flavonoids and isoflavones increased with both pressure and temperature. When pressure rose from 20 to 30 MPa, selectivity of each compound exhibited only a mild increase. However, a further rise to 35 MPa and 50 °C led to almost a twofold increase in phenolics (29.81–69.96 mg GAE/g dry extract), flavonoids (34.32–58.47 mg QE/g dry extract) and isoflavone (44.42–89.76 mg (F+BA)/g dry extract) selectivity. This trend may be related to the liquid phase being richer in water and having stronger affinity for a wider range of solutes and impurities, thereby allowing for more concentrated extracts.

5.2 Discussion

This work focused on validating the methodology of Mamani et al. [52] for recovering high-value polar compounds from plant matrices, applied here to red clover isoflavones. The data suggest that both recovery (Figure 5.6) and purity (Table 5.4) of extracts can be enhanced by suspending the solid substrate in a hydroethanolic mixture, which is partially miscible with the supercritical solvent and provides an effective medium for isoflavone transport. Cell wall disruption during sample preparation releases isoflavones into the static cosolvent or suspension, which subsequently transfer by solubility into the $scCO_2$ stream during extraction, i.e. the two-step transfer mechanism proposed by del Valle et al. [49]. In general, higher ethanol content in the overall solvent mixture enhanced extraction yields for both packed-bed and suspension extraction. This trend was also reported by Seabra et al, [46] in anthocyanin extraction from suspensions. For suspensions, phenolics (Figure 5.4B, Table 5.2) and flavonoids (Figure 5.4B, Table 5.2) clearly exhibited this trend. Meanwhile, isoflavone extraction was also dependent on pressure and temperature (Figure 5.6B, Table 5.3). The two main isoflavones extracted were formononetin and biochanin A, but very little amounts of genistein and daidzein were extracted (See Appendix D), which agrees with several authors about the overall isoflavone profile of red clover [9, 24, 65].

It is a commonly reported trend that the overall solid extraction, or total yield, increases with higher pressure for packed-bed extractions [27, 32]. This is associated to higher density of the $scCO_2$, which determines its solvent power [61], as seen in Figure 5.3A. For suspensions, this was not the case as the highest pressure had the lowest overall solid yield. However, pressure and temperature were both increased at the same time, which has a both positive (pressure) and negative (temperature) effect in the solvent density (Table 5.1). In general, packed-bed and suspension extractions exhibited contrasting trends. Yet, both highlighted ethanol's enhancing effect on red

clover phenolics recovery.

Building on the evident differences between the extraction methods, the transfer mechanism from suspensions can be rationalized through three phenomena:

1. The presence of a liquid phase competes with the CO_2-modified solvent for heavier or polymerized compounds, which may explain the lower solid yield obtained with suspensions. Appendix D supports this hypothesis, along with similar findings reported in the literature [31].

2. Ethanol enhanced the extraction yield of every analyzed component (figures 5.3, 5.4 and 5.5), as the benefits of CXLs are stronger with "type II" liquids. These solvents, like ethanol, undergo drastic changes in physical properties, such as, diffusivity, viscosity and density when saturated with pressurized CO_2 [16], thereby accelerating the otherwise slow diffusion step from the solid substrate. By contrast, water is less capable of dissolving CO_2 under high pressure and temperature and does not undergo substantial changes in these properties.

3. Pressure and temperature exert an indirect effect, as the extraction yield arises from the combined contributions of liquid and gas compositions, which determine the transfer rate. This explains, for example, why the least dense gas condition had the highest overall yield of total solids (Figure 5.3B) and isoflavones (Figure 5.6B). Yields could be correlated with the solvent physical properties and/or compound solubility. However, neither of these aspects were evaluated in this work.

Nevertheless, these statements remain speculative and call for deeper insights into isoflavones, flavonoids and phenolics solubility in $scCO_2$, phase equilibrium compositions at high pressure, distribution coefficients and mass transfer data.

