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DEVELOPMENT OF NOVEL AUTOMATED SAMPLE TREATMENT TECHNIQUES COUPLING WITH CHROMATOGRAPHIC METHODS FOR DETERMINATION OF FLAVONOIDS IN NATURAL AND MANUFACTURING MATRICES

Mohamad Subhi Sammani

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WE DECLARE:

That the thesis entitled "Development of novel automated sample treatment techniques coupling with chromatographic methods for determination of flavonoids in natural and manufacturing matrices" presented by Mohamad Subhi Sammani to obtain a doctoral degree has been completed under our supervision and meets the requirements to opt for the mentioned degree.

For all intents and purposes, we hereby sign this document.

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Palma de Mallorca, 25 May 2021

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DECLARAN

Que la tesis doctoral que lleva por título “Development of novel automated sample treatment techniques coupling with chromatographic methods for determination of flavonoids in natural and manufacturing matrices” presentada por Mohamad Subhi Sammani para la obtención del título de doctor ha sido dirigida bajo nuestra supervisión y que cumple con los requisitos necesarios para optar al mencionado grado.

Y para que quede constancia de ello firmamos este documento

Dr. José Manuel Estela Ripoll

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Palma de Mallorca, 25 May 2021

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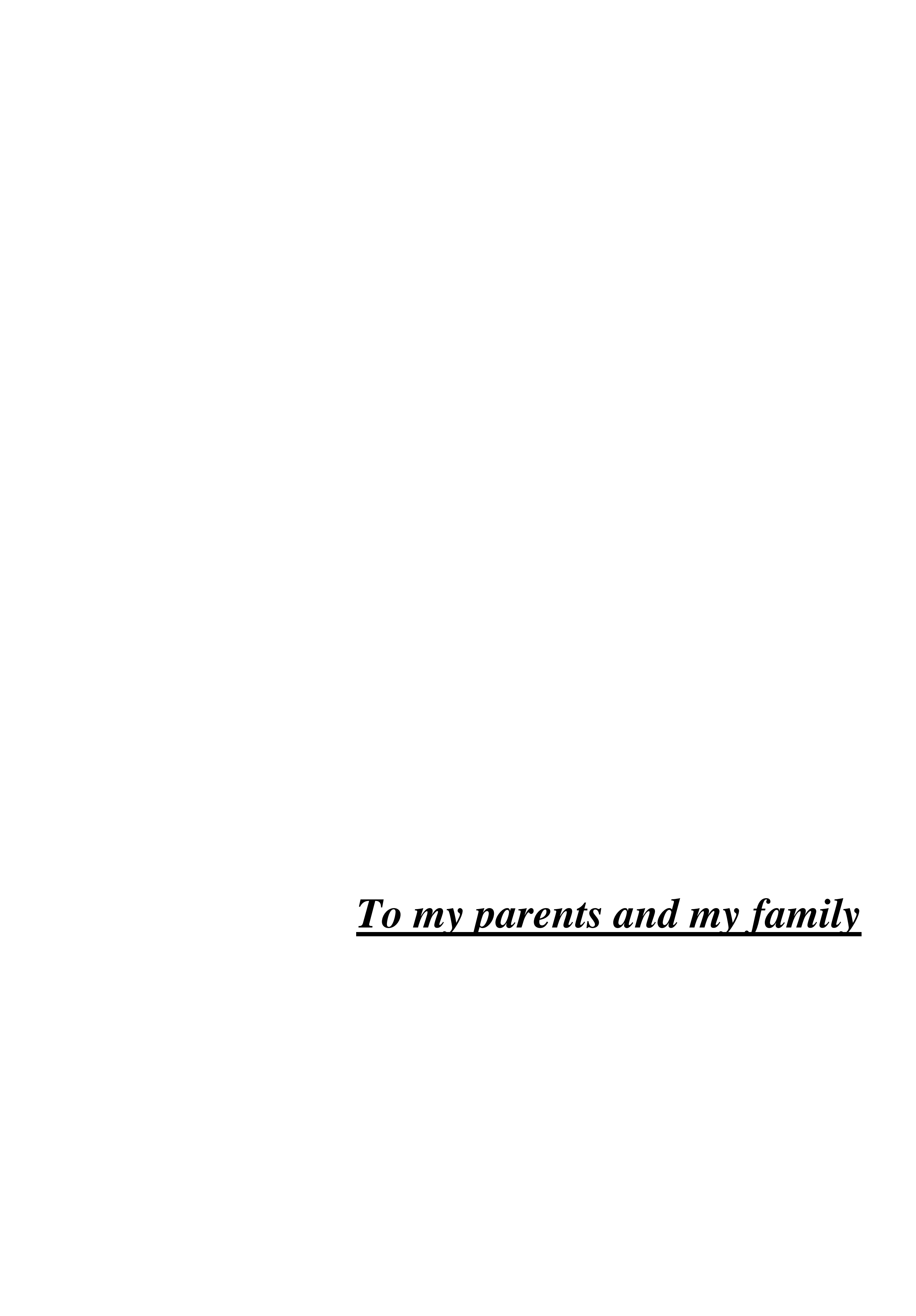

<u>To my parents and my family</u>

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ABBREVIATIONS

2DLC	2-dimensional liquid chromatography
2DTLC	Two-dimensional-TLC
AD	Amperometric detector
AdSV	Adsorptive stripping voltammetry
ACN	Acetonitrile
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
API	Active pharmaceutical ingredient
ASE	Accelerated solvent extraction
BBD	Box-Behnken design
Beta-CD	Beta-cyclodextrin
C	Circuit
CAD	Computer-aided design
CC	Central channel
CCD	Central composite designs
CCE	Chiral capillary electrophoresis
CD	Coulometric detector
CDS	Circular dichroism spectroscopy
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CET	Conventional extraction technique
CP	Central port
CTs	Chromatographic techniques
CV	Cyclic voltammetry
CVI	Chronic venous insufficiency disease
COV	Middle-Est Respiratory Syndrome-Coronavirus
CPFs	Citrus peel-flavonoids
CZE	Capillary zone electrophoresis

D	Desirability function
d_i	Individual desirability function
DIO	Diosmin
DLLs	Dynamic link libraries
DM	Doehlert matrix designs
DM-SPE	Dispersive magnetic solid phase extraction
DMSO	Dimethyl sulfoxide
DOE	Design of experiments
DPV	Differential pulse voltammetry
DSHEA	Dietary Supplement Health and Education Act
DTIN	Diosmetin
ED	Electrochemistry detector
ET	Extraction tank
FAT	Flow analysis technique
FCC	Fused core column
FDA	U.S. Food and Drug Administration
FDM	Fused deposition modelling
FFD	Full factorial design
FFSs	Flavonoids-food supplements
FIA	Flow injection analysis
FID	Flame ionization detector
FL	Fluorometric spectroscopy
FT-IR	Fourier transform infrared
GBL	Ginkgo biloba leaves
GC	Gas chromatography
HC	Holding coil
HES	Hesperidin
HF-LPME	Hollow fibre liquid phase micro-extraction
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HPTLC	High performance-TLC

HSCCC	High-speed counter-current chromatography
HTIN	Hesperetin
HRE	Heat reflux extraction
HSI	Hyperspectral imaging spectroscopy
HWBE	Hot water bath extraction
ILs	Ionic liquid solvents
IS	Isorhamnetin
IV	High-pressure injection valve
KA	Kaempferol
LC	Liquid chromatography
LLDME	Liquid-liquid dispersive micro-extraction
LLE	Liquid-liquid extraction
LLME	Liquid-liquid micro-extraction
LOV	Lab-on-valve
M	Motor
MAE	Microwave-assisted extraction
MC	Monolithic column
MCFIA	Multicommutated flow analysis
MCPE	Mixed cloud point extraction
MECE	Micellar electrokinetic capillary electrophoresis
MeOH	Methanol
MET	Modern extraction technique
MIP	Molecularly imprinted polymer
MPV	Multiposition valve
MS	Mass spectroscopy
MS^n	Tandem mass spectrometry
MSA	Magnetic-stirring-assistant
MSC	Multi-syringe chromatography
MSFIA	Multisyringe flow injection analysis
MSPDE	Matrix solid-phase dispersion extraction
NARIN	Naringenin

NAR	Naringin
NIR	Near-infrared
NIH	National Institutes of Health
NMR	Nuclear magnetic resonance spectroscopic technique
NOE	Nuclear overhauser effect
NP	Normal phase
NPs	Nanoparticles
OVAT	One-variable-at-a-time
PC	Paper chromatography
PEF	Pulsed electric field
PJ	PolyJet
PLE	Pressurized liquid extraction
PP	Peristatic pump
PMFs	Polymethoxy flavones
PTFE	Poly(tetrafluoroethylene)
PVDF	Polyvinylidene fluoride
PVP	Polyvinylpyrrolidone
QU	Quercetin
SAE	Sonicated-assisted extraction
SARS	Severe Acute Respiratory Syndrome
SE	Soxhlet extraction
SFA	Segmented flow analysis
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SIA	Sequential injection analysis
SIC	Sequential-injection chromatography
SI-LOV	Sequential injection lab-on-valve
SLA	Stereolithography
SLE	Solid-liquid extraction
SP	Syringe pump
SPE	Solid-phase extraction

SPDME	Solid phase dispersive micro-extraction
SPME	Solid phase micro-extraction
STs	Spectrophotometric techniques
SWC	Subcritical water chromatography
SWE	Subcritical water extraction
RC	Reaction coil
ROS	Reactive oxygen species
RP	Reversed phase
RRCL	Rapid resolution liquid chromatography
RS	Raman spectroscopy
RU	Rutin
TAN	Tangeretin
TFA	Trifluoroacetic acid
THz	Terahertz
TLC	Thin-layer chromatography
UHPLC	Ultra-high-performance liquid chromatography
Ultem	Polyetherimide
UV/VIS	Ultraviolet/Visible radiation
V	Solenoid valve
XR	X-ray crystallography
ZIC	Polymeric zwitterionic polymer

ABSTRACT

The increasing knowledge of the massive importance of flavonoids as strong antioxidant substances located in the plants, foods, and their manufactured products, as well as their pharmacological behaviour in the prevention and treatment of numerous diseases in the human and animal bodies, are lead to increase the interesting of the development of many products in the markets such as pharmaceutical and dietary supplement products besides the food-rich flavonoids products such as beverages, jams, and juices.

This swiftly growing of the flavonoids commercial industry needs to the adjustment of these products' quality, detecting the impurities, degradation, and the real concentration of the interesting compounds.

However, the complexity of the natural matrices and the sensitivity of flavonoids to the processing factors such as the temperature, mechanism's harvest, and storage conditions pose difficulties in the extraction and the accurate determination of these compounds, especially when the interesting analytes are present in a trace concentration as in the case of impurities or the decomposition of the analytes. In this sense, sample pretreatment steps are almost mandatory prior to the detection technique application. The new trend is focused on the development of sample pretreatment methods that can be saved in solvents, sample, and time consumptions being as protecting the environment.

Nowadays, microextraction techniques and the automation of the sample pretreatment procedure are the trends these days. Flow analysis techniques can be facilitating these interested procedures achieving efficient, fast, economical, workers and environmental protections, besides the increase of the throughput of the sample and the method's robustness.

In this thesis, different automatic micro-extraction methods based on flow techniques for the flavonoid's matrices sample treatments, and HPLC methods have been developed for

the determination of flavonoids in their natural sources and commercial products. Flow systems, in particular, were depended on the lab-on-valve, multi-position valve, and multisyringe flow injection analysis followed by HPLC-DAD analysis for exploiting fully automated analytical methods. The potential of flow analytical techniques has proven by the development of two sample pretreatment systems benefiting from the great features of the grafted micro-extraction column of the lab-on-valve platform, multi-position valve, and also from the 3D printer technology. The developed systems have been implanted for the extraction, preconcentration, and determination of flavonoids in pharmaceuticals, dietary supplements, citrus fruits, citrus hand squeezing juices, and their commercial products. All the developed systems proved the significant reduction of sample and solvents consumptions and enhancing the sample throughput, sensitivity, and reducibility of the analysis compared with the previous classical methods. The hyphenated HPLC and flow techniques attained fast, selective, and robust fully automated analytical systems. Additionally, a comprehensive review compared effectively between the developed methods and the works that have been existing in the literature.

The developed works included in this thesis are listed below:

1. A comprehensive review dealing with the classical and the state-of-the-art sample pretreatment and analysis techniques that have been employed for the extraction, preconcentration, and determination of flavonoids in natural, pharmaceuticals, and dietary supplements highlighting their advantages and disadvantages. In addition, the trend of coupling flow systems to chromatographic techniques for an automatic flavonoids analysis.
2. A rapid and high-sensitive high-performance liquid chromatographic method has been developed for the determination of hesperidin, diosmin, and their related natural impurities, hesperetin, and diosmetin, in bulk and pharmaceutical formulations. The sensitivity of the obtained method was able to detect, for the first time, the impurity "hesperetin" in studied drugs.

3. Micro-extraction system based on the lab-on-valve platform coupled with a high-performance liquid chromatographic system and ultraviolet diode array detection has been established for the automatic extraction and determination of hesperidin and diosmin in citrus edible part, hand squeezing juice, and commercial products. The proposed hybrid system provides a fully automated system for the determination of these two flavonoids located in citrus samples.
4. A solid-phase extraction resin support fabricated using 3D printing technology has been employed into the hyphenation system between a multi-position valve system and high-performance liquid chromatographic tool for the simultaneous extraction and determination of six flavonoids (hesperidin, hesperetin, naringin, naringenin, diosmetin, and tangertin) in citrus external peel (flavedo). The fabricated device is an effective solution to overcome the main drawback of the using the traditional extraction column into the flow system.
5. Sensitive and selective HPLC method for the determination of rutin, quercetin, kaempferol, and isorhamnetin in several dietary supplements and pharmaceutical formulations such as syrups, tablets, capsules, and oral ampoules has been developed. The selectivity of the developed method was able to separate similar flavonoids structures in their natural complex matrices without the need for an advanced sample pretreatment procedure.

RESUMEN

El creciente conocimiento de la enorme importancia de los flavonoides como potentes sustancias antioxidantes ubicadas en plantas, alimentos y sus productos manufacturados, así como su comportamiento farmacológico en la prevención y tratamiento de numerosas enfermedades en el cuerpo humano y animal, han llevado a incrementar el interés del desarrollo de muchos productos en los mercados tales como productos farmacéuticos y suplementos dietéticos, además de productos ricos en flavonoides como bebidas, mermeladas y zumos.

Este rápido crecimiento de la industria comercial de flavonoides necesita el ajuste de la calidad de estos productos, detectando la presencia de impurezas, la degradación y la concentración real de los compuestos de interés.

La complejidad de las matrices naturales y la sensibilidad de los flavonoides a los factores de procesamiento, tales como la temperatura, el mecanismo de recolección y las condiciones de almacenamiento, plantean dificultades en la extracción y determinación precisa de estos compuestos, especialmente cuando los analitos están presentes a niveles de trazas como en el caso de impurezas o productos de descomposición de los analitos. En este sentido, los pasos de pretratamiento de la muestra son casi obligatorios antes de la aplicación de la técnica de detección. La nueva tendencia se centra en el desarrollo de métodos de pretratamiento de muestras más amigables con el medio ambiente. Es por ello que las técnicas de microextracción y la automatización del procedimiento de pretratamiento de muestras son la tendencia de estos días. Las técnicas de análisis en flujo pueden facilitar estos procedimientos logrando métodos más eficientes, rápidos, robustos económicos y seguros para los trabajadores y el medio ambiente.

En esta tesis se han desarrollado diferentes métodos automáticos de microextracción basados en técnicas en flujo para los tratamientos de muestras de matrices de flavonoides, y métodos de cromatografía líquida de alta resolución (siglas en inglés HPLC) para la

determinación de flavonoides en sus fuentes naturales y productos comerciales. Los sistemas basados en flujo, en particular, se basan en el uso de inyección en válvula solenoide, válvulas de múltiples posiciones y de múltiples jeringas conectadas a sistemas HPLC con detección ultra violeta y arreglo de diodos (siglas en inglés DAD) a fin de desarrollar métodos analíticos totalmente automatizados. El potencial de las técnicas analíticas en flujo se ha demostrado mediante el pretratamiento de muestras en micro columnas de extracción en fase sólida insertadas en sistemas Lab-On-Valve (LOV, laboratorio en válvula), así como a través del uso de la tecnología de impresión 3D para la elaboración de dispositivos de extracción en fase sólida entre otros accesorios. Los sistemas desarrollados han sido implantados para la extracción, preconcentración y determinación de flavonoides en productos farmacéuticos, complementos dietéticos, frutos cítricos (incluyendo pulpa, zumos y piel) y sus productos comerciales. El acoplamiento de las técnicas de flujo y HPLC han permitido desarrollar sistemas analíticos completamente automatizados rápidos, selectivos y robustos.

Los trabajos desarrollados incluidos en esta tesis se enumeran a continuación:

1. Una revisión exhaustiva donde se expone el estado del arte, en cuanto a tratamientos de muestra y técnicas de análisis que han sido utilizadas en la extracción, preconcentración y determinación de flavonoides en fuentes naturales, farmacéuticas y suplementos alimentarios, destacando sus ventajas y desventajas, así como también las últimas tendencias en el acoplamiento de sistemas en flujo con cromatográficos para el desarrollo de métodos totalmente el línea
2. Un método rápido y selectivo utilizando cromatografía líquida en alta resolución para la determinaciñon de hesperidina, diosmina, hesperitina y diosmetina en formulaciones farmacéuticas. La sensibilidad del método permitió la cuantificación de la hesperitina por primera vez.
3. Un sistema totalmente en línea de extracción en fase microsólida en un dispositivo LOV acoplado a un cromatógrafo líquido con DAD utilizado para la determinación de hesperidina y diosmetina en pulpa, zumo y productos comerciales de diversos cítricos.

4. Un sistema en línea para la determinación de seis flavonoides: hesperidina, diosmina, naringenina, diosmetina y tangertina en pieles de frutos cítricos, utilizando para la extracción en fase sólida un dispositivo impreso en 3D recubierto con resina C_{18}, acoplado a un cromatógrafo líquido con DAD, mediante un sistema en flujo con una valvula multiposición. Dicho sistema permitió resolver los inconvenientes que presenta analizar este tipo de muestras de forma manual, dando lugar a resultados más exactos y precisos.
5. Un método selectivo y sensible para la determinación de rutina, quercetina, kaempferol e isorhamnetina en diversos suplementos dietéticos y formulaciones farmacéuticas. La selectividad del método permitió separar flavonoides de estructuras similares, mediante un tratamiento de muestra sencillo.

RESUM

El creixent coneixement de l'enorme importància dels flavonoides com a potents substàncies antioxidants ubicades en plantes, aliments i els seus productes manufacturats, així com el seu comportament farmacològic en la prevenció i tractament de nombroses malalties en el cos humà i animal, han portat a incrementar l'interès del desenvolupament de molts productes en els mercats, com ara productes farmacèutics i suplements dietètics, a més de productes rics en flavonoides, com begudes, melmelades i sucs.

Aquest ràpid creixement de la indústria comercial de flavonoides necessita l'ajust de la qualitat d'aquests productes, detectant les impureses, la degradació i la concentració real dels compostos interessats. No obstant això, la complexitat de les matrius naturals i la sensibilitat dels flavonoides als factors de processament, com ara la temperatura, el mecanisme de recollida i les condicions d'emmagatzematge, plantegen dificultats en l'extracció i determinació precisa d'aquests compostos, especialment quan els analits d'interès són presents a nivells de traces com en el cas d'impureses o els productes de descomposició dels analits. En aquest sentit, els passos de pretractament de la mostra són gairebé obligatoris abans de l'aplicació de la tècnica de detecció. La nova tendència se centra en el desenvolupament de mètodes de pretractament de mostres més amigables amb el medi ambient.

Les tècniques de micro-extracció i l'automatització del procediment de pretractament de mostres són la tendència d'aquests dies. Les tècniques d'anàlisi de flux poden facilitar aquests procediments aconseguint métodes eficients, ràpides, econòmiques i més segurs per als treballadors i el medi ambient.

En aquesta tesi s'han desenvolupat diferents mètodes automàtics de micro-extracció basats en tècniques de flux per als tractaments de mostres de matrius de flavonoides, i mètodes de cromatografia líquida d’alta resolució (sigles en anglès HPLC) per a la determinació de flavonoides en les seves fonts naturals i productes comercials. Els sistemes basats en flux,

en particular, es basen en l'ús d'injecció en vàlvula solenoides, vàlvules de múltiples posicions i de múltiples xeringues connectades a sistemes HPLC amb detecció ultraviolada d'arregle de díodes (sigles en anglès DAD) per a l'explotació de mètodes analítics totalment automatitzats. El potencial de les tècniques analítiques de flux s'ha demostrat mitjançant el desenvolupament de sistemes de pretractament de mostres que es beneficien de les excel·lents característiques de la columna de micro-extracció inserida en un LAB-On-Valve (LOV, laboratori en vàlvula), la vàlvula de múltiples posicions i també de la tecnologia d'impressora 3D per a l'elaboració de diversos accessoris. Els sistemes desenvolupats han estat implantats per l'extracció, preconcentració i determinació de flavonoides en productes farmacèutics, complements dietètics, cítrics, espremedores manuals de cítrics i els seus productes comercials. Tots els sistemes desenvolupats han demostrat una reducció significativa dels consums de mostres i dissolvents, i millorat el rendiment de la mostra, la sensibilitat i la reproductibilitat de l'anàlisi en comparació amb els mètodes clàssics anteriors. L'acoblament de les tècniques de flux i HPLC han permès desenvolupar sistemes analítics completament automatitzats ràpids, selectius i robustos. A més, s'ha realitzat una revisió exhaustiva per comparar els mètodes desenvolupats a la tesis amb els treballs que hi ha a la literatura.

Els treballs desenvolupats inclosos en aquesta tesi s'enumeren a continuació:

1. Una revisió exhaustiva on s'exposa l'estat de l'art, quant a tractaments de mostra i tècniques d'anàlisis que han estat utilitzades en l'extracció, preconcentració i determinació de flavonoides en fonts naturals, farmacèutiques i suplements alimentaris, destacant els seus avantatges i desavantatges, així com també les últimes tendències en l'acoblament de sistemes en flux amb cromatogràfics per al desenvolupament de mètodes totalment el línia
2. Un mètode ràpid i selectiu utilitzant cromatografia líquida en alta resolució per a la determinació de hesperidina, diosmina, hesperitina i diosmetina en formulacions farmacèutiques. La sensibilitat del mètode va permetre la quantificació de la hesperitina per primera vegada.

3. Un sistema totalment en línia d'extracció en fase microsòlida en un dispositiu LOV acoblat a un cromatògraf líquid amb DAD utilitzat per a la determinació de hesperidina i diosmetina en polpa, suc i productes comercials de diversos cítrics.
4. Un sistema en línia per a la determinació de sis flavonoides: hesperidina, diosmina, naringenina, diosmetina i tangertina en pells de fruits cítrics, utilitzant per a l'extracció en fase sòlida un dispositiu imprès en 3D recobert amb resina C_{18}, acoblat a un cromatògraf líquid amb DAD, mitjançant un sistema en flux amb una valvula multiposición. Aquest sistema va permetre resoldre els inconvenients que presenta analitzar aquest tipus de mostres de manera manual, donant lloc a resultats més exactes i precisos.
5. Un mètode selectiu i sensible per a la determinació de rutina, quercetina, kaempferol i isorhamnetina en diversos suplements dietètics i formulacions farmacèutiques. La selectivitat del mètode va permetre separar flavonoides d'estructures similars, mitjançant un tractament de mostra senzill.