With a biphasic system, phase equilibrium knowledge is essential to accurately interpret the extraction results. The distribution coefficients reported by Sato et al. [55] showed that, at 8-20 MPa and 40–60 °C, both pressure and overall water content increased the K-values of flavone, 6-methoxiflavone and 7-hydroxyflavone on a CO_2:ethanol:water solvent mixture. In suspensions, their extraction yields should follow a similar behavior. However, for red clover, the yields of phenolics, flavonoids and isoflavones did not change significantly with increasing temperature or pressure. Moreover, higher overall water content hindered the extraction yield in suspension systems. This highlights the difficulty of predicting and analyzing SFE yields in biphasic systems without reliable phase equilibrium data for the main red clover constituents. To date, no thermodynamic data have been reported for the quaternary system CO_2:ethanol:water:(formononetin/biochanin A) under typical SFE conditions, let alone for more complex five-plus multicomponent systems.

Regarding phenolic and flavonoid yield, additional experiments were carried out to investigate a potential underestimation of total phenolics and flavonoid content and analyze the unusual results, where TFC and TIC were greater than TPC (Table 5.2 and Table 5.3). These experiments analyzed the score of the main isoflavones in red clover and compared their response to those of the standards used for TPC (gallic acid) and TFC (quercetin). For TPC analysis, the results show a 93% underestimation for formononetin and 50% for biochanin A (See Appendix B). Some have criticized the Folin-Ciocalteu TPC assay, due not being specific to fenolic compounds [62]. Reducing sugars, ascorbic acid and reducing amino acids can interfere with the absorbance readings in sample analysis, some of which can be found in red clover [66].

Overall phenolic, flavonoid, and isoflavone content in red clover strongly depends on agronomic factors such as cultivar, growing conditions, maturity stage, and plant part (flowers, leaves, stems, or roots) [22, 65]. Mikulić et al. [65] reported that leaves contained more isoflavones than flowers and stems combined. Moreover, cultivars from different countries exhibited up to 1.5-fold higher isoflavone content. Hidalgo et al. [2] stated that red clover extracts were gaining popularity due to standardized isoflavone extraction processes and consistent medicine composition. Andres et al. [67] reported that the isoflavone content in red clover supplements generally matched the amounts advertised by manufacturers. These supplements allegedly provide 40, 50 or 80 mg of total isoflavones per capsule (approximately 80% F, 16% BA, and 4% D and G). Actual analyses showed deviations ranging from 0.4% to 12.8%. However, a short literature survey in extraction of red clover shows that results from different studies reveals substantial differences in isoflavone yields (Table 5.5).

Table 5.5: Comparison of the maximum yield/content of formononetin (F), biochanin A (BA), genistein (G), daidzein (D) and total isoflavones (TIC), expressed as mg/g dry leaves (L), flowers (Fl) or stems (St), between this work and other reported extraction methods of red clover extracts: (S-SFE) Supercritical fluid extraction of suspensions; (WSE) Water–solvent extraction; (ESE) Ethanol–solvent extraction; (PB-SFE) Packed-bed supercritical fluid extraction.

Substrate	Extraction method	F mg/g	B mg/g	G mg/g	D mg/g	TIC	Ref.
L	S-SFE	4.72	2.69	-	-	7.41	-
L, St, Fl	-	0.8-11	-	-	-	10-25	[5]
F	WSE						[9]
	ESE	0.661	1.252	0.357	-	2.59	[21]
L	PB-SFE	3.14	2.174	-	-	5.31	[25]
	ESE	5.82	3.03	-	-	8.85	

Based on the work of Andres et al. [67], the selectivity achieved in this work with extractions from suspensions is a strong indicator that this novel methodology can yield commercially viable products. Isoflavone selectivity from suspensions ranged from 40.92 to 89.76 mg (F+BA)/g

dry extract. Assuming that a supplement capsule is primarily composed of red clover extract, this methodology reaches and surpasses the average isoflavone content reported for commercial supplements. In contrast, packed-bed and conventional solid–liquid extractions did not reach the mean benchmark of 40 mg isoflavones/g dry extract. Thus, the markedly higher selectivity of suspensions highlights their potential as an attractive alternative to conventional extraction techniques.