LIST OF PUBLICATIONS

This thesis is presented as a thesis by publication. The publications included in this thesis are:

[1] Mohamad Subhi Sammani, Sabrina Clavijo, Lindomar Portugal, Ruth Suárez, Hassan Seddik, Víctor Cerdà, *Use of multiresponse statistical techniques to optimize the separation of diosmin, hesperidin, diosmetin and hesperitin in different pharmaceutical preparations by high performance liquid chromatography with UV-DAD,* Talanta 167 (2017) 695-702. DOI: 10.1016/j.talanta.2017.02.069. IF: 5,339

[2] Mohamad Subhi Sammani, Sabrina Clavijo, Alba González, Víctor Cerdà, *High-Performance Liquid Chromatographic Method for the Simultaneous Determination of Four Flavonols in Food Supplements and Pharmaceutical Formulations,* Analytical Letters 52 (2019) 1298-1314. DOI: 10.1080/00032719.2018.1536138. IF: 1.467

[3] Mohamad Subhi Sammani, Sabrina Clavijo, Alba González, Víctor Cerdà, *Development of an on-line lab-on-valve micro-solid phase extraction system coupled to liquid chromatography for the determination of flavonoids in citrus juices,* Analytica Chimica Acta 1082 (2019) 56-65. DOI: 10.1016/j.aca.2019.06.032. IF: 5.977

[4] Mohamad Subhi Sammani, Sabrina Clavijo, Víctor Cerdà, *Recent, advanced sample pretreatments and analytical methods for flavonoids determination in different samples,* Trends in Analytical Chemistry 138 (2021) 116220. DOI: 10.1016/j.trac.2021.116220. IF: 9.801

[5] Mohamad Subhi Sammani, Sabrina Clavijo, Andreu Figuerola, Víctor Cerdà, *3D printed structure coated with C_{18} particles in an online flow system coupled to HPLC-DAD for the determination of flavonoids in citrus external peel*, Microchemical Journal 168 (2021) 106421. DOI: https://doi.org/10.1016/j.microc.2021.106421. IF: 3.594

[6] Mohamad Subhi Sammani, Víctor Cerdà**,** *Sample Pre-treatment and Flavonoids Analytical Methodologies for the Quality Control of Food and Pharmaceutical Matrices*, The Book of Flavonoids, NOVA publisher, USA, (2021) pp.117. (Submitted chapter)

LIST OF CONFERENCES

The conferences that are participated due to the development of this thesis are:

[1] *An Optimized and validated HPLC-DAD method for determination of four flavonoids in pure form and in pharmaceutical preparations*, presented as a poster in the **20th International Conference on Flow Injection Analysis and Related Techniques**, Palma de Mallorca, Spain (2nd - 7th October 2016).

[2] *An Optimized and validated HPLC-DAD method for determination of four flavonoids in pure form and in pharmaceutical preparations***,** presented as a poster in the **VIII Workshop de Quimiometria in Campus da UFBA**, Ondina, Brazil (24th - 27th Abril 2017).

[3] *Development of on-line LAB-ON-VALV solid phase extraction (LOV-SPE) coupled with HPLC-DAD method for determination of flavonoids in Citrus juices*, presented as a poster in the **19th International Symposium on Advances in Extraction Technologies**, Santiago de Compostela, Spain (27th - 30th June 2017).

[4] *High performance liquid chromatographic method for simultaneous determination of four flavanols in different food supplements and pharmaceutical formulations***,** presented as a poster in the **21st International Conference on Flow Injection Analysis and Related Techniques**, Saint-Petersburg, Russia (3th - 8th September 2017).

[5] *An online LOV-µSPE coupled with HPLC-DAD for determination of flavonoids in citrus juices***,** presented as a poster in the *14th* **International Conference on Flow Analysis**, Bangkok, Thailand (2nd - 7th December 2018).

[6] *A 3D printed C_{18} disc used for online solid-phase extraction through a flow injection system coupled to HPLC-DAD for the determination of flavonoids in citrus flavedo*, presented as a poster in the **23rd International Symposium on Advances in Extraction Technologies**, Alicante, Spain (29th - 2nd July 2021).

CHAPTER 1

INTRODUCTION

In this chapter, a general overview of flavonoids in nature (fruits, medicinal plants) and their distribution in commercial products (juices, beverages, food supplements, and pharmaceutical formations) is presented, as well as the biological and pharmacological behaviour of flavonoids in plants and the human body. In addition, the main traditional and modern analytical techniques and sample pretreatment techniques which have been used for flavonoids determination in citrus fruit (edible and inedible parts), food supplement, and pharmaceutical drugs are extensively discussed. Finally, a detailed description of flow techniques and their evolution, together with their role in the automation of the sample pretreatment and its combination with separation techniques are shown.

1.1. Flavonoids

In 1930, a new flavonoid was isolated from the orange by Szent-Gyorgyi and was classified as vitamin P [1]. Later, this substance was known as an individual chemical group namely: flavonoids. About 9000 different compounds of flavonoids occur naturally in the plant kingdom as a larger secondary metabolism bio-compounds [2]. All of them share 15 carbon atoms in the phenylbenzopyran (C_6-C_3-C_6) framework [3], which consists of two benzene rings (A and B) linked through a heterocyclic pyran ring (C) (Figure 1.1).

Figure 1.1 General flavonoids skeleton.

The classification of flavonoids is based on the oxidation and saturation degree on position 3 and/or position 4 in the C-ring and can be distinguished into many major subclasses: flavanes, flavones, isoflavones, flavanones, flavanonols, flavanols, flavonols, anthocyanidins, and chalcones (Figure 1.2).

Figure 1.2 The major subclasses of flavonoids

The variation of flavonoids structure mainly regards their hydroxylate in positions 3, 5, 7, 3′, 4′ and 5′ which are frequently found methylated, prenylated, acetylated, sulphated, or glycosylated [4]. Naturally, flavonoids can reside in the plants as aglycone, or more stable form “glycoside” linked with one sugar molecule or more [5].

1.1.1. Flavonoids in nature

Flavonoids occur only in the plant kingdom and commonly are present in the vegetables, fruits, grains, and especially in the green leaves, located in their cell vacuoles in all plant parts (flowers, leaves, roots, seeds, and fruits). These substances take place as the most important bioactive compounds in the plants regarding their direct responsibility for the colour and aroma of fruits and flowers, playing role in the interaction between the plant and the environment, and considering the first line of defense against foreign surrounding factors and pollution as UV-filters, temperature acclimation, frost hardiness, and antibacterial functions [6].

1.1.2. Flavonoids in the human body

Flavonoids are not only playing important roles in the plants growing life, but also have countless biological and pharmacological activities in the human and animal bodies, which cannot synthesize biologically [7]. These activities range between prevention, protection, treatment, and reduction of the risk of numerous chronic diseases [8]. The great property of almost all flavonoids is a powerful antioxidant capacity to protect the body against reactive oxygen species (ROS) and free radicals that threatened by the damage of body cells and tissues continuously [9]. Thus prevent several human diseases such as chronic inflammatory disorders, cancer, atherosclerosis, ischemic injury, ageing, and neurodegenerative diseases [10]. Also, they show bio-activities such as antiulcer [11], antidiabetic [12], antitumor [13], vasorelaxant agent [14], antimicrobial activity such as antibacterial [15], antifungal [16], and antiviral activity, such as against Herpes simplex virus type 1 and 2 [17] and HIV-1 and HIV-2 [18], Severe Acute Respiratory Syndrome (SARS), and Middle-East Respiratory Syndrome-Coronavirus (COV) [19] and much more others [8].

Regarding all these considerable actions, these phytochemical compounds should be included in the human and animal daily diet, focusing on the food-rich flavonoids or on

food supplements-rich flavonoids that facilitate the obtaining of the daily required quantity of these antioxidant compounds.

1.1.3. Flavonoids in food and commercial products

Nutraceutical is a term that defined food or dietary supplements that provide medical or health benefits, including the prevention and treatment of diseases [20]. The major active nutraceutical ingredients in plants are flavonoids. Regarding the great benefits of flavonoids in both humans and animals mentioned before in section 1.1.2, they have been relying on them as the main part of their daily diet. Flavonoids are found mainly in herbs, citrus fruits, onions, legumes, parsley, berries, oils, wines, and beverages.

The distribution of flavonoids sub-classes in different types of fruit is not the same, also their concentration is dissimilar in different parts of the same kind of fruit. For instance, flavanones are the main flavonoids sub-classes found generally in the albedo of citrus fruits, while flavones and polymethoxy flavones (PMFs) are the major flavonoids in flavedo [21]. Otherwise, several manufactured products can be found from the albedo and flavedo of citrus fruits, such as commercial natural juices, nectars, jams, food flavours, active pharmaceutical ingredients (API), and reference analytical standard materials. Moreover, flavonoid substances that are already present in the original source, their quantity might be changed affecting by the product process, such as the applied pressure, temperature, storage temperature, and processing and storage time [22]. The distribution of flavonoids in albedo, flavedo and commercial products of citrus fruits will be discussed extensively in chapters 5 and 6.

On the other hand, the recommended daily intake dose of flavonoids for an average human adult is between 70 and 170 mg/day [23]. This recommended dose still difficult to daily intake because of the low concentration of flavonoids in nature, as well as their low absorption in the site of action inside the human body. Ensuring the required daily amount of these antioxidants is obtained, the dependence on food supplements and pharmaceutical forms have increased in recent years.

Flavonoids-food supplements (FFSs) are the concentrated natural source of flavonoids that are provided in pharmaceutical dose forms (tablets, capsules, syrup, or oral ampoules) facilitating the obtainment of the daily recommended flavonoids. FFSs can be pure extracted flavonoids, such as quercetin (QU) (3,3',4',5,7-pentahydroxyflavone), rutin (RU) (quercetin 3-rutinoside), hesperidin (HES) (hesperetin-7-rutinoside), and diosmin (DIO) (diosmetin 7-neohesperidoside) that exist individually or combined, in high concentration that might reach up to 500 times higher than they can found in their natural sources. Hence, the dried powder of edible plant-rich flavonoids is another important source of FFSs. The most famous medicinal plant used in FFSs is Ginkgo biloba leaves (GBL) that highly contains a concentration of flavonols aglycons mainly: QU, kaempferol (KA) (3,4',5,7-tetrahydroxyflavone), isorhamnetin (IS) (3,4',5,7-tetrahydroxy-3'-methoxy-flavone), and their derivatives glycosides.

Pharmaceutical flavonoids have been founded to treat many diseases. To date, regarding the DrugBank database [24], there are 11 approved flavonoids as drugs. In addition to, 33 and 50 ones in the investigation and experimental stages. For instance, the most famous drug used for chronic venous insufficiency disease (CVI) treatment contains DIO, normally in a dose of 500 mg, or combined with another flavonoid as HES, added in 10% *w/w* [25], these both flavonoids are extracted from citrus peel especially orange peel.

Therefore, flavonoids considered as a food and plant quality indicator. Controlling the quality of flavonoids’ natural and commercial products and detecting the exact concentration of each existing flavonoid in these matrices are essential to improve agricultural production, knowing the biological influence of each individual flavonoid located in the sample and their impact concentration on the host body. The variety of natural sources of flavonoids and their commercial products, and the difference between their concentration in nature lead to there is no suitable analytical method for all flavonoids and all matrices. Thus, the continuous development of suitable analytical methods that able to determine the interested flavonoids without endogenous negative effect on the accuracy of the analysis and reducing the required time, cost, and environmental pollution are the main aim in the analytical chemistry field.

1.2. Analytical methods for flavonoids determination

Knowing the existing flavonoids' classes and their concentration is important to exactly specify the therapeutic and pharmacological behaviour of these sources, in addition, estimating their quality that is related directly to the type and the concentration of these presented flavonoids.

Numerous analytical methodologies have been developed in the last decades took into account the vast difference characterization of flavonoids compounds in nature, such as the molecular weight, polarity, bonded compounds, differentiate their ability to dissolve in aqueous and organic solvents and complexity of the matrices. Furthermore, the goal of the analysis and the concentration of target flavonoids in the sample also play a significant role in the choosing of the appropriate analysis method. In general, flavonoids determination methods based on spectrophotometric (STs) and chromatographic (CTs) techniques. STs usually used for quick, simple, and low-cost estimation of total flavonoids content, total phenolic content, and total biological activity of fruits, plants, and pharmaceutical products as the case of concern [26]. While the CTs are a separative, sensitive and selective analysis technique for an individual flavonoids estimation in all their sources [27].

The most common analytical techniques used for flavonoids determination in their natural and commercial sources are discussed in the article review attached at the end of this chapter.

1.2.1. Spectrophotometric Assays

Several STs have been used for flavonoids determination for decades. Both absorption and vibrational spectroscopies have been employed for this purpose. In absorption ones, ultraviolet/visible radiation spectroscopy (UV/VIS) [26, 28], fluorescence spectroscopy (FL) [29], and nuclear magnetic resonance spectroscopic (NMR) [30] are the most common STs for flavonoids evaluation in their sources, while X-ray crystallography (XR) [31], and circular dichroism spectroscopy (CDS) [32] are the less often used in this field. In

vibrational spectroscopy, near-infrared (NIR) [33], Raman spectroscopy (RS) [34], terahertz (THz) [35], and hyperspectral imaging spectroscopy (HSI) [36] have been widely employed for flavonoids structural elucidation and prediction concentration. Along with the important application of Mass spectroscopy (MS) [37] in the field of flavonoids estimation has not been messed.

However, the principle flavonoids framework has two important absorption bands I (240-285 nm) from the adsorption of ring A and II (300-560 nm) from the absorption of ring B (Figure 1.1) [38]. Almost all flavonoids have at least one maximum adsorption wavelength in the range of these bands. For that, UV spectroscopy is a widespread technique that has been carried out to evaluate the flavonoids in their different matrices.

This technique is characterized by its fast, cost-effectiveness, ease of use, and not required advanced sample preparation technique. On the contrary, overlapping absorption of flavonoids linkage groups, the close of the maximum wavelength absorption of the most flavonoids groups and unexpected absorptions of the related matrix substances make this technique non-selective for individual flavonoids determination. Hence, it still useful for a quick and cheap evaluation of total flavonoids and total biological capacity study, especially, when the knowing information of individual flavonoids is not the target [39]. In practice, several works have been developed using the direct UV technique for the determination of total flavonoids directly after dissolving and filtrating the sample [26, 27, 40].

To increase the sensitivity of the analysis, the formation of coloured complex compound between flavonoids and shifting reagents is frequently used. Complexing agents such as aluminium chloride ($AlCl_3$), 2,4-dinitrophenylhydrazine [41], 4-aminoantipyrine [42], and oxidation with Cu (IV) [29] are employed for these reactions. The flavonoids-Al^{3+} complex is the most common coloured compounds implemented for the determination of the total flavonoid in plant samples using methanol (MeOH) [43], ethanol [44] or alkaline medium [45]. Nevertheless, the complex formation is not selective and several flavonoids group can be reacted with these shifting substances. As an example, all anthocyanidins can form an

intensive blue compound chelating with Al^{3+}[46]. Regarding that, this method is only utilized for total flavonoids estimation in natural samples.

FL has been also employed to determinate flavonoids. This technique is based on the natural fluorescence behaviour of some flavonoids' groups, such as isoflavonoids, PMFs, and flavonoids with a free hydroxyl in C_3 position (Figure 1.1). In these groups, FL is important to increase the selectivity and the sensitivity of their determination in complex samples. Furthermore, non-fluorescent flavonoids also can be determined by FL after their reacting with high-fluorescent chelating ions, such as aluminium (Al^{+3}) [47], terbium (Tb^{+3}) [48] or copper (Cu^{+2}) [29] to form a fluorescent complex. This fluorescent complexation formation required additional sample preparation time that can reach up to 60 min, plus this formation is not selective to individual flavonoids, but might form complexation with all flavonoids in the same classification group. Though FL notably improves the sensitivity and the selectivity of the detection of flavonoids, and it has been widely attained for the determination of total flavonoids in plants, foods, and human fluids.

Recently, NMR has been applied in flavonoids research as a powerful technique used to elucidate unknown structure and properties of flavonoids. In this field, several NMR experiments have been used i.e. one dimensional NMR ^{1}H and ^{13}C, homonuclear 2D NMR, heteronuclear 2D NMR, nuclear overhauser effect (NOE), and solid-state NMR [30]. The main advantages of NMR are the quickness, the ability to determinate all sample kinds (gas, liquid, and solid), no sample preparation is required, and quantitative and qualitative analysis of known and unknown flavonoids in the sample can be carried out [49].

MS is the highly selective and sensitive spectroscopic identification technique [50]. In the case of flavonoids, these compounds are polar, nonvolatile and thermally labile, which limit the application of MS without a prior derivatization process. These limitations can be overcome by the hyphenation with chromatographic techniques [51].

Other less frequency absorption spectroscopic technique that has been utilized for flavonoids elucidation are X-ray and CDS. The main limitation application of these techniques are these substances only form crystals in sporadic conditions and the

crystallization state is essential in XR to be performed [52], while CDS is unable to provide quantitative information and just used for quantitative elucidation of flavonoids [53].
Vibrational spectroscopic techniques are a non-invasive, non-destructive, very fast operation, almost not required previous sample treatments, can be implemented quantitively and qualitatively, accurate, chemical-free, and environmentally friendly methods. All types of vibrational spectroscopic techniques have been effectively utilized for the analysis of flavonoids in food samples [54].

In this field, IR and RS are the most utilized techniques for the elucidation of new flavonoids molecules in natural samples. The difference between them is, IR range is very sensitive to the OH bands stretching vibration, thus the determination of flavonoids in aqueous samples faced a lot of overlapping bands and make the detection of flavonoids difficult, while RS is not sensitive to the hydrogen bands so it can give accurate results with the existing of water into the sample [55].

HIS is less often applied, mainly, because of its long processing and classification time that required high computer hardware and software capabilities, which means additional high cost [56]. THz is similar to IR regarding the sensitivity to the OH bands which is not suitable for the determination of flavonoids in food that the content of the moisture is high [57].

In general, the main drawbacks of the application of vibrational spectroscopic techniques for the quantitative predictions of flavonoids are the required to special chemometric algorithms applications to extracted and processed the obtained raw data, however, these programmes are expensive and required specialists in the field of vibrational spectroscopy who can read, extract, and compare the bands in the spectral data.

Particularly, absorption spectroscopic techniques attain good a sensitivity for screening and routine analysis of total flavonoids in pharmaceutical and food industries enhancing the labour-time and cost-effectiveness. However, their weak differentiation between flavonoids substances (non-selective technique), in addition, the need for additional sample treatment

using reagents in some cases to form coloured or fluorescent complex make them an undesirable technique for selective determination of flavonoids. Otherwise, vibrational spectroscopic techniques are not direct estimation techniques and required intensive knowledge in the field, but all applied techniques provide highly accurate results, quick process, and solvent-free applications.

1.2.2. Chromatographic Assays

Several CTs techniques have been applied for individual flavonoids determination in all sources such as paper chromatography (PC), thin-layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE), liquid chromatography (LC) especially high-performance liquid chromatography (HPLC), and supercritical fluid chromatography (SFC).

The requirement of sample treatment, the sensitivity, and the selectivity of each technique are different. PC and TLC are the cheapest CTs techniques applied for flavonoids determination, but their low sensitivity and selectivity due to the difficultly to differentiate the overlapping bands and the poor detectors diversity makes them not able for selective flavonoids separation [58]. Furthermore, GC achieves good sensitivity and selectivity, but it is occasionally applied for flavonoids estimation because of the need for an additional laborious sample treatment for changing the flavonoids to a volatile shape [59]. CE is a novel separative technique for flavonoids achieving excellent selectivity, high resolution, and short analysis time [60]. Its low reproducibility, higher detection limit, and the cost of CE equipment are overcome by HPLC with its diverse stationary phase and detectors. HPLC attains high selectivity, reproducibility, and sensitivity for flavonoids separation and determination [60]. The played role of each one of these techniques on the flavonoid determination field is described in the following sections.

1.2.2.1. Paper and thin-layer chromatography

PC and TLC have been applied for simple and inexpensive isolation and purification methods of flavonoids from natural matrices, such as fruits and vegetables [61]. The main disadvantages of these methods are the difficulty to separate and identify the overlapping spots, its detection limit is quite high, affected with the temperature and the humidity as its direct contact with the environmental factors (open chromatography), and the impossibility of its coupling to detection systems such as MS, NMR, and FT-IR [58]. Improving the sensitivity, selectivity of flavonoids analysis, and reducing the analysis time, and solvents consumption than traditional TLC, two-dimensional-TLC (2DTLC) [62] and high performance-TLC (HPTLC) [63] have been employed.

More recently, the innovative combination of TLC and vibration spectroscopic techniques provides rapid, accurate, and sensitive determination technique of flavonoids. In practice, TLC-NIR [64], and 2D-TLC-Raman [65] achieved better sensitivity from the traditional TLC, as well as better detection efficiency and less analysis time.

1.2.2.2. Gas chromatography

GC is the alternative separation technique for flavonoids determination in their different sources. Despite the high resolution and high sensitivity of GC, it is not the main technique used for flavonoids determination because of the non-volatility properties of flavonoids and their high own boiling point (more than 300 °C). In this sense, converting these compounds to volatile derivative shapes and enhance the thermal stability required the applying of a derivatization process before GC injection [59]. The boiling point of studied flavonoids in this thesis will be presented in Tables 6.2 and 7.1. In spite of some GC applications have been done without a prior derivatization process [66], but most works have been carried out silylation, acetylation or methylation as common derivatization reagents of flavonoids [59]. The derivatization process is the main limitation of using GC for the determination of flavonoids. It is tedious, consuming time and solvents, increases the detection error by forming multiple derivatives due to the numerous hydroxyl groups of flavonoids [60]. On

the other hand, the non-polar fused stationary phase has been used widely for flavonoids separation, such as 100% dimethylpolysiloxane [66], 5% phenyl 95% dimethylpolysiloxane [67], and 1,4-bis(dimethylsiloxy) phenylene dimethylpolysiloxane [68]. While polar and highly polar stationary phases are less frequently utilized for this purpose corresponding to the polarity of flavonoids [69]. Then, the separated flavonoids have been detected by means of the MS as the most popular detection system coupled to GC, followed by flame ionization detector (GC-FID) [66].

1.2.2.3. Capillary electrophoresis

CE is a quite novel separation technique that has been employed for flavonoids determination in natural sources due to its high-resolution power, low sample and solvents consumptions, quick analysis, and the charging of flavonoids molecules in usually basic pH range [70]. Among different modes of CE, three modes have been used for flavonoids analysis, capillary zone electrophoresis (CZE) [71], micellar electrokinetic capillary electrophoresis (MECE) [72], and capillary electrochromatography (CEC) [73]. Flavonoids have been successfully detected by several detectors coupled with CE i.e. UV [74], electrochemistry detector (ED) [75], fluorescence detector (FL) [76], amperometric detector (AD) [77], and MS detector [78]. Nevertheless, the lower sensitivity of CE, trends to sample overload, and less quantitative reproducibility of CE, besides, the long equilibration time of the used column make it the second option behind HPLC for flavonoids analysis [79].

1.2.2.4. High-performance liquid chromatography

HPLC is the most convenient technique that has been employed for the simultaneous separation and determination of flavonoids, without the need for the preliminary derivatization, with high sensitivity, selectivity, and reproducibility.

In the separation stage, both normal (NP) and reversed phase (RP) have been applied. In this field, NP is not often used for flavonoids separation because of the polarity of these

substances that have hydroxyl groups, besides, the linkage polar groups such as saccharide molecules. Nevertheless, some works were developed to separate less polar flavonoids, such as aglycons by using a silica column [80], polystyrene divinylbenzene [81], diol [82], and cyanopropyl-bonded phase after flavonoids acetylation [83]. Generally, NP is not suited to flavonoids separation due to their limited solubility in the typical NP mobile phase, the length of analysis time, and the probability of the polar coextracted matrix substances irreversibility retained inside the column [84]. Thus, RP is the best alternative for flavonoids separation, usually is carried out by using C_8 and C_{18} columns [85, 86] regarding their power in separation different polarities of all sub-classes of flavonoids during short analysis time. This separation often conjugated with an acidified aqueous mobile phase in the case of maintaining the non-charge molecules of flavonoids and MeOH or/and acetonitrile (ACN) as organic modifier [86, 87].

Recently, two novel column technologies have been developed and used effectively for flavonoids separation from their complex samples. Fused core® (FCC) and monolithic column (MC) technologies are the ones that can reduce diffusion path peak and mass transfer, thus, increase the separation power, the selectivity, and decrease the analysis time and the generated backpressure comparing to the packed traditional columns. FFC consists of a fused-core silica substrate with a layer of coated porous silica. It has been applied for ten flavonoid aglycones determination in plant materials using FCC-C_{18} [88], and four glycosides in food supplements using FCC-amid [89]. MC composes of a porous silica rod. In the literature, MC has been widely used for flavonoids separation from different sources [90, 91].

Several detectors coupled with HPLC have been attained for flavonoids identification. UV/VIS detector is the most favourable detector for flavonoids determination due to the phenolic rings of their skeleton since effectively absorb the UV radiation as that detailed in section 1.2.1. This detector is relatively cheap, easy to use, and can be applied for all sub-classes of flavonoids [92]. FL detector is rarely used for flavonoids determination since only a few flavonoid groups have a slightly native fluorescence [93]. Non-fluorescent flavonoids also can be detected in FL after online post-column derivatization with chelating

ions [94], resulting in a higher detection level of these flavonoids as that mentioned in section 1.2.1. MS detector has been also employed for flavonoids detection. MS provides high sensitivity and extreme specificity in the detection of flavonoids [30]. It is widely utilized for the detection of trace concentration in natural or biological samples, as well as in the case of the reference standards are not available. Moreover, some flavonoids are electroactive compounds [95], which can be easily detected sensitively and selectively employing a coulometric detector (CD) [96] and AD [97]. Recently, NMR has been coupled to HPLC for the identification of unknown structures of flavonoids [98]. This combination significantly improves the prediction and elucidation of flavonoids.

Moreover, HPLC derivative techniques have been developed to enhance the separation power and improving the peak capacity. For instance, 2-dimensional liquid chromatography (2DLC) that depends on the passing of the sample through two separation columns, hydrophilic interaction liquid chromatography (HILIC) that uses polar stationary phase and mobile phase to separate polar substances [99], and the combination between these techniques (HILIC×RP-HPLC) have been used effectively for flavonoids analysis in their complex matrices [100] reducing the need to advanced sample pretreatments as the normal HPLC required [101].

The availability and flexibility of HPLC technique to separate flavonoids from different complexity matrices, and the diversity of its stationary phase and coupling detectors make the HPLC the dominant methodology to determinate flavonoids in all their sources with accurate results.

1.2.2.5. Supercritical fluid chromatography

SFC is very similar to HPLC, but CO_2 in supercritical conditions is the main component in the mobile phase. These conditions provide many advantages over HPLC as separation speed, lower viscosity of the mobile phase that means lower backpressure, high flow rate, and environmentally friendly technique [102]. Pure CO_2 as a mobile phase has a highly non-polar property, thus, the limitation to apply it for flavonoids separation. This

disadvantage can be overcome by adding a polar additive such as alcohols [103] or ionic liquids (ILs) [104]. Otherwise, the main limitations of this technique are the non-commercially available of different SFC polar columns and the requirement for special expensive vessel's material to maintain a high operational pressure in the system. Despite these limitations, SFC can effectively be applied to the analysis of flavonoids in short analysis time, high selectivity, and reliability [105].

1.3. Sample pretreatment

Sample preparation depends on the complexity of the sample, analytes concentration, chemical structure, and chemical behaviour of the interested substances. It is an important step that emphasizes the accuracy of the results and enhances the sensitivity of the analysis. However, the sample preparation step causes a major delay over all other analytical steps [106].

From the analytical point of view, sample preparation of flavonoids is quite diverse. Direct dissolving and filtering is the common sample pretreatment in the case of pharmaceutical formations where the flavonoids are present in a pure form as an API and with relatively adequate concentration to be detected [107], while more advanced sample preparation work such as solid-liquid extraction (SLE) [108], liquid-liquid extraction (LLE) [109], and solid-phase extraction (SPE) [110] are required in the case of more complex matrices, such as food, dried plants, and natural extract in food supplements. These natural matrices might contain several derivatives of each flavonoid and maybe the target flavonoid exists in a trace concentration. The article review posted at the end of this chapter detailed the advantages, disadvantages, and the difference between the required sample pretreatment techniques of natural flavonoids samples, such as medicinal plants, fruits, vegetables, their beverages, and also in the manufactured sources as pharmaceutical and dietary supplements formulations before their determination by different analytical techniques.

In this thesis, the details of the complexity of studied matrices, the faced difficulties in their sample preparation, sample pretreatment and analytical techniques that have been applied

for this purpose will be presented in the following chapters, such as pharmaceutical formations (Chapter 4), citrus juice, edible parts and their commercial products (Chapter 5), citrus external peel (Chapter 6), and food supplement formulations especially the dried powder of GBL (Chapter 7).

Sample pretreatment techniques can be divided into conventional extraction techniques (CET) and modern extraction techniques (MET). Conventional ones are the most consuming time and solvents and provide relatively low extraction yields such as Soxhlet (SE) [111], hot water bath extraction (HWBE) [112], heat reflux extraction (HRE) [113], and sonicated-assisted extraction (SAE) [114]. The moderns are the ones that have been developed for increasing the extraction efficiency, decrease the degradation of flavonoids, reduce time, cost and environmental pollution, as for example, pressurized liquid extraction (PLE) [115], microwave-assisted extraction (MAE) [116], LLE [117], SPE [118] and their derivatives, supercritical fluid extraction (SFE) [119], and high-speed counter-current chromatography (HSCCC) [120].

SE was the most common traditional extraction method for flavonoids because of its ease of use and its cheap equipment. Hence, its huge sample, solvent, energy, and time consumptions, and its low extraction yield due to the possibility to decompose the flavonoids concerning the high temperature and long extraction processing time [111] lead to it not often utilize these days. Likewise, HWBE, HRE and SAE also perform at high temperature for a long processing time that also affects negatively on the flavonoids' extraction efficiency. PLE and MAE have been developed to overcome the disadvantages of CETs by controlling the temperature and pressure, in addition, to carry out the extraction in a shorter time, that prevent the degradation of flavonoids [112, 116].

LLE has been developed to minimize solvents and time consumptions of CETs. LLE can be used directly to extract the aglycons using ethyl acetate or ACN containing a small amount of acid as a common LLE extraction solvent. If aglycones are the analytes of interest, the hydrolysis process is usually performed prior to LLE extraction using hydrochloric acid or formic acid due to ≥ 60 min of processing time and temperature between (80-100 °C) [90],

or enzymatically by β-glucuronidase [121] or β-glucosidase [122]. Observing that, the hydrolysis process consumes time and energy, and usually is carried out by refluxing solvents before the application of LLE [123]. It is worthy to mention that here, in the case of glycosides, the hydrolysis process must be avoided.

ILs are the innovative extraction solvents that have been used in LLE for flavonoids extraction regarding their unique features of tunable viscosity and miscibility with both water and organic solvents, high ionic conductivity, wide electrochemical windows, low volatility, high polarity, and chemical and thermal stability [124]. Alkylimidazolium type is the most common ILs that has been applied for both glycosides and aglycons extraction from plant and food matrices [125, 126].

SPE is an extraction technique that is effectively used for the extraction of flavonoids from their different complex matrices which observes wide applicability, short extraction time, minimize solvents consumptions, diversity available sorbents, ease of use, and ease its automation [110]. Several extraction resins have been used for flavonoids extraction, the most common one is C_{18} [118] with its own properties, such as strong hydrophobicity, non-polarity and functionality on a wide polarity range of flavonoids, Oasis HLB [110] has also been implemented for the same purpose as a reversed-phase capability with a special “polar hook” for enhanced capture of polar analytes and excellent wettability. Molecularly imprinted polymer (MIP) as a novel SPE sorbent has been applied practically to clean-up of flavonoids from endogenous matrix components [127] relating to its special characteristic of recognition sites for the target molecule. Even though, the presence of OH groups in flavonoids structure are the source of the hydrogen bonding and no localized electrostatic interactions between OH groups. This is the main drawback of MIP due to flavonoids extraction [128].

In order to miniaturize the sample pretreatment, improving the extraction recovery, decreasing the consumption of solvents, time, and cost, LLE and SPE derivatives techniques have been carried out successfully for flavonoids extraction, such as solid-phase and liquid-liquid micro-extraction (SPME) (LLME) [129], solid-phase and liquid-liquid

dispersive micro-extraction (SPDME) (LLDME) [130], matrix solid-phase dispersion extraction (MSPDE) [131], and hollow fibre liquid-phase micro-extraction (HF-LPME) [132]. The extensive details of these extraction techniques in the case of flavonoids extraction can be found in the article review attached at the end of this chapter.

SFE provides a relatively green extraction method of flavonoids from their natural sources. The use of supercritical fluids provides unique physical features than previous extraction techniques. The density and diffusivity values of these fluids affected by temperature and pressure changes in the system. The optimizing these two factors, these values can be located between liquid and gas properties thus highest extraction efficiency of flavonoids can be obtained. Furthermore, the need to apply high temperature and pressure in the system might lead to flavonoids decomposition [119].

HSCCC is a technique, similar to HPLC, used for the isolation and purification of flavonoids from their complex matrices such as the medicinal plant matrices. It depends on the separation of compounds by a fully liquid separation system without containing a solid stationary phase which avoiding the loss of the extracted substances due to irreversible interaction with the solid phase, hence provides completely sample recovery [133]. Unlike, the HSCCC equipment is expensive, and the procedure time of flavonoids isolation is varying and can be ranged between 100 min and 500 min [120, 134].

Nowadays, reducing the sample pretreatment processing time, miniaturize and automate this procedure is the main target of the analytical chemists for reducing the sample, solvents, time, and cost of the analysis, and protect the environment.

1.4. Advancement of flow analytical techniques

Flow analysis (FAT) are the techniques that depend on flowing the solvents and samples through a closed circuit, providing less samples and solvents evaporation phenomena, thus less losing sample, higher accurate results, less environmental pollution, and less contact with the toxic organic solvent and their vapour by the analysts. FATs in all founded

generations aim to automate the sample pretreatment procedure, the analytical procedure, reducing the solvents and samples needs per analysis and increasing the throughput of the analysis. FATs have been widely applied not only for environmental and radiochemical analysis, but also for food and biological ones.

Segmented flow analysis (SFA) was the first invented FAT. It mainly consists of a peristaltic pump (PP) for a continuous sample and reagent aspiration through a flexible plastic tube that connects the sample reservoir to the detector [135] (Figure 1.3). In this technique, samples are segmented by air bubbles which are inserted into the system and then removed before reaching the detector. The main disadvantages of SFA are the complexity of the design allowing the insertion and removing the bubbles. Further, the relative high solvent consumption due to the used PP pushes the solvents and the samples continuously.

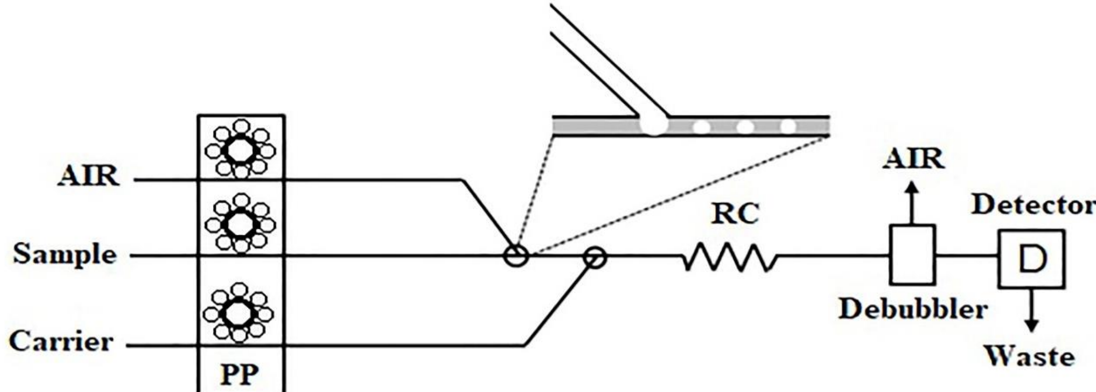

Figure 1.3 Scheme of a segmented flow analysis system. RC: reaction coil, PP: peristaltic pump.

Flow injection analysis (FIA) [136] was invented to overcome the drawback of SFA. The direct sample insertion through an injection valve into the carrier reagent is the main advantage of FIA. In this case, FIA reduces the carry-over into the system, sample, and solvent consumptions. Figure 1.4 shows the FIA manifold.

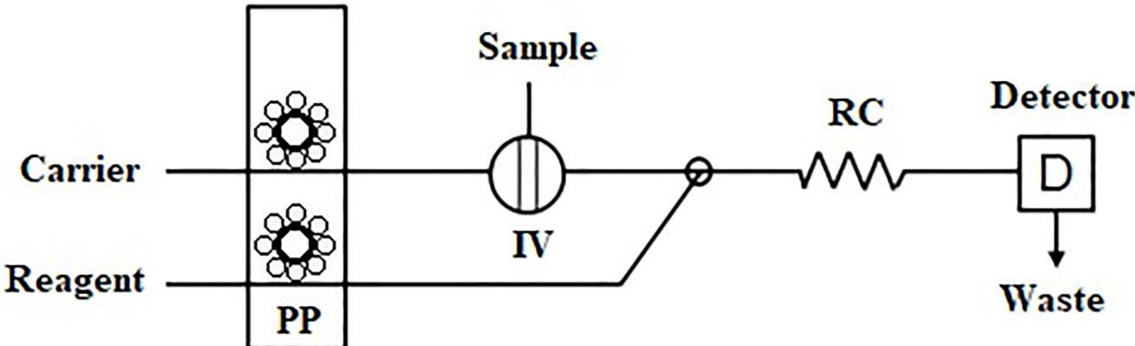

Figure 1.4 Schematic representation of a generic manifold of a flow injection analysis. PP: peristaltic pump, IV: injection valve, RC: reaction coil.

Moreover, sequential injection analysis (SIA) [137] consists of a switching valve with a central (CP) and sides ports. The CP of the valve connects to a two-way piston pump, while the sides ports can be used for connecting to the regents, samples, detector, waste and other devices i.e. mixing chamber, photo-oxidation system, or microwave oven. Figure 1.5. shows the typical SIA system. Essential features of SIA are its computerized control, acquisition, and processing of the results using specific software. Avoiding continuous pumping is dramatically reducing the consumption of the sample and reagents comparing to PP which continuously propels the solutions. Conversely, its main disadvantage is the hinder mixing of the sample and reagents from the way plugs are stacked.

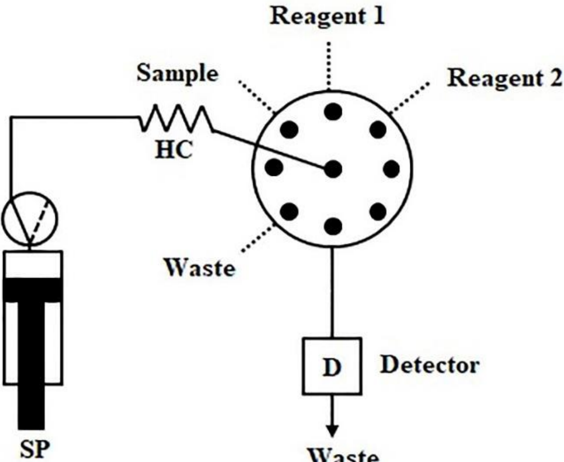

Figure 1.5 Scheme of a sequential injection analysis system. SP: syringe pump, HC: holding coil, RC: reaction coil.

Multicommutated flow analysis (MCFIA) [138] depends on fast-switching three-way solenoid valves and PP (Figure 1.6). PP aspirate continuously the reagents, while the fast solenoid valves return them to their reservoirs (OFF mode) or inserted them into the system (ON mode) resulting in reducing the consumption of the reagents and increase the throughput of the analysis. Its main drawback is the vulnerability of PP tubing against aggressive reagents.

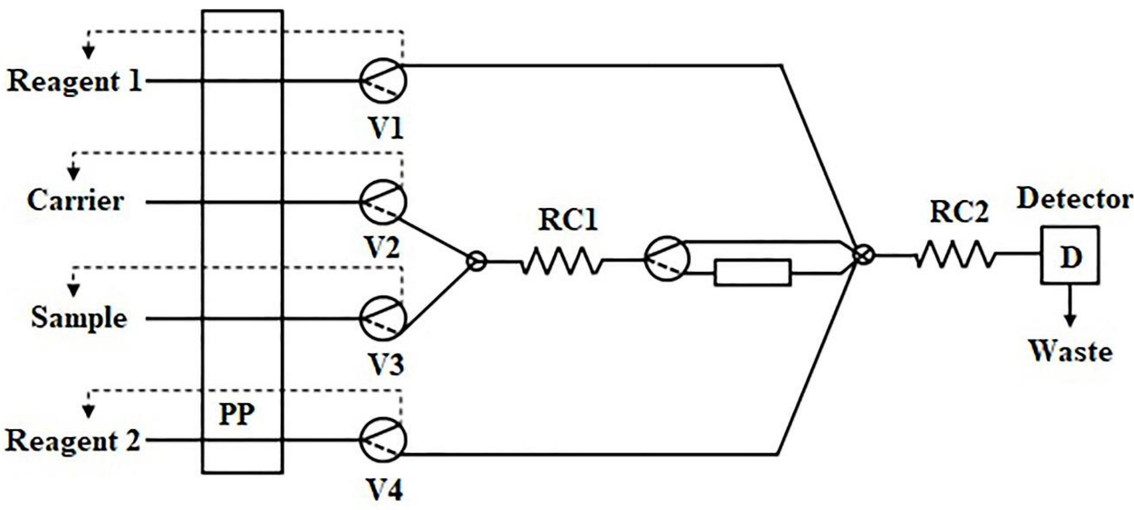

Figure 1.6 Scheme of a multicommutated flow injection analysis system. V: valve, RC: reaction coil.

Multisyringe flow injection analysis (MSFIA) was developed by Cerdà et al. [138]. The system includes a conventional automatic titration burette headed with a fast-switching solenoid valve (V) adapted with one motor that can move simultaneously a metal bar accommodated with up to 4 syringes (Figure 1.7). Its main advantages are high throughput, robustness, low sample and reagent consumption, high flexibility, and the ability to change the position of solenoid valves rapidly without the need to stop the pistons.

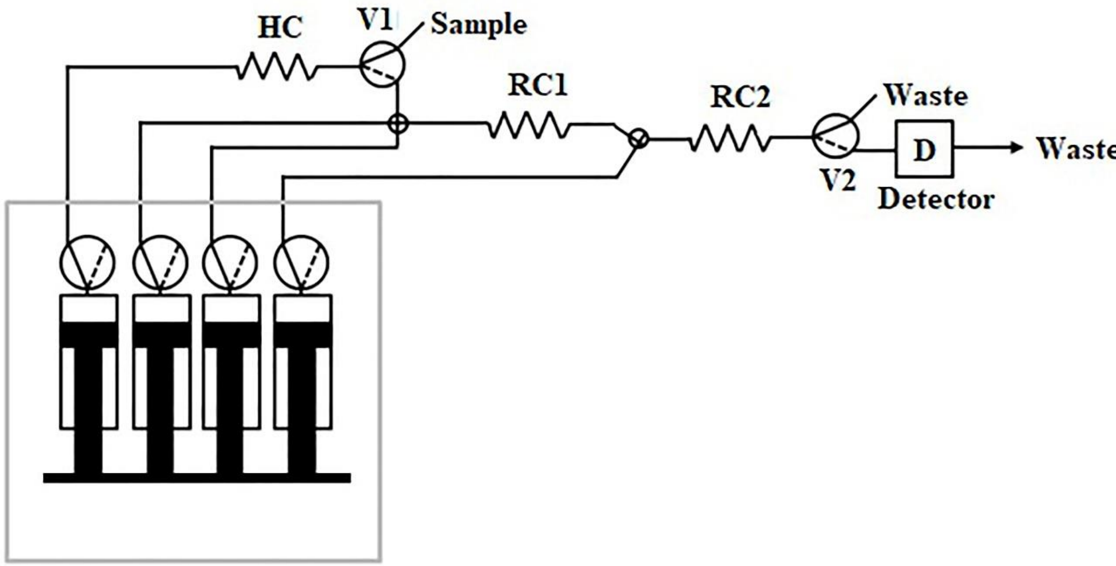

Figure 1.7 Scheme a multisyringe flow injection analysis system. HC: holding coil, RC: reaction coil, V: three-way solenoid valve.

Lab-on-valve (LOV), the third generation of flow techniques [139], is a device that can be integrated with the head of a switching valve of multiposition valve (MPV) manifold. Basically, The LOV platform consists of a valve with engraved microchannels. Each channel connected from one side (the inner port) to CP and from another one (surrounding ports) can be connected with the sample, reagents reservoirs, and maybe with other devices (Figure 1.8). Usually, LOV is made of PEEK or ULTEM for improved chemical resistance. The main features of LOV are the possibility to miniaturize and automatize the sequential injection operation mode, reduce the sample and solvents consumption, significantly facilitating the integration of various analytical units in the valve, providing great potential for miniaturization of the entire instrumentation, the robustness and repeatability under its computerization [140].

Different designs have been proposed, the original one was developed to accommodate sample metering, dilution, reagent addition, mixing, incubation, separation, and detection within a miniaturized device [139]. Moreover, LOV works in MPV mode, hence, precise volumes of sample and reagents are aspirated sequentially into a holding coil (HC) through a central channel (CC) using a syringe pump (SP) to propel the solutions in the manifold.

Then the mixture is propelled by reversing flow towards detection. Hence, LOV is the perfect manifold for the automation of sample pretreatment, especially for SPE as it will be explained in more detail in section 1.5 and chapter 5 as examples in automatization of the SPE depending on the LOV manifold.