Currently, the supercritical extraction of suspensions remains an underdeveloped approach for isolating high-value compounds from solid matrices. Nevertheless, it has shown promising results in terms of overall yield and selectivity of antioxidants [46, 51, 53] and algae extracts [47, 48, 50] from various substrates. Although current applications of this methodology are mainly focused on assessing yield and selectivity relative to regular modified-CO_2 SFE, it is expected to serve as a first step toward a packed-column CCSFE process for solid plant matrices. Naturally, such an application would present its own technical challenges, including packing fouling due to the presence of solids, tube clogging and others.

Chapter 6

Concluding remarks

This work evaluated the supercritical extraction of red clover isoflavones with modified $scCO_2$, using a hydroethanolic suspension as a static cosolvent throughout the entire extraction process. The effects of pressure, temperature and water content in the solvent mixture were assessed on the total solid yield, total phenolic yield, total flavonoid yield, total isoflavone yield, process selectivity and antioxidant activity of the extracts. The results obtained with this methodology were compared with those from a control solid–liquid red clover extract and from packed-bed supercritical extraction under the same conditions as the suspension. It was hypothesized that suspending disrupted red clover prior to supercritical CO_2 extraction would surpass the overall yield and selectivity of isoflavone from the packed-bed format. The data confirmed this hypothesis, showing that isoflavone recovery was 1.0–1.79 times higher and selectivity 3.0–10.6 times greater than those obtained with the packed-bed configuration.

Extracts containing penolic compounds, flavonoids and isoflavones were recovered from suspended red clover using modified $scCO_2$. Isoflavone yield peaked at 7.41 mg (F+BA)/g substrate at 20 MPa, 40 °C and 55 wt% water in the liquid phase (CO_2-free), which gratly differed with the packed-bed isoflavone yield by 44%. Maximum solid, phenolic, and flavonoid yields were observed at 30 MPa, 40 °C, and 35 wt% water in the liquid phase, reaching 20.13 wt%, 9.68 mg GAE/g substrate, and 7.80 mg QE/g substrate, respectively, with only the solid yield surpassed by that of packed-bed extractions. All conditions studied were more selective for phenolics, flavonoids and isoflavones than the packed-bed approach.

The applied methodology of Mamani et al. [52] performed as expected. Equilibrium compositions were reached at 30-60 min dynamic extraction, as composition of liquid extracts showed (Figure 5.2). In most conditions, the CO_2 dragged ethanol, enriching the liquid phase with water, except at 30 MPa, 40 °C and 35 wt% water (CO_2-free) in which the opposite happened. Although an odd result, the overall equilibrium compositions behaved similarly to those of reported in the literature.

Extraction curves were compared between packed-bed extractions and extraction of suspensions.

Packed-bed extractions exhibited an S-shaped curve for total solids, which is related to slow diffusion in the initial stage of extractions, except the condition with the highest cosolvent flow rate. This was not seen for suspensions, due a 30-min static period that reached the enhanced transport properties of the CO_2-expanded suspension. In general, ethanol content was the most influential factor, compared to pressure and temperature. It enhanced the overall extraction performance for each format, due higher affinity with the targeted compounds than water. However, opposing trends were seen between packed-bed and suspensions under the same conditions. This shows how their mass transfer mechanism fundamentally differs from each other and it would be mostly dependent on both liquid and gas phase composition. Lastly, AA was greater for packed-bed extractions. It peaked at 40.33 µmol TE/g substrate at 35 MPa and 50 °C, likely due higher overall phenolic and flavonoid content in the extracts and higher amounts of -OH functionalized compounds.

To further understand the mass transfer kinetics of the extraction of suspended red clover, more research is needed in solubility of isoflavones in water- and ethanol-modified $scCO_2$ at typical extraction conditions: 20-40 MPa and 40-50 °C. Then, distribution coefficients could be correlated and predicted using the same PC-SAFT model. Moreover, it will be useful to quantify mass transfer coefficients for each step of the extraction (2-step process). This methodology is expected to be applied in a CCSFE of PCs, although its industrial application still needs to undergo a detailed techno-economic assessment.

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Appendix A

Table A.1: Experimental conditions used in Mamani et al. [25]'s work, where T and P are the extraction temperature and pressure; w'_{3G} is the mass fraction of water in the gaseous phase on a CO_2-free basis during dynamic extraction; and Q_{CO_2} and Q_{CS}^{PB} are the mass flow rate of CO_2 and the volume flow rate of the cosolvent mixture in the extraction of suspensions during dynamic extraction, respectively.

				Dynamic extraction	
P MPa	T °C	$100 \cdot w'_{3L}$ wt%	Q_{CO_2} g/min	Q_{CS}^{PB} cm^3/min	$100 \cdot w'_{3G}$ wt%
20	40	55	10	0.90	16.0
30	40	85	20	0.69	36.7
30	40	35	10	0.93	17.2
30	40	55	10	2.04	15.2
35	50	55	10	0.91	18.7

Appendix B

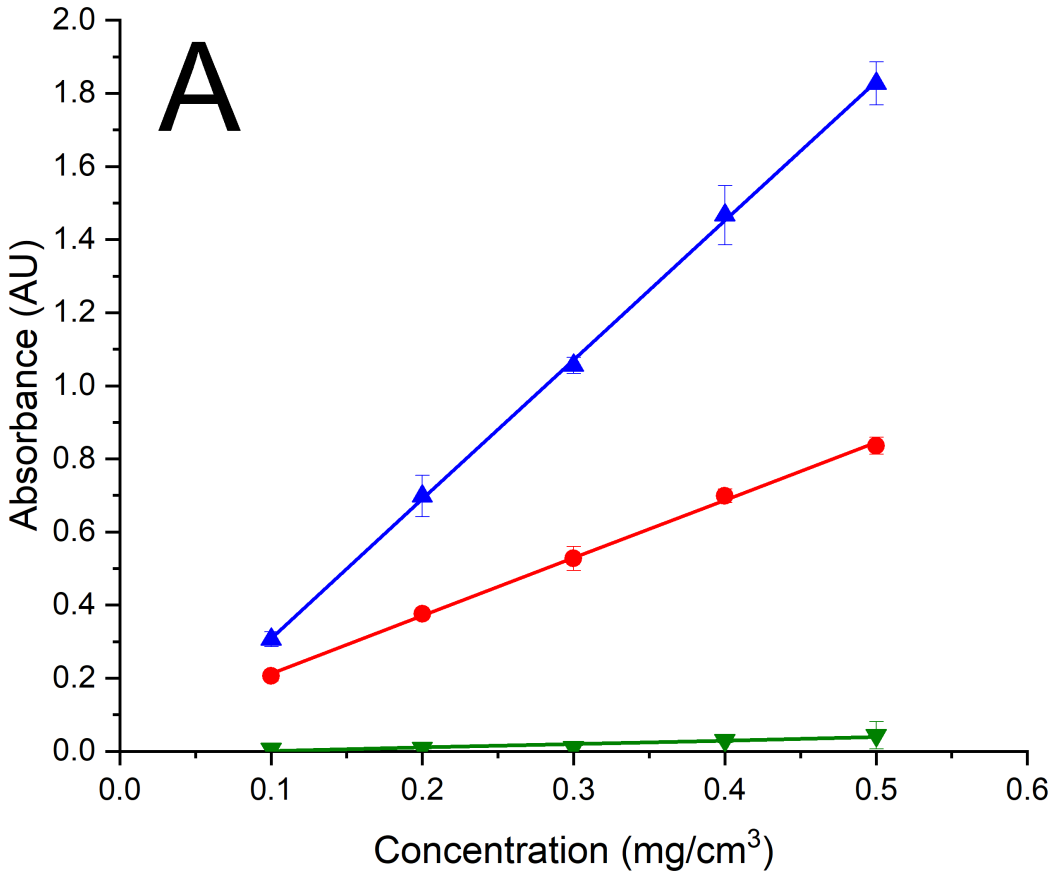

Figure B.1: Calibration curves for gallic acid (▲), biochanin A (●) and formononetin (▼). The curves for the three standards were made using the methodology for the assay for total phenolic content described in the manuscript.