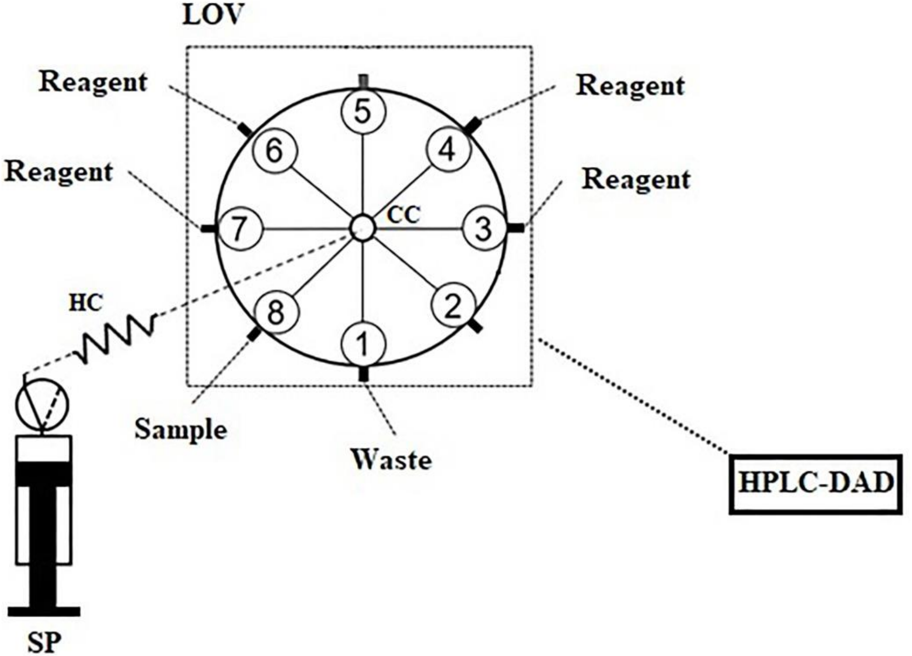

Figure 1.8 Scheme of a lab-on-valve system. SP: syringe pump, HC: holding coil, CC: central channel.

1.5. Automation of solid-phase extraction

The development of miniaturized, economical, and green analytical methods has become an issue of great concern. However, sample pretreatment is the most necessary step in the complex matrices prior to their analysis in order to clean-up, isolate, and concentrate the target analytes. This pretreatment step leads to an increase in the selectivity of the method by removing potential interferences from the sample and increase the analytical sensitivity by concentrating the analyte, thereby providing a robust and reproducible method, and protecting the analytical instrument [141].

FATs provide the ideal solution to automatize and miniaturize the SPE procedure with the possibility to apply all SPE support's invented shapes, such as disks [142], mini- and micro-columns [129], and recently 3D printed supported devices [143]. Comprehensive reviews on packed extraction columns coupled with or integrated into the LOV platform have been published, while this integrated technique scarcely applied for flavonoids extraction. In practice, C_{18} magnetic silica beads were placed in a coupled extraction micro-column into sequential injection lab-on-valve (SI-LOV) for matrix removal and preconcentration of QU in urine and red wine [118]. In the development of this thesis, LOV-µSPE depending on a grafted micro-column to perform the preconcentration of HES and DIO in citrus juice, edible part, and their commercial products [144] will be detailed in chapter 5.

Generally, the LOV-SPE extraction procedure is based on five main steps, filling the column with SPE solid beads, conditioning the stationary extraction phase, loading the sample, removing the sample interferences, and finally eluting the reminded target compounds [145]. The main advantages of the hybrid technique are not only the power of LOV to automatize all steps of the SPE technique but also it is online connecting to detection or analytical techniques that provide fully automated analytical methods and lead to minimizing the solvent and sample consumptions, reducing the analysis time, and the dangers of human exposure to solvents. Likewise, a packed SPE column can be used many times before its renewing by reconditioning it after each extraction time which increases the repeatability and decreases the consumption of the resin.

Otherwise, this combination presents the major drawback of producing high backpressure in long-term operation due to the progressively tighter packing or clogging of the column material [144]. Further drawbacks of online SPE with reusable surfaces are analyte carry-over effects, shrinking or swelling of the sorbent bed, malfunctions of the active entities including loss of functional moieties, resulting in deactivated surface [146]. To avoid these problems, the usability of the packed column should be optimized, then, the renewable protocol can be carried out automatically by the LOV [139].

1.6. Coupling of flow techniques to chromatographic techniques

Chromatography as a separation technique normally required a prior sample pretreatment to increase its selectivity, sensitivity, and the robustness of the analysis, thus, protecting the separation column, connections, filters, and the detector from clogging or damage via the impurities that might exist in the matrices and the interferences substances in the matrix that can be affected on the chromatogram.

FATs are non-separative techniques. They facilitate the sample pretreatment required by chromatography. In this context, the online combination between these two techniques is attractive as they allow the whole analytical sequence to be run automatically. For instance, this combination provides many advantages, i.e. extensively reducing the time of the analysis, consumption of solvents and samples, hence, reducing the analysis waste, the procedure required the least number of staff due to the automation of the whole procedure and reducing the staff intervention. Taking a benefit from all these advantages, many FATs approaches have been coupled online to chromatographic ones i.e. SIA [147], MSFIA [148] and LOV [144]. In addition to a new hyphenated system between FATs and chromatographic column such as the combination between SIA or MSFIA with a monolithic column, namely: sequential-injection chromatography (SIC) and multi-syringe chromatography (MSC), respectively [149]. Figure 1.9 shows the typical coupling between FAT and the main separation techniques. Unfortunately, these hybrid techniques have been utilized yet in the field of flavonoids determination.

In the improvement of this thesis, two online coupling flow techniques with HPLC are developed. Performing sample pretreatment using SPE approach-based flow systems, then the online determination of the interested flavonoids in citrus juices, albedo, flavedo, and their derivatives products using HPLC. These online coupling techniques will be detailed in chapters 5 and 6.

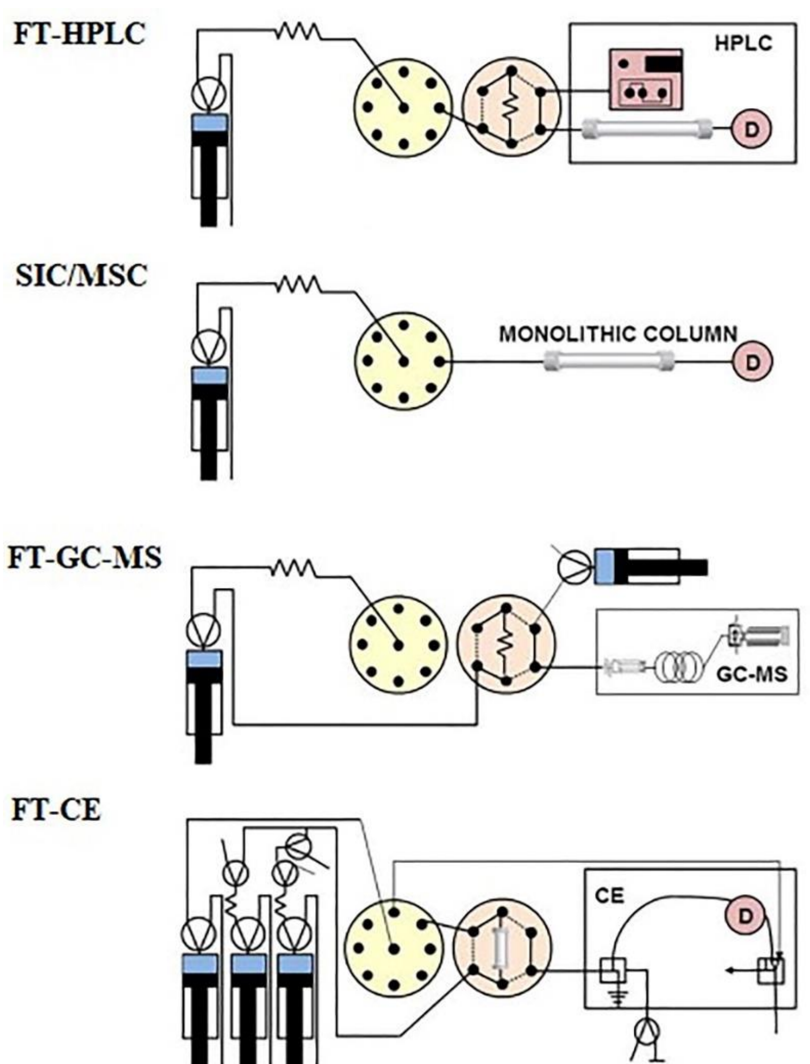

Figure 1.9 The online combination between flow analysis techniques devices and different chromatographic techniques.

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1.8. Original paper

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CHAPTER 2

OBJECTIVES

In this chapter, the general and specific objectives of this thesis are presented.

2. OBJECTIVES

The main purpose of the present thesis is the development of novel fully automated, robust, accurate, precise, reliable, and rapid analytical systems for the determination of main flavonoids present in pharmaceutical formulations, food supplements, natural and commercial citrus juice, their edible part, and the external peel of these fruits exploiting hyphenated systems of microextraction SPE-based flow techniques with HPLC.

Likewise, the systems were designed aiming to fulfil the simplicity, stability, and maximal robustness. In addition to achieving the minimal reagents, samples, and processing time consumptions, as well as a waste generation. The systems were applied to treat the real samples.

The specific objectives are given below, itemized per chapter.

Chapter 4

- Develop a simple, sensitive, and cost-effectiveness HPLC with a DAD detection method for the determination of two main flavonoids in pharmaceutical formulations.
- Use of symmetric C_{18} column for the separation.
- Possibility to detect some trace-related impurities of flavonoids that may exist in these drugs.
- Apply multivariate optimization design to study the influence of critical factors that affect the chromatographic separation behaviour.
- Apply the developed method to determine target flavonoids in two pharmaceutical raw materials and seven drugs from the Spanish and Syrian markets.

Chapter 5

- Develop an online μSPE method exploiting an in-syringe-LOV system for sample pretreatment and preconcentration of two flavonoids in citrus juice samples.
- Use multivariate optimization design to optimize the developed system.

- Coupling online LOV system with HPLC-DAD for the determination of these flavonoids in-hand squeeze citrus juice, commercial products, and citrus edible parts.
- Apply the developed methodology to determine the interested flavonoids in fourteen real samples of three kinds of citrus fruits (orange, mandarin, and lemon).

Chapter 6

- Develop a new 3D printed device made by stereolithography 3D printing technology.
- Employ a combination immobilized technique, Stick & Cure and Post-Cure techniques, to coat the 3D printed device with an extraction resin.
- Use the immobilized 3D printed device in the MPV tool for the clean-up and preconcentrate six flavonoids present in the external citrus peel.
- Hyphenate the developed MPV system with HPLC-DAD for the determination of extracted flavonoids.
- The separation of target compounds using a C_{18} monolithic column has been employed.
- Implementing multivariate optimization design for the optimization of all the developed system stages.
- Apply the developed system for the determination of the target flavonoids in the external peel of eight samples of three citrus kinds (orange, mandarin, and lemon).

Chapter 7

- Develop a simple, robust, selective, and sensitive HPLC-DAD for the separation and determination of four flavonoids in natural preparations of pharmaceutical formulations and food supplements.
- The separation is done using a symmetric C_{18} column.
- Apply the developed method for the determination of these flavonoids in fourteen different sample preparations.

CHAPTER 3

MATERIALS AND METHODS

In this chapter, the instrumentations, software, and multivariate optimization designs that have been used in this thesis are presented.

3. MATERIALS AND METHODS

3.1. Flow analysis instrumentation

The flow analysis instrumentations used in the development of the thesis are constituted by a multisyringe burette (BU4S, Crison Instrument, Barcelona, Spain) (Chapters 5 and 6), and a multiposition selection valve (Crison Instruments) system (Chapter 6) headed with a lab-on-valve platform (Sciware System, Bunyola, Spain) (Chapter 5). These instruments were employed for the development of automation methodologies for the extraction and determination of flavonoids in real samples.

Both Sciware and Crison instruments were connected to a computer for remote operational control via a serial RS232 interface and controlled with the AutoAnalysis software. The instruments used during the development of this thesis are described in detail below.

3.1.1. Lab-on-valve and multi-position selection valve instruments

The LOV platform was fabricated from Ultem® (polyetherimide), a resistant material in front of a variety of organic solvents. This device encompassing eight integrated microchannels (1.5 mm i.d./ 16.0 mm length), excepting two channels corresponding to the extraction beads charge and the microextraction column, respectively, which are made with (3.2 mm i.d.), (Chapters 5).

Each channel connects one side port to the CP at a time (Figure 3.1). The surrounding ports are connected to the reagents, sample, and extraction beads reservoirs by means of poly(tetrafluoroethylene) (PTFE) tubes (Chapter 5). The CP connects to a syringe using a PTFE holding coil.

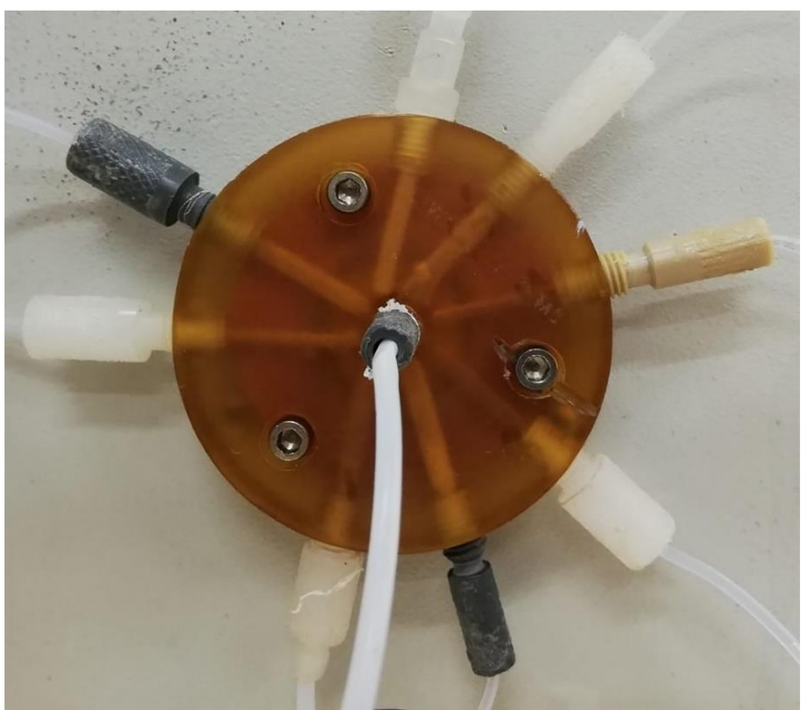

Figure 3.1. Basic lab-on-valve platform.

The LOV platform was mounted atop of a MPV equipped with an 8-port rotary multiposition valve made with chemical-resistant polymers PEEK (stator) and PTFE (rotor) (Figure 3.2). The valve allows the connection between the CP and the side ones via an integrated channel in the rotor of the selection valve. The rotation of this channel can be controlled by the AutoAnalysis software.

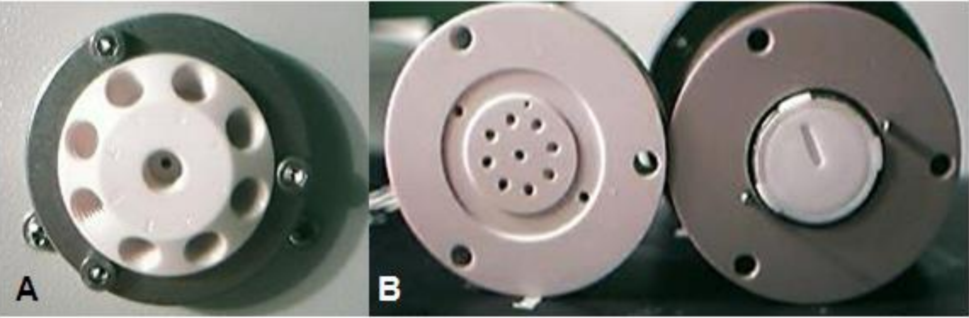

Figure 3.2 A) Multiposition valve cover view used in MPV system, and B) inner parts view of the valve cover; rotor view on the right. The LOV substitutes the cover A.

3.1.2. Multi-syringe flow injection analysis instrumentation

The front face of the MSFIA employed in this thesis (Chapters 5 and 6) consists of a conventional titration burette which can be equipped with up to four syringes (plastic or glass syringes), (Figure 3.3 A). Pistons of all syringes are mounted on a one-steel bar driven by a single stepper motor. Thus, all pistons are moved simultaneously and unidirectional for either liquid dispensing or aspirating. Each syringe is headed with a three-way V (N-Research, Caldwell, NJ, USA), which facilitates the application of multicommutation schemes. The first way of V is connected to the syringe port, the others are coded by ON that are usually connected to the manifold system and OFF commonly connected to the waste or it might be also connected to the reagent reservoir, that allows four liquid displacements: ON/OFF-dispense, and ON/OFF-pick-up, (Figure 3.3 B).

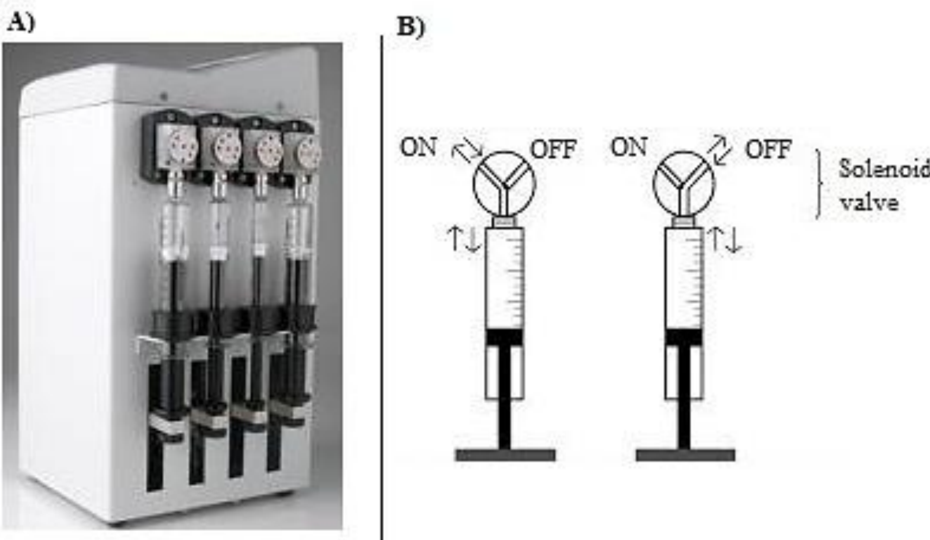

Figure 3.3 A) Multisyringe flow injection analysis, equipped by four syringes and B) the scheme of ON/OFF solenoid valve position.

This type of V achieves a very short time for switching between the positions (ON and OFF), only 35 ms, which allows changing the valve position even during flow operation. The valves can tolerate a back pressure of up to 2 bars. High chemical robustness is provided by the use of PTFE (piston heads, poppet flaps).

Wide syringe volume can be implemented, i.e. 0.5, 1, 2.5, 5, 10, and 25 mL, enabling a wide range of combinations. One glass syringe of 5 mL (Hamilton, Switzerland) was equipped in the developed works, (Chapter 5 and 6). The stepper motor can reach total displacement corresponding to 5000, 16000, or 40000 steps, depending on the burette model. In this thesis 5000 steps multisyringe burette was used achieving total displacement between 8-329 s, allowing precise volume handling and a wide flow rate range between (0.9-38.5 mL min^{-1}).

On the other hand, the rear face of the instrument obtains four connection ports (V5-V8), (Figure 3.4 A) which enable the power of additional external multicommutation valves, micropumps, motors, or other instruments either directly or via a protection platform (Figure 3.4 B) allowing remote control by software. This amplifies the possibility to construct sophisticated flow networks. Each port provides 12 V with a maximal current of 0.5 A. In addition to a one serial connection port "peripheral" that permits the connection to another instrument, i.e. MPV, injection valve or autosampler. In the development of this thesis, the motor (M) was connected through a relay to an auxiliary supply port (V6) of the MSFIA module employed to activate the M. The MPV connected to the serial port of the MSFIA.

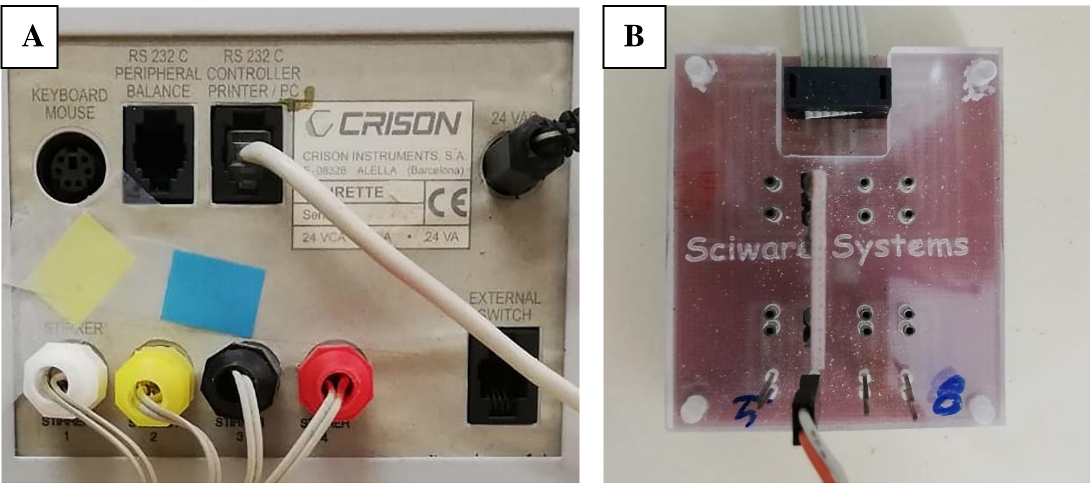

Figure 3.4 A) The rear side of multisyringe flow injection analysis instrument and B) solenoid valve protection platform.

The high-pressure injection valve (IV), (Figure 3.5 A) used is a 6-port rotary injection valve (Sciware System). IV performs with two different positions, load position (Figure 3.5 B) allows the filling of the external loop with the sample and inject one (Figure 3.5 C) allows injecting the sample filled in the loop into the carrier stream. The IV used as the interface between the flow system and the separation instrument as HPLC.

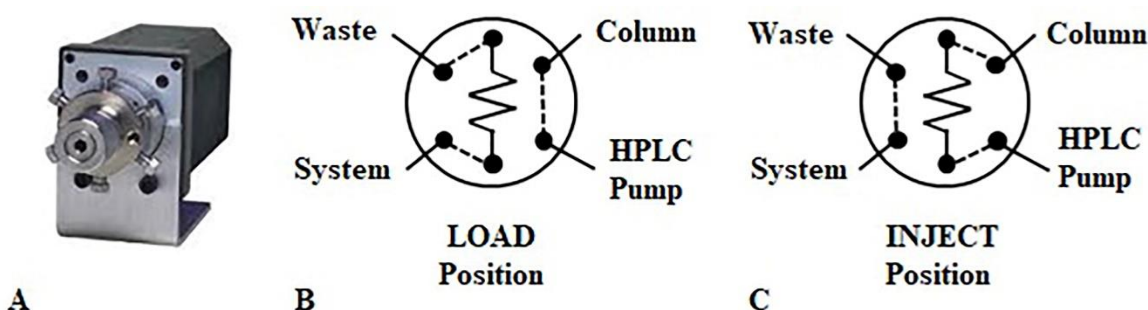

Figure 3.5 A) Injection valve, B) schematic representation of load position, and C) inject position.