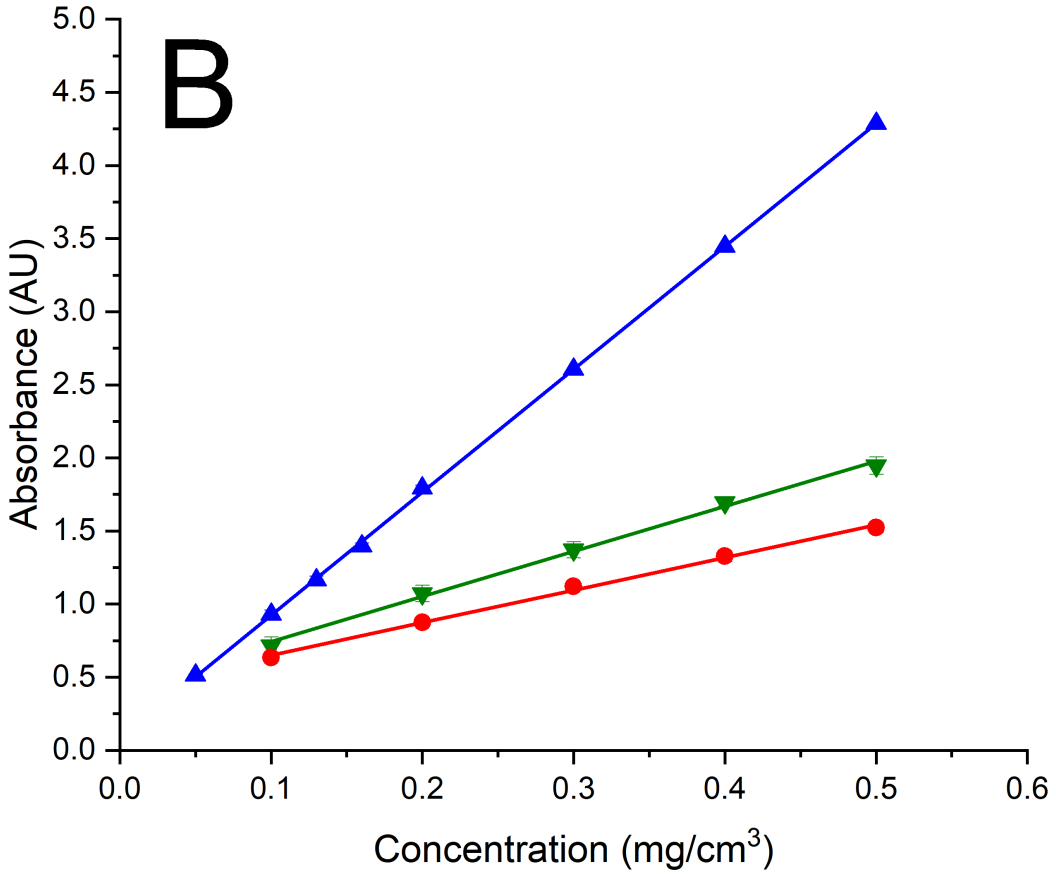

Figure B.2: Calibration curves for quercetin (▲), formononetin (▼), and biochanin A (●), and. The curves for the three components were made using the assay for total flavonoid content described in the manuscript.