3.1.3. Magnetic-stirring instrumentation

In this thesis, two pretreatment sample methods were developed for the isolation and preconcentration of interested flavonoids from real samples. Microextraction was done through a grafted column into the LOV device (Chapter 5), and with a 3D printed device that extracted the target flavonoids by stirring it with the flavonoids solution inside the homemade extraction tank (ET) (Figure 3.6 A) (Chapter 6). For that, magnetic-stirring-assisting instrumentation (MSA) is required to increase the interacting surface between the solution and the 3D printed device. This step is controlled automatically by the AutoAnalysis software.

The MSA system used in the work of chapter 6 has been developed in our laboratory, and it consists of an external magnetic stirring ring placed around the ET, holding two small neodymium magnets facing each other, creating a rotating magnetic field around the ET body (Figure 3.6 B), M (Figure 3.6 C) forces the rotation of the external device, and a circuit (C) which controls the ON / OFF and the engine revolution through one of the auxiliaries exits in the back-face of the MSFIA. The external magnetic ring had a groove for a rubber band, which connects to a low-cost DC motor with the external ring and allowed propel it with the M (Figure 3.7).

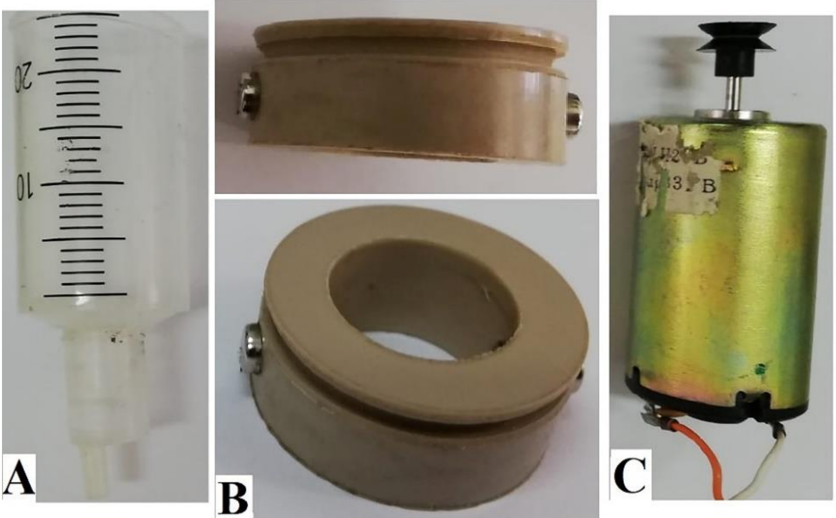

Figure 3.6 A) The homemade extraction tank, B) external magnetic ring, and C) a motor.

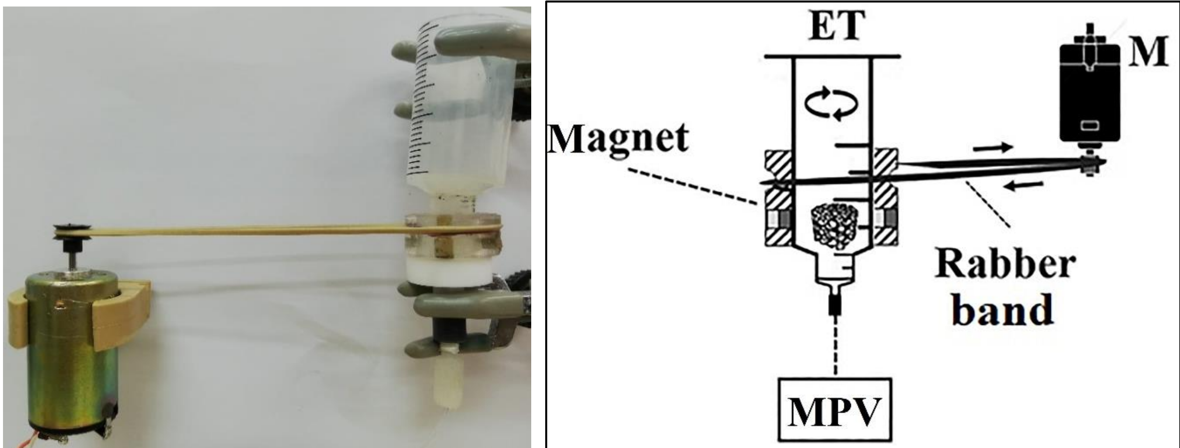

Figure 3.7. The image and the scheme of the magnetic-stirring-assisting system.

Homemade ET has also been developed in our laboratory to facilitate the 3D extraction process. ET has been designed with the smallest possible diameter in the bottom with a wider one above. In this case, the 3D printed device can be floated in the biggest sample volume, while the remained flavonoids on the extraction 3D printed device can be eluted with the smallest quantity of the eluent (Figure 3.6 A). Thus, the extraction factor has been improved.

The MSA let the 3D printed device rotates with the M by the force of the magnetic field on the thin metallic wire (0.5 mm id, 17.60 mm length) that was inserted into the 3D printed device. The 3D printed device will be discussed in detail through section 3.2. The image and the scheme of the MSA show in Figure 3.7.

3.1.4. Manifold components

The flow network of the manifold was constructed from flexible tubes of chemical-resistant PTFE of 0.8 mm i.d. except for the HC that is made of 1.5 mm i.d. with a volume of 4 mL. All connections were made by means of polyvinylidene fluoride (PVDF) screws. Figure 3.1 shows the connection tubes and the connectors.

3.1.5. AutoAnalysis software

The software AutoAnalysis 5.0 was developed by our group of Analytical Chemistry, Automation and Environment and it is commercialized by Sciware Systems S.L. The software is fundamentally described by Becerra et al. (1999) [1]. The processing data and instrumental control are carried out by the software written in Delphi and Visual C++ and offers a window-based graphical surface.

The possibility of using a single and versatile application without further modification for whatever instrumentation and detection system needed is performed by the distinctive feature of this software which is based on dynamic link libraries (DLLs) of 32 bits. It involves a basic protocol that allows the implementation of specific and individual DLLs, addressing the configuration of the assembled flow analyser. Thus, it is a very flexible tool, easy to handle by non-specialized users.

3.1.5.1. Hardware configuration

The installing and loading of connection hardware, the incorporation, configuration, and the establishment of a communication protocol, i.e. RS232, RS485, and USB that are executed via DLLs are required for each system forming an individual configuration. In addition, a minimum effort is required for the modification of instrumentation assembly. The AutoAnalysis is only limited by the availability of the required DLLs. To date, seven communication channels and thirty devices are available including atomic fluorescence, molecular fluorescence, spectrometric and electrochemical detectors, autosamplers, syringe-, peristaltic-, solenoid-micropumps, valve modules, and I/O, A/D, or D/A PC digital cards for the connection and communication with other devices (Figure 3.8).

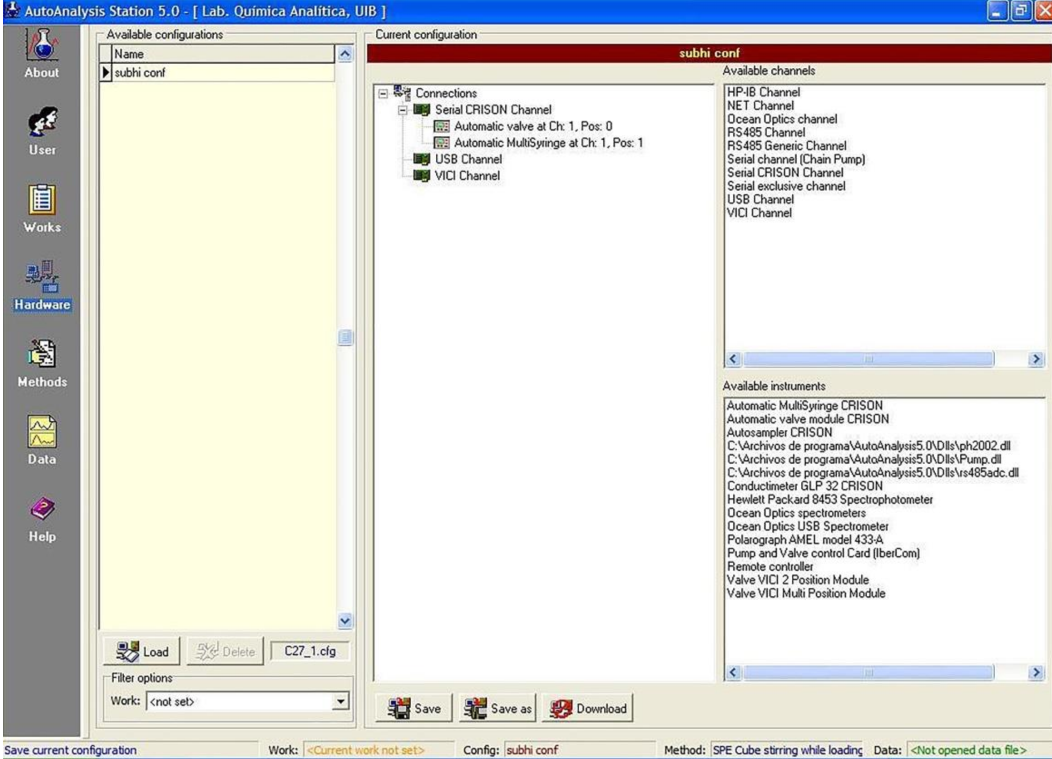

Figure 3.8 Configuration window of hardware, channels, and instruments in AutoAnalysis software.

Due to the development of this thesis, DLLs for the RS232 communication channel of Serial Crison was used and the DLLs for the following instruments control were employed such as MSFIA Crison, MPV Crison, and IV Sciware. The employed instruments can be addressed in each method after loading the required configuration for the connected instruments and will be available in the corresponding commend forms in the editor window. However, the software provides the possibility to process the data directly without the need to incorporate a new instrument into the system that led to minimizing the time and the efforts required. The AutoAnalysis processing data has not been used during the development of this thesis’s works because of the online coupling of the flow system to the chromatographic technique and processing the resulted data through HPLC software.

3.1.5.2. Method edition

The “Editor” window of the AutoAnalysis may create an analytical procedure by sequencing functions or instructions from the drop list menu for the configurated instruments (Figure 3.9). This window provides variability of programming functions including procedures, loops, indexing, basic calculations, variables, user input, waiting steps, events marking, comments, conditional inquiries, and online data processing. In addition, allows testing the method execution, definition of detection thresholds, manual data processing, such as calibration, data smoothing, spectral information processing, peak adjustment, and data export.

On the other hand, the “Execute” window enables the creation and optimization of highly versatile applications regarding the features that it has. For example, method initiation, pause, stop, control of the execution, continuous data acquisition, zoom, scale, and shift functions.

Otherwise, AutoAnalysis allows multitasking operations such as simultaneous method execution and data processing, multiple loading, and working with other programs running at the same time. Procedures are pre-created instruction protocols, which can be loaded by all methods within the same hardware configuration. The main advantages are higher method clearness by apparent shorting, module-like programming, and repeated application in the same method.

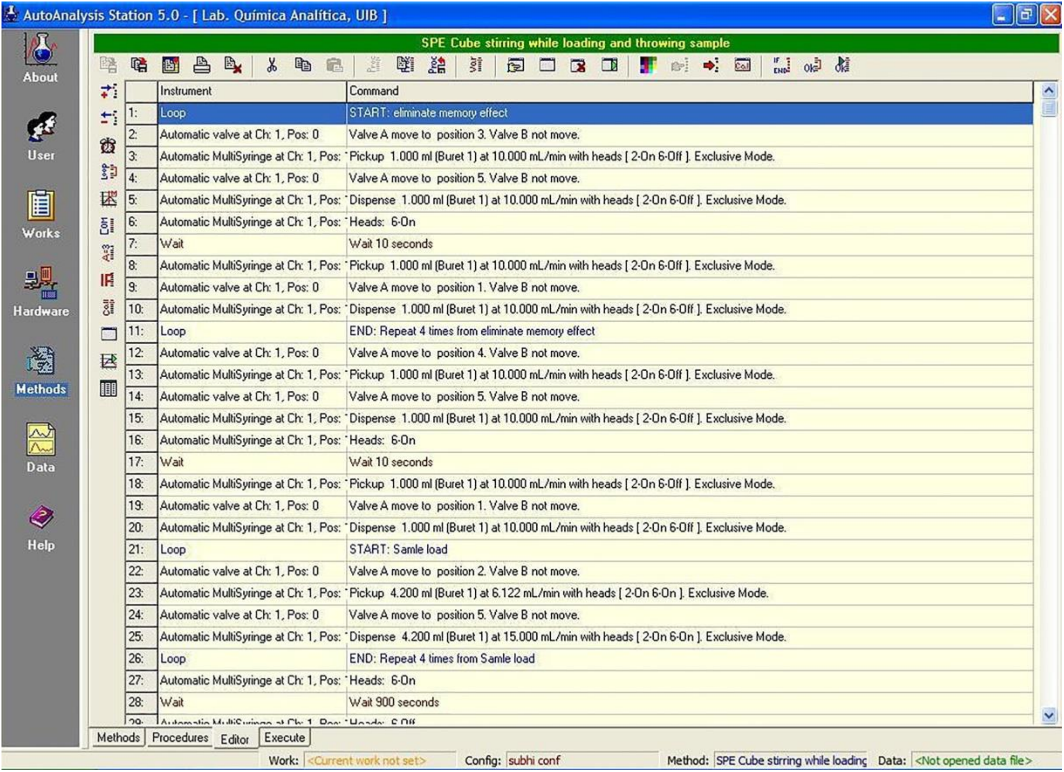

Figure 3.9 Method edition window.

3.2. 3D printer instrumentation

A 3D printer from Formlabs Inc., Somerville, USA, model Form 2 (Figure 3.10) integrated with the Perform Software (Formlabs) was used in the work presented in chapter 6.

Form 2 3D printer device (≤ 3.600 €) is the ease of use, robust, accurate, and high-resolution stereolithographic 3D printer device which is capable of printing with a resolution of 25µm of each layer's thickness, in addition, it can use a great diversity of the resin materials that allow printing models with a variety of their physical properties depending on the resin selection. Reliable printed prototypes are achieved with the existence of a responsive heating system that continuously warms the resin to a consistent required temperature [2].

Figure 3.10 Formlabs Form 2 3D printer instrument.

This instrument [3] works in the technology of stereolithography (SLA) which forms 3D models or prototypes layer-by-layer through the use of photopolymerization, a method that causes chains of molecules to link and form polymers through the use of a UV light source. To design the model, one has to use computer-aided design software (CAD). In chapter 6, Rhinoceros 5.0 SR11 32 software (McNeel & Associates, USA) was used. Then, the SLA file is created and imported to the Perform software to optimize the print process and get the design ready in minutes. The file is then uploaded to the Form 2 printer to start building. Next, the produced raw 3D printed device is cured to complete the polymerization resin by means of a UV crosslinker camera (Upland, California, USA) [4]. In this stage, extraction resin can be immobilized on the printed device before the curing process or after that, as will be detailed in section 6.2. Figure 3.11 shows the scheme (A), the printed device without covering (B), and after covering it by the extraction resin (C).

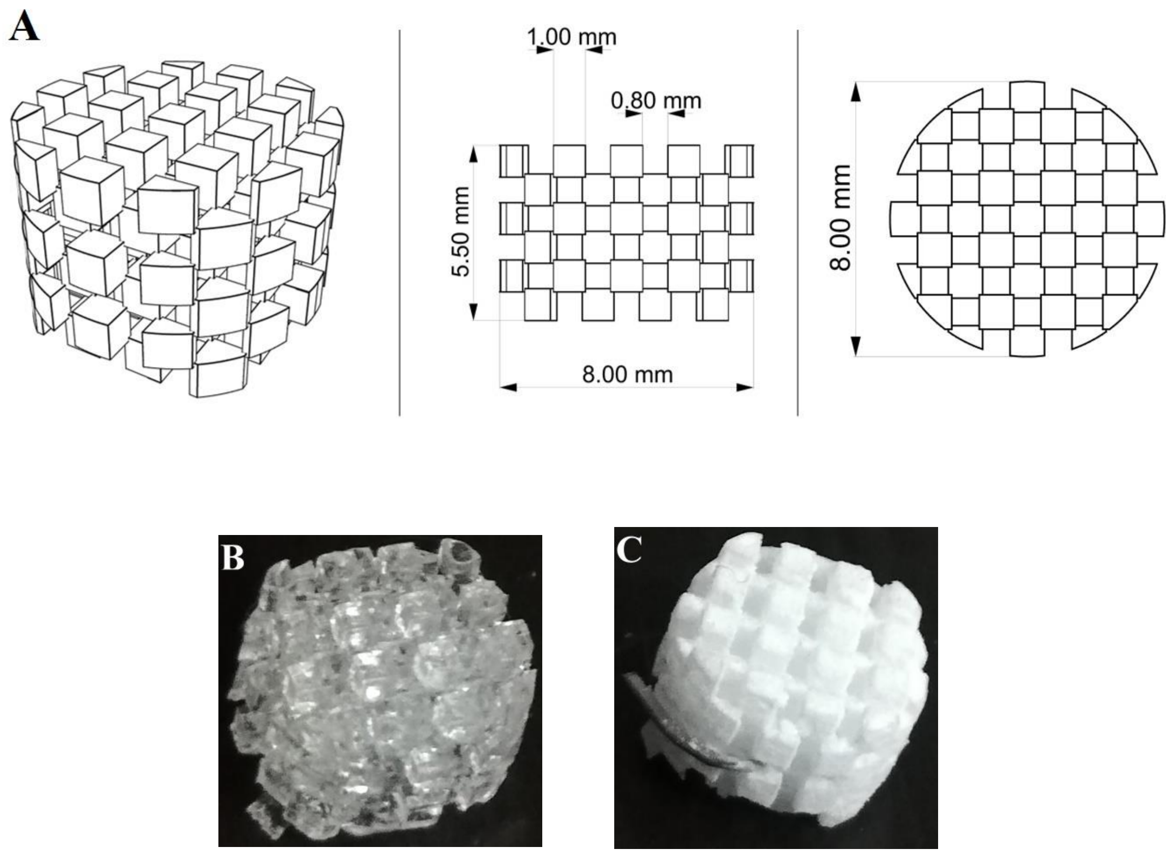

Figure 3.11 A): The scheme of a designed device, B): the printed device without covering by an extraction resin and C): after covering by the extraction resin.

3.3. High-performance liquid chromatographic instrumentation

The HPLC instrument (Figure 3.12) used in all works included in this thesis was a Waters LC System (Waters, Milford, MA, USA) equipped with a quaternary pump (600), a UV/vis detector (2996), and a column oven. Separations were performed in both Symmetry® C_{18} analytical column (250 mm x 3 mm id 5 µm) preceded by a C_{18} guard column (5 mm x 4.6 mm id), both from Waters (Torrance, CA, USA) (Chapters 4, 5 and 7), or in Phenomenex® Onyx monolithic C_{18} column (25 mm x 4.6 mm) (Torrance, CA, USA) (Chapter 6). Both used columns have a limitation pH range between (2.0-8.0) [5] and (2.0-7.5) [6],

respectively. The HPLC system and data management were controlled by Empower software (Waters).

Figure 3.12 Waters HPLC System.

3.4. Multivariate optimization design

The optimization step is the most important step in the development of the analytical methods and also the most time-consuming during the development of an analytical method. There are two different optimization strategies, one is a traditional approach known as one-variable-at-a-time (OVAT) and another one is a statistical approach depending on the chemometric tools known as design of experiments (DOE).

OVAT depends on changing one factor while keeping the others constant. This approach consumes time, a lot number of tests, resulting in solvent consumption, and unable to study the interaction between the studied factors. In addition, it might fail since the effect of one

variable depends on the level of the others involved in the optimization [7]. Normally, the OVAT method is used for individual factor optimization that has not had interaction with each other. On the contrary, DOE is a more modern approach allowing to evaluate accurately the interactions among various factors while reducing the optimization time due to less number of running tests executed, resulting in less solvents, cost, and laboratory work consumptions [8].

On the other hand, the DOE optimization approach involves two types of variables: factors are the studied variables and responses are the dependent variables that depend on the levels of the factors. DOE requires establishing the studied experimental domain of each factor regarding knowing the minimum and the maximum values for each factor to be investigated during the optimization procedure. They can be screened firstly to see the tendency of the system and which factors have a significant effect upon the response using normally full factorial designs (FFD) and fractional factorial designs. Then, the optimum operating conditions are attained by using more complex experimental designs such as. Box–Behnken design (BBD), Doehlert matrix (DM), and central composite design (CCD).

Therefore, the optimization results can be performed in two ways. Response surfaces can be determined individually for each response and these surfaces can be analyzed simultaneously, or a model for a single composite function that considers all responses can be determined to obtain a single response surface.

The most famous treating multiple responses is the desirability function (D), which was proposed by Derringer and Suich in 1980 [9]. To obtain D, the individual desirability of all responses should be first determined. Thus, each response y_i(i=1, 2, 3, …, n) is transformed into an individual desirability function (d_i), which ranges between 0 (an unacceptable response) and 1 (a completely desirable one). d_i is normally calculated depending on equations 1 and 2. Where L and H are the lower and upper responses for each factor and y_i is the response value. Equation 1 is used in the case of the desired value of the response increased ascendingly such as the responses during the optimization of the chromatographic method, the peak area, and the resolution between the peaks (the desirable value when the

peak area and the resolution are the better the higher). On the contrary, Equation 2 is employed in the case of the desired value of the response increased discerningly such as the optimization of the analysis time factor of the chromatographic method (the desirable value of the analysis time is the minimum value that can be observed). The D was calculated by determining the geometric mean of individual desirability (Equation 3), where k is the number of responses.

$$d_i = (y_i - L)/(H - L) \quad \text{Equation 1}$$

$$d_i = (H - y_i)/(H - L) \quad \text{Equation 2}$$

$$D = (d_1 \times d_2 \times d_3 \dots d_k)^{1/k} \quad \text{Equation 3}$$

Otherwise, multivariate optimization design of analytical methods should fulfil the following procedure:

(i) Chosen one of a statistical design to investigate the study of the interesting experimental domain.

(ii) Perform the experiments in random chronological order

(iii) Perform analysis of variance (ANOVA) on the regression results so that the most appropriate model with no evidence of lack of fit can be used to represent the data.

In this context, modern commercial statistical computer programs are available to carry out all these steps. In this thesis, the Statistica software (Statsoft, Tulsa, USA) has been used to carry out the multivariate optimization design (Chapters 4-6).