Appendix C

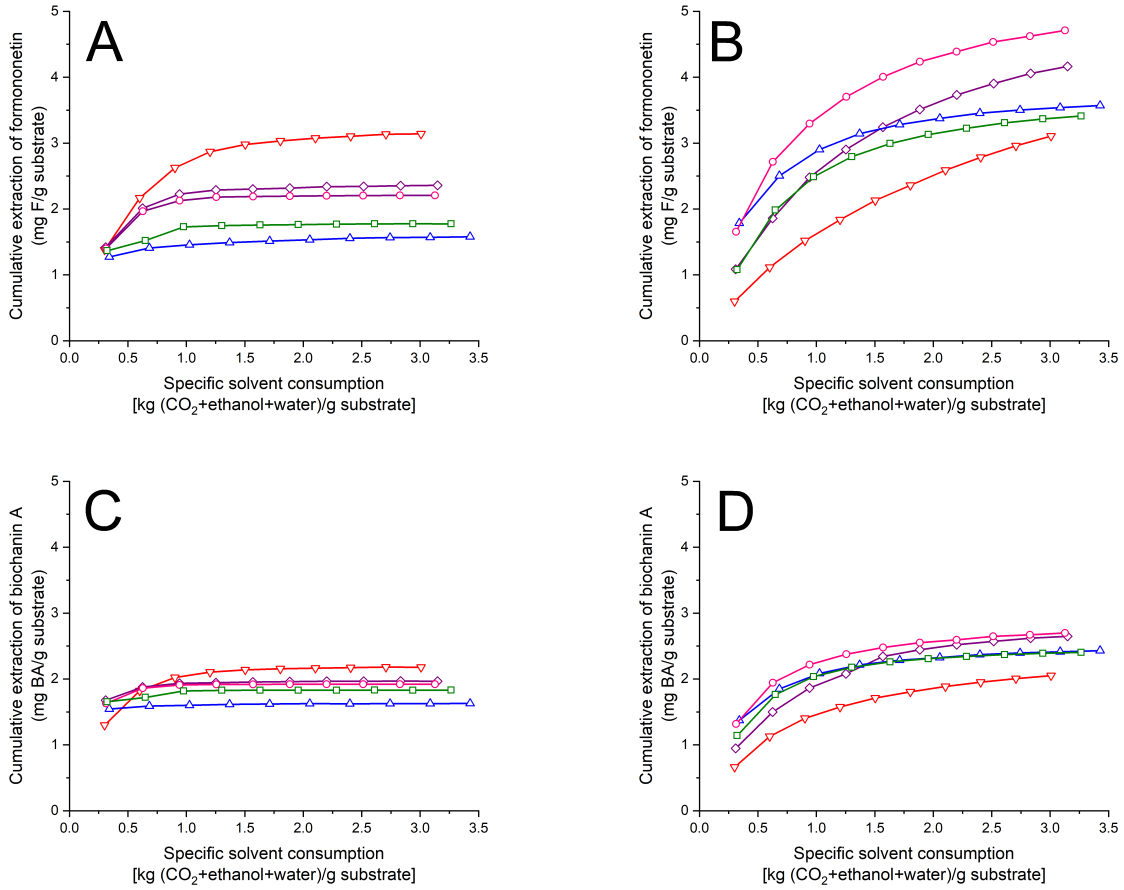

Figure C.1: Cumulative extraction curves for formononetin (A, B) and biochanin A (C, D) from packed-bed extractions (A, C) and the extraction of suspension (B, D) during the dynamic extraction stage. Symbols represent experimental measurements corrected to account for mass balance during full experiments when using 55 wt% water (CO_2-free basis) in the water-rich liquid phase at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; or (□) 35 MPa and 50 °C. When performing extractions at 30 MPa and 40 °C using a liquid phase containing (▽) 85 wt%; (◊) 55 wt%; and (△) 35 wt% water (CO_2-free basis).