3.4.1 Full factorial design

FFD is the most popular and first-order design owing to its simplicity and relatively low cost. Usually, it is used for a preliminary screening study to determine simple and linear responses in all investigated factors, and normally it is used to determinate not only which the most important factors to investigate are, but also which factors have a significant effect on the studied domain on the experimental results “responses”. It can also be employed to

determine a simple response surface that assessment of the linear influence of the individual factors and also the influence of factor interactions [10]. For a three factors case, the response surface is given by the linear model (Equation 4), where *y* is the response, *bi* is a linear term. Figure 3.13 shows the schematic representation of two levels of FFD design for three factors.

$$y = b_0 + b_1x_1 + b_2x_2 + b_{1,2}\, x_1\, x_2 \qquad \text{Equation 4}$$

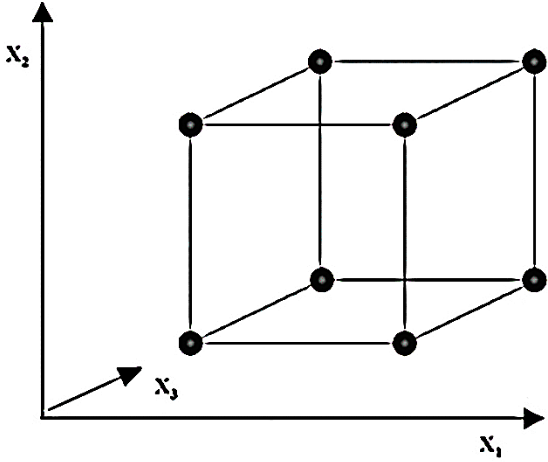

Figure 3.13 Schematic representation of two levels FFD design for three factors.

If the interaction term is negligible, then the response surface is planar. The more important the interaction term, the greater is the degree of twisting that the planar response surface experiences [11].

On the other hand, if the linear model is not sufficient to represent the experimental data adequately, more experiments can be performed which their results can be determined in a quadratic response surface (Equation 5). For instance, CCD and BBD [8].

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}\, x_1^2 + b_{22}\, x_2^2 + b_{12}\, x_1\, x_2 \qquad \text{Equation 5}$$

3.4.2. Box-Behnken design

BBD is a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs. For three factors, its graphical representation is seen in two forms: a cube that consists of the central point and the middle points of the edges (Figure 3.14 A) or a figure of three interlocking 2^2 factorial designs and a central point (Figure 3.14 B).

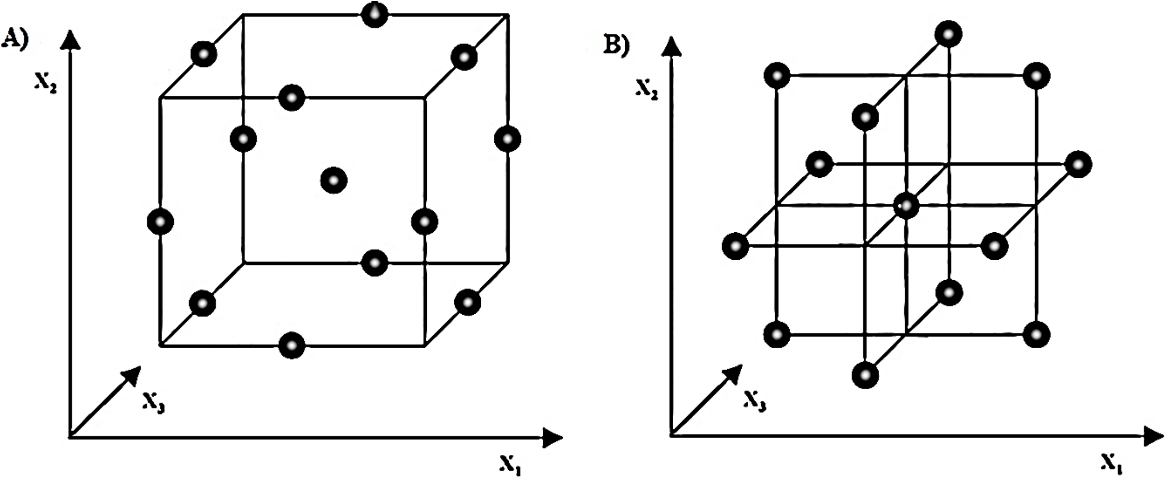

Figure 3.14 Graphical representations of Box-Behnken design for its three factors. A) the cube; and B) the interlocking 2^2 factorial designs.

BBD achieves some advantages over other DOE designs such as it is more efficient where each factor is studied at only three levels, all factors studied at their higher or lower levels avoiding unsatisfactory results may occur under extreme conditions, plus replicates at the central point and it requires less number of experiments that can be given in Equation 6, where N is the required number of experiments for the development of BBD, k is the number of factors, and C_0 is the number of the central point [12].

$$N = 2k(k-1) + C_0 \quad \text{Equation 6}$$

BBD has been frequently applied to optimize the analytical processes such as extraction and preconcentration steps [13-16], derivatization reactions [17], and chromatographic

methods, i.e. GC [18] and HPLC [19]. BBD is employed as a second optimization stage in chapters 4 and 6.

3.5. References

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CHAPTER 4

DEVELOPMENT OF A SIMPLE HPLC METHOD FOR FLAVONOIDS DETERMINATION IN PHARMACEUTICAL PREPARATIONS

4.1. Flavonoids in pharmaceutical preparations

In general, pharmaceutical drugs are usually used to cure diseases. In this context, U.S. Food and Drug Administration (FDA) defined that “drugs are substances intended to diagnose, treat or prevent diseases and they must pass clinical trials before being released to the public and the tests need to prove each drug is safe” [1]. Otherwise, it must be manufactured under carefully monitored conditions and packaged with complete information on the best dose, route, and schedule.

The antioxidant and the pharmacological behaviour of flavonoids in medicinal plants have been paid attention to from a long time ago for the protection and treatment of a number of diseases. For treatment purposes, pharmaceutical formulations are the best idea for facilitating the intake of these compounds in their natural powdered plants or the extracted and purred substances form.

DIO and its similar flavonoid HES are the most famous flavonoids that have been used in pharmaceutical formulations. The clinical investigations have been demonstrated that they can be used in the treatment of venous leg ulcers, improve venous tone, and enhance microcirculation [2] mainly, CVI and hemorrhoidal diseases [3], in addition, its anti-inflammatory activities have been demonstrated in neuronal cells [4, 5]. They also improve lymphatic drainage by increasing the frequency and intensity of the lymphatic contraction and by increasing the total number of functional lymphatic capillaries [6]. In this context, one of the most famous medicines that have been used for the treatment of CVI symptoms is Daflon® and its derivative medicines that obtain the same composition. This drug consists of DIO 90% and 10% of flavonoids expressed as HES [7].

For pharmaceutical manufacturing, HES is extracted and purified from citrus peel, while DIO is semi-synthesized from HES [8]. For this reason, HES accompanied DIO as a main natural impurity in its bulk pharmaceutical material [9]. Further, six impurities usually combined with DIO are cited in the European Pharmacopoeia including HES and diosmetin (DTIN) [10]. In this sense, the manufacturing of drug substances and drug products requires strict control of impurities that naturally exist in the raw material or formed as by-products during the

manufacturing process, or arise as degradation products during storage [11, 12]. Normally hesperetin (HTIN) and DTIN are the main natural impurities of HES and DIO raw material, respectively due to the possibility of their decomposition under restricted storage conditions [13].

4.2. Detection techniques of hesperidin, diosmin and their related substances in pharmaceutical preparations

The presence of HES and DIO in pharmaceutical dosage as the pure substances (Daflon®) or as active compounds in natural medicinal plants was detailed above. The existence of DTIN and HTIN in pharmaceutical forms limited to the related impurities to HES and DIO. For that, there are a few works that have studied the existence of DTIN in bulk and finished drug products. The official methodology for the determination of DIO and its related substances is expressed in the European Pharmacopoeia [10]. HPLC-UV is the used separation technique with a C_{18} column and the combination of MeOH, ACN, and glacial acetic acid as a mobile phase. Some modifications of this method have been introduced and used for quality control of DIO bulk drug substances and finished products [14]. Whereas, there is no official method for the determination of HES in pharmaceutical forms, as well, no reports of the study of HTIN in raw material and its finished products.

Due to the similarity in the chemical structure of HES and DIO, it represents the difficulty in determining one or both of them when present together in pharmaceutical dosage forms. In this context, some techniques have been carried out for their determination individually or simultaneously in pharmaceutical products that include the use of non-separative techniques such as UV, colourimetric, FL, FT-IR, and separative ones as HPLC and TLC.

UV spectroscopy has been developed for the fast and simple determination of HES and/or DIO in raw material and finished products. This technique is carried out by dissolving of HES in 60% of MeOH and measuring the absorption at 283 nm [15], or the combination of these two flavonoids being dissolved in 0.2 N of NaOH and then measuring them at 285 and 268 nm as the maximum wavelengths of HES and DIO, respectively [4]. In these applied methods, the obtained LODs were ≤ 0.139 and 0.048 $\mu g\ mL^{-1}$ of HES and DIO, respectively.

The colourimetric spectroscopic technique has been performed for the determination of DIO but has not been used for the determination of HES yet. This technique was applied for the determination of DIO in pharmaceutical forms by its reaction with 4-aminoantipyrine in the presence of hexacyanoferrate (III) in an alkaline medium and subsequent formation of a purple-coloured product, LOD of DIO in this method was 0.1 $\mu g\ mL^{-1}$ [16]. Another developed work was based on the reaction between DIO with sodium nitroprusside and hydroxylamine hydrochloride in a basic medium to give a green-coloured product with the LOD of 0.05 $\mu g\ mL^{-1}$ [17]. Also, DIO was measured based on the reduction of Ag^{+} cations to silver nanoparticles (Ag-NPs) by the effect of DIO in the presence of polyvinylpyrrolidone (PVP) as a stabilizing agent which results in the production of intense brown colour at the reaction time of 40 min. Its LOD was 0.26 $\mu g.\ mL^{-1}$ [18].

As a result, the colourimetric technique provides an alternative method for the determination of DIO in drug products due to the formation of a coloured complex between the DIO and the reagent. On the other hand, these methods are still not capable to determine the combination of DIO and HES, the reaction is not selective to DIO only but can react with other phenolic compounds present in the same sample. However, the attained sensitivity of these methods is lower than the direct UV methods applied.

FL technique has been also carried out to enhance the selectivity and the sensitivity of the analysis of both DIO and HES in the combination. The detection was exploited after separating the target analytes through the sample pretreatment by dissolving the HES, firstly in pure MeOH then the residual that contains DIO was dissolved in 0.1 N NaOH, assisted by an ultrasonic bath at 30 min for each solution, then the formation of the ternary complex of each flavonoid with Tb^{3+} in the presence of Tris buffer was performed [19]. The developed method is simple but required several sample pretreatment steps that might increase the detection errors. LOD of DIO and HES were 2.91×10^{-4} $\mu g\ mL^{-1}$ and 1.40×10^{-4} $\mu g\ mL^{-1}$, respectively. The fluorescent complex formation between HES and Al^{3+} ions was also studied with attaining LOD of 0.01 $\mu g\ mL^{-1}$ [20]. FL developed methods have significantly improved the sensitivity of the analysis compared to both direct and colourimetric determinations.

IR provides a useful way for the identification of drugs and the helping of Fourier transform (FT-IR) permits continuous monitoring of the spectral baseline and simultaneous analysis of the different components of the same sample. FT-IR spectrometric method was developed for the measurement of DIO in different pharmaceutical drugs. The method features are the rapid and direct quantitative determination of DIO applying commercial software involving chemometric approaches for the differentiation between the main signal of the standard and unknown studied substance. Thus, the capability to the qualitative calculation of the analytes due to 15 min including sample preparation and spectral acquisition. The proposed method is simple, precise, and not time-consuming [21]. Nevertheless, it lacks sensitivity and selectivity, in addition, it is based on special software for the predicted quantitative determination which is mean expensive and unavailable program.

Flavonoids contain phenolic hydroxyl groups which can be oxidized under proper conditions and yields an electrochemical signal. For that, electrochemical determination has been widely exploited for the determination of one compound DIO or HES without any mention of the combination between them. DIO was estimated at a glassy carbon electrode using cyclic voltammetry (CV) and differential pulse voltammetric (DPV) techniques in a Britton-Robinson buffer (pH 2.0 - 10.0) with obtained LOD of 0.21 µg mL^{-1} [5]. HES was determined using mesoporous SiO_2 modified electrode, the LOD was 152.5 µg mL^{-1} [22]. Adsorptive stripping voltammetry (AdSV) using a hanging mercury drop electrode was also reported for the determination of HES with a LOD of 0.18 µg. mL^{-1} [23]. Electrochemical developed methods observed the simple and fast detection features, but the obtained sensitivity was lower than the other techniques, plus these methods detect just one substance neither its combination.

TLC has also been applied to pharmaceutical determinations, DIO was separated on the plate of silica gel 60 with LOD of 0.02 µg/spot due to 20 min of run time [24], while the combination of HES and DIO was separated by HPTLC with LOD of 0.5 µg/spot [25].

HPLC technique coupled with UV [26] has been widely used for the determination of DIO and/or HES and their related substances in pharmaceutical raw materials and finished products employing C_{18} [20, 26] or C_8 [27] as a separation column. The mobile phase used normally

consists of the combination of water or acidified water with an organic modifier such as MeOH, ACN, or tetrahydrofuran. However, IL solution has also been added in small percentages, such as 0.025%, to the mobile phase to improve the chromatographic peaks' symmetry and shorting the analysis time of the combination (HES and DIO) to 10 min [9]. Usually, HPLC separation time varied between 12 and 40 min for the estimation of one or both flavonoids (HES and DIO) with the obtained value of LOD less than 2.5 µg mL^{-1} and 1.2 µg mL^{-1} of DIO and HES, respectively.

In conclusion, all developed spectroscopic techniques are carried out for the estimation of just one compound due to one analysis run in the pharmaceutical drugs, excepting one UV work that simultaneously determined the combination of HES and DIO [4]. Chromatographic techniques, especially HPLC, provide an effective way for the determination not only the combination of HES and DIO, but also to study their related substances and their impurities that indicate the transformation of these active compounds due to the non-suitable storage conditions and that might be affecting the pharmacokinetics of the drug.

4.3. Preconcentration and extraction techniques of hesperidin and diosmin from pharmaceutical samples

The difficulties of dissolving HES and DIO in aqueous solutions and most organic solvents limit the use of DMSO or NaOH alkaline solution as solvents. In the case of HES, also MeOH represents an alternative extraction solvent, that could be used for making a selective dissolution of this substance.

Additionally, the huge and rapid production of pharmaceutical industries and the necessity to control the quality of each lot encouraged the exploration of simpler and faster analytical methods. The most developed methods are based on the direct SLE procedure. Normally, conducted using MeOH [19], DMSO [24], mixing between them [9] or NaOH [16, 28] assisted by ultrasound [5, 19, 22, 27], shaking [15, 24], or refluxing [26] assisting techniques. These extraction procedures were performed in time less than 30 min. Otherwise, the use of either NaOH or pure DMSO for flavonoids extraction is not preferable because the strong alkaline such

as NaOH might convert HES and DIO into their corresponding chalcone derivatives [29], While the high viscosity and polarity of pure DMSO can stick and damage the connection tubes and vessels of analytical and detection used techniques such as HPLC connections and its conjugated detectors. For these reasons, the preferred extraction solvents of DIO and/or HES is a mixture of DMSO and MeOH.

4.4. Development of proposed HPLC method for the determination of hesperidin, diosmin and related flavonoids in the bulk and finished pharmaceutical products

The development of simple and fast analytical methods that can be effective in the determination of all combined pharmaceutical active substances in one run, in addition, the ability to detect the impurities that are present in a trace concentration are the main aim in pharmaceutical analytical chemistry.

In the current work, the HPLC method that allows the separation of four flavonoids (HES, DIO, HTIN, and DTIN) as a quaternary mixture in their combined pharmaceutical tablets was developed. In addition to, the possibility of detecting the trace presence of HTIN impurity in its related drugs. Table 6.2 identifies the physiochemical characteristics of the studied flavonoids. The separation was achieved in 22 min. Good precision, high sensitivity, cost-effectiveness, ease of operation, and high selectivity between the analytes and the excipients or impurities present in the pharmaceutical formulations were observed. The LODs of the detected flavonoids were 1.06×10^{-2}, 1.56×10^{-2}, 0.15×10^{-2}, and 0.15×10^{-2} $\mu g\ mL^{-1}$ of HES, DIO, HTIN, and DTIN, respectively. Two experimental design methodologies (FFD and BBD) were applied to study the factors that affected the optimization of the chromatographic separation with less number of required experiments, lower solvents consumption, and considerably less laboratory works.

The extraction procedure was simple. The sample was dissolved in DMSO with just 15 min ultrasonic bath-assisted extraction, then the extract was diluted with MeOH to the appropriate concentration of the analytes.

The developed method is convenient and simple to be applied in routine pharmaceutical quality-control laboratories to determine the active flavonoids, as same as keeping track of the quality of their products through detecting the related impurities in one run.

Experimental results of this proposed method and the detailed information are presented below in a research paper published in an international journal.

4.5. References

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4.6. Original paper

Title: Use of multiresponse statistical techniques to optimize the separation of diosmin, hesperidin, diosmetin and hesperitin in different pharmaceutical preparations by high performance liquid chromatography with UV-DAD

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CHAPTER 5

LAB-ON-VALVE MICRO-SOLID-PHASE EXTRACTION COUPLED TO HPLC-DAD FOR FLAVONOIDS DETERMINATION IN CITRUS JUICE AND THEIR COMMERCIAL PRODUCTS

5.1. Flavonoids in citrus juice

Citrus fruits are the most ubiquitous crops around the world. Spain is the biggest citrus producer in the European Union with a yield of about 21 million boxes per year. The most significant cropped citrus fruits are orange, mandarin, grapefruit, lemon, and lime.

The citrus edible fresh fruits contain flavanones and flavones that are present almost exclusively in citrus, in addition to some herbs such as mint [1]. Flavanones of citrus fruits found mainly as O-glycoside that derivative from mainly three aglycons: HTIN, naringenin (NARIN), and eriodictyol [2]. The main flavones aglycones in citrus juice are acacetin, DTIN, luteolin, QU, and KA [3]. These aglycones formed many glycosides that bound to both sugar molecules rutinose or neohesperidose. The main orange flavanone glycosides are HES, neohesperidin, narirutin, naringin (NAR), eriocitrin, and poncirin [4]. Likewise, the main existing flavone glycosides are DIO, neodiosmin, rhoifolin, and RU [5]. Table 5.1 summarizes the main flavonoids aglycons, their related glycosides, and their general structures.

On the other hand, hand-squeeze juice may have less concentration or even undetectable trace concentration of edible fruit part existing flavonoids, while the commercial citrus juice might be reached by the PMFs, the class of flavonoid placed in the citrus peel, during the industrial squeezing process [6]. Otherwise, flavonoids contribute to fruit and juice quality by the influence on the taste, appearance, and nutritional value of the fruits and their products. For instance, HES shares in the formation of sediments that result in undesirable cloudiness, while the NAR markedly influences the bitterness of the juice [3].

Generally, one drink of orange juice can provide the human with 25 to 80 mg of flavanones aglycons, but consuming the whole fruit can even supply five-fold higher amounts of these components [7] because the fruit fibre might be work as entrapping to flavonoids [8]. Therefore, the concentration of citrus flavonoids varied depending on the growing location, the surrounding environmental conditions, as well as the post-treatment process, such as the harvesting mechanism, the production treatment, and the storage conditions [9-13].

However, Epidemiological data observed that the frequent consumption of citrus fruits is associated with several health benefits such as reducing the risk of cardiovascular disease, cerebral infarction, ischemic stroke, improve cognitive function, anti-inflammatory, cholesterol-lowering, and immune system modulation [4, 14-17]. HES and NAR have been reported as effective compounds on plasma and hepatic cholesterol reduction [18, 19]. In these turns, they prevent cardiovascular diseases, protect from cancers of the colon, bladder, skin, and esophagus [20-22].

Table 5.1 The main flavonoids aglycons present in citrus juice and their related glycosides

	Flavonoid name	Flavonoid nomenclature	Related glycoside	Glycoside nomenclature
Flavanons	Hesperetin	5,7,3′-trihydroxy-4′-methoxy-flavanone	Hesperidin	Hesperetin-7-rutinoside
			Neohesperidin	Hesperetin 7-O-neohesperidoside
	Naringenin	5,7,4′-trihydroxyflavanone	Narirutin	Naringenin-7-rutinoside
			Naringin	Naringenin-7-neohesperidoside
	Eriodictyol	5,7,3′,4′-tetrahydroxyflavanone	Eriocitrin	Eriodictyol 7-rutinoside
	Isosakuranetin	5,7-dihydroxy-4′-methoxy-flavanone	Poncirin	Isosakuranetin 7-neohesperidoside
	Taxifolin	5,7,3',4'-flavan-on-ol	-	-
Flavones	Acacetin	5,7-Dihydroxy-4′-methoxyflavone	-	-
	Diosmetin	3′,5,7-Trihydroxy-4′-methoxyflavone	Diosmin	Diosmetin 7-neohesperidoside
			Neodiosmin	Diosmetin 7-O-neohesperidoside
	Luteolin	3′,4′,5,7-Tetrahydroxyflavone	-	Luteolin 7-O-rutinoside
	Quercetin	3,3',4',5,7-pentahydroxyflavone	Rutin	Quercetin 3-rutinoside
	Kaempferol	3,4',5,7-tetrahydroxyflavone	-	-
	Apigenin	4′,5,7-trihydroxyflavone	Rhoifolin	Apigenin 7-O-neohesperidoside

(-) data not available

5.2. Determination techniques of flavonoids in citrus juice

Regarding the complexity of citrus samples, the determination of flavonoids placed in citrus juice and their commercial products are mainly dependent on the separation techniques for qualitative and quantitative determination simultaneously. The most common separation technique used for the determination of flavonoids in citrus juice is HPLC that has been coupled with UV/VIS [23-25], MS [26-28], CD [29, 30], FL [31]. The majority of HPLC separations have been performed using RP-column such as C_{18} [23, 26, 32-34] and C_8 [35, 36]. Isocratic eluting mode [37] has been rarely employed for the separation of flavonoids in citrus juice due to the difficulty to separate the closed flavonoid molecules. For this reason gradient mode has been applied broadly [38] using ACN and/or MeOH, or tetrahydrofuran as an organic modifier [36], combined with acidified water, such as acetic acid [36] or formic acid [30].

HPLC's derivatization techniques have also been used for this purpose, such as ultra-high-performance liquid chromatography (UHPLC) coupled with UV [39] or MS [40] detector, employing C_{18} column with a particle size of 2.6 μm [39] or 1.8 μm [41]. Also, the 2DLC technique was reported in this field by coupling two separation columns, such as C_{18} column (D1) and carboxymethylated beta-cyclodextrin (beta-CD)-based column (D2) [42], and polyethylene glycol silica narrow-bore column (D1) coupled to a superficially porous C_{18} column (D2) [43] for the determination of flavanones in citrus juice. HILIC-MS using the polymeric zwitterionic stationary phase (ZIC-pHILIC) [44], and rapid resolution liquid chromatography coupled to ESI-MS^n detector (RRLC–ESI-MS^n) [45] have been established for the separation and determination of flavonoids located in citrus juice.

On the other hand, many techniques based on chiral separation have recently been employed to increase the effectivity and the selectivity of citrus flavanones enantiomers separation, such as NAR, HES, neohesperidin, NARIN, HTIN, pinostrobin, isosakuranetin, eriodictyol, and homoeriodictyol. HPLC chiral separation on Chiralcel® OJ-RH column [46], chiral capillary electrophoresis (CCE) performing on an uncoated fused-silica capillary [47], MEKC using γ-Cyclodextrin and sodium cholate as chiral modifiers [48], and CE using sulfobutyl ether-β-cyclodextrin in the background

electrolyte [49] were developed and used for the determination of flavonoids in citrus juice.

5.3. Preconcentration and extraction techniques of flavonoids from citrus juice matrices

The determination of flavonoids in citrus juice and their commercial products have been mainly previously preceded by a conventional sample preparation such as the sample dilution, filtration, and direct analysis [33, 50-52]. Despite the advantages of direct sample preparation, inconveniences such as the decreasing of the concentration of target analytes led to the lack of sensitivity. In order to increase the sensitivity in direct sample preparation procedure, some steps should be considered, such as the use of selective organic solvents [28, 34, 36, 53], assisting with extraction techniques, for example, shaking [53], ultrasonic bath [45], hot ultrasonic bath [54], or magnetic stirring system [23] to accelerate their dissolution. In this field, ethyl acetate has been used as flavonoids dissolving solvent [23, 28], but in the case of LC determination, this solvent required extra procedure such as evaporate the solvent to dryness and re-dissolve the residual with ACN or MeOH to fulfil the character of RP-LC. Likewise, refluxing extraction technique has been also applied for both hydrolysis and flavonoids extraction from citrus juice using hydrochloric acid [30], or trifluoroacetic acid (TFA) [55] as hydrolysis solution, while the above mentioned organic solvents have been exploited as extraction solvents. All previously mentioned conventional extraction techniques that have been used up to date for the extraction of flavonoids from citrus juice matrices consume huge quantities of solvents, sample and need a long processing time, thus, increasing the environmental contamination and the analyst exposure to the performed solvents hazard.

As has been exposed before in this manuscript, the SPE technique significantly reduces the consumption of solvents and running time comparing with the conventional extraction techniques. Additionally, the automation and miniaturization of the SPE approach have been recently focused on reducing the time and solvents that the manual SPE approach consumed and decrease the exposure of analysts to the solvents and prevent them from their hazards. SPE has widely been used for the extraction of flavonoids from citrus fruits, employing polyamide [47], C_{18} [31, 32, 38], XAD-2,

XAD-4, XAD-16N made from styrene-divinylbenzene [39] or mesoporous molecular sieve SBA-15 [41] as an extraction resin. Whereas, ACN [32, 38], or MeOH [31] are the chosen eluting solvent. Otherwise, SPE derivatives techniques have also carried out for this purpose, such as C_{18}-MSPDE [35], dispersive magnetic-SPE (DM-SPE) based on hemi/ad-micelles-Fe_3O_4/SiO_2 nanoparticles extraction resin [24].

5.4. Automation of the lab-on-valve micro-solid-phase extraction coupled to HPLC-DAD for flavonoids determination in citrus juice and their related products

The automation of citrus juice sample pretreatment is important to decrease the total solvent consumption, thus, protecting the environment and the working staff. Flow techniques facilitate not only the automation of sample pretreatment, but also the online transferring of the eluant to the analysis apparatus [56].

The first trial of the online determination of one flavonoid “HES” in the orange juice and peel was done employing the FIA system for the online mixing of the HES with the Al^{+3} in a micellar medium for the formation of fluorescent complex, then the online determination of this complex was done by FL [57]. The main disadvantages of using the FIA system are the basing on the PP that pushes continuously the reagents resulting in the huge consumption in solvents and sample. Otherwise, FIA is not computer-controlled obtaining the need to make the changes manually, such as starting and stopping the pump, as well as the impossibility to automatically process the analytical results.

The proposed work presents at the first time an online LOV system coupled with HPLC-DAD for the accurate and sensitive determination of two flavonoids (HES and DIO) in citrus juice, Table 6.2 presents the physicochemical characteristics of the studied analytes. The µSPE is performed automatically and the analytes were eluted with 0.27 mL of ACN, from which a fraction of 20 µL is online injected into the chromatographic system. Up to 35 extractions can be carried out before the renewal of the C_{18} micro-column, which is also performed in an automated way.

The developed system allows the determination of target analytes since the LODs and LOQs are lower than 0.1 µg mL^{-1} and 0.3 µg mL^{-1}, respectively. The method was successfully applied to the analysis of 14 natural and commercial samples from three different kinds of citrus fruits. The main advantages obtained by the proposed methodology are its good precision and sensitivity with minimum sample and solvents consumptions, which leads to cost-effectiveness compared with off-line approaches. Besides, reagent handling is reduced since all analysis steps are carried out automatically, which brings about a safer method that can be performed unattended.

Experimental results of this developed method and more detailed information are presented below in a published paper in a research international journal.

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5.6. Original paper

Title: Development of an on-line lab-on-valve micro-solid phase extraction system coupled to liquid chromatography for the determination of flavonoids in citrus juices

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CHAPTER 6

3D PRINTED DEVICE FOR THE EXTRACTION OF FLAVONOIDS FROM CITRUS FLAVEDO THROUGH FLOW INJECTION ANALYSIS COUPLED TO HPLC-DAD

6.1. Flavonoids in citrus peel

Citrus fruit is divided into edible and inedible (peel) parts. During the term of that, the inedible part is subdivided into albedo (the white inner middle layer) and flavedo (the external-coloured part) (Figure 6.1).

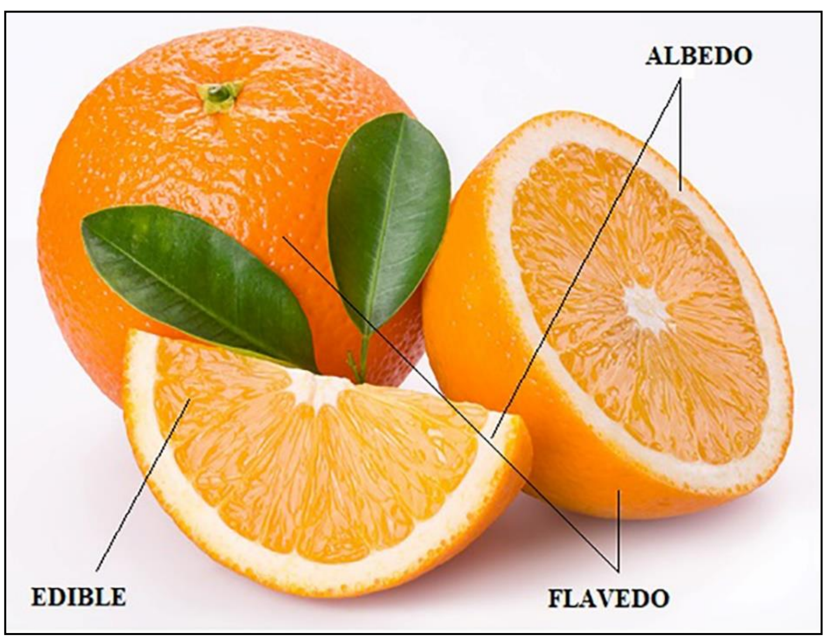

Figure 6.1. Layers of the citrus fruit

Citrus fruits, especially oranges are the most consuming fruits in the world as commercial production of juice. Oranges formed about 60% of the total consuming citrus fruits yearly [1]. This production produces tons of by-products residual citrus peel, about 50% of used fruits [2]. Some researchers have been focused on the biotransformation products of citrus peel, such as producing plastic polymer through the combination of some major compounds located in the citrus peel and atmospheric carbon dioxide, producing biogas, citric acid, and activated carbon [3]. Otherwise, citrus peel reaches by vitamin C, essential oils, minerals, phenolic compounds, flavonoids, and among others [4], which can be used as animal feed [5]. Another interesting application of this by-product is as the source of natural flavonoids, which can constitute 10% of the dry weight of the citrus fruit [6]. Many developed works have been studied the possibility to extract these flavonoids and use them in dietary

supplement, pharmaceutical drugs and even as standard analytical compounds regarding the availability of citrus peel, low-cost of production and their extensive quantity of flavonoids [7, 8]. For instance, the first flavonoid commercially produced from orange peel was HES [9] that can be used as an analytical standard or as API in the pharmaceutical drug [10]. NAR was the first flavonoid separated from grapefruit peel and commercially used to induce a bitter taste in beverages, jams, and as a raw material to produce rhamnose, p-coumaric acid, phloglucinol, and dyes [11]. Also, citrus peel is rich in PMFs such as nobiletin, tangeretin (TAN), sinensetin, and neohesperidin. In addition to flavanones and flavones aglycons and their related glycosides such as NAR, narirutin, rhoifolin, isorhoifolin, poncirin, didymin, DIO [12-16], and HES that consider as the major flavonoids in citrus peel [17]. Table 6.1 reports the main PMFs, flavanones, and flavones located in citrus peel.

Citrus peel-flavonoids (CPFs) are dissimilar in all citrus fruit classes, for example, neoeriocitrin, NAR, and neohesperidin are the main flavanones found in the peels of bergamot, lemon, and orange. On the other hand, HES and narirutin are the most abundant flavonoids in sweet orange, whereas NAR is the most bountiful in grapefruit and bitter orange peels [18].

Many therapeutic remedies of CPFs have been observed such as cure scurvy [19], antioxidant agents [20], prevention of degenerative diseases, anti-cancer [21], antiviral, anti-tumour, and anti-inflammatory activities, reduce capillary fragility, restrict human platelet aggregation, reduced the risk in the development of neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and multiple sclerosis [22, 23].

Table 6.1. The main flavonoids aglycons present in citrus peel, related glycosides, and their structures.

	Flavonoid name	Flaovnoid nomenclature	Related glycoside	Glycoside nomenclature
Flavanones	Hesperetin	5,7,3′-trihydroxy-4′-methoxy-flavanone	Hesperidin	Hesperetin-7-rutinoside
			Neohesperidin	Hesperetin 7-O-neohesperidoside
	Naringenin	5,7,4′-trihydroxyflavanone	Narirutin	Naringenin-7-rutinoside
			Naringin	Naringenin-7-neohesperidoside
	Isosakuranetin	5,7-dihydroxy-4′-methoxy-flavanone	Poncirin	Isosakuranetin 7-neohesperidoside
			didymin	Isosakuranetin-7-O-rutinoside
Polymethoxyflavone	Nobiletin	5,6,7,8,3',4'-hexamethoxyflavone		
	Tangeretin	4',5,6,7,8-pentamethoxyflavone		
	Sinensetin	3',4',5,6,7-pentamethoxyflavone		
	Quercetogetin	3,3',4',5,6,7-Hexahydroxyflavone		
Flavones	Apigenin	4′,5,7-trihydroxyflavone	Isorhoifolin	Apigenin-7-O-rutinoside
			Rhoifolin	Apigenin 7-O-neohesperidoside
	Diosmetin	3′,5,7-Trihydroxy-4′-methoxyflavone	Diosmin	Diosmetin 7-neohesperidoside

(-) data not available

6.2. 3D printed devices in analytical chemistry

When 3D printer apparatus became available to the public, it is poised to revolutionize manufacturing and research in many fields i.e. medicine, automotive, aerospace, military, chemistry, and biochemistry. Many 3D printer technologies have been developed such as SLA, PolyJet (PJ), and fused deposition modelling (FDM), and others. Among all, SLA is fast, low cost, simple, and enables highly accurate printed complex structures. It allows the

building of a 3D polymer structure that relies on a layer-by-layer photopolymerization of a liquid photopolymer resin based on a focused laser or UV light source [24].

The interest in the 3D printing techniques has been increased in the analytical chemistry field with the objective to design, prototype and print functional devices, and even for teaching aids [25]. However, in flow analysis field and sample pretreatment, 3D printer apparatus has been playing a considerable role in the development of new platforms, SPE supports and even used directly as SPE extraction resin. In this context, several 3D printed applications have been developed in the case of sample pretreatment. In spite of the small surface area of 3D printed materials and their limitation of extraction capacity, it used in some works as a SPE sorbent [26].

Otherwise, two techniques have been developed in order to immobilize commercial sorbents onto the surface of 3D-printed devices to circumvent the limitation of these materials. First, benefit from the less crosslinked monomers and sticky external surface "green state" of newly SLA printed devices to immobilize the sorbent before curing the device under UV crosslinker to compete for the polymerization and the immobilized material permanently physically attached to the device, namely: Sticky & Cure technique [27]. In this sense, the advantages of this immobilization techniques are reagent-free and no additional preparation time and skills are required [28]. Second, disperse the sorbent with a polymer into a solvent's mixture, then apply drops of the mixture onto the 3D printed device after the UV curing stage, namely: Post-printing technique [29]. Thus, the functional material can be reached the whole effective area of the device.

To date, the application of the 3D-printed device in sample pretreatment applications based on the FATs has been limited in environmental pollution determinations [27-35]. In this work, the application of C_{18} resin immobilized onto 3D printed device is performed by the combination of two immobilized techniques above mentioned, in the case of combining between their advantages, based on the flow system to automatically pretreat the flavonoids from citrus peel matrices.

6.3. Separation techniques of citrus peel-flavonoids

Several selective methods have been developed for the determination of individual interested flavonoids in the citrus peel. PC and TLC are the simplest ones that used for the isolation and determination of flavonoid glycosides in citrus peel [16]. More recent techniques such as HPTLC have been used for the separation of HES from different varieties of citrus peel samples using silica gel 60 as a stationary phase [36]. HPLC, μHPLC [37], and UHPLC [38] have also been applied widely coupling to DAD [39-41], ECD [37], or MS [42] detection system. C_{18} [43] or C_8 [17] employed as a stationary phase and acidified water with an organic modifier as a mobile phase. RP-column rarely used for this separation objective, NP-LiChrosorb Si 60 packed column with a mobile phase of n-heptane-ethanol or n-heptane-isopropanol [44] was reported.

Subcritical water chromatography (SWC) [45] and SFC [46] that use hot water or carbon dioxide, respectively, instead of organic solvents as a mobile phase have been carried out for the separation and identification of PMFs. These techniques are considerably faster than HPLC with good resolution and an adequate accuracy for the quantitative identification of PMFs, but they attain less sensitivity than HPLC [46].

Slight works have been developed for CPFs estimation performing GC with an OV-1 column [47], coupled with CE-ED [48], or ^{1}H NMR that was developed for the identification of the possible stereoisomers of flavanone glycosides placed in citrus peel such as NAR, HES and neohesperidin [49].

6.4. Preconcentration and extraction techniques of citrus peel-flavonoids matrices

The success of CPFs analysis depends on the sample pretreatment procedure and the extraction quality of the interested analytes. Although, the peel sample required preconditioning of raw materials either taken in fresh [50], frozen [42, 51], or in dried [41, 52] form, that required more complexity treatment procedure than the dealing with juice matrices. However, frozen or dried form is the easiest way for ground and homogenization

of the sample than the fresh one, as well removing the water content from the matrix (exist in the range of 70-90%) and lead to concentrate the flavonoids in the sample [6].

After preconditioning the sample, SLE is a common conventional extraction method of CPFs (the simplest extraction method) followed by shaking extraction [53], refluxing extraction [41], and Soxhlet extraction method [54]. MeOH [37], ethanol [52], acetone, and ethyl acetate [55] have been employed as extraction solvents. Whereas, the main drawbacks of these methodologies are a huge time and solvents consumptions and their low extraction yield belonging to the possibility to flavonoids degradation due to the required high operation temperature and long processing time.

In recent years, several modern extraction methods have been developed to minimize the undesired consumption and increase the enhancement extraction factor. MAE [56], UAE [39, 40, 57, 58], accelerated solvent extraction (ASE) [59] the PLE technique [60], high-pressure solvent extraction [61, 62], and subcritical water extraction (SWE) [45, 63-66]. fluids. The main sharing disadvantage of all the above-mentioned extraction methods is the applying the elevated temperature and pressure due to a long extraction time that might cause degradation of CPFs and change them to another form such as the transformation of glycoside into its related aglycon. This behaviour might be observed as a negative error in the case of the determination of glycosides forms, on the contrary, attains a positive error in the case of aglycon determination.

The SPE technique using C_{18} as an extraction resin and MeOH as an eluting solvent has been attained for this extraction purpose. Resulting in the reduction in the solvent and operation time and increases the effectivity of the extraction [50, 51]. MSPDE as a modern purification method has been used with Florisil sorbent and 1-butyl-3-methylimidazolium tetrafluoroborate as the IL elution solvent for the extraction of flavonoids from orange fruit peel [38]. Molecular sieve SBA-15 with MeOH as the eluent has also performed [43].

Otherwise, a semi-online extraction method was developed by mixing C_{18} gel with the powdered sample and packed into a guard column, which in turn, is connected to the

QTOF-MS/MS system for the extraction and determination of flavonoids isomers. The interested analytes eluted by MeOH / 0.1% formic acid as an elution solvent [67].

Pulsed electric field (PEF) assisted extraction was tested as a promised technology for increasing the extraction yield of CPFs using the pressing extraction technique. PEF facilitates the release of intracellular compounds from the cells by inducing permeabilization of the cytoplasmatic membranes. 7 kV/cm electric field might be increased the extraction yield of NAR and HES up to 159% due to 30 min of pressurization at 5 bars [68]. On the other hand, this technique consumes energy and time, in addition to, it needs especial expensive equipment that might be not existed in each laboratory.

6.5. Automation of the flow injection analysis technique coupled to HPLC-DAD method for flavonoids determination

The tediousness of the necessary sample preparation step prior CPFs determination can be solved by the automation of the analytical protocol exploiting FATs, thus, achieving lower detection limits, lower solvents, and sample consumptions, improve tolerance to potential interferences, and enhances the method throughput [69]. The applicability of FATs for CPFs sample pretreatment has not been focused on yet, except, the development work by Pérez-Ruíz et al. [70] that was explained in the previous chapter in section 5.4 and our proposed system for SPE extraction of citrus juice flavonoids exploiting the LOV [71], as reported in chapter 5.

The combination of the features of the flow analysis system and 3D printer technology can add big value to the automation of the SPE method, taking into account the high backpressure observed in the case of using an extraction column into flow techniques.

The proposed work presents a cheap fabricated 3D printed device coated with an extraction resin “C_{18}”, applied through an online coupling system between MPV and HPLC-DAD for the accurate and sensitive determination of six main flavonoids (NAR, NARIN, HES, HTIN, DTIN, and TAN), Table 6.2 presents the main physicochemical characteristics of

these interesting flavonoids. The procedure is performed automatically allowing decreasing the consumption of the solvents since just 0.3 mL of ACN was used as eluent. Then 10 µL of eluent is online injected into the chromatographic system. It has to be taken into account that the automation of the determination of CPFs applying 3D printed device into flow system leads to enhance the reproducibility, the throughput of the method, reducing the solvents and sample consumptions through the use of the 3D printed device over 19 times before its renewal, in addition to 75 injection fractions that can be carried out by each extraction time. Also, the developed system allowed improving the detection sensitivity of the interested analytes due to their LODs and LOQs which were lower than 4.69×10^{-2} µg mL^{-1} and 1.88×10^{-1} µg mL^{-1}, respectively. The applicability of the method was successfully tested by analyzing eight citrus flavedo samples from three different kinds of citrus fruits.

Experimental results of this developed method and more detailed information are presented below in a published paper in a research international journal.

Table 6.2. The physiochemical characteristics of studied flavonoids of chapters 4, 5 and 6

Flavonoid name	Flavonoid structure	Molecular Formula	Molecular weight (g/mol)	Boiling point (°C)	Solubility
Hesperidin		$C_{28}H_{34}O_{15}$	610.6	576.2	Insoluble in water; slightly soluble in glycerol; soluble in ethanol, MeOH and DMSO
Diosmin		$C_{28}H_{32}O_{15}$	608.5	926.8	Practically insoluble in water and alcohol; soluble in DMSO and alkali hydroxide
Hesperetin		$C_{16}H_{14}O_{6}$	302.3	586.2	insoluble in water; partly soluble in dilute alkalis, and ethanol; soluble in DMSO
Diosmetin		$C_{16}H_{12}O_{6}$	300.3	576.7	insoluble in water; soluble in DMSO, chloroform, dichloromethane and acetone
Naringin		$C_{27}H_{32}O_{14}$	580.5	559.4	Soluble in water, alcohol, acetone, DMSO and warm acetic acid
Naringenin		$C_{15}H_{12}O_{5}$	272.3	335.3	Practically insoluble in water; soluble in ethanol and DMSO
Tangeretin		$C_{20}H_{20}O_{7}$	372.4	565.3	Insoluble in water; soluble in MeOH, DMSO and ethyl acetate

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6.7. Original paper

Title: 3D printed structure coated with C_{18} particles in an online flow system coupled to HPLC-DAD for the determination of flavonoids in citrus external peel

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CHAPTER 7

HPLC METHOD FOR FLAVONOIDS DETERMINATION IN PHARMACEUTICAL AND FOOD SUPPLEMENTS

7.1. Flavonoids in food supplements

Contrary to the restricted conditions required to produce pharmaceutical drugs, dietary supplements are not compulsory to be controlled for safety, according to FDA under the Dietary Supplement Health and Education Act (DSHEA) definition [1]. Food supplements are considered as food that taking daily for supplementing the diet. Their main function is to provide a specific nutrient through the diet along with food and used for preventing or protecting the human from diseases.

Increasing the consumption of food-rich flavonoids is consistently recommended to reduce the risk of several chronic diseases. In this context, the interest in the development of dietary supplement products has been recently focused on as the alternative to easily boost the required antioxidant compounds to the consumer. Food supplements normally consist of pure flavonoids and/or plant-rich flavonoids that might be dried and purified. These concentrated flavonoids intake can be supported the bioavailability of dietary flavonoids, as well their absorption and ultimately their high concentration in the plasma and tissue levels [2].

The recommended daily intake of flavonoids is in amounts that are many times higher than those levels that can normally be taken from diet-rich flavonoids. In this sense, currently, there is an extensive range of flavonoids supplements in the market to provide this recommended dose [3]. The most worldwide botanical dietary supplements-rich flavonoids are soybean-isoflavones and GBL so far. These crops are not available in many countries, for this reason, the transformation of these crops into products that can be easily exported around the world, attracts the food supplements industry. QU is an example of widely intake flavonoids supplements, the recommended dose of QU is up to 1g [4], while the daily QU intake from food is in the range of 10-100 mg [5]. In this context, 1g of purified QU and/or concentrated dried powder of GBL (the main source of QU and other important flavonoids such as RU, KA, and IS) are marketed [6].

The extract of GBL is standardized to contain not less than 24% of flavonoids [7] and widely used for treating peripheral and cerebral circulation disorders, fighting memory loss, reducing lipid peroxidation in vascular walls and nerve cells, and as a potential drug for Alzheimer's disease [8, 9]. This plant is classified as a dietary supplement according to the DSHEA. Under this act, dietary supplements do not require review or approval by the FDA as mentioned above. This fact has led to significant variations in the quality of food supplement products and open the door to the possibility of the fabrication of adulterated products [10].

7.2. Determination techniques of rutin, quercetin, kaempferol, and isorhamnetin in food supplements

Normally, dietary supplements are performed in several dosage forms such as tablets, pills, capsules, oral solution, and oral ampoules, as mentioned in section 1.1.3. These products can be a dried natural plant material, or purified compounds, in addition, the combination between them as well as other additive substances might be added such as vitamins, minerals, metals, and fish oil. As same as the pharmaceutical drug production, to form this dosage product, the manufacturer must add some additive materials "excipients" to give these dosage forms their final stable shape, their taste, and appearance. All these additive compounds make the food supplement samples more complex and, in most cases, require a purification step before their analysis.