Appendix D

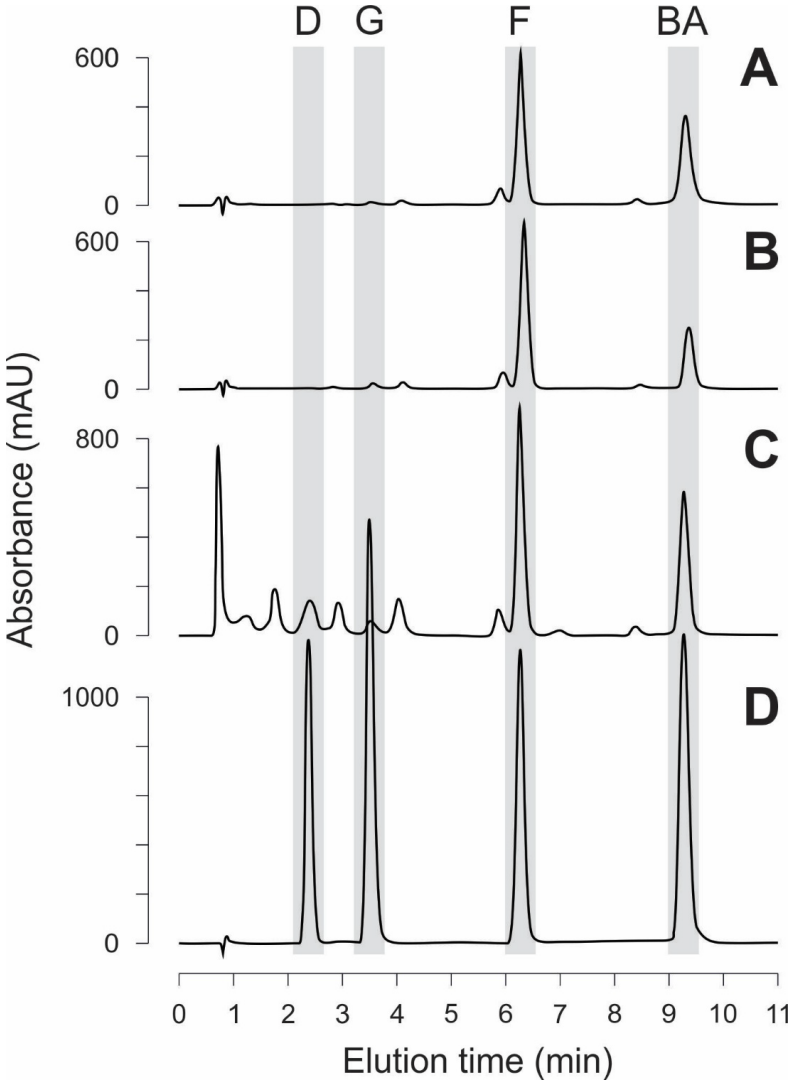

Figure D.1: HPLC chromatographs of red clover extract obtained by (A) SFE of an hydroethanolic suspension, (B) packed-bed SFE at 30 MPa and, 40 °C and 45 wt% diluted ethanol, (C) ethanol-solid-liquid extraction and (D) standard solution of (D) daidzein, (G) genistein, (F) formononetin and (BA) biochanin A.

Appendix E

Next page shows the evolution of phenolic (Figure E.1-A.B), flavonoid (Figure E.1-C.D) and isoflavone (Figure E.1-E.F) selectivity throughout dynamic extraction of packed-bed and suspensions.