In this case, performing an effective sample preparation procedure that can be able to eliminate matrix interferences with less time and solvent consumptions and it being as simple as possible to be employed by non-expert staff. In addition, the adjustment of the quality of dietary supplements and determine the exact quantity of their active compounds, saving the analysis time is the main challenge of the analytical chemistry researchers.

Total flavonoids in food supplements have been estimated for biological or pharmacological purposes carrying out the colourimetric spectroscopic detection after flavonoids reaction with Al^{3+} [11, 12] or Cu^{2+} [13] chelating ions. However, the

Association of Official Analytical Chemists (AOAC), under contract to the FDA and the National Institutes of Health (NIH) of the USA has developed a group of fully validated methods for the estimation of active substances in raw and in final products of dietary supplements [14]. The AOAC's official method for the determination of flavonoids in GBL supplements is based on the calculation of total glycosides depending on three primary aglycones in the GBL products (QU, KA and IS) formed after acid hydrolysis with HCL and the heating at 90°C/60 min. Analytes are estimated by HPLC-UV. Total flavonol glycoside concentration is then calculated using individual conversion factors for these three aglycones [15]. This method is so similar to the official method presented in the pharmacopoeias for the determination of total flavonols in the pharmaceutical products of GBL with the difference in the acceptable concentration range between (22.0 and 27.0)% of flavonols, and the hydrolysis process performance is 135 min by refluxing in a hot water bath [16-18]. As clearly observes that these official standardization methods are simple and widely acceptable, but on the other hand, they consume a relatively long sample preparation time ≥ 60 min and attain weakness to identify the derivatives of these flavonols and the real concentration of located aglycons and glycosides due to the hydrolysing of all existing flavonoids glycosides into their related aglycon form. Otherwise, the adulteration of flavonoids content in herbal finished products could not be revealed by hydrolysis process application [19].

Additionally, the importance of presenting glycosides in herbal products is the possibility to employ them as a quality control indicator of the finished products because they can be converted into the related aglycons under incorrect storage conditions. Therefore, the calculation of the percentage of glycoside/aglycon gives the information on the quality of the product that it is impossible to calculated applying the hydrolysis procedure. For these reasons, several methods have been developed to ensure comprehensive standardisation and improve the real quality control techniques of these flavonoids in food supplement matrices depending on the HPLC technique [20]. For this determination, this technique has been coupled with UV [9, 21-24], diode array [25, 26], MS [27, 28] and ED [14]. The separation exploited on RP-columns such as C_{18} [9, 14, 21-23] using, in some cases, water with MeOH as organic modifier [21], while acidified water is the most aqueous mobile phase

that has been used for flavonoids determination such as acetic acid [22], formic acid [25], and acetate buffer [9]. The using of microemulsion mobile phase consisted of 1.2% (v/v) of Genapol X-080 was applied for RU and QU separation [29]. NP-columns such as diol [14], and phenyl [14] have also been used. Almost most HPLC-developed methods required on average between 30 and 60 min for the separation of up to 10 flavonoids.

Recently, UHPLC has been applied for flavonoids determination in food supplements using normal [30] or monolithic C_{18} [24] column. MC technique allows the separation of up to 23 flavonoids at a time ranged between 5 and 28 min [31, 32], taking into account the selectivity that improved by using smaller particle size as same as new technologies of separation column such as a monolithic column that was detailed in section 1.2.2.4.

7.3. Sample pretreatment of food supplement matrices

Sample pretreatment procedures that have been performed prior to the determination of RU, QU, KA, and IS in food supplement matrices mainly depend on the direct SLE using ethanol [33], MeOH [9], or the mixture between MeOH and DMSO [21], assisted by ultrasound at a variety processing time up to 120 min [20, 24, 26, 34-36], or refluxing at the time ranged between 30 min and 12 h at the approximate operating temperature of 90 °C [12, 33, 37, 38].

Usually, the hydrolysis process is preceded in the flavonoids' analysis in food supplements. This procedure normally exploits during the refluxing extraction using hydrochloric acid at the same refluxing time and temperature [8, 33, 38], or enzymatic hydrolysis that required less time and temperature degree, such as the treatment of flavonoids in the seeds of *Cuscuta Chinensis Lam.* at 45 °C/45 min [39].

Other extraction techniques have not been much often applied for the extraction of flavonoids from food supplements, i.e. there are a few works that performed MSPDE using silica resin as an absorbent and MeOH as eluent for the extraction of GBL ingredients including 10 flavonoids prior to their UHPLC-MS determination. The procedure takes

place in 20 min [32]. Mixed cloud point extraction (MCPE) as a sample treatment with non-ionic surfactant Genapol X-080 and cetyl-trimethyl ammonium bromide as an extraction solvent has been used for the determination of six flavonoids from *Apocynum venetum* leaf [40]. The procedure was made in 20 min at 55 °C after refluxing the sample with ethanol for 1.5 h. Also, SFE applying pure carbon dioxide as a carrier without organic modifier has been used for the extraction of total flavonoids from *Maydis stigma* at 50 °C and 41 MPa of temperature and pressure, respectively, with an extraction time at 120 min [11]. MAE was also applied by refluxing with MeOH at 12h [12].

As already mentioned, the available extraction methods prior to the analysis of flavonoids may apply different conditions such as temperature, pressure, and time. In our point of view, sample pretreatment before the determination of flavonoids in food supplements and natural pharmaceutical formations is totally depending on the matrix and the significant influence of related matrix compounds on the determination of these compounds. The preferable extraction method should be carried out without hydrolysis, high temperatures, and done in the shortest possible time. In this case, the flavonols neither are decomposed nor converted to other forms, thus, they can be detected individually estimating their real concentrations, as well reliable evaluation of the biological and pharmacological behaviour of each one.

7.4. Development of the HPLC-DAD method for flavonols determination

The development of a simple HPLC-DAD method for the simultaneous determination of four flavonols (RU, QU, KA, and IS) has been achieved in the proposed work. The physiochemical characteristics of studied flavonols are presented in Table 7.1. The chromatographic separation was attained with high sensitivity, good precision, cost-effectiveness, ease of operation, and selective separation between the analytes and the excipients or impurity present in the matrices with low LOD and LOQ, lower than 0.03 µg. mL^{-1} and 0.08 µg. mL^{-1}, respectively. The method was successfully applied to different food supplements and pharmaceutical preparations. In addition to the pharmaceutical excipients, the studied samples contain other adding substances such as vitamins and

naturally extracted plants and fruits. Satisfactory selectivity and recovery values prove the potential of the developed method for the determination of the analytes in these kinds of samples. Besides, the method is suitable for the application in the quality control routines due to its ease of operation to determine the active flavonols, as well as it ensures the quality of products through detecting the artificial flavonols that could be added to these products.

Table 7.1. The physiochemical characteristics of studied flavonols

Flavonoid name	Flavonoid structure	Molecular Formula	Molecular weight (g/mol)	Boiling point (°C)	Solubility
Rutin		$C_{27}H_{30}O_{16}$	610.5	576.1	Practically insoluble in water; soluble in pyridine, DMSO, DMF
Quercetin		$C_{15}H_{10}O_7$	302.2	363.3	Insoluble in water; soluble in DMSO ether, MeOH, ethanol, acetone, pyridine, acetic acid
Kaempferol		$C_{15}H_{10}O_6$	286.2	582.1	Insoluble in water; slightly soluble in chloroform; soluble in acetic acid, alkalis, DMSO, ethanol, ethyl ether and acetone
Isorhamnetin		$C_{16}H_{12}O_7$	316.3	599.4	Sparingly soluble in water; soluble in acetone, MeOH, ethyl acetate, DMSO

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7.6. Original paper

Title: A High-Performance Liquid Chromatographic method for the simultaneous determination of four flavonols in food supplements and pharmaceutical formulations.

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CHAPTER 8

CONCLUSIONS

8. Summary and conclusions

The proposed thesis has been related to new automated approaches for the micro-extraction and estimation of flavonoids substances located in edible plants exploiting together and separated, flow and separative techniques. The developed methods have been implemented especially for the quality control purposes of the food, dietary supplements, and pharmaceutical formulations industries.

In general, HPLC as a separation technique in the presented analytical methods achieved super-effective methods for the separation of the studied flavonoids. The UV-DAD detection system coupled with HPLC provided a good selectivity and sensitivity. These proposed methods were rapid, simple, accurate, sensitive, selective, compared with other previously reported. Therefore, they are appropriate for the routine analysis in pharmaceutical and dietary supplements' laboratories to estimate APIs and monitor the quality of the bulk and the finished products. Further, using FATs to automate the different stages on the developed methods, maximized robustness, enhanced stability, and simplicity, resulting in accurate and precise data. Additionally, provided saving time and reagents, minimized the handling of the solutions, improved the extraction throughput, maintained environmental sustainability, and reduced waste production.

Specifically, in chapter 4, a simple HPLC-DAD method was effectively developed and utilized for the determination of two main flavonoids (HES and DIO) as APIs in pharmaceutical formulations and their two related flavonoids (HTIN and DTIN) as the natural impurities in the studied drugs, respectively. The proposed methodology achieved high selectivity to separate similar flavonoids' structures. The sensitivity of the developed method allowed detecting the flavonoid HTIN for the first time in trace concentration. Moreover, a simple sample preparation procedure was carried out. Direct SLE assisted with ultrasonic bath tool was employed for this purpose. As a lot of factors have a direct influence on analytes separation, DOE was successfully applied to optimizing them. DOE is a rapid and effective tool for optimizing the interaction between the HPLC significant factors. The

proposed method is useful for detecting the existing impurities in the drugs, thus assuring the quality of these products.

In chapter 5, a μSPE procedure was described performed at a LOV platform where a grafted C_{18} microextraction column was employed for the extraction of HES and DIO from citrus juice samples. The system was online hyphenated with the HPLC-DAD tool for the automatic determination of the extracted flavonoids. Using the developed system, all sample pretreatment steps were completely carried out automatically, the extraction resin was filled directly into the extraction column, conditioned, the matrix endogenous substances were removed, the remained analytes were eluted using the minimum quantity of eluting solvent, and then the analytes were transferred automatically to the HPLC separation column. The extraction column can be used up to 35 times before its renewal automatically and each eluant fraction can be injected up to 8 times, that can increase the repeatability and the reproducibility of the results, decrease the time and cost per analysis. The μSPE-LOV-HPLC-DAD system was successfully exploited for the effective extraction of HES and DIO from three different kinds of citrus hand-squeezed juice, edible part, and their related commercial products. The developed method gave extensive information about the distribution of the studied flavonoids in juice, edible part, citrus fruit's white layer, and also in commercial juices. This conjugated system has boosted the robustness, reducibility, increased the analysis throughput, the extraction factor, and reduced the reagents, sample, and extraction resin consumptions. The proposed system is convenient for the food industries routine quality control analysis to adjusting the quality of products during their production steps and the finished products.

In chapter 6, determination of six flavonoids, mainly located in the external peel of citrus fruits (NAR, NARIN, HES, HTIN, DTIN, and TAN) was made. 3D printing technology was performed for creating a SPE supporting device that coated with a C_{18} as an extraction resin. The printed extraction device was attained exploiting the MPV apparatus helping by the MSA system in term of the automation of the extraction. The used flow system was online coupled with HPLC-DAD for the analysis of extracted flavonoids applying C_{18} monolithic column as a powerful technology for the separation of closed flavonoid structures.

The combination between two immobilization procedures increased the loading of extraction beads onto all printed device surfaces even the interconnected surfaces, hence, increases the extraction power and decreases the necessity to build a bigger device that can be able to load more quantity of extraction beads, decreasing the printing cost, the waste of printing resin, and the printing time. Each printed disc (each device costs approximately 0.12 €) can be employed up to 19 times before its renewal that leads to minimizing the analysis cost . On the other hand, the development of the MSA system and the extraction tank reservoir in our laboratory have facilitated the connection between the extraction device and the analytes in the sample solution, improved the mixing efficiency, preconcentration factor, minimized solvent consumption, and speeded up the extraction procedure. The final collected elution volume allowed up to 75 injections per extraction, that increased repeatability and minimize the required time, money, and solvent consumptions per analysis. The capability of the hyphenated MPV-SPE (3D printed device)-HPLC-DAD system was demonstrated for the fully automated extraction and determination of the interested flavonoids in three various types of citrus external peel. The method was simple, robust, and allowed an efficient, rapid clean-up, reduced matrix-induced effects, and increased the potentiality of the industrial routine analysis. Furthermore, the developed system provided thorough information about the concentration of the studied flavonoids only in the external peel of citrus fruit without the white layer, contrasting all previously applications. Also, it showed the distribution of the flavonoids into the different kind of citrus species.

The synchronism of the sample pretreatment and chromatographic analysis increased the analysis throughput due to the extraction and separation steps that were carried out simultaneously at the same time. The MPV-SPE (3D printed device)-HPLC-DAD method can be successfully used in the food industries for routine analysis, as same as in the chemical standard materials manufactories that extract these flavonoids from the citrus peel.

In chapter 7, The complex dietary supplement, and natural pharmaceutical forms have been studied. A powerful HPLC method was developed for the separation of four main flavonoids (RU, QU, KA, and IS) in these natural-based matrices. The proposed method was able to separate accurately the target flavonoids from the countless impurities that usually present in these natural-based samples, also the additive vitamins, the mixture of other bioflavonoids,

and other fruit natural extracts that have been added to the finished product were not affected the accuracy. Furthermore, the main flavonols of Ginkgo biloba leaves were estimated without applying the hydrolysis procedure prior to HPLC analysis. A simple and direct extraction procedure was followed before sample analysis. The extraction method fulfilled high recoveries of all interested flavonoids in pharmaceuticals and dietary supplements. Regarding that, the developed method provides an effective tool for the estimation of the real flavonols concentration in these products, discovering the possible adulteration, and detecting the quality of the finished products during the time of storage.

In conclusion, applying separation techniques such as HPLC, provides selective and sensitive analytical methods for the separation and determination of flavonoids. On the other hand, the implementation of microextraction techniques in flow-based systems and their hyphenation to the HPLC technique allowed improving the selectivity and the sensitivity of the methods. Moreover, the automation of sample pretreatment enhanced reproducibility, analysis throughput, and reduced analysis costs, waste production, and protects the environment and the analysts Thereby, more efficient analytical methods are obtained in terms of precision, environmental impact, and cost-effectiveness.

8. Resumen y conclusiones

La tesis propuesta presenta nuevos enfoques automatizados para la microextracción y determinación de flavonoides en plantas a través del uso, tanto por separado como en conjunto, de técnicas de análisis en flujo (siglas en inglés FAT) y separación. Los métodos desarrollados pueden ser implementados especialmente para el control de calidad de estos analitos en industrias de alimentos, suplementos dietéticos y formulaciones farmacéuticas.

En general, el uso de la cromatografía líquida de alta resolución (siglas en inglés HPLC) como técnica de separación en los métodos analíticos presentados resultó será muy eficaz para la separación de los flavonoides estudiados. El sistema de detección ultravioleta y arreglo de diodos (siglas en ingles UV - DAD) junto con HPLC, proporcionó una buena separación entre los analitos con una buena selectividad y sensibilidad. Los métodos propuestos son rápidos, simples, precisos, sensibles, y selectivos en comparación con otros reportados en la literatura y por lo tanto, son apropiados para el análisis de rutina para estimar los ingredientes farmacéuticos activos (siglas en inglés API) y monitorizar la calidad de los productos terminados. Además, el uso de FAT para automatizar las diferentes etapas del procedimiento analítico en los métodos desarrollados maximizó la robustez, la estabilidad y la simplicidad proporcionando resultados con mayor precisión y exactitud. Además, con dichos métodos se consiguió un ahorro de tiempo y reactivos, así como también se mejoró el rendimiento de extracción y se redujo la producción de desechos, lo cual conlleva a la obtención de métodos más amigables con el medio ambiente

Específicamente, en el capítulo 4, se desarrolló y utilizó eficazmente un método simple con HPLC-DAD para la determinación de dos flavonoides principales hesperidina y diosmina como API en formulaciones farmacéuticas y sus dos flavonoides relacionados hesperitina y diosmetina como las impurezas naturales de estos compuestos activos respectivamente. La metodología propuesta logró una alta selectividad para separar las estructuras de flavonoides similares. La sensibilidad del método desarrollado permitió la detección de la hesperitina por la primera vez en concentraciones traza. Además, se llevó a cabo un sencillo procedimiento de preparación de la muestra. Para este propósito se empleó un baño de ultrasonidos para

asistir la extracción sólido-liquido. Por otra parte, ya que en las separaciones cromatográficas, son muchos los factores que influyen en la separación, se empleó el diseño experimental (siglas en inglés DOE) el cual se aplicó con éxito para su optimización. El DOE es una herramienta rápida y eficaz para optimizar la interacción entre los factores importantes. El método propuesto es útil para detectar las impurezas existentes en los medicamentos, asegurando así la calidad de estos productos.

En el capítulo 5, se describió un procedimiento con extracción en micro fase sólida (siglas en inglés µSPE) realizado en la plataforma laboratorio-en-válvula (siglas en inglés LOV) donde se empleó una columna de microextracción de C_{18} insertada dentro de la unidad, para la extracción de hesperidina y diosmina de muestras de zumo de diversos cítricos. El sistema se acopló en línea con HPLC-DAD para la determinación en línea de los flavonoides extraídos. Usando el sistema desarrollado, todos los pasos de pretratamiento de la muestra se llevaron a cabo de manera completamente automática, la resina de extracción se cargó directamente en la columna de extracción, se acondicionó, se eliminaron las sustancias endógenas de la matriz, se eluyeron los analitos restantes utilizando la cantidad mínima de solvente de elución, y luego los analitos se transfirieron automáticamente a la columna de separación por HPLC. La columna de extracción se puede utilizar hasta 35 veces antes de su renovación automática, cada fracción de eluyente se puede inyectar hasta 8 veces, lo cual aumenta la repetibilidad y la reproducibilidad de los resultados, así como también disminuye el tiempo y el costo por análisis. El sistema µSPE-LOV-HPLC-DAD fue utilizado con éxito para la extracción eficaz de hesperidina y diosmina en zumos de tres diferentes tipos de cítricos exprimidos a mano, así como también de la parte comestible y sus productos comerciales relacionados (refrescos, zumos a partir de concentrados). El método desarrollado proporcionó amplia información sobre la distribución de los flavonoides estudiados en los diferentes tipos de muestra. El sistema propuesto puede emplearse para análisis de control de calidad de rutina de las industrias alimentarias y ajustar la calidad de los productos durante sus etapas de producción y los productos terminados.

En el capítulo 6, Se realizó la determinación de seis flavonoides que se localizan principalmente en la piel externa de los cítricos (narengina, narenginina, hesperedina,

hesperitina, diosmetina, y tangertina). Se utilizó la tecnología de impresión 3D para crear un dispositivo de soporte de SPE, que se recubrió con un C_{18} como resina de extracción. El dispositivo de extracción se acopló a un sistema en flujo de multiposición de válvulas (siglas en inglés MPV) para poder mejorar la extracción a través agitación magnética (siglas en inglés MSA) y también poder automatizar el tratamiento de la muestra. El sistema de flujo utilizado se acopló en línea con HPLC-DAD para la separación y cuantificación de los flavonoides extraídos a través de una columna monolítica C_{18} permitiendo la separación de estos analitos con una buena resolución y sensibilidad.

La combinación entre dos procedimientos de inmovilización en el recubrimiento del dispositivo de extracción impreso en 3D, aumentó la carga de perlas de extracción en todas las superficies del dispositivo impreso, incluso las interconectadas, lo cual aumenta el poder de extracción y disminuye la necesidad de construir un dispositivo más grande, disminuyendo el costo de impresión, el desperdicio de resina de impresión y el tiempo de impresión. Cada disco impreso (cada dispositivo cuesta aproximadamente 0,12 €) y se puede emplear hasta 19 veces antes de su renovación lo que conlleva a minimizar los costes del análisis. Por otro lado, el empleo de un sistema MSA y la automatización del sistema, mejora la eficiencia de mezcla, aumenta el factor de preconcentración, minimiza el consumo de solvente y acelera el procedimiento de extracción. El volumen de elución final permitió hasta 75 inyecciones por extracción, lo que aumentó la repetibilidad y minimizó el tiempo, el dinero y el consumo de disolventes requeridos por análisis. Se demostró la capacidad del sistema MPV-SPE (dispositivo impreso en 3D)-HPLC-DAD para la extracción y determinación totalmente automatizadas de los flavonoides de interés en tres tipos distintos de cáscaras externas de cítricos. El método es simple, robusto, reduce los efectos de matriz y aumentó la potencialidad del sistema en el análisis de rutina industrial. Además, el método proporcionó una información completa sobre la concentración de los flavonoides estudiados en la matriz, contrastando con las aplicaciones realizadas anteriormente. Además, mostró la distribución de los flavonoides en los diferentes tipos de especies de cítricos. El sincronismo del pretratamiento de la muestra y el análisis cromatográfico aumentó el rendimiento del análisis debido a los pasos de extracción y separación que se llevaron a cabo simultáneamente. El método MPV-SPE (dispositivo impreso en 3D)-HPLC-DAD se puede utilizar con éxito en las industrias para análisis de rutina.

En el capítulo 7, se desarrolló un poderoso método de HPLC para la separación de cuatro flavonoides principales (rutina, quercetina, kaempferol, e isorhamnetin) en complementos dietéticos complejos y las formas farmacéuticas naturales. El método propuesto fue capaz de separar los analitos de las innumerables impurezas que suelen estar presentes en estas muestras, así como también de las vitaminas aditivas, las mezclas de otros bioflavonoides y otros extractos naturales de frutas que son agregados al producto terminado. Además, se estimaron los principales flavonoles en las hojas de Ginkgo biloba sin aplicar el procedimiento de hidrólisis antes del análisis por HPLC. Para ello, se siguió un procedimiento de extracción simple y directo antes del análisis de la muestra. El método de extracción logró altas recuperaciones de todos los flavonoides en las muestras. En este sentido, el método desarrollado proporciona una herramienta eficaz para la estimación de la concentración real de flavonoles en estos productos, descubriendo la posible adulteración y detectando la calidad de los productos terminados durante el tiempo de almacenamiento.

En conclusión, la aplicación técnica de separación tales como HPLC proporcionan métodos analíticos selectivos y sensibles para la separación y determinación de flavonoides. Por otro lado, la implementación de técnicas de microextracción en sistemas basados en flujo y su acoplamiento con la técnica de HPLC ha permitido mejorar la selectividad y la sensibilidad de los métodos expuestos. Además, la automatización del pretratamiento de muestras mejora la reproducibilidad, el rendimiento y reduce los costos de análisis, la producción de desechos y protege el medio ambiente y a los analistas. De esta manera, se obtienen métodos analíticos más eficientes en términos de precisión, impacto ambiental y rentabilidad.

DOCTORAL THESIS

DEVELOPMENT OF NOVEL AUTOMATED SAMPLE TREATMENT TECHNIQUES COUPLING WITH CHROMATOGRAPHIC METHODS FOR DETERMINATION OF FLAVONOIDS IN NATURAL AND MANUFACTURING MATRICES

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