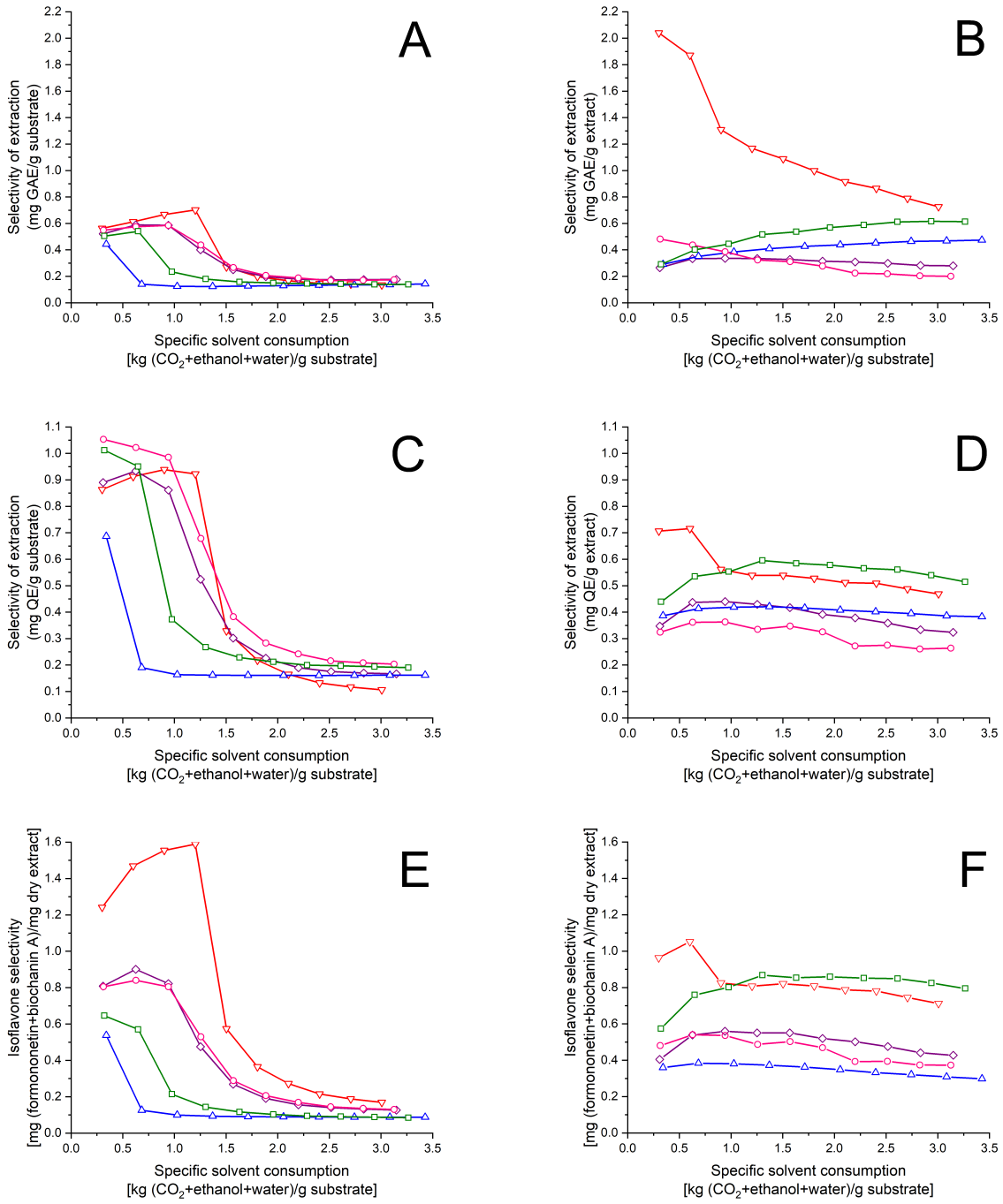

Figure E.1: Selectivity for the supercritical extraction of phenolics (A, B), flavonoids (C, D) and isoflavones (E, F) from red clover for (A, C, E) packed-bed extraction and (B, D, F) extraction of suspensions using aqueous-ethanol-modified $scCO_2$. Symbols represent experimental measurements when using 55 wt% water, on a CO_2-free basis, at (○) 20 MPa and 40 °C; (◊) 30 MPa and 40 °C; or (□) 35 MPa and 50 °C. When performing extractions at 30 MPa and 40 °C using a liquid phase containing (▽) 85 wt%; (◊) 55 wt%; and (△) 35 wt% water (CO_2-free basis).